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# STATUS OF RESISTANCE TO ANTIMICROBIAL AGENTS OF *STAPHYLOCOCCUS AUREUS* STRAINS AT THE LABORATORY OF MICROBIOLOGY OF THE HU-JRA ANTANANARIVO

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**Introduction: On contact of antibiotics, *S. aureus* has gradually acquired multiple antibiotic resistances, including the methicillin (MRSA) and without lose its virulence. The aim of the present study was to report the evolution of resistance of *S. aureus* to different common antibiotics and to determine the antibiotics active against MRSA.**
**Materials and methods: This is a retrospective and descriptive study for 10 years from January 2005 to December 2014 at the Laboratory of Microbiology of the HU-JRA Antananarivo, the biggest academic hospital located in the capital of Madagascar. All demands for standard bacteriological examination were registered in the laboratory for various bacteriological exams or from samples taken from hospitalized patients and we included all positive cultures for *S. aureus*. The variables selected and used for the study were community or nosocomial sources of patients and results of susceptibility testing.**
**Results: A total of 906 results from 282 (31.12%) community-acquired and 624 (68.88%) nosocomial infections were studied an average of 100±25strains by year of study. Overall, the prevalence of MRSA was 13.83% (39 of 282 isolates)for community-acquired strains, and 15.70% (98 of 624) for nosocomial infections (p> 0.05)with a total of 29.53%. Resistance rate to trimethoprim-sulfamethoxazole was significantly higher in nosocomial infection than in community-acquired. No significant difference was observed in other antibiotics. Of the 137 MRSA, except vancomycin, fusidic acid is the antibiotic that worked the most in 114 cases (83.21%) followed by gentamicin in 96 cases (70.07%). Apart from ciprofloxacin and tetracycline that we have noticed an increase in resistance rates in 2012 and 2013, almost all antibiotics tested have a stable rate of resistance.**
**Conclusion: The antibiotics tested showed extremely high rates of resistance and that the problem of antibiotic resistance in *S. aureus* is effective in our center.**
**Key words: Resistance -antibiotics- *S. aureus* - HU-JRA Antananarivo**

# ETAT DES LIEUX DE LA RÉSISTANCE AUX ANTIBIOTIQUES DES SOUCHES DE *STAPHYLOCOCCUS AUREUS* DANS LE LABORATOIRE DE MICROBIOLOGIE DE L'HU-JRA ANTANANARIVO

**Résumé**
**Introduction: Au contact des antibiotiques, *S. aureus* a progressivement acquis de multiples résistances, y compris la méthicilline (SARM) et sans perdre de sa virulence. Le but de la présente étude est de rapporter l'évolution de la résistance de *S. aureus* à différents antibiotiques et de déterminer les antibiotiques actifs contre les SARM.**
**Matériels et méthodes: C'est une étude rétrospective et descriptive pendant une période de 10 ans allant de Janvier 2005 à Décembre 2014 réalisée au Laboratoire de Microbiologie de l'HU-JRA Antananarivo. Les échantillons ont été prélevés sur des patients hospitalisés ou non et nous avons inclus toutes les cultures positives à *S. aureus*. Les variables étudiés ont été l'origine communautaire ou nosocomiale des échantillons et les résultats de l'antibiogramme.**
**Résultats: Un total de 906 résultats dont 282 (31,12%) d'origine communautaire et 624 (68,88%) provenant des infections nosocomiales ont été étudiés avec une moyenne de 100±25 souches par année d'étude. La prévalence des SARM était de 13,83% pour les infections communautaires, et de 15,70% pour les infections nosocomiales (p> 0,05). Le taux de résistance à la triméthoprime-sulfaméthoxazole était significativement plus élevé dans les infections nosocomiales que dans les infections communautaires. Sur les 137 SARM, à part la vancomycine, l'acide fusidique est l'antibiotique qui marche le plus dans 114 cas (83,21%), suivie par la gentamicine dans 96 cas (70,07%). La ciprofloxacine et la tétracycline ont montré une augmentation des taux de résistance en 2012 et 2013, alors que presque tous les antibiotiques testés ont un taux de résistance stable.**
**Conclusion: Les antibiotiques testés ont montré des taux de résistance extrêmement élevés et que le problème de la résistance aux antibiotiques de *S. aureus* est effective à l'HU-JRA Antananarivo.**
**Mots clés: résistance - antibiotiques - *S. aureus* - HU-JRA Antananarivo**

## INTRODUCTION

*Staphylococcus aureus* is a Gram-positive bacterium that can be apart of the normal flora on the skin and in the nose, but is another of the most important human pathogens. *S. aureus* is an important cause of serious infections in both hospitals and the community. *S. aureus* can cause a variety of infections, most notably skin, soft tissue, bone and bloodstream infections. It is also the most common cause of postoperative wound infections [1].

On contact with antimicrobial agent, *S. aureus* has gradually acquired multiple resistances, including

the methicillin (MRSA) and without lose its virulence. And for the main causes of these resistances, it was reported high consumption of antimicrobial agent, their misuse and the poor implementation of individual and collective hygiene rules within the services including those of intensive care [2].

MRSA infections have always occupied an important place in hospitals and are responsible for a high rate of morbidity and mortality especially in pediatric and surgical services, and is currently admitted that these infections cause a significant lengthening of durations of stay and hospital costs. To this end, *S. aureus* strains resistant to antimicrobial agent represent a major public health problem that Madagascar does not escape and are responsible for a hospital chronic endemic and epidemic globally. They also have the ability to spread like an epidemic in hospitals and care facilities, but it was also shown that they can be more and more frequently from the community[3.4]. Data concerning resistance of *S. aureus* to antimicrobial agent in Madagascar are rare.

The aim of the present study was to report the evolution of resistance of *S. aureus* to different common antimicrobial agent and to determine the antibiotics active against MRSA, to make an update on the susceptibility of *S. aureus* isolates from the laboratory of Microbiology of the University Hospital Joseph Ravoahangy Andrianavalona (HU-JRA) Antananarivo to various drugs and therefore to improve the empirical approaches to the therapy of serious infections.

**MATERIALS AND METHODS**

This is a retrospective and descriptive study for 10 years from January 2005 to December 2014 at the Laboratory of Microbiology of the HU-JRA Antananarivo, the biggest academic hospital located in the capital of Madagascar. It features several specialized services and a medical laboratory. Patients in the HU-JRA come from all regions of Madagascar.

All demands for standard bacteriological examination were registered in the laboratory for various bacteriological exams or from samples taken from hospitalized patients and we included all positive cultures for *S. aureus.* We excluded from our study the results of susceptibility testing which are not complete, because sometimes, there is a lack of antibiotics discs in the laboratory as procurement was inadequate.

The variables selected and used for the study were community or nosocomial sources of patients and results of susceptibility testing.

Criteria for nosocomial infection were all infections developed in a patient after 48 hours of hospitalization. Strains were considered as community-acquired when isolated from patients thathave not been hospitalized recently or from patients before 48 hours of hospitalization.

Initial identification was based on colony morphology, Gram staining, catalase and agglutination tests with Pastorex Staph. Susceptibility to antibiotics was assessed by the disk diffusion technique on Mueller-Hinton agar. An inoculum was prepared as recommended by the Antibiogram Committee of the French Microbiology Society (CASFM). After 24 hours at 37°C, the zone of inhibition was measured. Concerning the detection of methicillin resistance, we have followed the guidelines of the French Committee for the Antibiogram which recommend to use oxacillin on Mueller Hinton agar + 2% NACL, with incubation at 37°C for 24 hours.

The following 14 antibiotics were tested: oxacillin, penicillin, amoxicillin + clavulanic acid, erythromycin, lincomycin, clindamycin, vancomycin, ciprofloxacin, tetracycline, trimethoprim-sulfamethoxazole, fusidic acid, clarithromycin, gentamicin, chloramphenicol and spiramycin. The breakpoints for resistance were those recommended by the CASFM.

The resistance rate was calculated as the number of non susceptible isolates divided by the total number of isolates. Comparison of resistance rate between nosocomial or community-acquired strains and between MRSA and methicillin-sensitive *S. aureus* (MSSA) was based on Chi square test of Pearson or exact test of Fisher according to the distribution p, significant level considered was $p < 0.05$.

**RESULTS**

A total of 906 results from 282 (31.12%) community acquired and 624 (68.88%) nosocomial infections were studied an average of 100±25strains by year of study. Strains were isolated from 401 females and 505 males (mean age: 28.10 years old 95%CI [25.9–30.2], range 1– 81 years old, sex-ratio M/F: 1.26).

Concerning the origin of the community-acquired isolates, 256 (92.46%) were from pus, 15 (5.32%) from liquid pleural, 3(1.06%) from genital tract infections, 1(0.35%) from urinary tract infections, 2 (0.71%) from ascites, 3 (1.06%) from LCR, and 2 (0.71%) from sperm.

For nosocomial strains, most (577) were isolated from pus (92.47%), 39 from blood culture (6.25%), 6 (0.96%) from drain and 2 (0.32%) from catheter.

MRSA were found in 130 samples of pus, 2 drains, 4 pleural fluids and one of the genital tract. Overall, the prevalence of MRSA was 13.83% (39 of 282 isolates)for community-acquired strains, and 15.70% (98 of 624) for nosocomial infections ($p > 0.05$)with a total of 29.53%.

The strains of MRSA resistant to other antibiotics tested are represented by Table 1.

**TABLE 1: RESISTANCE PHENOTYPE OF MRSA TO OTHER ANTIBIOTICS**

| Phenotype | CHL | ACF | GEN | TCY | ERY | CLA | LIN | CLI | SPI | CIP | SXT | VAN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sensitive | 90 | 114 | 96 | 33 | 80 | 86 | 90 | 87 | 88 | 72 | 35 | 137 |
| Resistant | 47 | 23 | 41 | 104 | 57 | 51 | 47 | 50 | 49 | 65 | 122 | 0 |

**CHL: Chloramphenicol, ACF: fusidic acid, GEN: Gentamicin, TCY: Tetracycline, ERY: erythromycin, CLA: Clarithromycin, Lincomycin, CLI: Clindamycin, SPI: Spiramycin, CIP: Ciprofloxacin, SXT: Trimethoprim–sulfamethoxazole, VAN: Vancomycin. p=0.14**

Of the 137 MRSA, except vancomycin, fusidic acid is the antibiotic that walketh the most in 114 cases (83.21%) followed by gentamicin in 96 cases (70.07%). On the other hand, tetracycline (33 cases) and trimethoprim-sulfamethoxazole (35) were the antibiotics that are most resistant with respectively 75.92% and 74.46% resistance.

Regarding the evolution of the prevalence of patients infected with MRSA, there is a decrease in MRSA rates since 2013 (Figure 1).

Rates of resistance of MSSA and MRSA to the other antibiotics tested for community or hospital acquired infections are shown in Table 2.

**TABLE 2: RATES OF RESISTANCE OF MSSA AND MRSA TO THE OTHER ANTIBIOTICS TESTED FOR COMMUNITY OR HOSPITAL ACQUIRED INFECTIONS**

| Molecules | Phenotypes | Community acquired (n/%) | Nosocomial infection (n/%) | P |
|---|---|---|---|---|
| Penicillin | Resistant | 247 (87.59) | 531 (85.10) | >0.05 |
| | Sensitive | 35 (12.41) | 93 (14.90) | |
| Oxacillin | Resistant | 39 (13.78) | 98 (15.73) | >0.05 |
| | Sensitive | 244 (86.22) | 525 (84.27) | |
| Fusidic acid | Résistant | 12 (4.26) | 22 (3.53) | >0.05 |
| | Sensitive | 270 (95.74) | 602 (96.47) | |
| Amoxiccilin + clavulanic acid | Résistant | 43 (15.25) | 113 (18.11) | >0.05 |
| | Sensitive | 239 (84.75) | 511 (81.89) | |
| Chloramphenicol | Résistant | 25 (8.87) | 74 (11.90) | >0.05 |
| | Sensitive | 257 (91.13) | 548 (88.10) | |
| Ciprofloxacin | Résistant | 68 (24.11) | 140 (22.44) | >0.05 |
| | Sensitive | 214 (75.89) | 484 (77.56) | |
| Erythromycin | Résistant | 42 (14.89) | 88 (14.13) | >0.05 |
| | Sensitive | 240 (85.11) | 535 (85.87) | |
| Clarithromycin | Résistant | 26 (9.25) | 55 (8.81) | >0.05 |
| | Sensitive | 255 (90.75) | 569 (91.19) | |
| Gentamicin | Résistant | 16 (5.67) | 41 (6.57) | >0.05 |
| | Sensitive | 266 (94.33) | 583 (93.43) | |
| Lincomycin | Résistant | 21 (7.50) | 57 (9.15) | >0.05 |
| | Sensitive | 259 (92.50) | 566 (90.85) | |
| Clindamycin | Résistant | 18 (6.38) | 49 (7.85) | >0.05 |
| | Sensitive | 264 (93.62) | 575 (92.15) | |
| Trimethoprim-sulfamethoxazole | Résistant | 211 (74.82) | 506 (81.09) | 0,02 |
| | Sensitive | 71 (25.18) | 118 (18.91) | |
| Tetracyclin | Résistant | 166 (58.87) | 358 (57.37) | >0.05 |
| | Sensitive | 116 (41.13) | 266 (42.63) | |
| Spiramycin | Resistant | 20 (7.09) | 43 (6.89) | >0.05 |
| | Sensitive | 262 (92.91) | 581 (93.11) | |
| Vancomycin | Resistant | 0 (0.0) | 0 (0.0) | - |
| | Sensitive | 282 (100) | 624 (100) | |

By table 2, 87.59% of community-acquired strains, and 85.10% of nosocomial strains have penicillinases. Resistance rate to trimethoprim-sulfamethoxazole was significantly higher in nosocomial infection than in community-acquired. Nosignificant difference was observed in other antibiotics (Table 2).Generally, fusidic acid remains the most active antibiotic with only 12% resistance to community-acquired infections and 22% resistance to nosocomial infections followed by gentamicin (16%, 41%), clindamycin (18%, 49%), spiramycin (20%, 43%), chlarythromycin (26%, 55%) and chloramphenicol (25%, 74%) (Table 2).

There were no significant differences in the resistance rates to any antibiotic according to the site of infection, the age group or the year of isolation of the strains (data not shown).

At the outcome of the antibiotic sensitivity testing during all these years, the highest resistance rates were recorded with trimethoprim-sulfamethoxazole where it reached a resistance rate of 98.29% followed by penicillin 95.73 % by the year 2013 in the center (Figure 1).

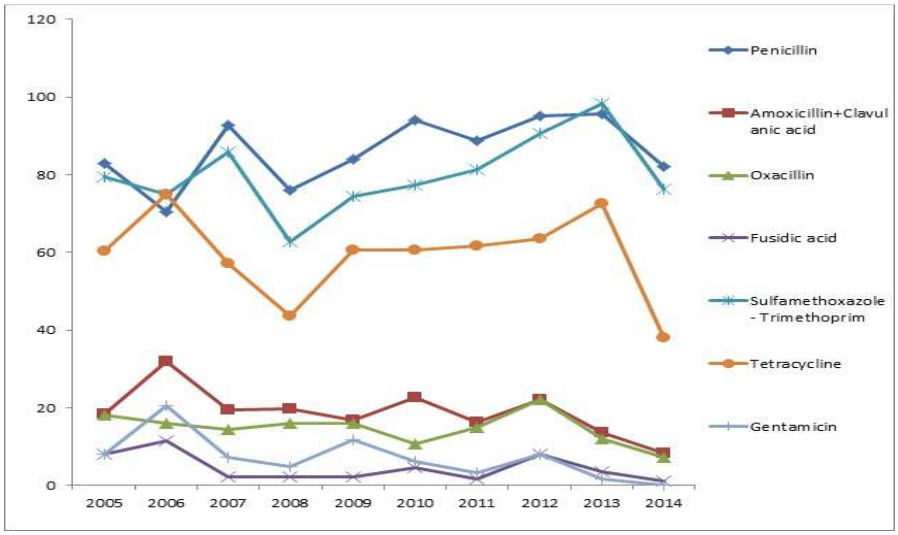


**FIGURE 1: CHANGES BY YEAR IN RESISTANCE OF *STAPHYLOCOCCUS AUREUS* TO ANTIBIOTICS**

Apart ciprofloxacin and tetracycline that we have noticed an increase in resistance rates in 2012 and 2013, almost all antibiotics tested have a stable rate of resistance. In 2014, we noted a remarkable decline in resistance to all antibiotics tested except for chloramphenicol but this result was not significant (Figure 1 and 2).

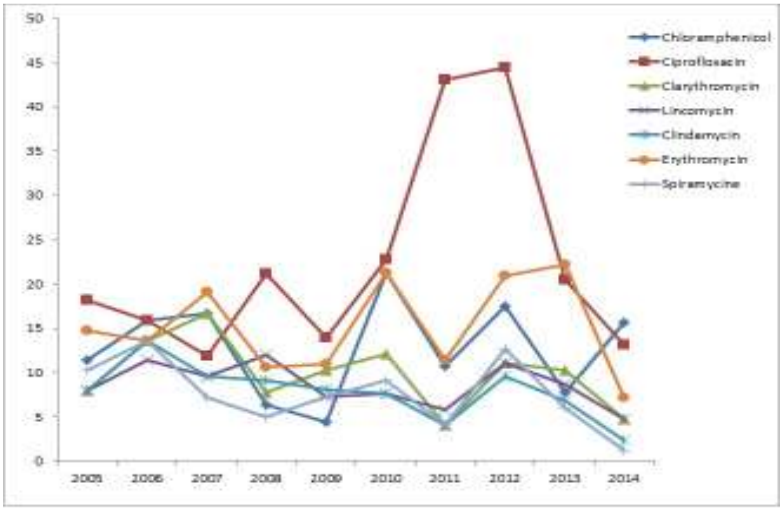


**FIGURE 2: CHANGES BY YEAR IN RESISTANCE OF *STAPHYLOCOCCUS AUREUS* TO ANTIBIOTICS**

**DISCUSSION**

We conducted this study to improve the therapeutic approach to infections due to *S. aureus* in Antananarivo. Our results are sometimes incomplete because there are moments when it is a rupture of antibiotic disk stock by lack of supply in our laboratory. So, there is resistance phenotypes that are not listed in our study thus limiting our study. Only 906 results are complete and usable.

Strains were isolated from 401 females and 505 males. The majority of strains isolated from pus of community origin or nosocomial respectively 92.46% and 92.47% followed pleural fluids (5.32%) and blood cultures (6.25%).

MRSA were found in 130 samples of pus. Overall, the prevalence of MRSA is 29.53% which 13.83% (39 of 282 isolates) for community-acquired strains, and 15.70% (98 of 624) for nosocomial infections ($p > 0.05$). Other authors have found MRSA in skin and soft tissue (50.2%) followed by urinary tract (38.2%) collected through a representative sample of French private-sector community-based medical laboratories (Labville network) [5]. The distribution

of MRSA nosocomial infections by infectious site in France in 2006 is dominated by infections of the skin and soft tissues [6], in contrast to other studies, where resistance rates are higher in nosocomial infections [7].

The prevalence of MRSA found in our study is high compared to a study conducted at the Pasteur Institute of Madagascar in 2014 (5.80%)[8]. Although rather low, the rate of resistance to methicillin has increased between 1997–1998 [9]. The very highest rates of methicillin resistance among *S. aureus* isolates have been noted in developed countries and especially in Western Pacific Regions, both in community acquired and nosocomial infections [7]. The prevalence of MRSA has increased worldwide, as it is evident from many surveillance studies [7, 10, 11]. However, there are considerable differences between countries. Most reported MRSA proportions exceed 20% in all WHO regions, and even exceed 80% in some reports [1].
We did not find any significant difference in the rates of resistance to most of antibiotic between strains isolated from nosocomial or community-acquired infections.
Over the past decade, community-acquired MRSA has increased significantly in a number of countries. Fortunately, many of these community-acquired MRSA strains have so far retained susceptibility to a number of non-beta-lactam antimicrobials, whereas most health-care associated MRSA infections are caused by difficult to treat multi resistant strains. For the latter, the treatment of last resort has been glycopeptides such as vancomycin (since the 1950s) and teicoplanin, which can only be given by injection and also needs careful monitoring to avoid adverse side-effects [1].Vancomycin does not yet exist at the pharmacy in Madagascar while its sensitivity is 100% of the tested strains.

Because of their low price and the low rate of resistance, fusidic acid or chloramphenicol in combination with gentamicin may be the more suitable treatment on MRSA strains.
Tetracycline and trimethoprim-sulfamethoxazole do not work that in almost 25% of cases of MRSA.

Resistance rate to trimethoprim-sulfamethoxazole was significantly higher in nosocomial infection than in community-acquired. This rise in resistance has several possible causes, there the free sale of these drugs to Madagascar, self-medication and also their misuse. In general, elevated rates of multidrug resistance may emerge from diverse isolates of *S. aureus* under antimicrobial pressure or as a result of widespread person-to-person transmission of multidrug-resistant isolates [12].

Other authors in Madagascar have found the same rates of resistance (75%) to tetracycline resistance whereas sulfamethoxazole-trimethoprimes is still 38.9% [8]. The resistance rates to cheaper antibiotics such as tetracycline and trimetoprim-sulfamethoxazole are higher than those observed in developed countries and are similar to that observed in African countries [1,7,13].
According to the evolution of antibiotic resistance testing, we noted a decrease in the prevalence of patients infected with MRSA since 2012. The percentage of *S. aureus* isolates reported as MRSA is now stabilizing or decreasing in most European countries, and the European union population-weighted mean MRSA percentage has decreased significantly over the last four years [14].

Sensitivity tests have shown that the problem of antibiotic resistance in *S. aureus* is effective in our center at extremely high rates, averaging 86% for penicillins. Inreality, these results reflect the situation of antibiotic resistance to penicillin in Antananarivo because another study conducted at the Pasteur Institute of Madagascar found a resistance rate to 87.7% [8]. This rate was also brought by other authors[15,16,17,18].
The highest resistance rates during all his years of study have been registered with trimethoprim-sulfamethoxazoles where it reached a 98.29% resistance rate monitoring penicillin 95.73% in the year 2013 in the center.Apart ciprofloxacin and tetracycline that we have noticed an increase in resistance rates in 2012 and 2013, almost all antibiotics tested have a stable rate of resistance. In 2014, we noted a remarkable drop in resistance to all antibiotics tested except for chloramphenicol but this result was not significant. This increase of chloramphenicol resistance may be due to the abuse of this medicine because chloramphenicol is from antibiotics which are still effective against MRSA in recent years in Madagascar. The other tested antibiotics such as spiramycin, clindamycin, lincomycin, and chlarythromycin, quite used to Madagascar were more active with sensitivity rate close to 92% respectively. These antibiotics are available but a fairly expensive cost to the population.

On the national level, real access to drugs began in 2007 as part of national health policy that combines a reinforcement of training of health personnel in quality and quantity with adequate distribution of qualified personnel in all health centers. This measure consists of ensuring at least 85% availability and accessibility of essential medicines, medical consumables, reagents in all health facilities by extending the range of Salama (essential drug supplier) for medicines and consumables hospital. This was accompanied by a rationalization of the prescription and also the rational use of drugs with the objective of improving health system in Madagascar until 2011.The best access to drugs and their rational prescribing in hospitals may explain the decrease in MRSA in our center during 2012. There was also a downward trend in resistance to almost all antibiotics tested but the differences are

not statistically significant. However, private firms, who escape to training and supervision of the Ministry of Health, continued in the irrational prescription of medicines, particularly antibiotics [19].

The decrease in the pharmacological pressure by changes in protocols also probably resulting led to the decline in resistance. Several studies have reported resistance decreases following the suppression or restriction in prescribing certain antibiotics [20].

**Conclusion**

In summary, methicillin resistance among *S. aureus* isolated at the Laboratory of Microbiology of the HU-JRA Antananarivo are mainly found in pus of community or nosocomial but the rate is still low compared to other countries. Other antibiotics tested showed extremely high rates of resistance and that the problem of antibiotic resistance in *S. aureus* is effective in our center. It is important for Malagasy doctors in case of suspected infection *S.aureus* to do sensitivity tests to antibiotics before prescribing drugs. However a nationwide survey should be undertaken to confirm these results and could be valuable for the selection of therapeutic alternatives and also to control the emergence and dissemination of antimicrobial resistance.

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## ORIGINAL ARTICLE

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# ANTIMICROBIAL SUSCEPTIBILITY OFEXTENDED-SPECTRUM BETA-LACTAMASE PRODUCING *ENTEROBACTERIACEAE* CAUSING URINARYTRACT INFECTIONS IN OUAGADOUGOU, BURKINA FASO

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**ABSTRACT**
**Objective: To determine the frequency of extended-spectrum beta lactamase producing *Enterobacteriaceae*(ESBL) and other antibioticsresistant bacteria in urinary tract isolates.**
**Study Design: prospective and experimental study.**
**Methodology: Place and duration of study :YalgadoOuedraogo University Hospital Center, Charles De Gaulle Pediatric Hospital Center, Saint Camille Hospital and National Public Health Laboratory, Ouagadougou, from November 2014 to October 2015.**
**All*Enterobacteriaceae*strains isolated from urinary samples of patients were identifiedusing API 20E chemical gallery (BioMerieux, France). All strains were subjected to an array of 14 antibiotics to study their drug susceptibility by using Kirby-Baeurdisk diffusion method. Detection of ESBL was carried out by double disk diffusion technique. Statistical analysis was performed by Microsoft Excel and Anova one-way GrapPad Prism version 5.01. Chi-square ($\chi 2$) test was used to determine significance. A $p < 0.05$was considered to be statistically significant.**
**Results: A total of 324 isolates of *Enterobacteriaceae* were identified during the study period, including211(65%) *E. coli*, 75 (23%)*Klebsiella* spp., 18 (6%) *Enterobacter* spp., 11 (3%)*Proteus* spp., 5 (2%) *Citrobacter* spp., *Serratia* spp. 3 (1%).All the clinical isolates were susceptible to imipenem. Resistance to amikacinwas 14% (45/324); gentamicin 54% (175/324); tobramycin 58% (187/324); nalidixic acid 72% (234/324),ciprofloxacin 63% (204/324) and to cotrimoxazole 83% (269/324).The overall rate of the EBSL producing strains was 35% (114/324). Their susceptibility to antibiotics was (imipenem,amikacin, cefoxitin and fosfomycin) 100% (114/114), 93% (106/114), 74% (84/114) and 84% (96/114) respectively. ESBL positivity within individual organism group was highest in*Escherichia coli* 64% (73/324) followed by*Klebsiella*spp. 28% (32/324), *Enterobacter*spp. 3% (4/324), *Proteus* spp. *and Citrobacter*spp. 2% (2/324).**
**Conclusion: The results showeda high frequency of ESBL producing *Enterobacteriaceae*, especially *Escherichia coli* and *Klebsiella*spp. The data points to theneed of routine detection and surveillance of ESBL producing bacteria in Burkina Faso.**

**Keywords: Antimicrobial susceptibility, *Enterobacteriaceae*, Urine, Burkina Faso**

# SENSIBILITE DES ENTEROBACTERIES PRODUCTRICES DE BETA-LACTAMASESA SPECTRE ELARGI ISOLEES DES INFECTIONS URINAIRES, OUAGADOUGOU, BURKINA FASO

**Résumé**

**Objectif : Déterminer la fréquence des entérobactéries productrices de bêta-lactamases à spectre élargi(BLSE)et la résistance aux autresantibiotiques utilisésle traitement des infections urinaires.**
**Type de l'étude : Etude prospective et expérimentale**
**Méthodologie : Lieu et période : Centre Hospitalier Universitaire YalgadoOuedraogo, Centre Hospitalier UniversitairePédiatrique Charles De Gaulle, Hôpital Saint Camille et Laboratoire National de Santé Publique, Ouagadougou, de Novembre 2014 à Octobre 2015.**
**Toutes les entérobactéries isolées des urines de patients et identifiées sur galerie API 20$^{E}$ (BioMerieux, France). Quatorze (14) antibiotiques ont été utilisés pour tester la sensibilité des souches cliniques par la méthode de diffusion des disques selon Kirby-Bauer. La détection des souches productrices de BLSE a été faite en utilisant la technique de test à double synergie. Le logiciel Excel et Anova one-wayGrapPadPrism version 5.01ont été utilisés pour l'analyse statistique et le test de $\chi 2$au seuil de $p < 0.05$ était considéré statistiquement significatif.**
**Résultats: Un total de 324 souches d'*Enterobacteriaceae* ont été collectées durant la période d'étude composées de 211 (65%) souches de *E. coli*, 75 (23%)*Klebsiella*spp. ,18 (6%) *Enterobacter*spp., 11 (3%) *Proteus*spp.,5 (2%)*Citrobacter*spp. , 3 (1%) *Serratia*spp.**
**Toutes les souches d'entérobactéries étaient sensibles àl'imipenème. La résistanceà l'amikacineétait de 14%(45/324); la gentamicine 54% (175/324); la tobramycine 58% (187/324); l'acidenalidixique 72% (234/324), la ciprofloxacine 63% (204/324) et le cotrimoxazole 83% (269/324). La fréquence des souches productrices de BLSE était 35% (114/324). Leurs taux de résistance aux antibiotiques étaient de 100% (114/114), 93% (106/114), 74% (84/114) et 84% (96/114) respectivement àl'imipeneme, à l'amikacine, à la cefoxitineetà la fosfomycine.Lafréquence des souches productrices de BLSE par espèceétaitélevée chez*Escherichia coli* 64% (73/324) suivie de*Klebsiella*spp. 28% (32/324), *Enterobacter*spp. 3% (4/324), *Proteus*spp.et*Citrobacter*spp. 2% (2/324).**

**Conclusion:35% des souches cliniques étaient productrices de BLSE. Une fréquenceélevée des entérobactéries productrices de BLSE aétéobservée chez l'espèce*E. coli* et *Klebsiella*spp parmi les souches cliniques testées. Les résultats montrent une nécessité de mettre en place un système de surveillance des souches productrices de BLSE au Burkina Faso.**
**Mots clés: Sensibilité aux antibiotiques, *Enterobacteriaceae*, Urine, Burkina Faso**

## INTRODUCTION

Urinary tract infections(UTI) is one of the most common infectious diseases ranking next to upper respiratory tract infection, it is an important cause of morbidity and mortality in human. Infected urine, renal calculi, obstructive uropathy, vesico ureteral reflux and avoiding disorders can lead to urinary stasis and may predispose to the development of UTIs and complications [1].It has been estimated that nearly 10% of the human population will experience an UTI during the life time [2, 3, 4]. Resistance to commonly-prescribed antibiotics is an expanding global problem and has been observed in both developed and developing countries[5, 6, 7, 8]. *Enterobacteriaceae* are the major causative organisms of UTIs and are responsible for more than 81% of UTIs cases. *Escherichia coli* is the most prevalent causative organisms of UTIsand is solely responsible for more than 69% of the infections [1,9,10].

Bacterial resistance to antibiotics has emerged even to newer, more-potent antibacterial agents [11]. A number of epidemics have recently occurred caused by multiple resistant organisms [12, 13].

In Burkina Faso, UTIs due to *Enterobactriaceae* are common and represent a frequent cause of morbidity in outpatients as well as a frequent cause of nosocomial infections in many hospitals. Most infections are treated on an empirical basis. Clinical experience has indicated the presence of numerous cases of infection resistant to conventional antibiotics therapy. Microbial resistance rates to commonly prescribed antibiotics have increased recently. Updated knowledge of urinary tract infections *Enterobacteriaceae*, the frequency of ESBL strains and the susceptibility patterns to other antibiotics is important for the proper selection and use of antibiotic and for the development of an appropriate prescribing policy. The aim of this study was to determine the frequency of ESBL strains and the susceptibility patterns of other antibioticresistant bacteria of clinical importance responsible for urinary tract infections in Ouagadougou, Burkina Faso.

## Materiel and methods

### Study population and settings

This study was an experimental study ofbeta-lactam and other antibiotics resistance expression of*Enterobacteriaceae*.The socio demographic data and*Enterobacteriaceae* strains were obtainedfrom patients who came for an etiological diagnosis for bacterial infectionfrom November 2014 to October 2015.

The *Enterobacteriaceae*strains were obtained from thefollowing 3 health centers in Ouagadougou: YalgadoOuedraogo University Hospital (CHU-YO), the largest public medical institution, Charles De Gaulle Pediatric University Hospital (CHUP-CDG), the referral public pediatric hospital with 120 bedsand

Saint Camille Hospital (HOSCO), the confessional hospital.

All *Enterobacteriaceae* strains collected at these 3 different sites were transported to the LNSP Bacteriology-Virology Laboratoryfor *Enterobacteriaceae* investigation. Strains identification was performed using API 20 E gallery (Biomérieux, Marcy- L'étoile, France) after 24 hours incubation at 37 ° C.

The isolated and identified strains were seeded on Mueller-Hinton (MH) agar for 18 to 24 hours in order to have young and pure colonies.All clinical isolates were stored at -30°C for future investigations at Institut Pasteur de Côte d'Ivoire (IPCI).

### Antibiotic Susceptibility Testing

All isolates were tested for susceptibility to 14 different antimicrobial agents using the disk diffusion method on Mueller-Hinton agar (BioRad, France) following the European Committee on Antimicrobial Susceptibility Instructions (EUCAST) guidelines (CA-SFM/EUCAST, 2016). *E. coli* ATCC 25922 and ATCC 35218 were used as a control. The antimicrobial disks (Bio-Rad, France) used were:gentamicin (15 µg), amikacin (30 µg), tobramycin(15µg), Amoxicillin (25µg), amoxicillin + clavulanic acid (20µg), cefepim (30µg), cefotaxim (30µg), ceftriaxon (30µg), cefoxitin (30µg), imipenem (10µg), ciprofloxacin (5µg), cotrimoxazole (1.25 / 23.75µg), nalidixic acid (30ug) and fosfomycin (50µg).

### ESBL strains screening

The double-disk synergy tests (DDST) to detect ESBL-producing isolates was performed on Mueller-Hinton agar, placingcefepim, cefotaxim, and ceftriaxone discs around the amoxicillin + clavulanic acid diskat a distance of 3cm from the center to center, according to theEuropean Committee on Antimicrobial Susceptibility Instructions (EUCAST) guidelines (CA-SFM/EUCAST, 2016) (14).

### Determination of multiple antibiotic resistance index

Multiple antibiotic resistance index (MAR) was determined using the formula MAR=*x/y*, where *x* was the number of antibiotics to which test isolate displayed resistance and *y* is the total number of antibiotics to which the test organism has been evaluated[15].

### Statistical analysis

Statistical analysis was performed with Excel and Anova one-way GrapPad Prism version 5.01. Chi-square ($\chi 2$) test was used to calculate probabilities and determine significance. A p-value of less than 0.05 was considered to be statistically significant ($p<0.05$).

## RESULTS

### Characteristic of the study population and distribution of strains

(162 men and 162 women) in 3 hospital centers in Ouagadougou, Burkina Faso.The mean age of these patients was 33.7 ± 2 years and the sex ratio 1.7.

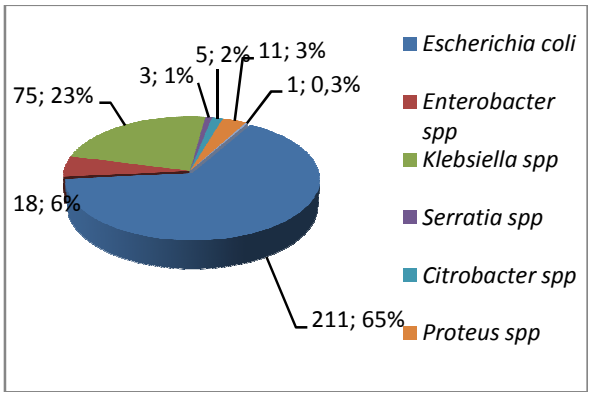



**FIGURE 1: DISTRIBUTION OF DIFFERENT SPECIES BELONGING TO*ENTEROBACTERIACEAE* FAMILY ISOLATED DURING THE STUDY**

**This figure showed that urine samples are the samples which predominant 65% (211)**

Out of 324 *Enterobacteriaceae* isolates, 211(65%)were *E. coli*, 75 (23%) *Klebsiella* spp., 18 (6%) *Enterobacter* spp., 11 (3%)*Proteus* spp., 5 (2%) *Citrobacter* spp., *Serratia* spp. 3 (1%)as shown inFigure 1.

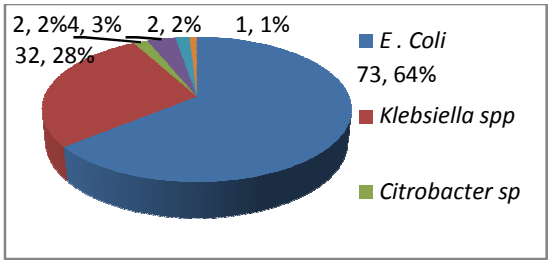



**FIGURE 2: ESBL PRODUCING ISOLATES WITHIN INDIVIDUAL STRAINS DIVIDED BY TOTAL ISOLATES OF THAT ORGANISM REPRESENT PERCENT ESBL ISOLATES. ESBL PRODUCING *E. COLI* IS DOMINANT**

The most frequent urinary *Enterobacteriaceae* isolated were *E. coli, Klebsiella species, and Enterobacter species.* Other urinary tract bacteria were isolated in relatively few number. These included *Citrobacter* spp., *Proteus* spp. *Serratia* spp. and *Providencia* spp.

A total of 324 clinical isolates belonging to *Enterobacteriaceae* family were obtainedfrom patients

**TABLE 1:AGE WISE DISTRIBUTION OF ESBL-PRODUCING *ENTEROBACTERIACEAE* IN DIFFERENT AGE GROUPS**

| Age groups (years) | Total number of isolates (n=324) | ESBL positive (n=114) | Percent ESBL positive | P-value |
|---|---|---|---|---|
| < 15 years | 67 | 28 | 42% | |
| 15-45 years | 135 | 44 | 33% | 0.5321* |
| 46-60 years | 38 | 12 | 32% | |
| >60 years | 84 | 30 | 36% | |

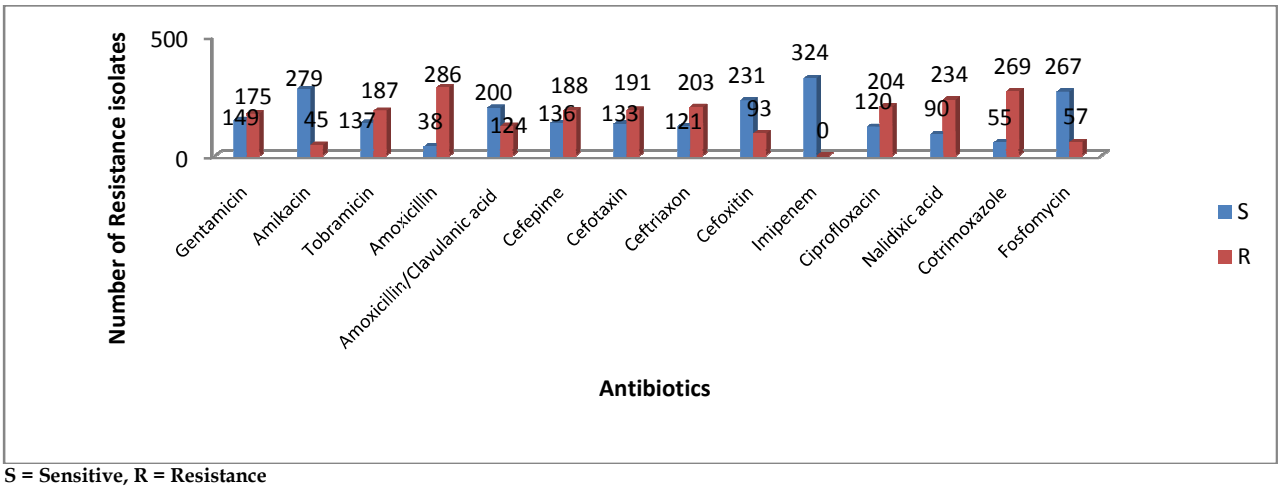
***$p$ = 0.5321, no significant statistically**

**TABLE 2:RESISTANCE RATE OF44 ESBL-PRODUCING *ENTEROBACTERIACEAE* TO ANTIBIOTICS IN OUAGADOUGOU, BURKINA FASO**

| Antibiotics | Resistance rate | |
|---|---|---|
| | I+R(%) | S(%) |
| Gentamicin | 37(84) | 7(16) |
| Amikacin | 1(2) | 43(98) |
| Tobramicin | 36(82) | 8(18) |
| Amoxicillin | 44(100) | 0 |
| Amoxicillin/ clavulanicacid | 9(20) | 35(80) |
| Cefoxitin | 8(18) | 36(82) |
| Ceftriaxon | 42(95) | 2(5) |
| Cefotaxim | 43(98) | 1(2) |
| Cefepime | 42(95) | 2(5) |
| Imipenem | 0 | 44(100) |
| Nalidixicacid | 38(86) | 6(14) |
| Ciprofloxacin | 40(91) | 4(9) |
| Cotrimoxazole | 44(100) | 0 |
| Fosfomycin | 12(27) | 32(73) |

### Antibiotics susceptibility test

The antibiotic resistance profile of the urinary tract isolates is shown in figure 3. All the strains isolated showed high resistance to amoxicillin 88% (286/324), amoxicillin/clavulanicacid 38% (124/324), gentamicin 54% (175/324), tobramycin 58% (187/324), ciprofloxacin 63% (204/324), nalidixic acid 72% (234/324) and to cotrimoxazole 83% (269/324). However, there was low resistance toamikacin 14% (45/324)and fosfomycin 18% (57/324) (Figure 3). All strains were susceptible toimipenem. *Escherichia coli and Klebsiella pneumonia* showed high rate of resistance to gentamicin, tobramycin, amoxicillin and all the 3rd generation cephalosporins (Figure 3).




**S = Sensitive, R = Resistance**

**FIGURE3: RESULTS OF OVERALL RESISTANCE AND SENSITIVITY OF ALL *ENTEROBACTERIACEAE*ISOLATES TO ANTIBIOTICS. AMIKACIN, IMIPENEM AND FOSFOMYCIN SHOWED THE BEST SUSCEPTIBILITY RATE.**

**Occurrence of ESBL-producing *Enterobacteriaceae***

The overall rate of ESBL-producing *Enterobacteriaceae* in UTIs in both male and female patients was found to be 35% (114/324). The frequency of ESBL strains within individual organism group was *E. coli* 64% (73/324),*Klebsiella*spp. 28% (32/324), E*nterobacter*spp. 3% (4/324), *Proteus* spp.and*Citrobacter* spp.2% (2/324)as shown in Figure 2.*E. coli* strains producingESBL were higher than those of the other species.

Considering the clinical isolates origin, ESBL strains rate was 24 % (78/324) at CHU-YO, 4% (13/324) at Saint Camille Hospital (HOSCO) and 7 % (23/324) at CHUP-CDG.The mean age of patients with ESBL producing organisms was 32.38 years compared to 27.66 years for patients with non-ESBL strains. ESBL production among various age groups ranged from 32% to 42%. However, there was no statistically significant difference between the age groups (Table 1) with respect to ESBL production ($p$ = 5321).ESBL producing strains showed high susceptibility rateswith imipenem 100% (114/114), amikacin 93% (106/114), cefoxitin 74% (84/114) and fosfomycin 84% (96/114) (Table 2)

**DISCUSSION**

In this study, we investigated the frequency of ESBL production by *Enterobacteriaceae* isolates in three hospitals in Ouagadougou, Burkina Faso.

A total of 324 clinical isolates belonging to *Enterobacteriaceae* family were obtained from urine samples from these3 centers in Ouagadougou, Burkina Faso from November 2014 to October 2015. These bacteriawere isolated from 162 females and 162 males. The patients mean age was33.7 ± 2 years and the sex ratio 1.7.

UTIs are the most common nosocomial infections, comprising about 35% of such occurrences in both hospitals and nursing homes [16].

More than 95% of UTIs are caused by a single bacterial specie and *Escherichiacoli* is by far the most frequent infecting organism in acute infections[17]. The spectrum of strains isolated from urinary samples in this study is not different from those reported in literature.

In this study, all of the 324 *Enterobacteriaceae* strains, *Escherichia coli* predominated followed by *Klebsiella* spp. and *Enterobacter*spp.*Citrobacter*spp. and*Proteus* spp. were less significant.

In several others studies in Burkina Faso,Sudan, India,Pakistanand Ivory Coast[18,19,20,21,27]the authorsreported that *Escherichiacoli* was also the most common isolate followed by *Klebsiella*spp., *Enterobacter*spp. and *Proteus* spp.

*E. coli* remains an essential bacterium in urinary tract infections. Wilson and Gaido[28]also reported that *E. coli*is the major bacterial etiology of urinary tract infections. This corroborates the high frequency of *E. coli* isolates reported in our study.

The antibiotic susceptibility tests revealed in our study high levels of resistance to certain molecules used in common practice.Our study also revealed that 88% (286/324)of isolates were resistance to amoxicillin, which may be due to the frequent and possiblemisuse of this antibiotic. The resistance rate to amoxicillin + clavulanicacid was38% (124/324).In contrast with the report of Adonis-Koffi*etal.*[30] in Ivory Coast who obtained a resistance rate of 68% to amoxicillin + clavulanic acid.

The high levelresistance of the *Enterobacteriaceae*to 3rd and 4th generation cephalosporin antibiotics were also observed (cefepim showed 58%, cefotaxim 59% and ceftriaxon 63%).

The most active molecule among the aminoglycosideantibiotic family was amikacinand the resistance rate of strain was 14%;BonniCisse et *al.* [27] did observe the same trend in Ivory Coast. Other aminoglycoside antibiotics tested were gentamicin and tobramicinwith resistance rates of 54% and 58%respectively.Leski et *al.*[29]in Sierra Leone however reported ahigh percentage (73%) resistance to gentamicin.

Furthermore, the clinical strains showed very high resistance to ciprofloxacin and nalidixic acid and the

resistance rates were 63% (204/324) and 72% (234/324)respectively.Results of our study is similar to that of Guessennd et *al*. [25] conducted from 2005 to 2006 in which the resistancerateto quinolones was 71% to ciprofloxacin and 77% tonalidixic acid. Fosfomycin had the bestactivity among antibiotics used, where strains showed 82% susceptibility. We observed high resistance rate tocotrimoxazolewith 83% (269/324), this is in line with reports of BonniCisse *et al*.[27] and Guessennd *et al*. [25]in which they reported 76% and 91% resistance to cotrimoxazole respectively in Ivory Coast. These high rates could be attributed to the use of cotrimoxazole in chemoprophylaxis in the treatment of opportunistic infections in immunocomprimised patients.

The resistance to antibiotics and ESBL production spares no country in the world.The frequencies of ESBL producing strains vary from country to country and from species to species in the world [22, 23].

Studies carried out by Ouedraogo et *al*. [24]in Burkina Faso. Mohantyet *al*. [26]in India reported the overall prevalence of 58% and 69% ESBL producing bacteria respectively. Our study showed a prevalence of 35% ESBL producing strain which is relatively high and is almost similar to other data which showed high prevalence.

This resistance is often associated with antibiotics such as aminoglycosides and quinolones (Table 2). The emergence of these Bacteria Multi-Resistances (BMRs) in hospitals of Ouagadougou could lead to therapeutic failures despite the administration of aminoglycosidesand 3rd generation cephalosporins. While the issue of antibiotic resistance has long been considered a concern in hospitals for nosocomial infections, in recent years the problem has been extended to include community medicine [32]. One of the reasons is the high consumption of antibiotics in human medicine, the illicit sale of antibiotics on the streets, and the increased movement of colonized or infected patients between hospitals and community settings [32].

However, the good susceptibility of the ESBL-producing*Enterobacteriaceae* to imipenem100% (114/114) and also to amikacin93% (106/114), cefoxitin74% (84/114) and fosfomycin84% (96/114) makes them the molecules of choice in alone treatment or in combination of other antibiotics (Table 2).

These results should act as an impetus for the establishment of antibiotic control policies. Indeed, currently there is no restriction in the use of antibiotics in Burkina Faso.

**Conclusion**

UTIs antibiotics therapyshould be guided by antimicrobial susceptibility as increasing numbers of urinary isolates are developing resistance to commonly use antibiotics. Increasing antimicrobial resistance of *Enterobacteriaceae* has led to reconsideration of traditional treatment of recommendations in many areas. This experimental study should be followed by several studies on antimicrobial resistance among patients in Burkina Faso hospitals and other regions of West Africa as there is relatively few data concerning the antibiotic susceptibility spectrum of bacteria isolated from patients with UTIs.

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## ORIGINAL ARTICLE

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# INCIDENCE MICROBIOLOGICAL PROFILE AND DRUG RESISTANCE PATTERN OF UROPATHOGENS CAUSING ASYMPTOMATIC BACTERIURIA AMONG BELOW POVERTY LINE DIABETIC MALE PATIENTS

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**ABSTRACT**

**Introduction: With the prevalence of DM increasing among rural population in developing countries, factors associated with diabetes and its complications also becomes more important. More than half of diabetic patients with ASB have upper urinary tract involvement and the frequency of symptomatic UTI had been significantly higher. Symptomatic UTIs tends to be more common in diabetic subjects with ASB than in those without ASB. Although ASB is of major concern in diabetic population, the long-term consequences of ASB in patients with DM are poorly documented, Almost all studies were performed among elderly women with type 2 diabetes and there is very little information on the occurrence of ASB among BPL diabetic males in our local setting. Hence in the present study the incidence and etiology of ASB among BPL diabetic male patients was monitored along with the resistance pattern of bacterial isolates to antimicrobial agents.**

**Materials and methods: Clean catch voided midstream urine samples were collected from 1131 BPL Diabetic male patients enrolled for the study. Wet film of centrifuged urine was performed to detect the presence of pus cells, epithelial cells, erythrocytes, microorganisms, cast. Culture was performed using standard loop method and antimicrobial susceptibility of the isolates was studied using Kirby Bauer disc diffusion method following CLSI guidelines.**

**Results: Out of 1131 BPL diabetic male patients screened for ASB, 155 (13.7%) were culture positive. Among the uropathogens Gram negative bacilli was the most commonest type (72.7%) and the most prevalent organisms isolated was *Klebsiella spp* (35.2%), *Enterococcus spp* (22.4%), followed by E. coli (19.4 %) ,Pseudomonas aeruginosa ( 7.3%), etc.,. 87.5% of *E.coli* isolated were ESBL, followed by 77.6 % of *Klebsilla spp* and 11.1 % *Enteroabacter spp. Pseudomonas aeruginosa* reported in this study were 100% ESBL and 16.6% Metallo β lactamase (MBL) producers.8.1 % of Vancomycin resistant Enterococcus (VRE) was also found in this study.**

**Conclusion: This study demonstrated a high occurrence of ASB in BPL diabetic males (13.7%). Klebsiella was the most commonest uropathogen found in our study followed by Enterococcus , E.coli and Pseudomonas. E.coli and Pseudomonas showed high rates of drug resistance. Nitrofurantoin and Amikacin was the most effective drugs for majority of the isolates. Hence routine monitoring and screening for ASB in this population is essential. Moreover patients in rural parts of developing countries with diabetes has to be sensitized about the complications of ASB and regarding maintenance of their glycemic control which is of major importance in prevention of the condition.**

# PROFIL MICROBIOLOGIQUE DE L'INCIDENCE ET LA RÉSISTANCE DES UROPATHOGÈNES CAUSANT LA BACTÉRIURIE ASYMPTOMATIQUE CHEZ LES DIABÉTIQUES SOUS LE SEUIL DE PAUVRETÉ CHEZ L'HOMME

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**RÉSUMÉ**

**Introduction: Avec la prévalence de DM en hausse chez les populations rurales dans les pays en développement, les facteurs associés au diabète et à ses complications devient aussi plus important. Plus de la moitié des patients atteints de diabète, à l'ASB ont voies urinaires et la fréquence de l'infection urinaire symptomatique a été sensiblement plus élevé. Infection urinaire**

**symptomatique a tendance à être plus fréquente chez les sujets diabétiques avec CNA que dans ceux sans BSA. Bien que l'ASB est une préoccupation majeure dans la population diabétique, les conséquences à long terme de l'ASB chez les patients atteints de DM sont mal documentés, presque toutes les études ont été effectuées chez les femmes âgées atteintes de diabète de type 2 et qu'il y a très peu de renseignements sur l'incidence du CNA entre les hommes diabétiques BPL dans notre contexte local. Par conséquent, dans la présente étude, l'incidence et l'étiologie de l'ASB chez les patients hommes diabétiques BPL a été suivie avec le patron de résistance des isolats bactériens aux agents antimicrobiens.**

**Matériels et méthodes : Nettoyer la route barrée de capture des échantillons d'urine ont été recueillis à partir de 1131 hommes diabétiques BPL patients recrutés pour l'étude. Urine centrifugé de film humide a été effectuée pour détecter la présence de pus, les cellules épithéliales, érythrocytes, micro-organismes, exprimés. La culture a été réalisée à l'aide d'une méthode de la boucle et à la sensibilité aux antimicrobiens des isolats a été étudiée à l'aide de méthode de diffusion disque Bauer Kirby qui suit les lignes directrices du CLSI.**

**Résultats: sur 1131 patients hommes diabétiques BPL de dépistage de l'ASB, 155 (13,7 %) étaient positifs à la culture. Parmi les uropathogènes bacilles Gram négatif a été la plus commune la plupart type (72,7 %) et le plus souvent organismes isolés a été Klebsiella spp (35,2 %), Enterococcus spp (22,4 %), suivi par E. coli (19,4 %), Pseudomonas aeruginosa ( 7,3 %), etc.,. 87,5 % d'E.coli BLSE ont été isolés, suivie par 77,6 % des Klebsilla Enteroabacter spp et 11,1 % spp. Pseudomonas aeruginosa rapportés dans cette étude ont été de 100 % et 16,6 % de BLSE Métallo β lactamase (MBL) les producteurs.8,1 % d'entérocoque résistant à la vancomycine (ERV) a également été constaté dans cette étude.**

**Conclusion: Cette étude a montré une fréquence élevée de l'ASB dans le BPL les hommes diabétiques (13,7 %). Klebsiella a été le plus plus commun uropathogen trouvés dans notre étude suivie d'E.coli et Enterococcus, Pseudomonas. E.coli et Pseudomonas ont montré des taux élevés de résistance aux médicaments. La nitrofurantoïne et l'amikacine a été les médicaments les plus efficaces pour la majorité des isolats. Par conséquent, la surveillance de routine et de dépistage de l'ASB dans cette population est essentielle. De plus les patients dans les régions rurales des pays en développement souffrant de diabète doit être sensibilisés sur les complications de l'ASB et concernant l'entretien de leur contrôle glycémique qui est d'une importance majeure dans la prévention de l'état.**

## INTRODUCTION

Diabetes, a silent epidemic has been diagnosed in about 31.7 million people in the world which comprises 10% of the world's population, where India stands as the "Diabetic Capital" of the world (1). With the prevalence of both Type I and Type II diabetes increasing worldwide, factors associated with diabetes and its complications also become more important (2). In 2030 the estimated amount of Diabetes in India is 79.4 million (3, 4) and the prevalence among rural areas of India ranges from 2.4% to 2.7% (5, 6). Poverty is found to be a major risk factor for kidney disease (7), hypertension (8), diabetes (9), UTI (10 ),etc., Diabetes to little knowledge is just an acute metabolic threat to life, but it actually leads to complications that are life threatening and one such complications is Urinary Tract Infection (UTI) (11). Reports on the association of diabetes mellitus (DM) and urinary tract infections (UTI's) has been increasingly found (12) and UTI being the most important cause of morbidity in these patients (11). UTI's generally commence asymptomatically which leads to the symptomatic phase and may lead to serious complications if not properly managed that requires treatment with antibiotics (13). Upper urinary tract infections and the frequency of symptomatic UTI has been significantly higher in half of diabetic patients with asymptomatic bacteriuria (ASB) (14). Diabetic patients have an increased risk of certain symptomatic UTIs such as pyelonephritis, acute cystitis, emphysematous infections, *Candidal* infections, renal and perinephric abscess (15). The important clinical concerns of ASB in diabetic individuals are its contribution to morbidity, either the short-term risk of developing a symptomatic UTI and its more serious complications or the longer-term risks of developing serious diabetic complications such as nephropathy (16). Complications of ASB include emphysematous cystitis, pyelonephritis and renal papillary necrosis which occurs more commonly in subjects with type II DM (17). The rate of 30% of ASB is a risk factor for development of pyelonephritis (18) and Symptomatic UTIs tended to be more common i.e., 2.8 % higher in diabetic subjects with ASB than in those without ASB (19). The incidence of ASB in diabetic women varied from 9% - 29% and 0.7% -11% in diabetic men (20). Prevalence of ASB is usually 3 times higher in diabetic population compared to non-diabetic population. This is because of the fact that there is metabolic derangement, impaired granulocyte function, neuropathic bladder, increased adherence of bacterial organism to bladder epithelial cells and increased glucose content of urine (21). Moderate and severe glycosuria enhances bacterial growth in-vivo, thereby glycosuria may be one factor contributing to the increased prevalence of the bacteriuria in patients with DM (22) .Uropathogenic bacteria possess specific virulence factors that enhance both invasion and colonization of UTI i.e P-fimbriae of certain strains of *E.coli* (23, 24). The higher prevalence of UTI in diabetic patients does not appear to be based on the difference in virulence of the causative microorganism but due to differences in host

response (24). *E.coli* being the most common pathogen in ASB with 80% of isolates (25). Infections with other Gramnegative bacilli such as *Klebsiella*, *Pseudomonas aeruginosa* and *Proteus mirabilis*, *Enterococcus* species, coagulase-negative *Staphylococci* and fungi like *Candida spp*, are also common organisms causing UTI in men (26, 27). Antibiotic resistance of uropathogens is increasingly being reported in Diabetic patients with high occurrence of multiple drug resistant strains (28). Higher percentage of resistance to the most commonly prescribed antimicrobials such as Amoxicillin, Nitrofurantoin, Trimethoprim/Sulfamethoxazole and Ciprofloxacin are reported in isolates from diabetic patients (29). Multi-Drug Resistant (MDR) *E. coli* has also been increasingly reported in UTI (30). Keeping in view the estimated prevalence of DM worldwide, with the increasing burden of DM in the rural areas and the emergence of MDR strains escalating in the developing countries. The only way to thoroughly clarify the significance of ASB in patients with diabetes is to perform high-quality prospective studies on screening and treating ASB (31). Moreover the long-term consequences of ASB in diabetic male patients are poorly documented and there is little data on the occurrence of ASB among Below Poverty Line (BPL) diabetic males in our local setting. Hence with this perspective the present study was undertaken with the objective to determine the incidence and etiology of ASB among BPL diabetic male patients along with the resistance pattern of the bacterial isolates.

**MATERIALS & METHODS**

A prospective study conducted over a period of 1 year from March 2015 to April 2016 in the Department of Microbiology and Diabetic Outpatient departmentSri Lakshmi Narayana Medical College & Hospital Pondicherry, India. This study was approved by the Institutional Human Ethics Committee and Informed consent was obtained from all participants included in the study. Accordingly all men included in this study were diabetic, BPL card holders of age > 35 years and had not been on any antimicrobials (oral or topical) within the previous 4 weeks. Diabetic male patients with any indwelling urinary tract catheters, history of UTI symptoms dysuria, frequency and urgency, hypertension, known congenital anomalies of urinary tract were excluded from the study. A total of 1131 BPL Diabetic men who attended the diabetic clinic were enrolled in this study. 1131 Clean catch voided midstream urine samples were collected in a sterile wide mouthed culture container from all participants enrolled in this study and it was processed in the microbiology laboratory within 1hr (IDSA 2005) following collection. Microscopic examination of wet film of centrifuged urine was performed to detect the presence of pus cells, epithelial cells, erythrocytes, microorganisms, cast, etc. Urine samples were cultured using standard loop method on to 5%sheep blood agar, MacConkey agar & CLED medium and incubated at 37˚ C for 24hrs and prolonged incubation was done for 48 hrs if there is no growth after 24 hrs. The growth was interpreted as sterile if no growth after 48 hrs, significant if the number of colonies corresponded to $10^5$ Colony forming Units (CFU)/ mL, insignificant growth if colony count was less than $10^5$ CFU/ mL and Mixed growth if > 2 types of colonies were present (32) The growth was identified based on Gram staining , Motility , Catalase test, Oxidase test & other routine biochemical tests like Indole, Methyl red test, Vougesproskauer test, Citrate , Urease , Triple sugar iron agar test & Coagulase test as per Cowan and Steels Manual.9. Antimicrobial susceptibility of the isolates was studied using Kirby Bauer disc diffusion method following CLSI guidelines (2012) on Muller Hinton agar plate, the antibiotics tested were Amikacin 30 mcg, Gentamycin 10 mcg , Nitrofurantoin 300 mcg, Ceftazidime 30 mcg, Amoxyclav 30/10 mcg, Cefepime 30 mcg, Co-trimoxazole 25 mcg, Ceftazidime-clavulanicacid 30/10 mcg, Norfloxacin 10 mcg, Clindamycin 2 mcg, Vancomycin 30 mcg, Cefoxitin 30 mcg, Imipenem 10 mcg, Meropenem 10mcg , Imipenem with EDTA , Aztreonam 50 mcg,Gentamycin 120 mcg and Erythromycin 15 mcg (33).

**RESULTS**

A total of 1131 BPL diabetic male patients were screened for ASB. Among 1131 patients tested 155 (13.7%) were culture positive and reported to have ASB. Out of 1131 urine samples screened 166 (14.7%) of urine samples showed insignificant bacteriuria, 78 (6.9%) samples showed mixed growth & about 732 (64.7%) samples showed No growth. (Table : 1 ) .

**TABLE 1: RESULTS OF URINE CULTURE**

| Results of culture | No. of cases<br>n = 1131 | Percentage (%) |
|---|---|---|
| Significant bacteriuria | 155 | 13.7 |
| Insignificant bacteriuria | 166 | 14.7 |
| Mixed Growth | 78 | 6.9 |
| Sterile | 732 | 64.7 |

**TABLE 2: AGE DISTRIBUTION OF CULTURE POSITIVE CASES**

| Age(yrs) | No. of culture positive cases<br><br>n = 155 | Percentage (%) |
|---|---|---|
| 36 – 45 | 22 | 14.2 |
| 46 – 55 | 13 | 8.4 |
| 56 – 65 | 70 | 45.2 |
| 66- 75 | 44 | 28.4 |
| > 76 | 06 | 3.9 |

**(The youngest among the cases studied was 36 years old and oldest was 86 years old)**

Of the 155 cases reported to have ASB, the majority of culture positive cases were in the age group 56 - 65 years (45.2%) followed by 66 - 75 years (28.4%) and 36 – 45 years (14.2 %) while the least (3.9 %) was seen in the age group above 76 years (Table : 2). The distribution of uropathogens isolated from ASB positive cases is listed in (Table :3) .

**TABLE 3: DISTRIBUTION OF UROPATHOGENS IN ASB POSITIVE CASES**

| S.No | Urine isolates | % (Percentage) |
|---|---|---|
| 1. | Gram negative bacilli | 72.7% |
| 2. | Gram positive cocci | 22.4% |
| 3. | Yeast | 4.8% |

Gram negative bacilli was the most commonest type (72.7%) isolated, followed by Gram positive cocci (22.4%) and then yeasts (4.8%). In the present study, 10 cases showed double growth and the most prevalent organisms isolated was *Klebsiella* spp (35.2%), *Enterococcus spp* (22.4%), *followed by E. coli* (19.4 %), *Pseudomonas aeruginosa* ( 7.3%), *Enterobacter spp* (5.5%), *Proteus mirabilis* (5.5%) and *Candida spp* (4.8%) (Table: 4).

The antibiotic susceptibility pattern of gram negative bacilli revealed, majority of the isolates were sensitive to nitrofurantoin & amikacin except 6 (50%) isolates of *Pseudomonas aeruginosa* and 1 (3.1%) isolate of *E.coli* which was found resistant to nitrofurantoin (Chart :5). 87.5% of *E.coli* strains isolated were Extended spectrum β lactamase (ESBL), followed by 77.6 % of *Klebsillaspp*& 11.1% of *Enterobacter spp* isolated in this study were ESBL.

**TABLE 4: MICROBIOLOGICAL PROFILE OF UROPATHOGENS IN CULTURE POSITIVE CASES**

| Organism Isolated | No. of isolates<br><br>n = 165 | Percentage (%) |
|---|---|---|
| *E.coli* | 32 | 19.4 |
| *Klebesillaspp* | 58 | 35.2 |
| *Enterobacter spp* | 09 | 5.5 |
| *Pseudomonas aeurigonosa* | 12 | 7.3 |
| *Proteus mirabilis* | 09 | 5.5 |
| *Enterococcus* | 37 | 22.4 |
| *Candida albicans* | 08 | 4.8 |

All the 12 isolates of *Pseudomonas aeruginosa* were found resistant to ceftazidime, ceftazidime - clavulanic acid & gentamicin. *Pseudomonas aeruginosa* reported in this study were 100% ESBL and 16.6% Metallo β lactamase (MBL) producing strains (Table: 6). *Klebsiella spp*. being the most prevalent organism in this study, showed maximum resistance to ampicillin (94.8%), norfloxacin (82.7%), followed by gentamycin (79.3%), ceftazidime (77.6%) and ciprofloxacin (75.9%) (Chart :5). There was 1 (3.1%) multidrug resistant *E.coli* found in this study. Among the gram positive organisms, 3/37 (8.1%) of *Enterococcus spp*isolates were resistant to vancomycin (Table: 6) and the highest percentage of resistance was seen to ampicillin (40.5%), erythromycin (29.7%), ciprofloxacin (27.0%) followed by low level resistance to clindamycin (10.8%).

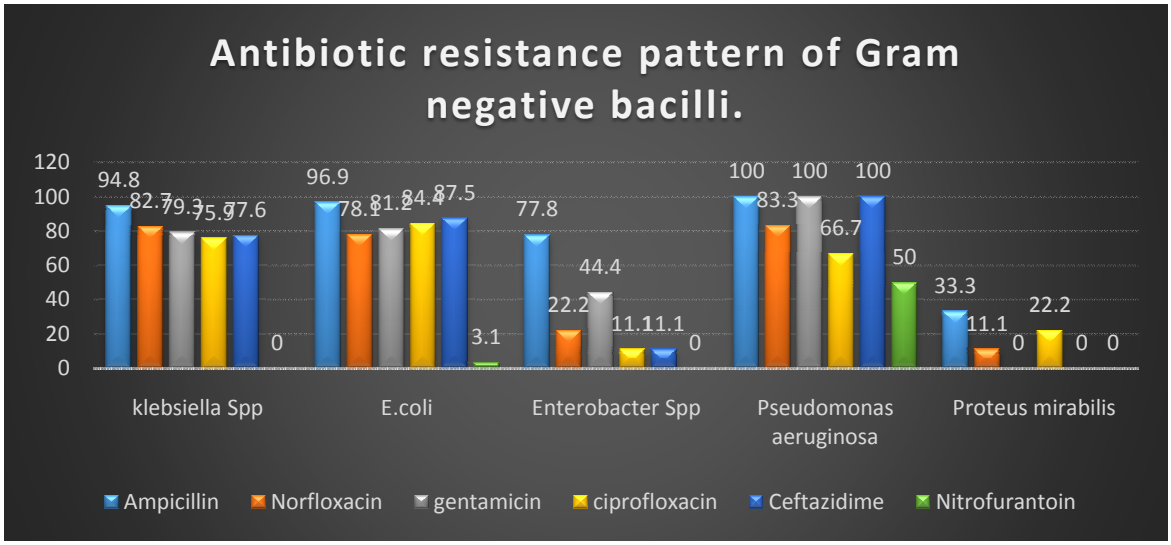


**CHART 5: ANTIBIOTIC RESISTANCE PATTERN OF GRAM NEGATIVE BACILLI.**

**TABLE 6: DRUG RESISTANCE PATTERN OF THE ISOLATED STRAINS**

| Organism Isolated | Total no. of isolates | Resistant strains (%) |
|---|---|---|
| *Enterococcus sps* | 37 | 3 VRE (8.1%) |
| *Pseudomonas aeurigonosa* | 12 | 12 ESBL (100%)<br>2 MBL (16.7%) |
| *E.coli* | 32 | 28 ESBL (87.5%)<br>1 MDR (3.1 %) |
| *Enterobacter spp* | 09 | 01 ESBL (11.1%) |
| *Klebsillaspp* | 58 | 45 ESBL (77.6%) |
| *Proteus mirabilis* | 09 | Nil |

**Foot Notes: VRE: vancomycin resistance Enterococci, ESBL: Extended spectrum β lactamases, MBL: Metallo β lactamase, MDR: Multi drug resistance.**

## DISCUSSION

The present study was undertaken to determine the incidence rate and etiology of ASB among BPL Diabetic male patients. This study demonstrated a high occurrence of ASB in BPL diabetic males with an overall incidence of (13.7%). This is in concurrence with many other studies as well (34,35, 36). In a study from North Indian Type 2 Diabetic Patients the prevalence of ASB in males was 17.3% (37). Whereas in Bharatpur the prevalence of ASB among diabetic males patients was reported to be 5.08% (38). Variations in prevalence have been attributed to factors such as geographical variations, ethnicity of the study participants and variation in the screening tests used (1, 34). Studies consistently state that Patients with DM have a higher prevalence of ASB and incidence of UTIs compared to patients without DM (39). Patients with DM had 8.7 % bacteremia and urinary tract was the commonest focus for these infections hence UTI in men should be considered complicated (40). It is well understood that lack of awareness about diabetics and its complications, their financial status and lack of time impedes their regular visit to heath care centers which contributes to the higher incidence in this population. Unfortunately, there is very poor awareness about the real dimension of the problem among the BPL male patients. Moreover the risk of end stage renal disease (ESRD) has been found to be increased in individuals with low income and in low income communities ( 41) and multiple studies have documented an association of poverty and diabetes with UTI and kidney disease (42, 43,44,45,46). Hence routine monitoring & screening for ASB in this population is essential.

*Klebsiella* spp. was the most prevalent pathogen 35.2 % in our study which correlated with earlier published reports of Alebiosu et al., were 42.4% was reported ( 47, 48). However this result was in contrast to majority of published reports where among the gram negative bacilli *Escherichia coli* was the most prevalent uropathogen isolated in diabetic patients with ASB and UTI (21,31,49,50,51). *Enterococcus and Pseudomonas* was also found to be higher in this study. The predominance of bacteria other than *E. coli* in the urinary tract is increasingly being reported (28,52).

The recent study in Nigeria has reported *Staphylococus aureus* to be the most common uropathogen in patients with DM (53) .The occurrence of ASB in BPL diabetic male was highest in the age group 56 – 65 years, but studies consistently document that the prevalence of ASB is not influenced by the age or type or duration of diabetes (20,54). Earlier studies show that symptomatic UTI occurred in 69.2 % of diabetic male patients out of 76.5% of diabetic males with ASB (20). The presence of ASB was found to be the major risk factor for developing symptomatic UTI in diabetic male patients, other risk factors include prostatic syndrome in men (20, 54). Hence further follow up studies are essential to prove or to assess the true incidence of ASB among BPL diabetic males across various age groups and their clinical progression into symptomatic UTI. Moreover there is also inadequate awareness about existing intervention for the prevention of disease in this population.

The antimicrobial sensitivity and resistance pattern differs from each community and each hospital. In our study nitrofurantoin and amikacin was the most effective drugs for the majority of isolates, except 6 isolates of *Pseudomonas aeruginosa* and 1 isolate of *E.coli*, which was found resistant to nitrofurantoin. High rates of drug resistance was found in our study with increased percentage of drug resistance shown by *Pseudomonas aeruginosa, E.coli &Klebsiella spp*. This is in accordance with other studies from developing countries (31, 47, 50, 55, 56 ). This may be due to indiscriminate use of antibiotics or previous exposure of these patients to antibiotics. Treatment of ASB is still an open issue with no clear guidelines. In U.S., the treatment of ASB is recommended, even though specific screening recommendations do not exist whereas in Europe, ASB is not treated (57). However, many of the patients with ASB can progress to symptomatic UTI (34, 58) and UTI in diabetic patients is more likely to cause complications than in non-diabetic patients (59). Hence screening and treatment of ASB in diabetics may be warranted. However more studies and Meta-analysis needs to be done before formulating guidelines and for more successful outcome it is essential to educate this population on ASB, its long term complications and importance of treatment adherence once diagnosed. Early diagnosis and prompt treatment of ASB will definitely pave way to reduce health care related expenditure and morbidity.

**Conclusion**: The incidence of ASB was found to be higher in BPL diabetic male patients. This is one of the major public health importance. Diabetic patients with ASB have a tendency to progress to symptomatic UTI and develop complications from UTI. Hence screening, monitoring and if needed treatment of these patients on a routine basis may be beneficial in such cases. Majority of the isolates in this study showed increased drug resistance. Therefore there is need to create awareness against antibiotic abuse in this population. Proper glycemic control is also of major importance in prevention of the condition. Moreover patients in rural parts of developing countries with DM has to be sensitized about the complications of ASB & UTI which can lead to dreadful consequences in terms of mortality and morbidity.

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## ORIGINAL ARTICLE

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# PREVALENCE AND ANTIBIOTICS SUSCEPTIBILITY PROFILE OF *ENTEROCOCCUS* SPP. ISOLATED FROM SOME HOSPITALS IN ABUJA, NIGERIA.

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**ABSTRACT**
**This study investigated the prevalence and antibiotics susceptibility of *Enterococcus* spp. isolated from patients and some selected hospital environment in Abuja, Nigeria. The samples included clinical and environmental. The clinical samples included stool, urine and wound swabs while the environmental samples included swabs samples taken from the health care givers hands, floor, beds, door handle, BP cuff, stethoscope, sink, toilet seats. The samples were cultured on bile aesculinazide agar and the isolates were identified with microgen test kit. The enterococcal strains isolated include *Enterococcus faecalis*, *Enterococcus faecium, Enterococcus mundtii, Enterococcus gallinarum, Enterococcus casseliflavus, Enterococcus dispar, Enterococcoushirae* and *Enterococcus avium*. The susceptibility testing was done with vancomycin, teicoplanin, gentamicin, streptomycin, linezolid, ampicillin, ciprofloxacin, chloramphenicol, doxycycline, nitrofurantoin, erythromycin and rifampin. More than 50% of the isolates were resistant to erythromycin, rifampin and doxycycline. E-test M.I.C confirmed 12 out of 34 strains to be intermediately resistant to vancomycin. *Enterococcus faecium*and *Enterococcus mundtii* exhibited more resistance than *Enterococcus faecalis*.**

**Key Word: *Enterococcus* spp., samples, Isolates, Hospitals, susceptibility, resistance, vancomycin.**

# Prévalence ET PROFIL DE SENSIBILITÉ AUX ANTIBIOTIQUES DES ENTEROCOCCUS SPP. Isolées DE CERTAINS HÔPITAUX À ABUJA, NIGERIA.

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**ABSTRACTThis étude examine la prévalence et la sensibilité aux antibiotiques des Enterococcus spp. isolées de patients et certains hôpitaux à Abuja, Nigeria. Les exemples inclus et de l'environnement clinique. Les échantillons cliniques inclus les selles, l'urine et d'écouvillons plaie tandis que les échantillons environnementaux inclus écouvillons prélevés sur des fournisseurs de soins de santé les mains, étage, lits, poignée de porte, un brassard, stéthoscope, lavabo, toilettes sièges. Les échantillons ont été mis en culture sur gélose bile aesculinazide et les isolats ont été identifiés avec microgen trousse d'essai. Les souches isolées d'entérocoques : Enterococcus faecalis, Enterococcus faecium, Enterococcus mundtii, Enterococcus gallinarum, Enterococcus casseliflavus, Enterococcus dispar, Enterococcoushirae et Enterococcus avium. La sensibilité a été fait avec la vancomycine, teicoplanine, la gentamicine, la streptomycine, le linézolide, à l'ampicilline, le chloramphénicol, la ciprofloxacine, la doxycycline, l'érythromycine, la nitrofurantoïne et la rifampicine. Plus de 50 % des isolats étaient résistants à l'érythromycine, la rifampicine et la doxycycline. E-test M.I.C confirmé 12 des 34 souches à intermédiaires résistantes à la vancomycine. Enterococcus Enterococcus mundtii feciumand ont présenté plus de résistance qu'Enterococcus faecalis.**

**Mots clés: Enterococcus spp., les échantillons, les isolats, les hôpitaux, la sensibilité, la résistance, la vancomycine.**

## INTRODUCTION

Enterococci are facultative anaerobic Gram-positive cocci that share their morphology and Lancefield antigenicity with group D streptococci. The genus *Enterococcus* includes at least 17 species, distinguished on the basis of pigment production, motility, and ability to produce acids from various carbohydrates (1). These coccoid-shaped bacteria are common in environments affected by animal and human faecal material. *Enterococcus* spp. could be spread via hand contact with open wounds containing the bacteria, or by touching contaminated environmental surfaces, where the organisms can survive for weeks. Recent years have witnessed increased interest in enterococci because of their ability to cause serious infections such as endocarditis, bacteraemia, intra-abdominal and urinary tract infection (UTI) and also because of

their increasing resistance to many antimicrobial agents (2).

Acquisition of microorganisms resistant to multiple antibiotics represents a threat to patients' safety. Enterococci easily acquire resistance when exposed to antibiotics or when they acquire genetic resistance factors from neighboring organisms (3). Therefore, VRE can spread through the population via human, environmental or animal reservoirs.The treatment problem such as prolong hospital stay by patients translates to increase healthcare bills and eventual death of the patients due to multi-resistant nature of VRE to antibiotics.

**METHODOLOGY**

Five hundred samples were collected fromKuje and Kubwa general hospitals which are secondary care hospitals; University of Abuja Teaching Hospital and National Hospital which are tertiary care hospitals. Ethical approval was obtained from the management of the hospitals. The 500 samples included 400 clinical and 100 environmental samples. The clinical samples collected included 100 stool, 240 urine, 60 wound swabs. From the 400 clinical samples, 97 strains were isolated while 5 strains were isolated from the environment. The procedure included inoculation of the stool, urine and swabs onto bile esculinazide agar, incubation for 24 hours at 37c$^{o}$, observation of the characteristic dark brown colonies is assumed presumptive of isolation of *Enterococcus* spp. The isolates were further subjected to growth at 45c$^{o}$, growth in 6.5% salt (NaCl) broth, growth on 40% bile agar, catalase test before being subjected to further confirmatory test with microgen test kit. The enterococcal strains isolated include Enterococcus faecalis, Enterococcus faecium, Enterococcus mundtii, Enterococcus gallinarum, Enterococcus casseliflavus, Enterococcus dispar, Enterococcoushirae and Enterococcus avium. Antibiotics susceptibility of the isolates were conducted using Kirby-Bauer disk diffusion method using vancomycin (30µg), teicoplanin, erythromycin (15ug), doxycycline (30ug), ampicillin (10ug), chloramphenicol (30ug), linezolid (30ug), rifampicin (5ug), (30ug), ciprofloxacin (5ug), nitrofurantoin (300ug), gentamicin (120ug) and Streptomycin (300ug).

RESULT

Table 1 shows the prevalence of the species isolated from the various hospitals. A total of 102 isolates made up of 8 *Enterococcus* spp. were isolated from the various hospitals. The various samples yielded 59(57.8%) *Enterococcusfaecalis,* 24(23.5%) *Enterococcus faecium,* 11(10.8) *Enterococcus mundtii*, 3(2.9%) *Enterococcus gallinarum*, 2(2.0%) *Enterococcus dispar*, 1(1.0%) each of *Enterococcus casseliflavus*, *Enterococcus avium* and *Enterococcus hirae*. Most of the isolates were from stool with 68, followed by urine with 24, wound and environmental swabs with 5 each. Table 2 shows the antibiotics susceptibility profile of the isolates from the various hospitals. Susceptibility of all the species to ampicillin (10µg) was 72.5%, 57.8% to ciprofloxacin(5µg), 20.6% to rifampin(5µg), 57.8% to linezolid (30µg), 66.7% to vancomycin(30µg), 25.5% to doxycycline(30µg), 65.7% to teicoplanin(30µg), 16.7% to erythromycin(15µg), 51.0% to chloramphenicol(30µg), 84.3% to nitrofurantoin(300µg), 70.6% to gentamicin(120µg), 57.8% to streptomycin(300µg).

**TABLE 1: PREVALENCE OF *ENTEROCOCCUS* SPECIES ISOLATED FROM SOME HOSPITALS IN ABUJA**

| Source | No. +ve for *Enterococcus* | E.f (%) | E.fc (%) | E.c (%) | E.g (%) | E.m (%) | E.a (%) | E.d (%) | E.h (%) |
|---|---|---|---|---|---|---|---|---|---|
| **Urine** | **24** | **22(91.7)** | **1(4.2)** | **0(0.0)** | **0(0.0)** | **0(0.0)** | **1(4.2)** | **0(0.0)** | **0(0.0)** |
| **Stool** | **68** | **29(42.6)** | **23(33.8)** | **1(1.5)** | **2(2.9)** | **11(16.2)** | **0(0.0)** | **1(1.5)** | **1(1.5)** |
| **Wound** | **5** | **5(100.0)** | **0(0.0)** | **0(0.0)** | **0(0.0)** | **0(0.0)** | **0(0.0)** | **0(0.0)** | **0(0.0)** |
| **Environmental** | **5** | **3(60.0)** | **0(0.0)** | **0(0.0)** | **1(20.0)** | **0(0.0)** | **0(0.0)** | **1(20.0)** | **0(0.0)** |
| **Total** | **102** | **59(57.8)** | **24(23.5)** | **1(1.0)** | **3(2.9)** | **11(10.8)** | **1(1.0)** | **2(2.0)** | **1(1.0)** |

**Key: +ve: positive, *E.f:E.faecalis, E.fc:E.faecium, E.c:E.casselliflavus, E.g:E.gallinarum, Em: E. mundtii, E.a:E.avium, E.d: E.dispar, E.h: E.hirae.***

Table 2 also shows that *E.faecalis* was the most susceptible of all the species while more resistance was exhibited by *E.feacium* and *E.mundtii* in this study. Table 3 confirmed 12 out of 34 strains that had resistance to vancomycin by disk diffusion method to be intermediately resistant by E-test minimum inhibitory concentration (M.I.C).

TABLE 2: SUSCEPTIBILITY PROFILE OF *ENTEROCOCCUS* SPP. ISOLATED FROM THE HOSPITALS

| Antibiotics | Sus | *E.faecalis* 59(%) | *E.faecium* 24(%) | *E.cas* 1(%) | *E.ga* 3(%) | *E.mundtii* 11(%) | *E.avium* 1(%) | *E.dispar* 2(%) | *E.hirae* (1(%) | Total 102(%) |
|---|---|---|---|---|---|---|---|---|---|---|
| AMP 10µg | R | 9(15.3%) | 10(41.7) | - | 1(33.3) | 6(54.5) | - | 1(50) | 1(100) | 28(27.5) |
| | I | - | - | - | - | - | - | - | - | - |
| | S | 50(84.7) | 14(58.3) | 1(100) | 2(66.7) | 5(45.5) | 1(100) | 1(50) | - | 74(72.5) |
| CIP 5µg | R | 10(16.9) | 9(37.5) | - | 1(33.3) | 4(36.4) | - | - | 1(100) | 25(24.5) |
| | I | 14(23.7) | 3(12.5) | - | - | - | - | 1(50) | - | 18(17.6) |
| | S | 35(59.3) | 12(50) | 1(100) | 2(66.7) | 7(63.6) | 1(100) | 1(50) | - | 59(57.8) |
| RIF 5µg | R | 37(62.7) | 18(75.0) | - | - | 11(100) | - | 1(50) | 1(100) | 68(66.7) |
| | I | 10(16.9) | 2(8.3) | - | - | - | 1(100) | - | - | 13(12.7) |
| | S | 12(20.3) | 4(16.7) | 1(100) | 3(100) | - | - | 1(50) | - | 21(20.6) |
| LIN 30µg | R | 13(22.0) | 13(54.1) | - | 1(33.3) | 9(81.8) | - | 1(50) | 1(100) | 38(37.3) |
| | I | 3(5.1) | 1(4.2) | - | - | - | 1(100) | - | - | 5(4.9) |
| | S | 43(72.9) | 10(41.7) | 1(100) | 2(66.7) | 2(18.2) | - | 1(50) | - | 59(57.8) |
| VAN 30µg | R | 10(16.9) | 12(50.0) | - | 1(33.3) | 9(81.8) | - | 1(50) | 1(100) | 34(33.3) |
| | I | - | - | - | - | - | - | - | - | - |
| | S | 49(83.1) | 12(50.0) | 1(100) | 2(66.7) | 2(18.2) | 1(100) | 1(50) | - | 68(66.7) |
| DOX 30µg | R | 34(57.6) | 16(66.7) | - | 3(100) | 9(81.8) | 1(100) | 1(50) | 1(100) | 65(63.7) |
| | I | 8(13.6) | 3(12.5) | - | - | - | - | - | - | 11(10.8) |
| | S | 17(28.8) | 5(20.8) | 1(100) | - | 2(18.2) | - | 1(50) | - | 26(25.5) |
| TEIC 30µg | R | 9(15.3) | 12(50.0) | - | 1(33.3) | 9(81.8) | - | 1(50) | 1(100) | 33(32.4) |
| | I | 2(3.4) | - | - | - | - | - | - | - | 2(1.96) |
| | S | 48(81.4) | 12(50.0) | 1(100) | 2(66.7) | 2(18.2) | 1(100) | 1(50) | - | 67(65.7) |
| ERY 15µg | R | 28(47.5) | 17(70.8) | 1(100) | 3(100) | 9(81.8) | 1(100) | 1(50) | 1(100) | 61(60.0) |
| | I | 21(35.6) | 1(4.2) | - | - | 2(18.2) | - | - | - | 24(23.5) |
| | S | 10(16.9) | 6(25.0) | - | - | - | - | 1(50) | - | 17(16.7) |
| CHL 30µg | R | 28(47.5) | 10(41.7) | - | 1(33.3) | 6(54.5) | 1(100) | - | - | 46(45.1) |
| | I | 2(3.4) | 1(4.2) | - | 1(33.3) | - | - | - | - | 4(3.9) |
| | S | 29(49.2) | 13(54.1) | 1(100) | 1(33.3) | 5(45.5) | - | 2(100) | 1(100) | 52(51.0) |
| NIT 300µg | R | 4(6.8) | 3(12.5) | - | - | 2(18.2) | - | - | - | 9(8.8) |
| | I | 3(5.1) | 3(12.5) | - | - | 1(9.1) | - | - | - | 7(6.9) |
| | S | 52(88.1) | 18(75) | 1(100) | 3(100) | 8(72.7) | 1(100) | 2(100) | 1(100) | 86(84.3) |
| GEN 120µg | R | 14(23.7) | 8(33.3) | - | - | 3(27.3) | 1(100) | 1(50) | - | 27(26.5) |
| | I | 1(1.7) | 2(8.3) | - | - | - | - | - | - | 3(2.9) |
| | S | 44(74.6) | 14(58.3) | 1(100) | 3(100) | 8(72.7) | - | 1(50) | 1(100) | 72(70.6) |
| STR 300µg | R | 24(40.7) | 12(50.0) | - | - | 4(36.4) | 1(100) | - | - | 41(40.2) |
| | I | 1(1.7) | - | - | - | - | - | - | 1(100) | 2(1.96) |
| | S | 34(57.6) | 12(50.0) | 1(100) | 3(100) | 7(63.6) | - | 2(100) | - | 59(57.8) |

**Key: E.ca: *E.casseliflavus*, E.ga: *E.gallinarum*, Sus: Susceptibility,R: Resistance, I: intermediate, S: susceptible, Cassel: *casselliflavus*, AMP: Ampicillin, CIP:Ciprofloxacin, RIF: Rifampicin,LIN: Linzolid, VAN:Vancomycin, DOX: Doxicyclin, TEC:Teicoplanin,ERY:Erythromycin,CHL:Chloramphenicol,NIT:Nitrofurantoin,GEN:Gentimicin,STR:Streptomycin.**

## DISCUSSION

Enterococci are part of human and animal intestinal flora which have emerged as community acquired pathogens and a leading cause of hospital acquired infections. In this study, we investigated the prevalence of *Enterococcus* spp. isolated from 500 samples collected from some selected tertiary and secondary care hospitals in Abuja, Nigeria. Eight different species were isolated with *E faecalis* as the majority with a percentage of 57.8 followed by *E.faecium* with percentage of 23.5, *E. mundtii* (10.8%), *E.gallinarum*(2.9%), *E.dispar*(2.0%), *E. casseliflavus*(1.0), *E.avium*(1.0%) and *E.hirae*(1.0%). This result is comparable to other work on *Enterococcus* spp. in other parts of the world where *E.faecalis* predominated followed by E.faecium while others account for less than 5% (4), (5) however Baragundi*et al*. (6), Anjana*et al*., (7) and Azza*et al*.,(8), reported more isolation of *E.faecium* in their studies. The more isolation of *E.faecium* could be responsible for the multidrug resistance reported in their studies as it has been implicated to be the

most causative agent of nosocomial infection and vancomycin resistance. This findings also confirmed the report of Cetinkaya*et al.*(9) where *E. gallinarum*, *E. casseliflavus*, *E. disparand E. avium* were isolated less frequently and account for less than 5% of clinical isolates. More isolation of *E.faecalis* (68) from stool in this study could be due to the normal floral nature of *Enterococcus* spp. in the gastrointestinal track of most organisms especially humans unlike the other samples in this study such as urine, wound that are sterile unless there is infection.

**TABLE 3: ZONE DIAMETER INTERPRETIVE STANDARDS AND EQUIVALENT MINIMUM INHIBITORY CONCENTRATION (MIC) BREAKPOINTS FOR *ENTEROCOCCUS* SPECIES**

| S/N | Isolate code | Sample | Strain | R(<14mm) | Etest Van MIC(ug/ml) <4 | 8-16 | >32 |
|---|---|---|---|---|---|---|---|
| 1 | Kw2 | Stool | *E.faecium* | 0 | 4 | - | - |
| 2 | Kw3 | Stool | *E.mundtii* | 0 | - | 8 | - |
| 3 | Kw4 | Stool | *E.mundtii* | 0 | - | 8 | - |
| 4 | Kw5 | Stool | *E.faecalis* | 0 | 2 | - | - |
| 5 | Kw6 | Stool | *E.faecium* | 0 | 2 | - | - |
| 6 | Kw10 | Stool | *E.hirae* | 0 | 4 | - | - |
| 7 | UA14 | Stool | *E.faecalis* | 0 | 1 | - | - |
| 8 | UA15 | Stool | *E.faecium* | 0 | 1 | - | - |
| 9 | UA17 | Stool | *E.faecium* | 0 | 1 | - | - |
| 10 | UA18 | Stool | *E.faecalis* | 0 | 4 | - | - |
| 11 | NH2 | Stool | *E.gallinarum* | 0 | - | 8 | - |
| 12 | NH4 | Stool | *E.mundtii* | 0 | 2 | - | - |
| 13 | NH5 | Urine | *E.faecalis* | 0 | 2 | - | - |
| 14 | NH7 | Stool | *E.mundtii* | 0 | 1 | - | |
| 15 | NH8 | Stool | *E.faecium* | 0 | - | 8 | - |
| 16 | NH9 | Stool | *E.mundtii* | 0 | - | 8 | - |
| 17 | NH10 | Stool | *E.faecium* | 0 | 2 | - | - |
| 18 | NH11 | Stool | *E.faecium* | 0 | 2 | - | - |
| 19 | NH12 | Stool | *E.faecium* | 0 | 4 | - | - |
| 20 | NH17 | Stool | *E.faecalis* | 0 | - | 8 | - |
| 21 | NH18 | Stool | *E.faecium* | 0 | 4 | 8 | - |
| 22 | NH19 | Urine | *E.faecalis* | 0 | 2 | - | |
| 23 | NH20 | Urine | *E.faecalis* | 0 | - | 8 | - |
| 24 | NH21 | Urine | *E.faecalis* | 0 | 4 | - | - |
| 25 | NH24 | Stool | *E.faecium* | 0 | 1 | - | - |
| 26 | NH25 | stool | *E.mundtii* | 0 | - | 8 | - |
| 27 | NH26 | stool | *E.faecium* | 0 | 4 | - | - |
| 28 | NH27 | stool | *E.faecalis* | 0 | - | 8 | - |
| 29 | NH31 | stool | *E.mundtii* | 0 | 1 | - | - |
| 30 | NH32 | stool | *E.mundtii* | 0 | 4 | - | - |
| 31 | NH33 | stool | *E.faecium* | 0 | - | 8 | - |
| 32 | NH34 | stool | *E.mundtii* | 0 | 4 | - | - |
| 33 | NH35 | stool | *E.dispar* | 0 | - | 8 | - |
| 34 | NH36 | urine | *E.faecalis* | 0 | 2 | - | - |

The susceptibility profile of the isolates shows above average susceptibility of the strains to commonly used recommended antibiotics by CLSI, 2014 (10). Out of the 12 antibiotics tested, 9 showed good activity against the strains except for rifampin, doxycycline and erythromycin that had more than 50% of the isolates resistant to them. The resistance to this 3 antibiotics could be associated to their abuse since they are over the counter medication and accessible to patients without doctor's prescription due to proliferation of patent medicine stores and pharmacies. Also, consumption of poultry or animal product reared with this antibiotics as growth supplement could have contributed to the resistance as the susceptibility profile is comparable to the work of Schwaiger*et al.*,(11) where *Enterococcus* spp. isolated from hens showed high resistance to rifampicin, erythromycin, fosfomycin and doxycycline. Good susceptibility to glycopeptides, ampicillin and high level aminoglycosides in this research gives reassurance for synergistic treatment of vancomycin resistant enterococcal infections such as endocarditis, urinary tract infections and bacteriamia. The above average activity of high level aminoglycoside (120ug gentimicin and 300ug of streptomycin) in this study is encouraging as ampicillin, penicillin, or vancomycin (for susceptible strains) can be combined, plus an aminoglycoside to work synergistically for the treatment of serious enterococcal infections, such as endocarditis, unless high-level resistance to both gentamicin and streptomycin is documented (10). The susceptibility profile of the isolates in this study showed *E.mundtii* and *E.faecium* to be more resistant than *E.faecalis*.

In this research, 33.3% of the enterococcal isolates were resistant to vancomycin by Kirby Bauer disk diffusion method. Most of the VRE isolates were isolated from National Hospital Abuja. Previous studies accounted for 100% susceptibility of *Enterococcus faecalis* to vancomycin(12) however most of our resistant strains were *E.mundtii* with 81.8% resistance and *E.faecium* with 50.0% resistance unlike *E.faecalis* that showed a lower percentage (16.9%) of resistance. It has been reported that

*E.faecium* is responsible for most vancomycin resistant enterococci (VRE) infections (1). The higher resistance of *E.mundtii* in this study could be because of its close relatedness to *E.faecium* by phylogeny (13). Minimum inhibitory concentration of the 34 resistant enterococcal strains confirmed 12(11.8%) strains to have intermediate susceptibility of≤ 8 µg/ml by E-test strips (oxoid) method using CLSI, 2014 antibiotics susceptibility interpretive guideline. E-test MIC confirmed 4(36.4%) *E.mundtii*, 3(12.5%)*E.faecium* and 3(5.1%)*E.faecalis* which are the most frequently isolated to have intermediate susceptibility of 8µg/ml each.Non was extremely resistant with MIC of ≥32 µg/ml. The possibility of acquisition of resistant genes and exposure to different antibiotics could have caused the emergence of low or intermediate enterococcal resistance to vancomycin in this study. Enterococci acquire drug resistance through plasmids, conjugative transposition or by mutations which leads to the rapid spread of multidrug resistant enterococcal infections (7). In Nigeria, VRE may soon become a great threat since 33.3% of the 102 isolates exhibited resistance to vancomycinby by disk diffusion method even though only 4 were phenotypically confirmed by minimum inhibitory concentration. Adequate measures aimed at curtailing its spread needs to be implemented.

## CONCLUSION

The result showed *E.faecalis* as the major isolates among the *Enterococcus* spp. isolated with stool urine, wound and environmental swabs as the major sources. Most of the isolates showed greater than 50% susceptibility to the antibiotics tested except for erythromycin, doxycycline and rifampicin with < 50% susceptibility.

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## ORIGINAL ARTICLE

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# ANTIMICROBIAL ACTIVITY OF MORINGA ON EAR, NOSE AND THROAT ASSOCIATED FUNGI, AND VANCOMYCIN RESISTANT COCCI ISOLATED AT AMINU KANO TEACHING HOSPITAL, KANO, NIGERIA

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**ABSTRACT**

**This study was aimed at evaluating the antimicrobial activity of Moringa on ear, nose and throat associated fungi and vancomycin resistant cocci. The plant material was extracted with methanol and petroleum ethe and screened for phytochemical contents. The microbial isolates were obtained from females and males patients (both adults and children) attending ear, nose and throat clinic at Aminu Kano Teaching Hospital. Coccal bacteria and fungi were isolated accordingly. The cocci were screened for vancomycin resistance. The antimicrobial assay was carried out using gradient double (12.5-100mg/mL) assay. The MIC, MBC/MFC and Brine shrimp toxicity test were also conducted. *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Candida albicans* and *Aspergillus fumigatus* were isolated. Up to 21.4% of *S. aureus* were vancomycin resistant, 20% of *S. pneumoniae* isolated were vancomycin resistant and 16.7% *S. pyogenes* were vancomycin resistant. The plant extracts showed zones of inhibition of 08mm-20mm at concentrations ranging from 12.5- 100mg/mL. The most susceptible organism to both extracts was *C. albicans* and the least susceptible was *S. aureus*. The MIC of the methanol extracts ranged from 0.78 to 50mg/mL but MBC/MFC ranged from 6.25 to 200mg/mL. The MIC of the petroleum ether was at 50 to 200mg/mL and the MBC/MFC was from 200 to 800mg/mL. The brine shrimp lethality assay showed $LC_{50}$ value of 93.48µg/mL for Moringa methanol extract while the $LC_{50}$ value for Moringa petroleum ether extract was 3.691µg/mL. Moringa methanol extract (100mg/mL), showed appreciable activity against the fungal isolates and vancomycin resistant cocci associated with Ear, Nose and Throat symptoms while Moringa petroleum ether extract showed activity only on the fungal isolate *C. albicans*. The study demonstrated that Moringa methanol extracts was more active than Moringa petroleum ether extracts. The search for novel cytotoxic ingredient in Moringa should be encouraged.**

**Keywords: Antimicrobial, Moringa, Ear, Nose, Throat,Fungi,Vancomycin, Resistant, Cocci**

# L'ACTIVITÉ ANTIMICROBIENNE DE MORINGA SUR L'OREILLE, NEZ ET GORGE CHAMPIGNONS ASSOCIÉS, ET D'ENTÉROCOQUES RÉSISTANTS À LA COCCI ISOLÉS À L'HÔPITAL, AMINU KANO Kano, Nigéria

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**RÉSUMÉ**

**Cette étude avait pour but d'évaluer l'activité antimicrobienne de Moringa sur l'oreille, nez et gorge champignons associés et cocci résistants à la vancomycine. L'usine a été extraite avec du méthanol et du pétrole ela et tamisée pour contenu phytochimique. Les isolats microbiens ont été obtenus à partir de les hommes et les patients (adultes et enfants) fréquentant l'oreille, nez et gorge clinique à l'Hôpital d'enseignement Aminu Kano. Coccal les bactéries et champignons ont été isolés en conséquence. Les coques ont été examinés pour la résistance à la vancomycine. L'antimicrobien a été réalisée à l'aide de double gradient 12.5-100(mg/mL). Le MIC, MBC/MFC et l'essai de toxicité d'artémia ont également eu lieu. Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Candida albicans et Aspergillus fumigatus étaient isolés. Jusqu'à 21,4 % des S. aureus étaient résistants à la vancomycine, 20 % de S. pneumoniae isolées étaient résistantes à la vancomycine et 16,7 % étaient S. pyogenes résistantes à la vancomycine. Les extraits de plantes ont montré des zones d'inhibition de 08mm-20mm à des concentrations variant de 12,5- 100mg/mL. Les plus sensibles à l'organisme les deux extraits a été C. albicans et le moins sensible était S. aureus. Le MIC de l'extrait au méthanol variait de 0,78 à 50 mg/mL mais MBC/MFC variait de 6,25 à 200mg/mL. Le MIC de l'éther de pétrole était à 50 à 200 mg/mL et le CBM/MFC a été de 200 à 800 mg/mL. La létalité d'artémia ont montré la valeur de LC50 93.48µg/mL pour le Moringa extrait méthanolique tandis que la CL50 pour le Moringa L'éther de pétrole extrait a été**

**3.691µg/mL. Le moringa extrait au méthanol (100mg/mL), a montré une activité appréciable contre les isolats fongiques et d'entérocoques résistants à la cocci associés à l'oreille, nez et gorge symptômes, tandis que l'éther de pétrole extrait de Moringa a montré que l'activité sur l'isolat fongique C. albicans. L'étude a démontré que les extraits de Moringa le méthanol a été plus active que l'éther de pétrole extrait de Moringa. La recherche de nouveaux ingrédients dans le Moringa cytotoxiques doivent être encouragés.**

## INTRODUCTION

Medicinal plants have been used from ancient time for their medicinal values as well as to impact flavor to food. Nowadays, the crude extracts and dry powder samples from medicinal and aromatic plants species are used for the development and preparation of alternative medicine and food additives (1) . ***Moringa***, native to parts of Africa and Asia, is the sole genus in the flowering plant family Moringaceae. Important medicinal properties of the plant include antipyretic, antiepileptic, antiinflammatory, antiulcerative (2) , antihypertensive (3) cholesterol lowering (4) antioxidant (5) anti diabetic, hepatoprotective (6) , (antibacterial and antifungal activities (5). Vancomycin is an antibiotic used to treat a number of bacterial infections (7). It is recommended intravenously as a first-line treatment for complicated skin infections, bloodstream infections, endocarditis, bone and joint infections, and meningitis caused by methicillin-resistant *Staphylococcus aureus* (8). In addition to natural circumstances, misuse of vancomycin has led to vancomycin resistance. The reasons for clinical failure of vancomycin are many and have been hypothesized to include poor penetration of the drug to certain tissues (9, 10).Wide varieties of Ear, Nose and Throat diseases are usually presented to the Otorhinologist (head and neck surgeons) (11). The pattern of these diseases may vary from community to community or hospital to hospital based on the availability of specialist personnel or facilities for the management of such diseases which are either congenital or acquired in origin. Ear, nose and throat diseases are serious public health problems with universal distribution affecting all age groups (12).

One of the research problems facing chemotherapy today is that microorganisms are now gaining resistance to vancomycin, which has been considered to be the reference standard for the treatment of bacterial infection. In Nigeria today, ear, nose and throat- related conditions constitute a major burden of infections. Yet the majority of the citizens are ignorant of ENT treatment options. Disease of the ear, nose and throat (ENT) affect the functioning of adults as well as children, often with significant impairment of the daily life of affected patients (13). Due to the emergence of vancomycin resistance which is the last resort antibiotic where other antibiotics have failed and ignorance of the severity of ear, nose and throat infections, there is need for an easy, effective and affordable means to cure infections of the ear, nose and throat (ENT). ***Moringa*** is known for its numerous medicinal properties one of which is its antimicrobial activity (14). It is very common worldwide to find people consuming this plant in combination or alone as remedies against symptoms believed to be associated with the selected microorganisms targeted in this work. There is a need to find out if this plant has potent antimicrobial activity against ENT fungi and vancomycin resistant cocci. This plant is easy to afford. Moringa can be included in our foods and drinks e.g. tea and soups (15).

## MATERIAL AND METHODS

### *Collection and Identification of Plant Materials*

The Moringa leaves were collected from Naibawa in Kano state. It was identified and compared to voucher specimen with voucher number (Moringa BUKHAN 0011) at the department of Plant Biology Bayero University, Kano Herbarium with the assistance of Baha'uddeen Said Adam(16).

### Processing and Extraction of Plant Materials

The ***Moringa*** leaves were thoroughly washed with distilled water and air dried in a shady environment for two weeks and made into powdered form using a clean pestle and mortar, then it was sieved through a mesh to obtain fine powder of approximately 20µm particle size. The powder was stored at room temperature in sealed container until required for use as demonstrated(17).[ Accordingly, one hundred grams (100g) of the powdered plant material was extracted separately with methanol and petroleum ether using soxhlet apparatus as demonstrated (18).

### Confirmation of the Bioactive Components of the Plants

Phytochemical screening was carried out to confirm the bioactive components of the plant as follows:

### Test for Alkaloids

This was carried out qualitatively as demonstrated (19). Using a pipette, 1.0 ml of the extracts was placed in two separate test tubes. Using a dropper, three drops of Meyer's reagent was added separately. A white precipitate with Meyer's reagent indicated the presence of alkaloids.

### Test for Saponins

This was carried out as demonstrated by the method reported (20). 0.5g each of the extracts was placed in

a test-tube, 5.0ml of sterile distilled water was added to the extract in the test-tubes and shaken vigorously. A froth that persisted for 15 minutes was an indication of the presence of saponins.

**Test for Steroids**
This was carried out as demonstrated by the method (19). 2g of each the extracts was placed in a test tube and evaporated to dryness. The extract was then dissolved in acetic anhydride followed by the addition of chloroform and then concentrated sulphuric acid was added by the side of the test tube. Appearance of a brown ring at the interface of the two liquids and the appearance of violet colour in the supernatant layer indicated the presence of steroids in the extract.

**Test for Reducing Sugar**
This was carried out as demonstrated by the method (20). Here, 1g each of the extracts was weighed and introduced into separate test tubes. The extracts were diluted with 2.0ml each of dimethyl sulphoxide (DMSO) and sterile distilled water respectively. Fehling's solution was added to the solution obtained, and then the mixture was warmed. A brick-red precipitate at the bottom of the test tubes indicated the presence of reducing sugar.

**Test for Tannins**
2ml of each of the plant was diluted with distilled water in separate test tubes and 2-3 drops of 5% ferric chloride ($fecl_3$) were added. A green-black or blue-black colouration indicated the presence of tannin as demonstrated (19).

**Test for Flavonoids**
This was carried out as demonstrated (21). A 4mg weight of the extracts and a piece of magnesium ribbon were added together followed by concentrated HCL drop-wise. A colour change from crimson to magenta indicated the presence of flavonoids in the extracts.

**Test for Terpenoids**
0.5ml of the extracts was added to 2ml of chloroform, 3ml of concentrated $H_2S0_4$ was added to form a layer. A reddish brown colouration at the interface indicates the presence of terpenoids as demonstrated by the method (21) .

**Test for Anthraquinone**
0.5ml of the extract was taken into a dry test-tube and 5ml of chloroform was added and shaken for 5mins. The extract was filtered and drops of ammonia solution were added. A pink violet or red colour in the ammonical layer (lower layer) indicates positive results. This is as demonstrated by the method (21).

**Tests for Phenol**
Few drops (0.5%) of dilute ferric chloride solution was added 0.5 ml of each of the extracts, the formation of a dark green colour shows the presence of phenol according to the method (22).

***Collection and Identification of Test Isolates***
The isolation was carried out in Aminu Kano Teaching Hospital after ethical clearance has been approved. The isolation was carried out under the supervision of a medical laboratory technician. Thirty three specimens were collected from patients attending ENT clinic in any age group. The organisms were isolated from the ear, nose and throat swabs. The specimen was cultured on Sabouraud Dextrose Agar (Manufacturing date, 2016; Expiring date, 2018) for the isolation of fungi. After 3-7days of incubation the fungi isolates were identified macroscopically and microscopically with the help of scheme (23).

The specimens were cultured on Chocolate agar for the isolation of cocci bacteria. The cocci were identified macroscopically in the culture plates after 24 hours of incubation, after which gram staining was carried out. This was followed by catalase and coagulase test to confirm the species of Staphylococci. Optochin, bacitracin disc and bile solubility test were used to further confirm the species of streptococci. The identified cocci were subjected to vancomycin sensitivity disc (30µg) and the cocci that were found to be resistant to the vancomycin were used for this study as demonstrated (24).

***BIOASSAY***

**Preparation of Extracts Concentrations**
This was carried out according to the method described by the method (25). Stock solution of moringa, methanol and petroleum ether crude extracts were prepared by dissolving 0.6g of each of the plant extracts in 6mL of dimetyhylsulphoxide (DMSO) in glass vial bottles. Therefore, each stock solution had concentration of 100000µg/mL (100mg/mL). The stock solution was double-diluted to get three varied extracts concentrations in addition to it to make them four different concentrations of 100mg/mL, 50mg/mL, 25mg/mL and 12.5mg/mL (26).

**Standardization of Inoculum**
The isolates were adjusted to 0.5 McFarland standard ($1.5 \times 10^8$ CFU/mL) turbidity for bacteria isolates and $1 \times 10^6$spores/mL for the fungi isolates by adding sterile normal saline. McFarland standards were used as a reference to adjust the turbidity of microbial suspension so that the number of microorganisms

will be within a given range. For the preparation of the 0.5 McFarland standard, 0.05mL of barium chloride ($BaCl_2$) (1.17% w/v $BaCl_2.2H_2O$) was added to 9.95mL of 0.18M $H_2SO_4$ (1.0% w/v) with constant stirring. To aid comparison the standard was compared against a white background with a contrasting black line (27).

**Preparation of Antibiotic Dilution**

The antibiotic ciprofloxacin and fluconazole were purchased from Lamco pharmacy Kano State, Nigeria and was reconstituted by dissolving 3g of the ciprofloxacin and fluconazole powder in 100ml of distilled water so as to get a concentration of 30mg/mL. The prepared dilution of the antibiotics was used for subsequent antimicrobial test as positive control (21).[21]

**Antimicrobial Assay**

The bioassay was carried out using the agar well diffusion method described by cheesbrough (2006). 0.1mL of the standardized inoculums ($1.5 \times 10^8$ CFU/ml) of *Staphylococcus aureus* was inoculated onto sterile prepared Mueller Hinton Agar and was spread with a sterile swab while *Streptococcus pneumoniae* was inoculated on chocolate agar and *Streptococcus pyogenes* was inoculated on sterile blood agar plates.

*Aspergillus fumigatus* and *Candida albicans* were inoculated on sterile Sabouraud dextrose agar plates. Six wells were made with a 6mm sterile cork borer into the agar plates containing the bacterial and fungal inoculums and 0.lmL of the four different concentrations from the stock solution of the extracts at concentrations (100, 50, 25, and 12.5mg/mL) were introduced into their respective wells. 0.1mL of DMSO was introduced into the fifth well to serve as negative control while 0.1mL of 30mg/mL of ciprofloxacin was introduced into the sixth well to serve as a positive control for the bacterial isolates and fluconazole was used for the fungal isolates. The inoculated plates were left to stand for about 30 minutes to allow diffusion of extract before incubating at 37◦C for 24 hours for the bacterial isolates and the fungal isolates were incubated at 37°C for 3 days. The zones of clearance produced around the wells after incubation was observed and measured using a vernier caliper and recorded (in mm). Each of the experiment was conducted thrice and mean results were taken for the test organisms (28).

**Determination of Minimum Inhibitory Concentration, Minimum Bactericidal and Fungicidal Concentration**

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that inhibited visible growth of microorganisms after overnight incubation (Andrews, 2002). The doubling macro dilution broth method was used to determine the MIC. Two (2) mL of the reconstituted crude extract at a concentration of 100000µg/ml was added to 2mL sterile Mueller Hinton broth for the bacterial isolates, 2mL of the reconstituted crude extract was added to 2mL of Sabouraud dextrose broth for the fungal isolates. Two (2) mL of this extract concentration was transferred to another test-tube and this dilution continued until the 12th test-tube was reached, giving extract concentrations ranging from 800-0.39mg/mL in different test tubes. 0.1mL of an 18h culture of bacteria and 3 days culture of fungi previously adjusted to 0.5 MacFarland standard was inoculated into each of the test tubes and the contents were thoroughly mixed. A test tube containing the broth and extract was used as positive control while a test tube containing the broth and bacteria/fungal inoculum was used as negative control. The inoculated culture tubes were incubated at 37°C and were observed for growth after 24 hours for the bacterial isolates and 3days for the fungal isolates. The lowest concentration of extract showing no visible growth when compared with the control was considered as the MIC as demonstrated by the method (27).

The minimum bactericidal/fungicidal concentration is the lowest concentration of antimicrobial agent that prevented the growth of an organism. 0.1mL aliquot from the tubes that showed no visible bacterial/fungal growth from the determination of minimum inhibitory concentration was inoculated on a sterile Mueller Hinton Agar for 24 hours at 37◦C for the bacterial isolate while the fungal isolates were inoculated on sterile Sabouraud dextrose agar at 37°C. The lowest concentration in which no growth occurred was taken as the minimum bactericidal concentration (MBC/MFC) as demonstrated (27).

***Assay for $LC_{50}$ of the Plant Extracts by Brine Shrimp Lethality Test***

The eggs were hatched in a hatching chamber containing ocean sea salt water (75ml). Natural light was allowed to penetrate into the hatching chamber for 48 hours so that the eggs will hatch into the shrimp larvae. Twenty milligram (20mg) of the extracts were separately dissolved in 2ml of methanol and equally a positive control of which 20mg of the extracts was dissolved in 2ml of distilled water. 500, 50 and 5ml of the solution equivalent to 1000, 100 and 10 µg/mL respectively was transferred into vials. A negative control which is simply the solvent (methanol) without the test extracts was also prepared. Each concentration were tested in triplicate,

therefore each extracts had 9 test tubes. The methanol in the test tubes containing the extracts were allowed activity against Brine shrimp larvae (*Artemia salina*). To each test sample vial, sea water was added and a drop of DMSO solvent was added in order to facilitate the solubility of each test samples in the sea water. Ten (10) shrimps were transferred using a Pasteur pipette and natural sea water was added to make a total volume of 5ml. After 24 hours, the number of surviving shrimps at each concentration was counted and recorded. Lc50 values were determined at 95% confidence intervals by analyzing the data on a computer loaded with a "Finney Programme." The Lc50 values of the brine shrimps obtained for extracts of the plants studied were recorded (29).

## *RESULTS AND DISCUSSION*

### Phytochemical *Moringa* Plant Extracts

Some Phytochemical components of ***Moringa*** plant extracts are presented in Table 1. The data showed that, phenol, steroids, reducing sugar, flavonoid, terpenoids, tannins anthraquinone were present in both Moringa methanol and petroleum ether extracts. Alkaloid and saponin were present in Moringa methanol but absent in moringa petroleum ether extracts.

### Inhibitory Activity of the *Moringa* Extracts on ENT Associated Fungi and Vancomycin-Resistant Cocci

The inhibitory activity of Moringa on the test organisms is presented on Table 2. Moringa methanol extract showed zones of inhibition ranging from 8 – 20mm at concentrations ranging from 12.5 – 100mg/mL on the test organisms. However Moringa petroleum ether extract was not active on all the organisms except *Candida albicans* and at a concentration of 100mg/mL with a 09mm zone of inhibition.

### MIC and MBC/MFC of the *Moringa* Extracts on the Test Organisms

The MIC and MBC/MFC of the methanol and petroleum ether extracts on the test organisms is presented in Table 3. From the data presented, the MIC for the test organism ranged from 0.78 to 50mg/mL while the MBC/MFC ranged from 6.25 to 200mg/mL. The MIC of the petroleum ether extracts ranged from 50 to 200mg/mL while the MBC/MFC ranged from 200 to 800mg/mL.

to evaporate to dryness for about 48 hours at room temperature and were subjected to test for their

### Assay for the $LC_{50}$ of Moringa extracts by Brine Shrimp Lethality Test

Brine shrimp lethality toxicity assay of the plant extracts is presented in table 4. The brine shrimp results in this study are interpreted as follows: $LC_{50}$ <1.0 µg/mL – highly toxic; $LC_{50}$ 1.0-10.0 µg/mL – toxic; $LC_{50}$ 10.0-30.0 µg/mL – moderately toxic; $LC_{50}$ >30 <100µg/mL – mildly toxic, and > 100µg/ml as non-toxic (Moshi *et al*., 2010). From the data presented in table 4, the $LC_{50}$ for MME is 93.48µg/mL while the $LC_{50}$ MPE is 3.691µg/mL, From this result, MPE is toxic while MME is mildly toxic.

In this study Moringa methanol was found to be more active than moringa petroleum ether extract. Moringa methanol inhibited the growth of all the organisms it was tested on. Moringa petroleum ether extract on the other hand inhibited only the growth of *Candida albicans* at a concentration of 100mg/mL which gave a 09mm zone of inhibition. However when the concentration was increased from 200-800mg/mL it was found to inhibit the growth of the organisms and it also had bactericidal and fungicidal effect. A similar study was conducted and moringa leaf petroleum ether extract was found to be active at this same concentration as reported (26). It was also revealed in this study that moringa methanol extract possessed all the phytochemicals it was screened for. Alkaloids and saponins were absent in moringa petroleum ether extracts, this could be the reason for its poor activity. However, a similar research carried out (30), reported the presence of Alkaloid and Saponin but in low amount. Alkaloids are naturally occurring chemical compounds containing basic nitrogen atoms. They often have pharmacological effects and are used as medications and recreational drugs (31). Saponins cause hemolysis of red blood cells (32).

**TABLE 1: PHYTOCHEMICAL COMPONENTS OF MORINGA IDENTIFIED**

| Test | MME | MPE |
|---|---|---|
| **Phenols** | + | + |
| **Alkaloids** | + | - |
| **Saponins** | + | - |
| **Steroids** | + | + |
| **Reducing Sugar** | + | + |
| **Flavonoids** | + | + |
| **Tannins** | + | + |
| **Terpenoids** | + | + |
| **Anthraquinone** | + | + |

**KEY:, MME= *Moringa* Methanolic Extract, MPE= *Moringa* Petroleum Ether Extract; + = Presence of Secondary Metabolite, - = Absence of Secondary Metabolite**

**TABLE 2: INHIBITORY ACTIVITY OF MORINGA (*MORINGA OLEIFERA*) EXTRACTS ON ENT FUNGI AND VANCOMYCIN RESISTANT COCCI**

| Organisms | Zones of inhibition (mm) MME | | | | MPE | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 100 | 50 | 25 | 12.5 | 100 | 50 | 25 | 12.5 | DMSO | Cip/Flu |
| *S. aureus* | 10 | 08 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 30 |
| *S. pyogenes* | 14 | 11 | 08 | 08 | 00 | 00 | 00 | 00 | 00 | 25 |
| *S. pneumonia* | 10 | 08 | 08 | 00 | 00 | 00 | 00 | 00 | 00 | 28 |
| *A. fumigates* | 19 | 12 | 12 | 10 | 00 | 00 | 00 | 00 | 00 | 00 |
| *C. albicans* | 20 | 16 | 10 | 00 | 09 | 00 | 00 | 00 | 00 | 10 |

**KEY: DMSO= Dimethyl sulfoxide, Cip= Ciprofloxacin, Flu= Fluconazole, MME = Moringa methanol extract, MPE = Moringa petroleum ether extra**

**TABLE 3: MIC AND MBC/MFC OF THE METHANOL AND PETROLEUM ETHER EXTRACTS ON ENT FUNGI AND VANCOMYCIN RESISTANT COCCI**

| Organisms | MME MIC | MBC/MFC | MPE MIC | MBC/MFC |
|---|---|---|---|---|
| *S. aureus* | 50 | 100 | 200 | 800 |
| *S. pyogenes* | 50 | 200 | 100 | 200 |
| *S. pneumoniae* | 6.25 | 50 | 100 | 400 |
| *A. fumigatus* | 6.25 | 25 | 100 | 200 |
| *C. albicans* | 0.78 | 6.25 | 50 | 200 |

**KEY: MIC= Minimum Inhibitory Concentration, MBC/MFC = Minimum Bactericidal Concentration/Minimum Fungicidal Concentration**

**TABLE 4: BRINE SHRIMP LETHALITY TOXICITY TEST**

| Extracts | Concentrations (µg/mL) | Total Survival | % Mortality | $LC_{50}$ |
|---|---|---|---|---|
| MME | 1000 | 11 | 26.7 | 93.48 |
| | 100 | 22 | 6.7 | |
| | 10 | 28 | 53.3 | |
| MPE | 1000 | 14 | 46.7 | 3.691 |
| | 100 | 16 | 26.7 | |
| | 10 | 22 | 26.7 | |

**KEY: MME = Moringa methanol extract, MPE = Moringa petroleum ether extra**

## RECOMMENDATIONS

1. The study demonstrated that Moringa extracts was active, therefore, the search for the novel cytotoxic ingredients in Moringa should be encouraged.
2. Individual compounds of the plants should be isolated, purified, characterized and tested.
3. Other solvents and extraction method should be used for the extraction of the plant material to see if the performance of the extracts will be better.
4. The plant extracts should be evaluated *in vitro* to ascertain their activity on ear, nose and throat fungi and vancomycin resistant cocci and also to evaluate their effect on vital organs of the body.
5. Combination of Moringa with plants with lower toxicity should be encouraged.

## *CONCLUSION*

The present study deduced that, Moringa methanol extract was more active than Moringa petroleum ether extracts, although Moringa petroleum ether extract was experimentally more active on *C. albicans* than on vancomycin resistant coccal bacteria. *Aspergillus fumigatus* was the most predominant fungus while *Staphylococcus aureus* was the most predominant cocci associated with ENT at the time of this study. Moringa methanol extract was more active on *S. aureus*, *S. pyogenes, S. pneumoniae, Aspergillus fumigatus* and *C. albicans*.

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# ISOLATION AND SCREENING OF FUNGAL ISOLATES FROM BAMBARA (*VIGNA SUBTERRANEA*) NUTS FOR TANNASE PRODUCTION

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**ABSTRACT**

Tannase (Tannin acyl hydrolase, EC 3.1.1.20) is an enzyme produced in the presence of tannic acid by various filamentous fungi. They are produced principally by fungi of the genus *Aspergillus* and *Penicillium*. The enzyme is used in the food and beverage industry as a clarifying agent for wines, beers and fruit juices. In Africa, billions of dollars are expended yearly on the importation of commercial enzymes for the food and pharmaceutical industries and this increases the cost of production and the finished goods. This study was carried out to isolate tannase producing fungal species using Bambara nuts as a substrate in a bid to finding alternatives to the importation of tannase. Fresh Bambara nuts were collected from different locations in Nigeria. They were cleaned, sorted and intermittently moistened with water to encourage fungal growth for fourteen days. The different fungi obtained after fourteen days were inoculated onto Potato Dextrose Agar plates and incubated at 25°C for five days. Subculturing of fungal isolates was carried out to obtain pure cultures of isolates. Tannilytic activity (hydrolysis of tannin) of isolates was assessed by inoculating them in media containing tannin. The plates were incubated at 25°C for 2-5 days after which the plates were observed and zones of hydrolysis measured. A total of eighteen isolates were obtained. They were all members of the *Aspergillus* genus. 56% (10) of the isolates were able to degrade tannin acid with mean zone of hydrolysis of 39mm ±23.7 mm (Range 10-70mm). This study established members of the *Aspergillus* genus isolated from Bambara nuts as viable fungi for application in the production of tannase. This study adds to existing reports on fungal production of tannase.

**Keywords: Tannase; Fungal isolates; *Aspergillus*; Bambara nuts; *Vigna subterranea***

# L'ISOLEMENT ET LA PRÉSÉLECTION DES ISOLATS FONGIQUES DE BAMBARA (VIGNA SUBTERRANEA) POUR PRODUCTION DE CHAMPIGNONS DE TANNASE.

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**ABSTRACT**
**Tannase acyle hydrolase, Tanin (EC 3.1.1.20) est une enzyme produite en présence d'tannicacid par divers champignons filamenteux. Ils sont produits principalement par des champignons de l'genusAspergillus et Penicillium. L'enzyme est utilisé dans l'industrie des aliments et boissons comme aclarifying agent pour vins, bières et jus de fruits. En Afrique, des milliards de dollars sont expendedyearly sur l'importation d'enzymes commerciales pour l'industrie alimentaire et pharmaceutique andthis augmente les coûts de production et les produits finis. Cette étude a été réalisée à l'aide d'espèces de champignons produisant des isolatetannase écrous Bambara comme substrat dans le but d'findingalternatives à l'importation de tannase. Les écrous Bambara frais ont été prélevés dans differentlocations au Nigéria. Ils ont été nettoyés, triés par intermittence et humidifié avec de l'eau croissance fongique toencourage pendant 14 jours. Les différents champignons obtenus après 14 jours de wereinoculated sur Potato Dextrose Agar plaques et incubées à 25°C pendant 5 jours. Sous-offungal isolats a été réalisée pour obtenir des cultures pures d'isolats. Activité Tannilytic hydrolysisof (tanin) des isolats a été évaluée en inoculant dans des milieux contenant du tanin. Le plateswere incubé à 25°C pendant 2-5 jours après laquelle les plaques ont été observées et les zones mesurées. ofhydrolysis Un total de 18 isolats ont été obtenus. Ils étaient tous membres d'theAspergillus genre. 56 % (10) des isolats ont été capables de dégrader l'acide tannique zone ofhydrolysis avec une moyenne de 39mm ±23,7 mm (10-70 mm). Cette étude a établi les membres de theAspergillus genre isolé de Bambara écrous comme champignons viables pour l'application dans la production de tannase Cette étude ajoute aux rapports existants sur la production de champignons de tannase.**

**Mots-clés : Tannase ; Aspergillus isolats fongiques ; noix ; Bambara Vigna subterranea**

**Significant Statement**

This study revealed that bambara nuts (*Vigna subterranea*) plays host to a variety of members of the genus *Aspergillus* that can be beneficial for tannase production. Previous researchers have established tannase production by microorganisms isolated from other legumes. This study hopes to diversify the use of bambara nuts for industrial enzyme production rather than consumption alone. Tannase production locally will help to reduce the overall cost of enzyme importation particularly in developing countries like Nigeria.

**Statement on Conflict of Interest: "none declared"**

## INTRODUCTION

Tannase can be obtained from plant, animal and microbial sources (1). It is present in tannin-rich plants mainly in their fruits, leaves, branches and barks of trees (2). For animal sources, the enzyme can be extracted from bovine intestine and the ruminal mucous (3).The microbial sources of tannase are mainly from bacterial and fungal origin and this represents most enzymes used in biotechnological applications (4). Tannase can be obtained from different sources such as tea waste dump sites, agro-residue waste sites and site nearby tannery industries (5). Microbial sources of enzymes are preferred to animal and plant sources (6). Tannase is produced by a number of microorganisms like fungi (*Aspergillus, Penicillium, Rhizopus sp*), yeast (*Candida* sp), and bacteria (*Bacillus* sp) (7 ; 8). Enzymes including tannase are used in almost all industries in Nigeria and the country spends #200 billion annually on importation of industrial enzymes (9). Tannase has several industrial applications; as clarifying agent in the manufacturing of instant tea, beer, fruit juices and some wines (10), for treatment of tannin-polluting industrial effluents and agricultural waste and for the hydrolysis of gallotannin to gallic acid which is an intermediate required for the synthesis of an antifolic antibacterial drug, trimethoprim (8). Bambara (*Vigna subterranea*) commonly known as 'Okpa' in Nigeria is widespread in Africa and is related to cow pea. *Aspergillus niger* is a unique organism compared to other organisms used in individual enzyme application because of its good fermenting capabilities, wide range of enzymes produced from degradation of plant cell wall polysaccharides, high level of protein secretion, more predictable and controllable enzyme content, it is relatively harmless compared to other filamentous fungi (11). There is paucity of information on the practical uses of tannase due to insufficient knowledge of its properties, optimal uses and purification processes (12). This study is therefore an approach to screen for fungal isolates from bambara nuts for tannase production in a bid to decrease the high cost associated with importation of enzymes for industrial uses in the developing economy of Nigeria.

## MATERIALS AND METHODS

### Sample Collection

Bambara nuts of two different colour coats (Red and Cream) (Fig. 1) were purchased from three different locations. The locations are Ota Market – Cream Bambara nuts, Bodija Market –Cream Bambara nuts and IITA Ibadan – Red Bambara nuts. Samples were transported to the Microbiology laboratory of the Department of Biological Sciences, Covenant University, Ota Nigeria for sorting, cleaning and analysis.

**Fig. 1: Cream and Red Colour Coat Bambara (*Vigna subterranean)* Nuts**

**Sample Processing**

A total of 35g of each sample was weighed into a sterile petri dish and 5mls of sterile distilled water was sprinkled on it according to a modified method described by (13). Petri dishes containing the samples were covered up and then sealed with paper tape. Sterile distilled water was sprinkled intermittently, every other day for Fourteen days. After fourteen days, moulds growing on the samples (Fig. 2) were harvested.

**Fig.2. Moulds growing on Cream Colour Coat Bambara nuts**

A second set of 35g each of the three samples (Fig. 3) were weighed and soaked inside a sterilized beaker containing 40mls of sterile distilled water and sealed with paraffin for 6h. The water was drained and the nuts were transferred into sterile petridishes, covered and sealed up with paper tape for fourteen days.

**Fig 3: Bambara Nuts Soaked in Sterile Distilled Water**

**Isolation of Fungal Isolates from Mouldy Bambara Nuts**

Moulds growing on Bambara nut samples were inoculated on Potato dextrose agar plates (Fig. 4) and incubated at 28°C for 72h. The plates were further sub-cultured until pure cultures were obtained from each of the plates.

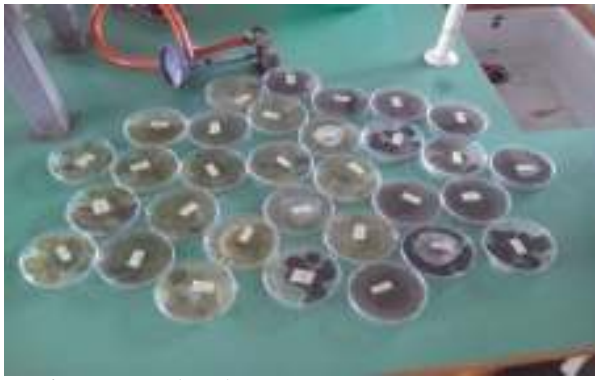

**Fig. 4: Fungal Isolates on Potato Dextrose Agar Plates**

**Screening of Fungal Isolates for Tannase Prodution**

The selection medium (g/l) consisted of $NaNO_3$-3.00, $KH_2PO_4$-1.00, $MgSO_4.7H_2O$-0.50, $FeSO_4.7H_2O$-0.01, Agar- 30.0 according to the method described by (13). It was autoclaved at 121°C for 15 min and supplemented with 1% tannic acid (10 ml) which was previously filter-sterilized through Whatman filter paper No1(pH- 4.0) and adjusted using 100 mM NaOH. Strains of *Aspergillus sp* isolated were then point-inoculated and incubated at 30°C for 96 h. The diameters (mm) of the colonies were measured at 24h intervals for seven days.

**RESULTS**

A total of 18 fungal isolates were obtained (Table 1). All the isolates were members of the genus *Aspergillus* (Table 2). Fungal isolates were obtained from soaked Bambara nuts only from two locations (Bodija Market and Ota Market) while none was obtained from IITA samples (Table 3). For the wet Bambara nuts fungal isolates were obtained from the nuts of all three locations (Table 3). All the 18 fungal isolates (*Aspergillus* genus) were screened for tannase production. The zone of hydrolysis for fungal isolates obtained from soaked bambara nuts from two locations (Bodija Market and Ota Market) is shown in Figure 5. The zones of hydrolysis for fungal Isolates obtained from wet Bambara nuts from the three sample locations is shown in Figure 6. Highest zones of hydrolysis for both wet and soaked bambara nuts (Fig.7).

**TABLE 1: FUNGAL ISOLATES OBTAINED FROM BAMBARA NUTS AND LOCATION**

| Sample Location | Isolate Codes | Colour Change | Sample Location | Isolate Codes | Colour Change | Sample Location | Isolate Codes | Colour Change |
|---|---|---|---|---|---|---|---|---|
| BODIJA MARKET IBADAN | $SBJ_{A11}$ | Brown | OTA MARKET | $SOT_{B11}$ | Black | IITA IBADAN | $WIIT_{A11}$ | Brown |
| | $SBJ_{B11}$ | Brown | | $SOT_{B12}$ | Green | | $WIIT_{C11}$ | Brown |
| | $SBJ_{A21}$ | Green | | $WOT_{A11}$ | Greenish yellow | | $WIIT_{C21}$ | Brown |
| | $SBJ_{A21}$ | Black | | $WOT_{A12}$ | Green | | $WIIT_{C22}$ | Brown |
| | $SBJ_{B21}$ | Greenish yellow | | $WOT_{B12}$ | Brown | | | |
| | $WBJ_{A11}$ | Brown | | | | | | |
| | $WBJ_{B12}$ | Greenish yellow | | | | | | |
| | $WBJ_{C11}$ | Brown | | | | | | |
| | $WBJ_{C21}$ | Greenish yellow | | | | | | |

**TABLE 2: MICROSCOPIC CHARACTERISTICS FOR IDENTIFICATION OF *ASPERGILLUS* SPECIES**

| Isolate codes | Colour Change | Shape | Surface | Conidia Surface | Identification |
|---|---|---|---|---|---|
| $SBJ_{A11}$ | Brown | Spherical | Rough walled | Smooth | *A. tamarii* |
| $SBJ_{B11}$ | Brown | Spherical | Rough walled | Smooth | *A. tamarii* |
| $SBJ_{A21}$ | Green | Glubose | Finely roughened | Distinctly rough | *A. parasiticus* |
| $SBJ_{A21}$ | Black | Glubose | Smooth walled | Very rough irregular | *A. niger* |
| $SBJ_{B21}$ | Greenish yellow | Glubose ellipsoid | Quietly spherical | Smooth finely roughened | *A. flavus* |
| $WBJ_{A11}$ | Brown | Spherical | Rough walled | Smooth | *A.tamarii* |
| $WBJ_{B12}$ | Greenish yellow | Glubose ellipsoid | Quietly spherical | Smooth finely roughened | *A. flavus* |
| $WBJ_{C11}$ | Brown | Spherical | Rough walled | Smooth | *A. tamarii* |
| $WBJ_{C21}$ | Greenish yellow | Glubose ellipsoid | Quietly spherical | Smooth finely roughened | *A.flavus* |
| $SOT_{B11}$ | Black | Glubose | Smooth walled | Very rough irregular | *A. niger* |
| $SOT_{B12}$ | Green | Glubose | Finely roughened | Distinctly rough | *A. parasiticus* |
| $WOT_{A11}$ | Greenish yellow | Glubose ellipsoid | Quietly spherical | Smooth finely roughened | *A.flavus* |
| $WOT_{A12}$ | Green | Glubose | Finely roughened | Distinctly rough | *A. parasiticus* |
| $WOT_{B12}$ | Brown | Spherical | Rough walled | Smooth | *A. tamarii* |
| $WIIT_{A11}$ | Brown | Spherical | Smooth walled | Smooth slightly rough | *A. nidulans* |
| $WIIT_{C11}$ | Brown | Spherical | Smooth walled | Smooth slightly rough | *A. nidulans* |
| $WIIT_{C21}$ | Brown | Spherical | Smooth walled | Smooth slightly rough | *A. nidulans* |
| $WIIT_{C22}$ | Brown | Spherical | Smooth walled | Smooth slightly rough | *A.nidulans* |

**TABLE 3: FUNGAL ISOLATES FROM SOAKED AND WET BAMBARA NUTS FROM DIFFERENT LOCATION**

| Soaked Bambara nuts | | | | | Wet Bambara nuts | | |
|---|---|---|---|---|---|---|---|
| **Sample Location** | **Sample** | **Colour** | **Identification** | **Sample Location** | **Sample** | **Colour** | **Identification** |
| Bodija Market Ibadan | $SBJ_{A11}$ | Brown | *A.tamarii* | Bodija Market Ibadan | $WBJ_{A11}$ | Brown | *A.tamarii* |
| | $SBJ_{B11}$ | Brown | *A.tamarii* | | $WBJ_{B12}$ | Greenish yellow | *A.flavus* |
| | $SBJ_{B11}$ | Green | *A.parasiticus* | | $WBJ_{C11}$ | Brown | *A.tamarii* |
| | $SBJ_{A21}$ | Black | *A.niger* | | $WBJ_{C21}$ | Greenish yellow | *A.flavus* |
| | $SBJ_{B21}$ | Greenish yellow | *A.flavus* | Ota Market | | | |
| Ota Market | $SOT_{B11}$ | Black | *A.niger* | | $WOT_{A11}$ | Greenish yellow | *A. flavus* |
| | $SOT_{B12}$ | Green | *A.parasiticus* | | $WOT_{A12}$ | Green | *A.parasiticus* |
| | | | | | $WOT_{B12}$ | Brown | *A.tamarii* |
| | | | | IITA Ibadan | $WIIT_{A11}$ | Brown | *A.nidulans* |
| | | | | | $WIIT_{C11}$ | Brown | *A.nidulans* |
| | | | | | $WIIT_{C21}$ | Brown | *A.nidulans* |
| | | | | | $WIIT_{C22}$ | Brown | *A.nidulans* |

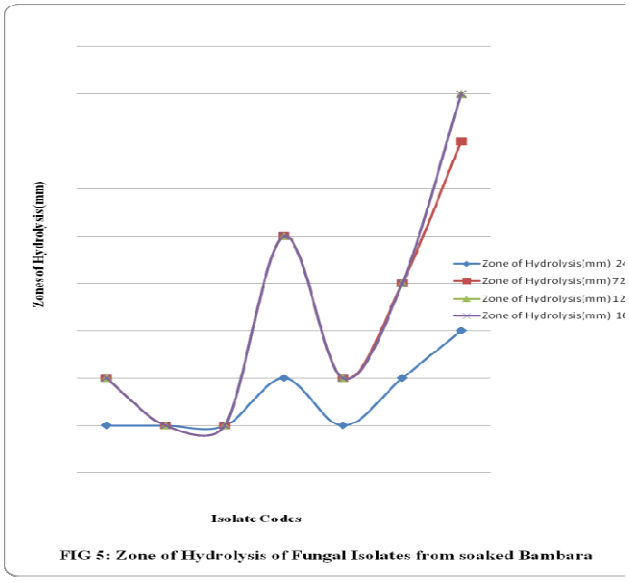


FIG 5: Zone of Hydrolysis of Fungal Isolates from soaked Bambara

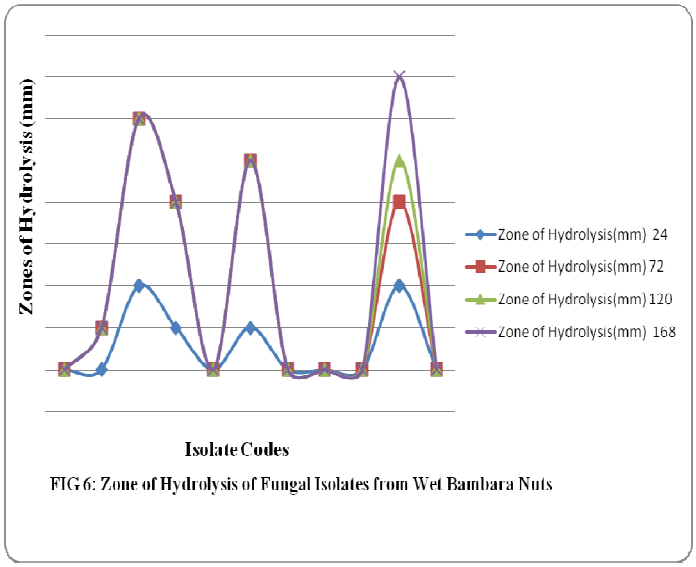


FIG 6: Zone of Hydrolysis of Fungal Isolates from Wet Bambara Nuts

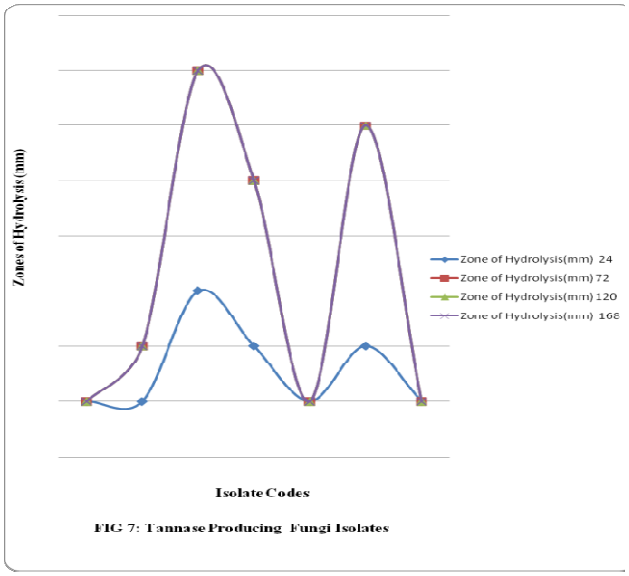


FIG 7: Tannase Producing Fungi Isolates

## DISCUSSION

This study revealed the isolation and screening of fungal isolates from bambara nuts for tannase production. Most of the fungi isolated were of the genus *Aspergillus* (14) had earlier isolated different species of *Aspergillus* from various legumes sampled. This correlates with the findings of (15) and (16) where fungi especially of the genus *Aspergillus* were employed successfully as sources of producing different enzymes. *A. niger* isolated from the soaked bambara nuts showed high tannase production. *Aspergillus niger* strains was isolated from bambara nuts of three different colour seed coats and the strain of *A.niger* from the red colour seed coat showed the highest potential for tannase enzyme (17). However, this is in contrast to the wet method whereby *A. flavus* showed the highest tannase production. Tannase production by *A. flavus* had been reported (18). Filamentous fungi of the *Aspergillus* and *Penicillium* genus have been widely used for tannase production (19). Tannase as an extracellular inducible enzyme produced in the presence of tannic acid by fungi, bacteria and yeast was reported (20).

## Conclusion

Despite the long history and numerous publications on tannase, it is still considered as one of the costly industrial enzymes. This study adds to existing reports on isolating high productive strains of tannase in view of the growing demand for tannase.


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## ORIGINAL ARTICLE

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# ALTERED PROTEIN AND IRON LEVELS OF PATIENTS WITH ACTIVE TUBERCULOSIS IN A NIGERIAN REFERENCE HEALTH FACILITY

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**ABSTRACT**
**Backgound: Tuberculosis as a state of chronic inflammation impacts on haematologic functions of the body.**

**Objectives: This study aimed at assessing iron parameters and serum protein levels of ninety tuberculosis patients aged fifteen to sixty years, enrolled from Dr Lawrence Henshaw Memorial Hospital, Calabar, Nigeria. Ninety apparently healthy individuals age and gender-matched served as control subjects.**

**Methods: Packed cell volume, haemoglobin concentration, serum iron, total iron binding capacity, total protein, albumin and serum ferritin were determined using standard methods. Transferrin saturation, globulin concentration and albumin-globulin ratio were derived by calculation. Data were analysed using student T-test on SPSS version 20 software. Statistical significance was set at P-value less than 0.05.**

**Results: Packed cell volume, haemoglobin concentration, serum iron, total iron binding capacity, transferrin saturation, albumin levels and albumin-globulin ratio of tuberculosis patients were found to be significantly lower while serum ferritin and globulin were significantly increased ($p<0.05$) as compared with control values. Serum ferritin improved towards control values as anti-tuberculosis therapy progressed.**

**Conclusion: A reduction in haemoglobin, serum iron, total iron binding capacity and transferrin saturation and increase in serum ferritin as well as altered serum protein levels, occur in tuberculosis infection.**

**Key words: Tuberculosis, iron, serum protein**

# PROTÉINE ALTÉRÉE ET NIVEAUX DE FER CHEZ LES PATIENTS ATTEINTS DE TUBERCULOSE ACTIVE DANS UN ÉTABLISSEMENT DE SANTÉ DE RÉFÉRENCE DE L'

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**RÉSUMÉ**
**Fond : la tuberculose comme un état d'inflammation chronique hématologique des impacts sur les fonctions de l'organisme.**

**Objectifs : Cette étude visait à évaluer les paramètres fer sérique et la teneur en protéines des quatre-vingt-dix patients âgés de quinze à soixante ans, inscrits par le Dr Lawrence Henshaw Memorial Hospital, Calabar Nigéria. Quatre-vingt-dix personnes apparemment en bonne santé appariés selon l'âge et le sexe ont servi de témoins.**

**Méthodes : l'hématocrite, du taux d'hémoglobine, le fer sérique, capacité totale de fixation du fer, protéines totales, albumine et la ferritine sérique ont été déterminées en utilisant les méthodes standard. La saturation de la transferrine, l'albumine et globuline globuline concentration ratio ont été obtenus par calcul. Les données ont été analysées à l'aide de student T-test sur le logiciel SPSS version 20. La signification statistique a été fixé à p est inférieur à 0,05.**

**Résultats : l'hématocrite, du taux d'hémoglobine, le fer sérique, capacité totale de fixation du fer, la saturation de la transferrine, l'albumine et l'albumine-globuline rapport entre les patients atteints de tuberculose ont été trouvés à être beaucoup plus faible alors que le taux de ferritine sérique et d'immunoglobuline humaine ont augmenté significativement ($p < 0,05$) par rapport aux valeurs des témoins. Le taux de ferritine sérique est améliorée vers les valeurs de contrôle comme thérapie anti-tuberculose progresse.**

**Conclusion : une réduction de l'hémoglobine, le fer sérique, capacité totale de fixation du fer et la saturation de la transferrine et la ferritine sérique en augmentation ainsi que le taux de protéines sériques modifié, se produisent dans l'infection par la tuberculose.**

**Mots clés : Tuberculose, fer à repasser, protéine sérique**

## INTRODUCTION

Tuberculosis (TB) is still a major concern of public health in Nigeria. Among the 22 high burden countries, Nigeria ranks eleventh [1]. Tuberculosis is primarily a pulmonary disease caused by *Mycobacterium tuberculosis* [2, 3]. It is an infectious disease which induces a state of chronic inflammation. The reference health facility for the diagnosis and treatment of tuberculosis in Cross River State, Southern Nigeria is Dr Lawrence Henshaw Memorial Hospital also called Infectious Disease Hospital located in the state capital, Calabar. This center is equipped to diagnose TB through microscopy, Gene Xpert, radiology and culture. The Directly Observed Therapy Short course (DOTS) program for the treatment of TB is fully implemented in this center. Iron is an essential micronutrient for all living organisms which plays a very crucial role in modulating the struggle for survival between mammals and pathogens. Its role is well known in metabolic processes such as cell respiration, growth and DNA synthesis [4]. It has been shown that iron overload promotes free radical induced tissue damage and organ failure, decreases immune protection and increases pathogen invasion. On the other hand, iron deficiency causes iron-deficiency anaemia with all its associated symptoms. An optimal level of iron is needed for natural immunity against the growth of pathogens [5]. Haematological abnormalities have been associated with tuberculosis infection. Decrease in packed cell volume and haemoglobin concentration and increase in erythrocyte sedimentation rate and relative plasma viscosity have been reported [6, 7]. It has also been reported that the majority of TB patients have significantly elevated level of antibodies against *Mycobacterium tuberculosis* in addition to acute phase response which involves release of plasma proteins as a consequence of inflammation [8, 9]. Hypoalbuminaemia, hyperproteinaemia and hyperglobulinaemia are findings associated with pulmonary tuberculosis [10]. There is paucity of information on iron parameters and serum protein levels in tuberculosis infection in our locality; hence this study was done to assess iron parameters and protein levels of patients with active tuberculosis in a reference health facility in Calabar, Nigeria.

## METHODS

A total of one hundred and eighty subjects were recruited for this study. This consisted of ninety tuberculosis patients of both gender aged between fifteen and sixty years, enrolled from Dr Lawrence Henshaw Memorial Hospital in Calabar, Cross River State, Nigeria. TB patients included newly diagnosed cases and those on anti-tuberculosis therapy. Anti-tuberculosis therapy under the directly observed therapy short course (DOTS) lasts for a period of six (6) months. Those patients on anti-tuberculosis therapy from day one up to less than two (2) months are in the intensive phase while two to six months constitute the continuation phase of treatment. Ninety apparently healthy individuals age and gender-matched who were negative for tuberculin (mantoux) test, selected from residents of Calabar metropolis were the control subjects. Ethical permission was given by the Cross River State Ministry of Health and all participants gave their consent. Packed cell volume and haemoglobin concentration was by microhaematocrit and Cyanmethaemoglobin methods respectively [11]. Serum iron (SI), total iron binding capacity (TIBC), total protein (TP) and albumin (ALB) were measured by colorimetric method with test kits manufactured by GIESSE Diagnostics, Italy and RANDOX Laboratories, United Kingdom. Serum ferritin (SF) was determined by enzyme linked immunosorbent assay (ELISA) using CALBIOTECH USA produced kit. Transferrin saturation (TS), globulin concentration (GLOB) and albumin-globulin ratio (ALB-GLOB) were derived by calculation. Student's t-test was used to analyze data on SPSS version 20 software. Statistical significance was set at P-value less than 0.05.

**Consent:** Informed consent was obtained from all participants in this study.

**Competing interest:** No competing interest exists.

**Authors' contributions:** *This work is a result of collaboration between all authors. Akpan PA designed the study and wrote the first draft of the manuscript. Okafor IM performed the statistical analysis. Anakebe S managed the literature searches. All authors were involved in the laboratory analysis; the final manuscript was read and approved by all authors.*

## RESULTS

Demographic data of TB patients and controls is presented in figure 1. Tuberculosis patients consisted of 63 males and 27 females while 60 males and 30 females served as control subjects. The TB patients as well as control were grouped into three based on age. Forty three (43) of the TB patients were aged 15-30 years with 36 and 11 aged 31-45 and 46-60 years respectively. Similarly, controls were 38, 40 and 12 in number for the 15-30, 31-45 and 46-60 years groups respectively.

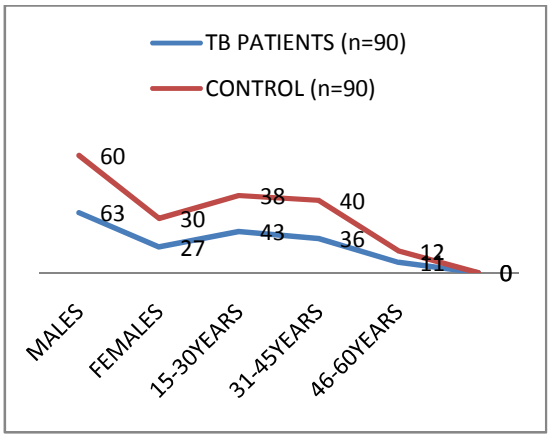


**FIGURE 1: DEMOGRAPHIC DATA OF TB PATIENTS AND CONTROL**

Table 1 shows iron parameters of TB patients and control subjects. The packed cell volume and haemoglobin of TB patients were observed to be significantly lower (p=0.004 and 0.001) than values obtained for control. Similarly, serum iron, total iron binding capacity and transferrin saturation were significantly lower (p=0.001) for TB patients when compared to control. Conversely, serum ferritin was significantly higher (p=0.001) for TB patients than their control.

**TABLE 1: IRON PARAMETERS OF TUBERCULOSIS PATIENTS AND CONTROL SUBJECTS**

| Variable | TB patients (n=90) | Control (n=90) | P-value |
|---|---|---|---|
| Packed cell volume (L/L) | 0.37±0.06* | 0.41±0.04 | 0.004 |
| Haemoglobin (g/L) | 1.04±0.16* | 1.33±0.13 | 0.001 |
| Serum iron (µg/dl) | 48.99±8.41* | 106.00±24.30 | 0.001 |
| Total iron binding capacity (µg/dl) | 187.10±37.00* | 257.95±47.00 | 0.001 |
| Transferrin saturation (%) | 26.28±4.32* | 41.62±8.13 | 0.001 |
| Serum ferritin (ng/ml) | 361.25±267.00* | 82.40±53.84 | 0.001 |

*: Significantly different from control subjects

In table 2, the serum protein levels of tuberculosis patients and control are presented. The TB patients and their control had similar values (p=0.226) for total protein. While the albumin concentration was significantly lower (P=0.001) for the TB patients, the globulin level was significantly higher (p=0.016) when compared to control values. The albumin-globulin ratio was significantly lower (p=0.001) for TB patients than their control.

**TABLE 2: SERUM PROTEIN LEVELS OF TUBERCULOSIS PATIENTS AND CONTROL SUBJECTS**

| Variable | TB patients n=90 | CONTROL n=90 | P-VALUE |
|---|---|---|---|
| TOTAL PROTEIN (g/L) | 94.46±21.33 | 88.95±22.71 | 0.226 |
| ALBUMIN (g/L) | 44.58±7.31* | 50.18±7.10 | 0.001 |
| GLOBULIN (g/L) | 49.46±20.42* | 38.78±21.20 | 0.016 |
| ALB-GLOB RATIO | 0.90±0.34* | 1.32±0.44 | 0.001 |

*: Significantly different from control subjects

Figure 2a shows iron and protein levels of TB patients in different phases of treatment. Fifty two (52) of the ninety TB patients were in the intensive phase while the remaining thirty eight (38) were in the continuation phase of treatment. The serum iron, total iron binding capacity, transferrin saturation, total protein, ALB and GLOB did not change significantly between TB patients in both intensive and continuation treatment phases. However, the serum ferritin levels reduced significantly (p=0.001) from the intensive to the continuation treatment phase. In figure 2b, the PCV, HB and albumin-globulin ratio were observed to be comparable in both phases of treatment.

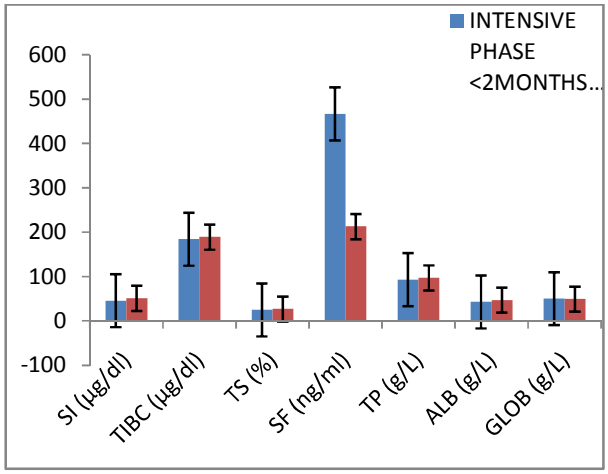


**FIGURE 2A: IRON AND PROTEIN LEVELS OF TB PATIENTS IN INTENSIVE AND CONTINUATION PHASES OF TREATMENT**

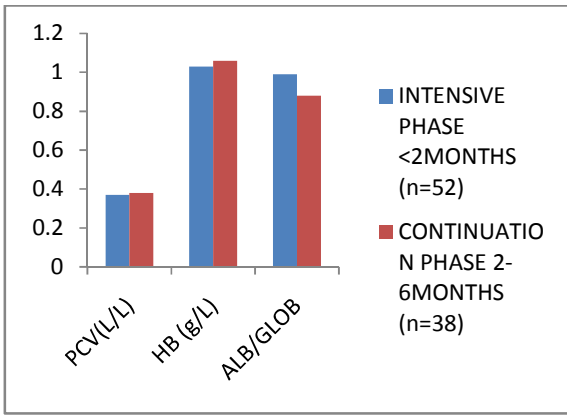


**FIGURE 2B: PCV, HB AND ALBUMIN-GLOBULIN RATIO OF TB PATIENTS IN INTENSIVE AND CONTINUATION PHASES OF TREATMENT**

## DISCUSSION

This study has shown (table 1) that the packed cell volume and haemoglobin concentration of TB patients were significantly lower when compared to that of apparently healthy (control) subjects. Tuberculosis is a chronic ailment which could influence haemopoiesis leading to a reduction in erythropoiesis [12]. Reduced haemoglobin concentration below the reference range (11.0g/dl) which is termed anaemia, has been reported in tuberculosis infection [13, 7, 14]. This anaemia is classified as normocytic normochromic type showing features of anaemia of chronic disease and inflammation [15]. Other factors such as decrease in red cell survival and reduced erythroproietin response by the bone marrow erythroid cells are also known to cause anaemia in infections [12].

The serum iron (SI) level of TB patients was significantly lower than the value obtained for control subjects. According to Dacie and Lewis [11], SI level is low in anaemia of chronic disease and infection. Tuberculosis being a chronic inflammatory condition, it interferes with iron metabolism resulting in a low serum iron. Similarly, a lower total iron binding capacity (TIBC) was observed for TB patients when compared to control values. TIBC has been reported to be lower than reference values in infections and particularly in anaemia of chronic disease [16, 11]. Low serum iron as well as total iron binding capacity in infection has been attributed to increased hepcidin production. Hepcidin is an acute phase protein which regulates the absorption of iron in the small intestine and its release from macrophages. In an inflammatory state such as tuberculosis, hepcidin level increases but its normal function is impaired resulting in low SI and TIBC levels [17, 18]. Furthermore, hepcidin induces certain alterations in the metabolism of iron like less iron absorption from the gastrointestinal tract as well as trapping of iron in macrophages [19]. Transferrin saturation (TS) of the TB patients was significantly lower than values obtained for control subjects. Since the TS is calculated from SI and TIBC, a low TS follows the pattern of the low SI and TIBC. The low values of these iron parameters imply that erythropoiesis is ineffective and this accounts for the anaemia observed in chronic disease and inflammation.

The serum ferritin level of tuberculosis patients (361.25±267.00) was significantly higher ($p=0.001$) compared to control value (82.40±53.84). Serum ferritin is a valuable factor in the measurement of iron reserve of the body however it is a positive acute phase reactant protein which could be increased in tuberculosis infection due to inflammatory state [4]. As a reaction to injury, neutrophils and macrophages (local inflammatory cells) secrete several cytokines particularly interleukins (IL) 1, 6 and 8 as well as tissue necrotic factor alpha (TNFα) into the bloodstream. As a result, acute-phase reactants are produced by the liver in large amounts [20]. Also, the observed increase in ferritin level could be as a result of iron sequestration mechanism of the body during pathogen invasion. Iron supplementation which is the current practice in TB treatment, may be implicated in increased ferritin levels as the body withholds iron from the invading pathogen. It is therefore recommended that iron supplementation should be discouraged in the management of TB patients.

In this study, the total protein levels of TB patients were similar to control values and also comparable to previous finding [21]. TB patients had a significantly lower albumin and a significantly higher globulin level ($p<0.05$) in comparison to control (table 2). Reduction in albumin level correlates with some reports [22, 23] and disagrees with increase in albumin reported in another study [24]. Low albumin may be due to inadequate protein intake while increased globulin concentration might be due to immune response by the body against the invading TB bacilli. The albumin/globulin ratio of the tuberculosis patients was found to be lower than that of apparently healthy subjects (control).

It was observed that serum iron, TIBC, TS, albumin as well as globulin improved (though not significantly) in the continuation phase as compared to the intensive phase. On the other hand, serum ferritin reduced significantly ($p<0.05$) towards control values with progressive treatment. This could be attributed to adherence to anti-tuberculosis therapy under the DOTS program and the effectiveness of the anti-TB drugs in reversing the acute phase response. It also stands to reason that the body no longer withholds iron in the form of ferritin since the offending pathogen *Mycobacterium tuberculosis* is being cleared by the anti-TB drugs. Packed cell volume, haemoglobin and albumin-globulin ratio of TB patients were comparable for both phases of treatment.

**Conclusion:** This study has shown that reduction in packed cell volume, haemoglobin, serum iron, total iron binding capacity, transferrin saturation and albumin and increase in serum ferritin and globulin concentration occur in active tuberculosis; also, iron parameters and protein levels improved as anti-tuberculosis therapy progressed. It is suggested that early diagnosis and initiation of anti-tuberculosis therapy and strict adherence will alleviate the changes in iron stores.

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