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**Review Article****Open Access****Candida species: the silent enemy**¹*Al-Laaiby, A., ²Ali, S., and ³Al-Saadoon, A. H.¹Department of Biology, College of Science, University of Basrah, Iraq²Department of Nursing Clinical Sciences, College of Nursing, University of Kirkuk, Iraq³Department of Pathological analyses, College of Science, University of Basrah, Iraq*Correspondence to: ayat200022@yahoo.com**Abstract:**

Candida species are known to cause serious infections in immunocompromised patients but uncommon cases have been reported in immunocompetent individuals regardless of the harmless co-existence of the fungi with the host. Recently, the incidence rate of candidiasis has increased dramatically alongside the emergence of antifungal resistance. Although conventional methods to ensure prompt diagnosis of candidiasis for effective therapy have been established, the scientific world is witnessing progress in the development of more accurate, timely and cost-effective methods that is coinciding with the molecular revolution and advanced DNA analysis. Moreover, the challenges of resistance of *Candida* to available antifungal agents are being met with the deployment of molecular techniques to investigate the mechanisms of resistance. This review is an attempt to provide up-to-date information on the persistent problems of *Candida* with highlights on the clinical importance, molecular diagnosis, and resistance to candidate antifungal drugs; azoles and echinocandins.

Keywords: *Candida*, resistance, molecular diagnosis, azole, echinocandin

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Copyright 2019 AJCEM Open Access. This article is licensed and distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution and reproduction in any medium, provided credit is given to the original author(s) and the source.**Espèce *Candida*: l'ennemi silencieux**¹*Al-Laaiby, A., ²Ali, S., et ³Al-Saadoon, A. H.¹Département de biologie, Collège des sciences, Université de Basrah, Irak²Département de sciences cliniques infirmières, Collège des sciences infirmières, Université de Kirkuk, Irak³Département de Pathological analyses, Collège des sciences, Université de Basrah, Irak*Correspondance à: ayat200022@yahoo.com**Abstrait:**

Les espèces de *Candida* sont connues pour causer des infections graves chez les patients immunodéprimés, mais des cas peu communs ont été rapportés chez des individus immunocompétents, indépendamment de la coexistence inoffensive des champignons avec l'hôte. Récemment, le taux d'incidence de la candidose a considérablement augmenté parallèlement à l'émergence d'une résistance antifongique. Bien que les méthodes conventionnelles permettant d'assurer un diagnostic rapide de la candidose en vue d'un traitement efficace aient été établies, le monde scientifique constate des progrès dans la mise au point de méthodes plus précises, plus rapides et plus rentables qui coïncident avec la révolution moléculaire et l'analyse avancée de l'ADN. De plus, les défis posés par la résistance de *Candida* aux agents antifongiques disponibles sont résolus par le déploiement de techniques moléculaires pour étudier les mécanismes de résistance. Cette revue tente de fournir des informations à jour sur les problèmes persistants de *Candida* en soulignant l'importance clinique, le diagnostic moléculaire et la résistance aux antifongiques candidats; les azoles et les echinocandins.

Mots-clés: *Candida*, résistance, diagnostic moléculaire, azole, echinocandine**Introduction:**

The scientific term *Candida* was derived from the Latin name 'candid', referring to its white colour (1). Anton van

Leeuwenhoek first observed yeast cells under the microscope in 1680 (2) and classified it into kingdom Fungi, phylum Ascomycota, class Saccharomycetes, order Saccharomycetales, family Saccharomycetaceae and

genus *Candida* (3). The significant feature of yeast is the ability to alter its morphology from unicellular ovoid cells to pseudohyphae and hyphae forms. They are therefore known as dimorphic fungi (4). The morphological transition of yeast is stimulated by surrounding conditions such as temperature, pH, blood, serum and nutrient abundance (4). They form chlamydospores in specific conditions (5) and reproduce non-sexually by forming buds or sexually through mating (4).

Candida dwells in human body as a commensal where they are regarded as normal flora. They inhabit different body sites including the skin, mouth, gastrointestinal tract and vagina, and are generally harmless in immunocompetent individuals, scarcely infecting healthy people with only mild infections (6). However, they become pathogenic opportunistic fungi in individuals with impaired immune system (7).

Methodology:

During the period, September 2018 to March 2019, we employed Google scholar engine to search for relevant published materials and bibliographic citations including original and review articles, books, and conference papers through the period 1985 to 2018. Keywords employed for the search were Candidiasis, *Candida* diagnosis, *Candida* classification, *Candida* resistance, *Candida* molecular diagnosis, and *Candida* treatment. One hundred and ninety four reference materials were identified but following assessment of the relevance of the materials, only 70 reference materials were selected for the review.

Clinical significance of *Candida*

Candida species of clinical importance include *Candida albicans* and non-*albicans* *Candida* species such as *Candida parapsilosis*, *Candida krusei*, *Candida glabrata*, *Candida dubliniensis*, *Candida tropicalis*, *Candida stellatoidea*, *Candida auris*, *Candida guilliermondii*, *Candida lusitanae* and *Candida kyfer* (8, 9). Clinically, *C. albicans* is of utmost significance because of its frequent isolation as a pathogenic and virulent species compared to other species (10). Genetically, *C. albicans* is diploid with heterozygous cells compared to *C. glabrata* that is haploid (10, 11). *C. albicans* is also an imperfect yeast (vegetative) as its life cycle contains a non-sexual phase only (10).

Candida species can cause infections ranging from simple superficial to life threatening systemic diseases (12). Superficial infections affect the skin, nails and hair in addition to mucous layer (13) and

include such diseases as athlete's foot, ring worm, oral and vaginal thrush (14,15). Studies have shown that approximately 75% of females suffer from vaginal *Candida* infections (vulvovaginal candidiasis) at least once during their life (16). *Candida* has been reported as the third or fourth cause of nosocomial infections in the United States (17) and as an insidious pathogen causing ocular candidiasis, cerebral candidiasis or candida meningitis in infants and susceptible adults (8).

Shetti and co-workers reported for the first time in 2011 that *Candida* treatment failure is an indicator of HIV infection (18). Similar to other pathogenic agents, opportunistic pathogenic *Candida* species possess virulence factors that enable colonization and host infection. The stages of infection involve inoculation, adherence to the host tissue, penetration and dissemination to other body sites through the bloodstream (6).

Risk factors for *Candida* infection

Candida infections are associated with multiple risk factors and the prevalence of *Candida* infection is related to the ability of the pathogen to adapt to environmental factors, for example, pathogenic species successfully grow and multiply at 37°C compared to non-pathogenic species (8,13). The risk factors can be categorized into two; (i) factors associated with medical management and (ii) factors related to host immune status.

Recent advances in health care management and medications have provided the opportunity of a suitable environment that supports *Candida* growth and colonisation of the host. Such factors include catheterization, parenteral nutrition, organ transplantation, prolonged hospitalization, and use of new medications (8). On the other hand, host factors such as age, gender, and immune status determine the incidence of candidiasis (8).

An *invitro* study showed that nicotine concentrations of cigarette smoke are associated with yeast growth and adherence in both biofilm and plankton forms (19). Others have shown an association between *Candida* infections and iron deficiency, owing to the relationship of iron and the strength of the immune system (20). Newborns are at risk of infection with *Candida* species because of the risk of transmission from the mother during childbirth and perinatal period (21). Moreover, patients with AIDS, oral cancer, cystic fibrosis, dentures, and recipients of lung, liver, stem cell or other solid organs are

highly susceptible to *Candida* infections (6, 22)

Laboratory diagnosis of Candidiasis

The accurate diagnosis of infection caused by pathogenic microorganisms is an important guide in the selection of appropriate treatment regimen (23). The diagnosis of fungi infection is complex due to overlapping symptoms and signs with other infections, patients’ age, health status and environmental factors (24). Advances in the field of mycology have led to the development of diagnostic methods for fungi infections (1). Two mycological laboratory approaches are employed in the diagnosis of

Candida infections; conventional standard (microscopy and culture) and non-culture methods such as serology and molecular techniques (25) (Fig 1).

In the era of molecular diagnosis, new techniques have been developed and used for accurate identification of fungi (26). However, the conventional methods are still employed in combination with the new advanced molecular techniques (27). As explained by Kurtzman *et al.*, the conventional methods are necessary for the understanding of the fungi and their ecological interactions in addition to obtaining accurate results through molecular analysis of their gene variations and modifications (28).

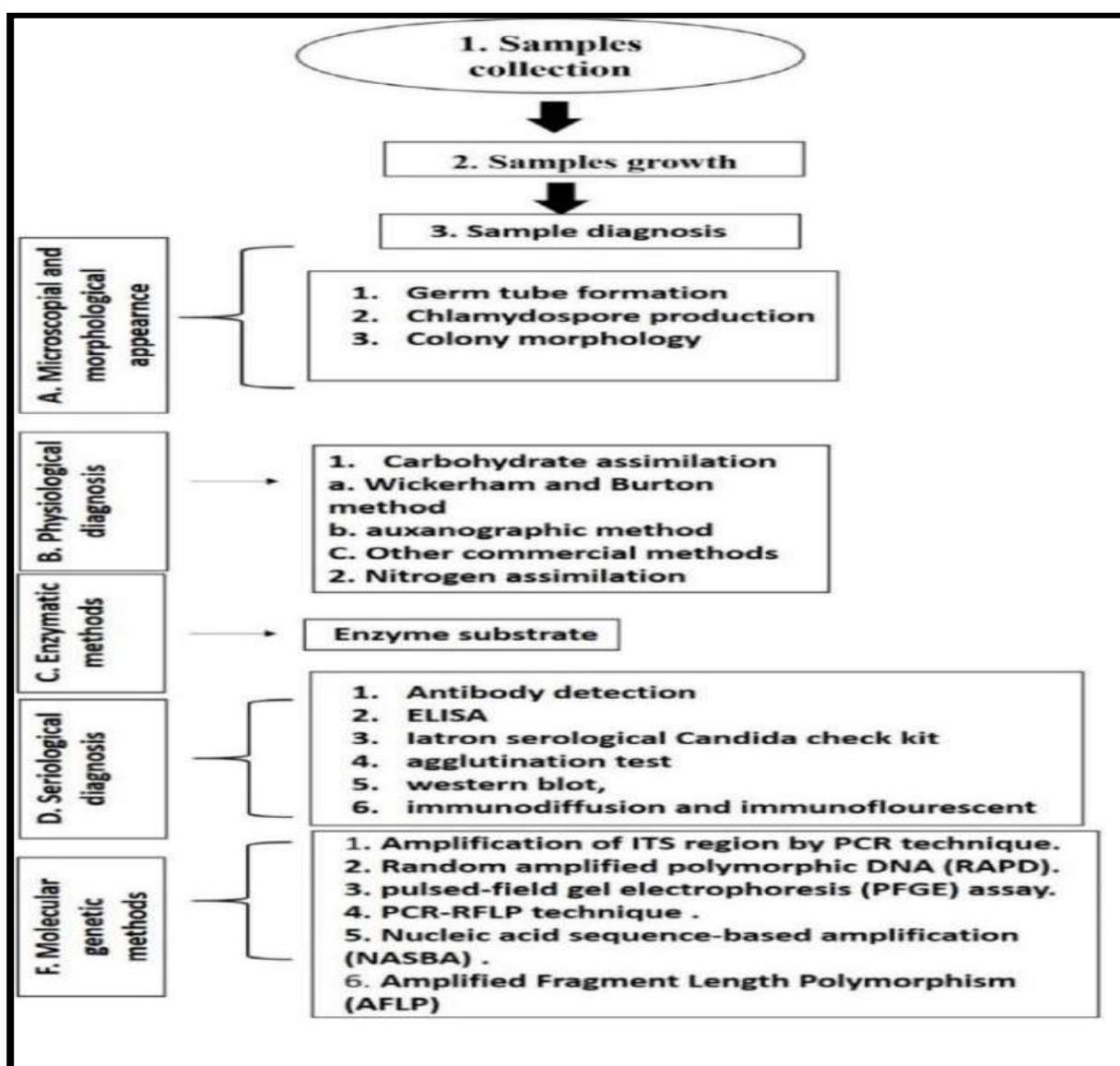


Figure 1: Schematic diagram summarizing important steps for laboratory diagnosis of *Candida*

Molecular identification of *Candida*

Polymerase chain reaction (PCR) assay

PCR technique is useful for basic molecular identification of the organism, however successful PCR depends on DNA extraction, purity and quantity (29). The amplification by PCR involves three phases of denaturation, annealing (hybridization) and extension. To amplify specific target DNA sequence in a conventional PCR, specific primers are designed to hybridize to the sequence of target and amplification carried out in master mix PCR reaction tubes inside a thermal cycler machine. PCR products are then separated on an agarose gel to visualize the DNA bands and confirm successful DNA fragment amplification (29).

PCR techniques are promising assays that evaluate and differentiate yeast and pathogenic fungi species. In particular, the multiplex PCR is a simple, timely and cost effective approach to identify different species of yeasts (30). Luo *et al.*, skipped the DNA extraction step and amplified DNA directly from the yeast colony. This modification reduced the turn-around-time for the assay and avoided the obstacle of DNA isolation which reduced the overall cost (30).

The conserved sequences in the genome of organisms exhibit divergence among different species, the knowledge of these variations have been utilized to determine relatedness between species (26). Kurtzman and Robnett investigated the relatedness between yeast species using the variations of D1/D2 domain in a large subunit (26S) of ribosomal DNA, while at the same time, amplifying and sequencing the Internal Transcribed Spacer (ITS) region with universal primers, and comparing this with the BLAST database (31,32).

There are two ITS regions, which are non-coding regions located between the small and large subunits of rRNA. In fungi, ITS1 and ITS2 are located in the rDNA gene complex between the 18s and 5.8s rRNA and 5.8s and 26s rRNA genes respectively (32). These regions are distinguished by their variations among fungal species and have therefore been selected as DNA barcode to differentiate the fungal species (33). Leaw *et al.*, have demonstrated the candidate ITS regions for *Candida* species identification. First, the length of the amplified ITS fragments differs among *Candida* species (34). However, the maximum length (size) of the DNA fragment amplified is less than 0.3 kb, with the exception of *Saccharomyces cerevisiae* with length of 3.7 kb and *C.*

glabrata with 0.4 kb (34). Secondly, the identification of yeast species, depending on the ITS regions (particularly ITS2) is reliable, accurate and timely (it can be completed within 24 hours) when used in identification of clinical isolates (34). Romeo *et al.* (35) used specific primers targeting the *HWP1* gene to differentiate between *C. albicans*, *C. africana* and *C. dubliniensis* by amplifying different sizes of DNA segments with sizes of 941 bp, 700 bp and 569 bp respectively (35).

Random amplified polymorphic DNA

The amplification of random fragments of genomic DNA to investigate genetic diversity among species is called random amplified polymorphic DNA (RAPD) and it has been used to study the relatedness of *Candida* isolates (36). The advantages of the RAPD assay is that of simplicity with no need for precise DNA sequence data, rapidity and lower cost. However, the problem of reproducibility is a major disadvantage because non-specific or arbitrary primers annealed to the DNA under demanding conditions (37,38). The study by Valério *et al* reported that RAPD may be unreliable due to the phenotypic switching in *Candida* species (39).

Pulsed-field gel electrophoresis assay

The investigation of the karyotypes of *Candida* species is an additional means of differentiating between *Candida* species which can be achieved with pulsed-field gel electrophoresis (PFGE) assay (38). However, this analytical assay is time consuming and labour intensive.

Nucleic acid sequence based amplification

Nucleic acid sequence based amplification (NASBA) is an alternative method to PCR technique that is used to amplify specific segments of rRNA using RNA polymerase to detect active pathogens (40).

Restriction fragment length polymorphism

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique has contributed to the precise diagnosis of *Candida* species. Mousavi *et al.*, identified five *Candida* species; *C. albicans*, *C. krusei*, *C. glabrata*, *C. tropicalis* and *C. guilliermondii* by PCR-RFLP technique (41). The technique involves amplification of DNA segments using universal primers ITS1 and ITS4, followed by digestion of the amplified products using *Msp* I restriction enzyme (41). The digested products are separated on agarose gel and the characteristic band patterns are interpreted according to established protocol.

Table 1: Summary of the targets and actions of antifungal agents (44)

Antifungal	Target	Action
Amphotericin B (polyene)	Cell membrane (ergosterol)	Targets the integrity of the cell membrane by binding to ergosterol, owing to its affinity to ergosterol more than any other sterol. The drug affects permeability by forming pores in the cell membrane.
Azole	Cell membrane (ergosterol)	Targets the synthesis of ergosterol by inhibiting lanosterol 14- α -demethylase, which converts lanosterol to ergosterol in the fungal cell membrane.
Echinocandin	Cell wall (glucan)	Targets the integrity of the fungal cell wall by inhibiting B-glucan synthase, leading to the inhibition of glucan synthesis.
Flucytosine	DNA and RNA synthesis	5-Flucytosin uptake is undertaken by the cytosine permease and this interferes with the pathways used for DNA and RNA synthesis.

Candida treatment and resistance

Four major classes of anti-fungal agents are approved by the United States Food and Drug Administration (FDA); amphotericin, azole, echinocandin and flucytosine (42). The mechanism of action of each class is summarised in Table 1. Compared to the wide range of antibacterial drugs, only a few antifungal drugs are available with noticeable side effects due to the similarity of fungal and human cell membrane (both are eukaryotic cells) which is the site of action of a large number of antifungal agents (43). Echinocandins and azoles are the most effective antifungal agents against candidiasis. Echinocandins tend to have fewer side effects than other antifungal classes due to the absence of a cell wall (its target of action) in the human cell (43).

In spite of the success of these classes of anti-fungals for treatment of yeast and mould infections, these fungal pathogens have developed resistance to these drugs (44). Resistance to anti-fungal agents occur through mutational changes in the genome of the fungi leading to modifications of the metabolic pathway of the drugs in the pathogen (44). There are two categories of resistance; primary (intrinsic) and secondary (acquired). Primary resistance is genetically inherited from the parent as seen in human pathogenic fungi such as *Lomentospora prolificans*, which is intrinsically resistant to all antifungal drugs (45). Secondary resistance is acquired from surrounding environment (46).

Molecular mechanisms of drug resistance in Candida

In more recent years, the determination of genes and their product functions have been carried out via molecular and genetic engineering techniques, such as through the determination of the gene mutations which cause clinical failure through gene sequencing or tracking gene expression

using real time PCR or microarrays (47,48). *Candida* species have developed various mechanisms to resist antifungal medications depending on the type of drugs, mechanisms of action and drug targets (44). The most commonly available antifungal drugs for treatment of candidiasis are azoles and echinocandins but *Candida* species have developed resistance to them (49).

Resistance to echinocandins

Echinocandin inhibits the enzyme, β -1,3-D-glucan synthase, leading to arrest in the biosynthesis of β -1,3-D glucan and disruption of the integrity of the fungal cell wall (43) (Table1). The enzyme is composed of two subunits; catalytic 'Fks' subunit and regulatory 'Rho' subunit though echinocandin targets the *Fks1* gene (43).

Candida species have developed resistance to echinocandin despite its effectiveness. The exposure of *C. albicans* to echinocandin induces the synthesis of chitin (50). Two signalling pathways; PKC Ca^{2+} -calcineurin and HOG, regulate the expression of the genes for chitin synthesis (51). Both signalling pathways are involved in the activation of transcriptional stimulation in response to echinocandins (50).

Additionally, presence of mutations in the *Fks1* gene is associated with resistance to echinocandin. For example, sequencing of resistant *C. auris* isolates showed substitution of serine to phenylalanine in the *Fks1* gene locus (9). Mutations in the conserved region of the *Fks1* gene are called 'hot spots' for acquisition of *Candida* resistance to echinocandin (52).

Resistance to azoles

Azole targets ergosterol biosynthesis (Table 1) but *Candida* has developed four mechanisms to circumvent the fungicidal mechanism of azole including an efflux pump to lower drug concentration within the cell, modification of the ergosterol target, elevating levels of production of the targeted enzyme, and the establishment of alternative pathways (44). Some strains showed cross

resistance to antifungal drugs. *C. tropicalis* carried more than one mutations leading to azole-polyene cross resistance (53).

Two types of transporter proteins, which export different elements such as carbohydrate include the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) (54). These proteins are encoded by the *CDR* and *MDR* genes respectively. The efflux pump encoding genes; *FLR1*, *AZR1* and *TPO1* are associated with resistance to flucytosine, ketoconazole and caspofungin respectively (55,56,57). Studies have shown that *Candida* utilised these genes to expel drugs taken by the cell outside of the cells thereby reducing the concentration within the cell to ineffective level (54). The upregulation of transporter genes is thus associated with azole resistance in *C. albicans* (58).

The transcriptional profiling of resistant *C. glabrata* isolates to azole has been compared with their counterpart susceptible isolates using DNA microarray technique. The results showed that the upregulation of *PDR1* genes (related to a single mutation in a different locus of *CgPDR1* ORF) is associated with azole resistance (59). Northern blot analysis revealed that *CDR1*, *CDR2* and *MDR1* genes were over-expressed in the resistance isolates compared to susceptible strains (60). The disruption of single or double genes involved in the efflux pump generated mutants sensitive to fluconazole rather than the resistance parent strains in the plankton (61).

Another mechanism in which the *Candida* species develop resistance to azole antifungal drugs is by modifying the drug targets (62). *ERG11* gene encodes lanosterol, 14- α -demethylase (Erg11p), which is targeted by the azole antifungal drug (62). The analysis of PCR products (products mediated by gene amplification from sensitive and resistance strains) was used to investigate point mutations of the *CYP51* gene in the resistance isolates that reduced the function of azole (63). Point mutation leads to amino acid substitution leading to decreased binding of the azole to its target. Flowers *et al.*, characterised 26 novel point mutations while Morio *et al.*, highlighted more than 140 mutations leading to amino acid substitutions. Mutations in the genome sequence, involving *ERG3* and *ERG11* genes of *C. tropicalis*, led to development of resistance to azoles (53).

The action of azole against the fungal cells interferes with the synthesis of functional ergosterol and produces a toxic intermediate compound called 14 α methyl-3,6-diol, blocking fungi growth (44).

However, *Candida* species have developed another mechanism to resist azole drugs by establishing an alternative pathway. For instance, a missense mutation in the *ERG3* gene of *C. tropicalis* leads to accumulation of the sterol intermediates, ergosta-7,22-dienol and ergosta-7-enol instead of ergosterol (64). *C. albicans* strains produce functional sterol 14 α -methylfecosterol instead of ergosterol, which leads to development of resistance to fluconazole (65). Another study reported that reduction of *C. krusei* susceptibility to azole drugs was related to up-regulation of Erg11p expression (66). The overexpression of the *ERG11* gene is associated with the mutation of the *UPC2* gene (zinc cluster transcription factor) (68). In a study that targeted the *UPC2* gene disruption, there was reduction in *ERG11* expression in the *UPC2* deficient mutant compared to wild type strains (67). It was shown that the mutation in the *UPC2* gene coinciding with the upregulation of *Erg11* expression was one of the mechanisms of increase, causing *ERG11* gene over-expression. However, other mechanisms do exist (69).

Conclusion:

Candida species cause a wide range of infections from mild to severe life threatening diseases. Meanwhile, they inhabit the human body as normal flora. It could therefore be said that these normal flora are silent enemies because they exploit opportunities and attack the body in the event of a perturbation of the immune system. Apart from this, *Candida* species have the ability to modify their genome to develop resistance to antifungal drugs. Although, azoles and echinocandins are the most common antifungal agents effective against *Candida* infections, resistance to these drugs are increasing.

In recent time, attempts have been made to develop methods of identifying *Candida* species by exploring certain variations in the conserved region of their genome to determine relatedness between species. To solve the problem of increasing resistance to antifungal agents, molecular techniques are being utilized to investigate gene modifications that overcome the action of antifungal drugs. These efforts aspire to develop more accurate, effective and less time-consuming methods of diagnosis in addition to developing new antifungal agents.

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**Original Article****Open Access****Experimental murine model of intra-abdominal infections caused by some non-albicans *Candida* species***¹Elkady, N. A., ²Elkholy, I. M., ³Mohammed, H. A., ¹Elmehalawy, A. A., and ¹Abdel-Ghany, K.¹Microbiology Department, Faculty of Science, Ain Shams University, Abbassia, 11566, Cairo, Egypt,²Clinical Pathology Department, Ain Shams University Specialized Hospital Ain Shams University, Abbassia, 11566, Cairo, Egypt³Zoology Department, Faculty of Science Ain Shams University, Abbassia, 11566, Cairo, Egypt*Correspondence to: nadiaelkady@sci.asu.edu.eg; 02-01008853275; ORCID: <https://orcid.org/0000-0001-5475-1326>**Abstract:****Background:** Even though intra-abdominal candidiasis (IAC) has been increasingly recognized, with associated high morbidity and mortality rates, its pathogenesis remains poorly understood. This model aims to study the pathogenicity and *invivo* susceptibility of non-*albicans Candida* species associated with IAC in human in order to predict the frequency of infections, outcome of clinical disease and response to antifungal therapy.**Methodology:** Both immunosuppressed and immunocompetent female CD-1 mice were challenged intraperitoneally with 5 x 10⁸ CFU/ml inoculum of five non-*albicans Candida* strains; *Candida glabrata*, *Candida parapsilosis*, *Candida lipolytica*, *Candida tropicalis* and *Candida guilliermondii*. Mice were closely observed for symptoms. Treated groups received voriconazole (40 mg/kg/day) or micafungin (10 mg/kg/day) 24 hours after infection depending on *invitro* susceptibility results. Survival rate, mean survival time and fungal tissue burdens were recorded for all groups.**Results:** All infected groups developed hepatosplenomegaly, peritonitis and multiple abscesses on intra-abdominal organs and mesenteries. *C. glabrata* and *C. lipolytica* represented the most and the least virulent strains respectively in terms of survival rate, mean survival time and fungal burden in both immunosuppressed and immunocompetent models. Following treatment, all immunocompetent animals survived the entire duration of experiments (0% mortality rate), while mortality rate was relatively high (20-60%) in immunosuppressed mice. Treatment failed to eradicate the infection in immunosuppressed mice despite significant decrease of the fungal burden and increase mean survival time.**Conclusion:** This study reports an increasing pathogenicity of non-*albicans Candida* species, with persistent infection among immunosuppressed animals.**Keywords:** Intra-abdominal, Candidiasis, non-*albicans*, *invivo*, mice.

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Copyright 2019 AJCEM Open Access. This article is licensed and distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution and reproduction in any medium, provided credit is given to the original author(s) and the source.**Modèle murin expérimental d'infections intra-abdominales causées par certaines espèces de *Candida* non albicans***¹Elkady, N. A., ²Elkholy, I. M., ³Mohammed, H. A., ¹Elmehalawy, A. A., et ¹Abdel-Ghany, K.¹Département de microbiologie, Faculté des sciences, Université Ain Shams, Abbassia, 11566, Le Caire, Égypte,²Département de pathologie clinique, hôpital spécialisé Ain Shams University, université Ain Shams, Abbassia, 11566, Le Caire, Égypte³Zoologie, faculté des sciences, université Ain Shams, Abbassia, 11566, Le Caire, Égypte*Correspondance à: nadiaelkady@sci.asu.edu.eg; 02-01008853275; ORCID: <https://orcid.org/0000-0001-5475-1326>**Abstrait:****Contexte:** Bien que la candidose intra-abdominale (CAI) soit de plus en plus reconnue, avec des taux de morbidité et de mortalité élevés associés, sa pathogenèse reste mal comprise. Ce modèle vise à étudier le pouvoir pathogène et la susceptibilité *in vivo* d'espèces de *Candida* non *albicans* associées à l'IAC chez l'homme afin de prédire la

fréquence des infections, l'évolution de la maladie clinique et la réponse au traitement antifongique.

Méthodologie: Des souris femelles CD-1 immunodéprimées et immunocompétentes ont été stimulées par voie intrapéritonéale avec un inoculum de 5×10^8 UFC / ml de cinq souches *Candida* non *albicans*; *Candida glabrata*, *Candida parapsilosis*, *Candida lipolytica*, *Candida tropicalis* et *Candida guilliermondii*. Les symptômes ont été observés de près chez les souris. Les groupes traités ont reçu du voriconazole (40 mg/kg/jour) ou de la micafungine (10 mg/kg /jour) 24 heures après l'infection, en fonction des résultats de sensibilité *invitro*. Le taux de survie, la durée de survie moyenne et la charge en tissu fongique ont été enregistrés pour tous les groupes.

Résultats: Tous les groupes infectés ont développé une hépatosplénomégalie, une péritonite et de multiples abcès aux organes intra-abdominaux et au mésentère. *C. glabrata* et *C. lipolytica* représentaient respectivement les souches les plus et les moins virulentes en termes de taux de survie, de durée de survie moyenne et de charge fongique dans les modèles immunodéprimés et immunocompétents. Après le traitement, tous les animaux immunocompétents ont survécu à toute la durée des expériences (taux de mortalité de 0%), tandis que le taux de mortalité était relativement élevé (20 à 60%) chez les souris immunodéprimées. Le traitement n'a pas réussi à éradiquer l'infection chez les souris immunodéprimées malgré une réduction significative de la charge fongique et une augmentation du temps de survie moyen.

Conclusion: cette étude rapporte une pathogénicité croissante des espèces de *Candida* non *albicans*, avec une infection persistante chez les animaux immunodéprimés.

Mots-clés: intra-abdominal, candidose, non *albicans*, *invivo*, souris.

Introduction:

Candida species are important fungal pathogens that can survive in different anatomical sites (1, 2). Invasive candidiasis is the most frequently encountered fungal disease among hospitalized patients. This includes candidaemia and intra-abdominal candidiasis (IAC), which manifests as peritonitis and intra-abdominal abscesses (3, 4). Despite the high frequency of IAC among critically ill patients, it remains understudied compared to candidemia (5-7). Over the last decades a progressive increase in the epidemiology of non-*albicans Candida* infections has been recorded (8-11). The expanding population of patients with severe illnesses or immunosuppression, abdominal surgeries, use of broad-spectrum antibiotics, intravenous catheters and parenteral nutrition are factors contributing to the changing epidemiology of these infections (11, 12).

The shift toward non-*albicans Candida* pathogens is accompanied with the emergence of resistance against commonly used antifungal agents rendering the treatment challenging especially in critically ill individuals (9-11, 13). IAC has been associated with poor outcomes in some patients (11, 14). Lack of adequate therapy and source control and severity of illness are the most important determinants of poor outcome (12). Research on IAC has several limitations including diagnosis difficulties due to the clinical heterogeneity of the disease (7, 14, 15). Moreover, treatment of this type of infection is challenging because it is usually based on

previous case reports and experience gained from other forms of fungal disease (5). In addition, the emergence of strains resistant to the available antifungals has further complicated the treatment of IAC (16).

Animal models simulating IAC will provide a useful tool for understanding of disease pathogenicity, host response and evaluation of the therapeutic strategies in the absence of clinical trials (14,17). Since most animal models of candidiasis have been used for disseminated infections through intravenous route (3), the present study aims to provide a model simulating IAC caused by non-*albicans Candida* species in both immunocompetent and immunosuppressed mice via intraperitoneal introduction of pathogen and for *invivo* study of the pathogenicity and evaluation of treatment outcomes.

Material and Methods:

Test strains

Five non-*albicans Candida* strains were selected among isolates obtained from patients previously diagnosed with IAC at Ain Shams University Specialized Hospital (ASUSH), Cairo, Egypt. Isolates were identified using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry analysis (MALDI-TOF MS) (VITEK® MS, bioMérieux Inc., Marcy l' Etoile, France). The *invitro* susceptibility of the strains to fluconazole, voriconazole, caspofungin, micafungin, amphotericin B and flucytosine was determined using the Vitek2 system (bioMérieux Inc., Marcy l' Etoile, France). (This was part of a surveillance

Table 1: non-albicans *Candida* species used in this study

<i>Candida</i> species	Clinical origin	Type of IAI	Patient type	AUMC no.
<i>C. glabrata</i>	Peritoneal fluid	Secondary peritonitis	Liver transplantation	13949
<i>C. parapsilosis</i>	Peritoneal fluid	Secondary peritonitis	Appendectomy	13952
<i>C. lipolytica</i>	Peritoneal fluid	Secondary peritonitis	Liver transplantation	13950
<i>C. tropicalis</i>	Peritoneal fluid	Secondary peritonitis	CAPD	13948
<i>C. guilliermondii</i>	Bile	Cholecystitis/cholangitis	Liver transplantation	13951

IAI = Invasive Abdominal Infection; AUMC = Assiut University Mycological Centre; CAPD = Continuous Ambulatory Peritoneal Dialysis

study on IAC previously presented as an e-poster in the 20th Congress of the International Society of Human and Animal Mycology, ISHAM, Amsterdam, 2018).

MALDI-TOF MS and Vitek2 assays were performed at The Children's Cancer Hospital Egypt 57357 (CCHE), Cairo, Egypt. All the isolates were deposited in the culture collection of Assiut University Mycological Centre (AUMC), Assiut University, Assiut, Egypt, and their numbers and identification are listed in Table 1.

Test animals

Pathogen-free female outbred CD-1 mice aged 4-5 weeks old (22-30 gm) purchased from The Nile Company for Pharmaceuticals and Chemical Industries, Cairo, Egypt; were used for the *in vivo* studies. The test animals were maintained in the animal facility of The Research and Training Centre on Vectors of Diseases, Faculty of Science, Ain Shams University, Cairo, Egypt. They were housed in a non-stressful environment in cages of 5 mice each representing a group and left to acclimatize for one week before infection, with access to food and water *ad libitum*.

Inoculum preparation

The yeast strains were cultured on CandiSelect™ 4 chromogenic media (Bio-Rad, Marnes la Coquette, France) to ensure purity, and then cultured overnight in yeast extract-peptone dextrose broth (YPD) (1% yeast extract, 2% peptone, 2% D-glucose) with shaking at 30°C. Yeast cells were harvested by centrifugation, washed twice with phosphate buffered saline (PBS) and adjusted to the desired concentration of 5×10^8 CFU/ml (3) on a haemocytometer using PBS. Dilutions of the inocula were plated on SDA plates to ensure the correct inoculum was injected.

Infection assay

Each yeast strain was tested on both immunosuppressed and immunocompetent mice. Mice were immunosuppressed by administering cyclophosphamide intraperitoneally at a dose of 200 mg/kg for 3 consecutive days prior to infection. Both immunosuppressed and immunocompetent mice were then infected intraperitoneally. A control group was injected with PBS only. Challenged mice were closely observed daily for signs of the disease. Infected mice were sacrificed following administration of a dose of 200 mg/kg sodium pentobarbitone intraperitoneally when infected mice show severely reduced mobility, inability to reach food or water, weight reduction, hunched posture and fur ruffling.

Antifungal therapy

For all treatments, therapy began 24 hours after infection. According to Vitek2 results for each test strain, treatment was initiated with intraperitoneal dosage of 40 mg/kg of voriconazole (Vfend; VRC, Pfizer Inc., Egypt) once daily for 7 consecutive days. Micafungin (Mycamine; Astellas Toyama Co., Ltd. Japan) was administered intraperitoneally with a dose of 10 mg/kg/day for 6 consecutive days in a 0.2 ml volume. The doses were determined according to previous studies (18, 19). To prevent bacterial infections, mice received 5mg/kg/day ceftazidime subcutaneously.

Fungal burden and histopathology

For tissue burden, mice were sacrificed on day 3 post-infection and one day after treatment was completed in treated groups. The kidney, liver, spleen and pancreas were aseptically excised and washed with sterile PBS and homogenized in 5 ml sterile PBS on ice. The serially diluted homogenates were plated

onto SDA and incubated at 37°C. Colonies were counted to determine the colony-forming units (CFU) per gram tissue. Counts were expressed as log₁₀ CFU per gram tissue. To assess the presence of peritonitis, the peritoneal cavity was washed with PBS, the effluent was collected, centrifuged and the pellet was re-suspended in 600 µl sterile water containing 0.01% bovine serum albumin. The undiluted suspension was plated on SDA plates and the CFU was counted (20).

For histopathological analysis, the liver, kidneys, spleen and pancreas were excised and immediately fixed in 10% neutral buffered formalin and embedded in paraffin. 5 µm sections were cut and stained with haematoxylin and eosin (H&E) for tissue morphology and Periodic Acid-Schiff (PAS) reagent to visualize fungal elements in tissue. Tissue sections were examined using light microscope (B-500T, Optika, Italy) and images were captured using a 3.1-megapixel eyepiece

USB camera (C-B3 Optika, Italy) with Scope Image 9.0 software.

Statistical analysis

Data were analyzed using IBM® SPSS® Statistics for Windows, Version 25.0 (2017, Armonk, NY: IBM Corp.) A one-way analysis of variance (ANOVA test) was used when comparing more than two means. For multiple comparisons between different variables, Post Hoc tests: Tukey HSD was done and *p*<0.05 was considered significant.

Results:

Five non-*albicans Candida* species used in this study were *C. glabrata*, *C. parapsilosis*, *C. lipolytica*, *C. tropicalis* and *C. guilliermondii*. Survival studies revealed that *C. glabrata* and *C. tropicalis* caused the highest mortality in both immunosuppressed and immuno-competent models (Table 2).

Table 2: Mean survival time (days), survival range (days) and mortality rates (%) among different study groups

	<i>C. glabrata</i>	<i>C. parapsilosis</i>	<i>C. lipolytica</i>	<i>C. tropicalis</i>	<i>C. guilliermondii</i>
<i>Immunosuppressed group</i>					
Mean ± SD	7.20±2.17	7.60±1.34	32.6±16.7 ^a	7.80±1.48	9.80±1.92
Range	(5-10)	(6-9)	(14-45)	(6-10)	(8-13)
Mortality %	100	100	40	100	100
<i>Immunosuppressed-treated group</i>					
Mean ± SD	29.00±14.68	32.60±16.99	40.00±11.18	39.60±12.07	33.00±16.45
Range	(16-45)	(13-45)	(20-45)	(18-45)	(14-45)
Mortality %	60	40	20	20	40
<i>Immunocompetent group</i>					
Mean ± SD	11.80±1.64 ^b	25.8±17.54	45.00±0.00 ^c	11.40±1.14 ^d	32.00±17.80 ^e
Range	(10-14)	(12-45)	45	(10-13)	(12-45)
Mortality %	100	60	0	100	40
<i>Immunocompetent-treated group</i>					
Mean ± SD	45.00±0.00 ^f	45.00±0.00	45.00±0.00	45.00±0.00 ^g	45.00±0.00
Range	45	45	45	45	45
Mortality %	0	0	0	0	0

^a vs other immunosuppressed *Candida* groups, *p*≤0.001.

^{b, d} vs *C. lipolytica* and *C. guilliermondii* immunocompetent infected groups, *p*<0.05.

^{c, e} vs *C. glabrata* and *C. guilliermondii* immunocompetent infected groups, *p*<0.001 and *p*<0.05 respectively.

^f vs *C. glabrata* immunocompetent infected group, *p*<0.001.

^g vs *C. tropicalis* immunocompetent infected group, *p*<0.001.

Candida parapsilosis and *Candida guilliermondii* caused 100% mortality rates among immunosuppressed mice but a relatively lower mortality rates among immunocompetent ones (60% and 40% respectively). *Candida lipolytica* caused the lowest mortality rates in both immunosuppressed and immunocompetent groups. From the study of mean survival time, *C. lipolytica* infected immunosuppressed mice had

significantly longer survival time ($p < 0.001$) while there was no significant difference in the mean survival time among other *Candida* species. Among the immunocompetent groups, mice infected with *C. lipolytica* and *C. guilliermondii* had the highest mean survival time while those infected with *C. glabrata* and *C. tropicalis* had the least mean survival time (Fig. 1).

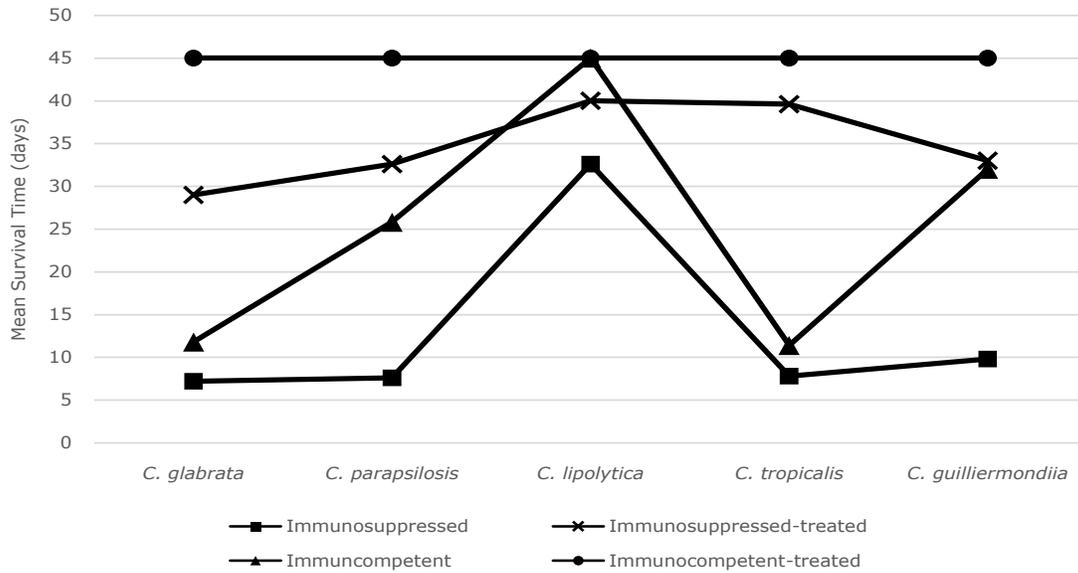


Fig. 1: Mean Survival Time of infected groups before and after treatment

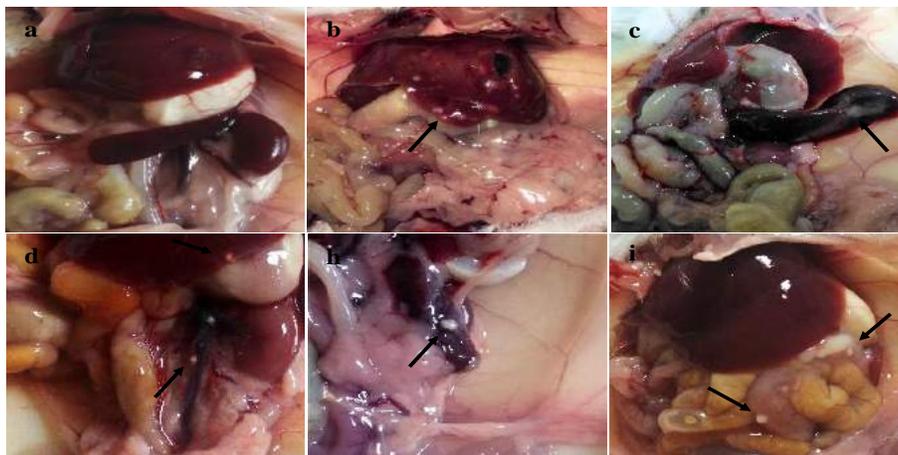


Fig. 2: Gross morphology of intra-abdominal organs of mice infected with *Candida* species. (a) normal control (b) multiple liver abscess (arrows) caused by *C. glabrata*, (c) severe spleen enlargement (arrows) due to *C. tropicalis* infection, (d) liver and mesenteric abscesses (arrows) caused by *C. parapsilosis* and (h) & (i) abscess formation (arrows) by *C. guilliermondii* and *C. lipolytica* respectively

On gross morphology of organs (Fig. 2), hepatosplenomegaly was the first notable sign in nearly all the infected immunosuppressed mice in all *Candida* groups. Enlarged, mottled, and markedly congested liver with multiple abscesses of varying sizes were observed especially in *C. glabrata*. However, some immunocompetent mice showed macroscopically normal livers even though microscopic abnormalities were seen on histological examination. Significant splenic enlargement was observed in all groups, with some mice showing severe congestion of the spleen accompanied with abscesses and white patches. Kidneys were slightly enlarged with white patches in some infected animals and color change in others. Few pancreatic abscesses were observed only in immunosuppressed animals infected with *C. tropicalis*. Abscesses were also observed on the peritoneum and mesenteric membranes.

Haematoxylin and Eosin (H&E) tissue preparations (Fig.3) revealed that kidneys from

all infected groups exhibited variable pathological changes represented in glomerular and tubular damage with marked infiltration of lymphocytes and interstitial haemorrhage. Liver sections also showed disorganized hepatic parenchyma with congestion and dilation of blood sinusoids and inflammatory cells infiltration, and diffuse Kupffer cells was observed between sinusoids and around congested central veins. Spleen of infected mice showed extravasation of RBC's with multifocal granulomatous inflammation and abundant neutrophilic infiltration. Pancreatic tissue preparations from *C. tropicalis* infected immunosuppressed mice revealed scanty epithelial cells with mixed acute and chronic inflammatory infiltrates (Fig. 4).

PAS-stained tissue sections confirmed the presence of extensive fungal elements in all organs of both immunocompetent and immunosuppressed infected groups (Fig. 5).

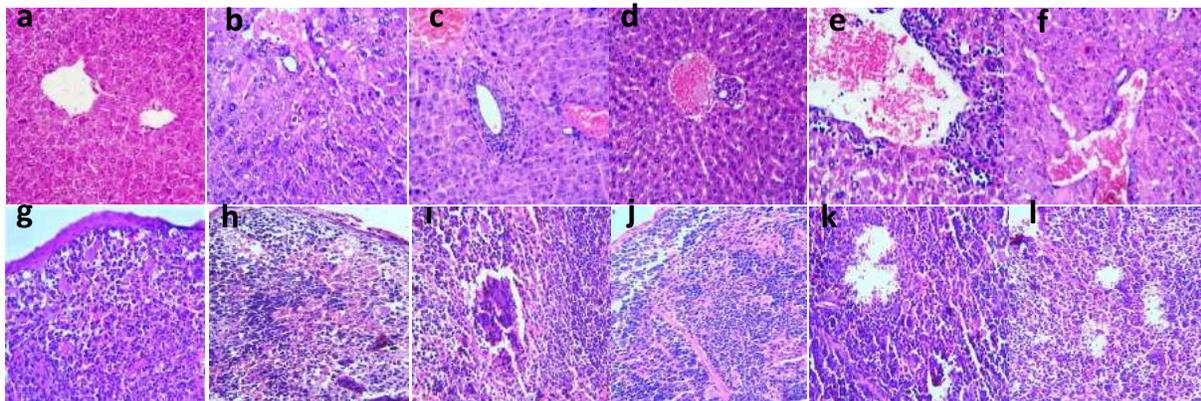


Fig. 3: Photomicrograph of the liver and spleen sections from different *Candida* groups. (a) Control mouse liver section, (g) Control spleen section, (b, h) liver and spleen sections from *C. glabrata* group, (c, i) liver and spleen sections from *C. parapsilosis* group, (d, j) liver and spleen sections from *C. lipolytica* group, (e, k) liver and spleen sections from *C. tropicalis* group, (f, l) liver and spleen sections from *C. guilliermondii* group (H&E 400x)

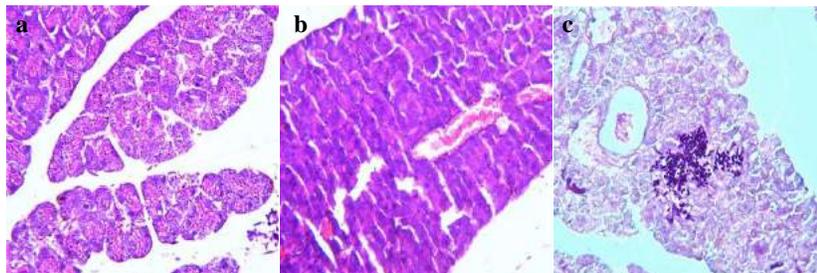


Fig. 4: photomicrograph of pancreatic tissue preparations, (a) control pancreas mouse section (H&E 400x), (b) pathological changes caused by *C. tropicalis* (H&E 400x), (c) fungal elements of *C. tropicalis* (PAS 400x)

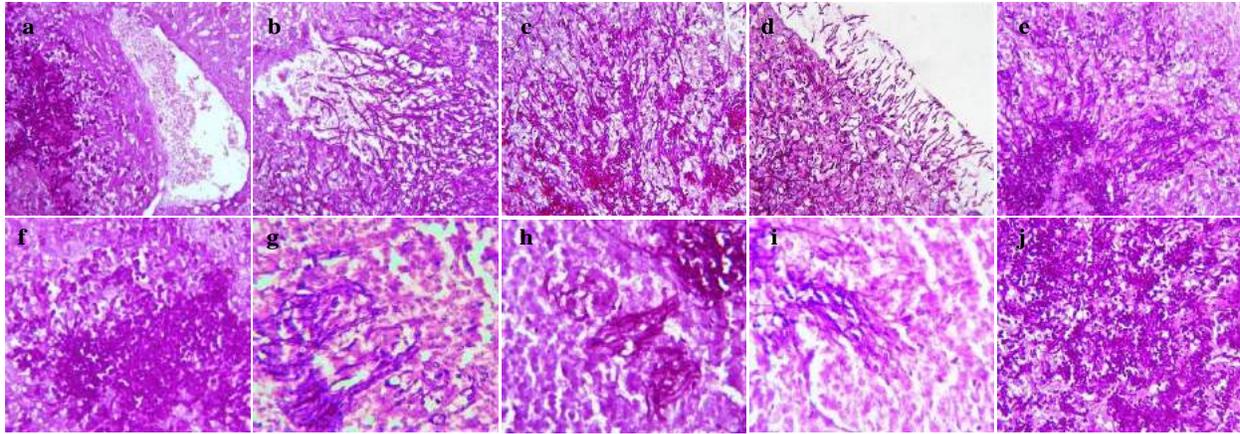


Fig. 5: Photomicrograph showing fungal elements in liver (a-e) and spleen (f-j) tissue preparations of infected mice. (a,f) *C. glabrata*, (b,g) *C. parapsilosis*, (c,h) *C. lipolytica*, (d,i) *C. tropicalis*, (e,j) *C. guilliermondii*. (PAS 400x)

Table 3: Fungal tissue burden of infected mice in all groups

Organ	Fungal Tissue Burden (Log ₁₀ CFU/gm)				
	<i>C. glabrata</i>	<i>C. parapsilosis</i>	<i>C. lipolytica</i>	<i>C. tropicalis</i>	<i>C. guilliermondii</i>
<i>Immunosuppressed</i>					
Kidney	6.60 ± 0.78 ^a	5.45 ± 0.17	3.86 ± 0.29 ^d	5.21 ± 0.45	5.19 ± 0.30
Liver	5.80 ± 0.67 ^b	4.94 ± 0.33	3.70 ± 0.47 ^e	4.48 ± 0.19	4.52 ± 0.37
Spleen	6.48 ± 0.69 ^c	5.22 ± 0.32	3.54 ± 0.27 ^f	5.14 ± 0.30	5.02 ± 0.36
Pancreas	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	2.09 ± 0.50 ^h	0.00 ± 0.00
Peritoneal wash	3.77 ± 0.35	3.60 ± 0.40	2.53 ± 0.3 ^g	3.83 ± 0.12	3.53 ± 0.05
<i>Immunosuppressed-treatedⁱ</i>					
Kidney	2.19 ± 0.99	1.64 ± 0.32	1.43 ± 0.21	1.69 ± 0.49	1.74 ± 0.37
Liver	1.92 ± 0.83	1.33 ± 0.29	1.52 ± 0.10	1.43 ± 0.33	1.37 ± 0.31
Spleen	2.15 ± 1.15	1.38 ± 0.34	1.57 ± 0.36	1.55 ± 0.52	1.88 ± 0.47
Pancreas	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Peritoneal wash	1.27 ± 0.06	1.22 ± 0.24	1.14 ± 0.06	1.07 ± 0.05	1.08 ± 0.63
<i>Immunocompetent</i>					
Kidney	4.97 ± 0.56 ^j	3.69 ± 0.19	2.26 ± 0.10 ⁿ	3.54 ± 0.35	3.72 ± 0.85
Liver	4.82 ± 0.15 ^k	3.53 ± 0.17	2.28 ± 0.12 ^o	3.20 ± 0.19	3.57 ± 0.26
Spleen	4.67 ± 0.08 ^l	3.63 ± 0.27	2.33 ± 0.07 ^p	3.65 ± 0.33	3.66 ± 0.10
Pancreas	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Peritoneal wash	3.86 ± 0.08 ^m	3.37 ± 0.06	2.16 ± 0.09 ^q	3.12 ± 0.14	3.41 ± 0.21
<i>Immunocompetent-treated^r</i>					
Kidney	0.09 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Liver	0.03 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Spleen	0.03 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Pancreas	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Peritoneal wash	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

(a-c) vs other immunosuppressed groups; $p \leq 0.005$, $p < 0.05$, $p \leq 0.001$ respectively
 (d-g) vs other immunosuppressed groups; $p \leq 0.001$, $p < 0.05$, $p < 0.001$ respectively
 (h) vs other immunosuppressed groups; $p < 0.001$
 (i) vs immunosuppressed groups; $p < 0.001$
 (j-m) vs other immunocompetent groups; $p < 0.001$
 (n-q) vs other immunocompetent groups; $p < 0.001$
 (r) vs immunocompetent groups; $p < 0.001$

Fungal burden results among immunosuppressed mice (Table 3) showed that *C. glabrata* had significantly higher kidney, liver and spleen burdens than other *Candida* species (Fig. 6). *Candida parapsilosis*, *C. tropicalis* and *C. guilliermondii* had statistically similar fungal burden in the kidneys but higher *C. guilliermondii* spleen burdens.

All tested *Candida* species were capable of causing peritonitis with no significant difference between their peritoneal wash burdens. *C. lipolytica* showed the least fungal burden of all organs among immunosuppressed mice. Of note, only *C. tropicalis* displayed pancreatic fungal burden.

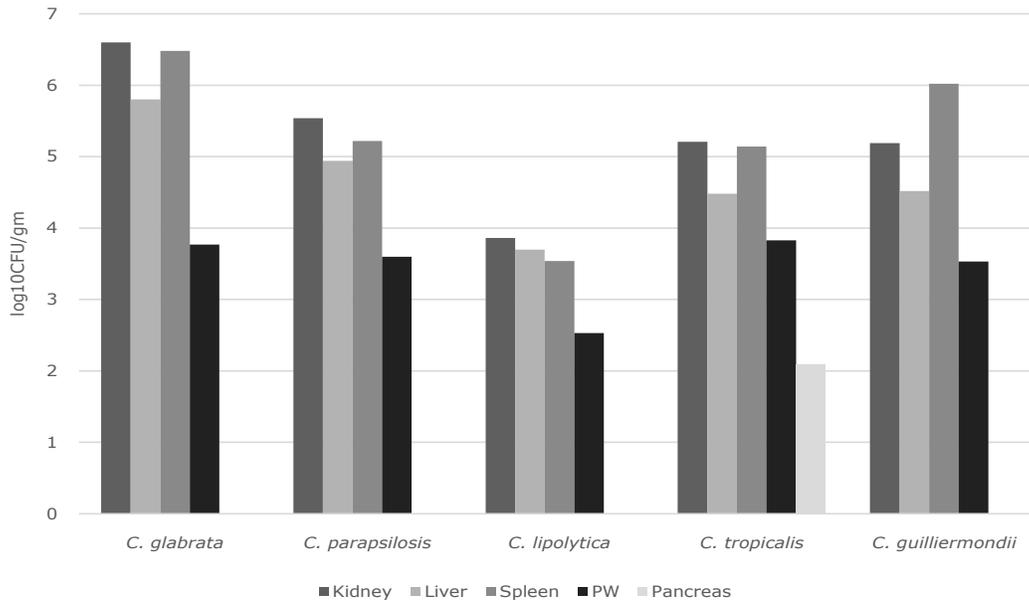


Fig. 6: Fungal tissue burden among immunosuppressed groups

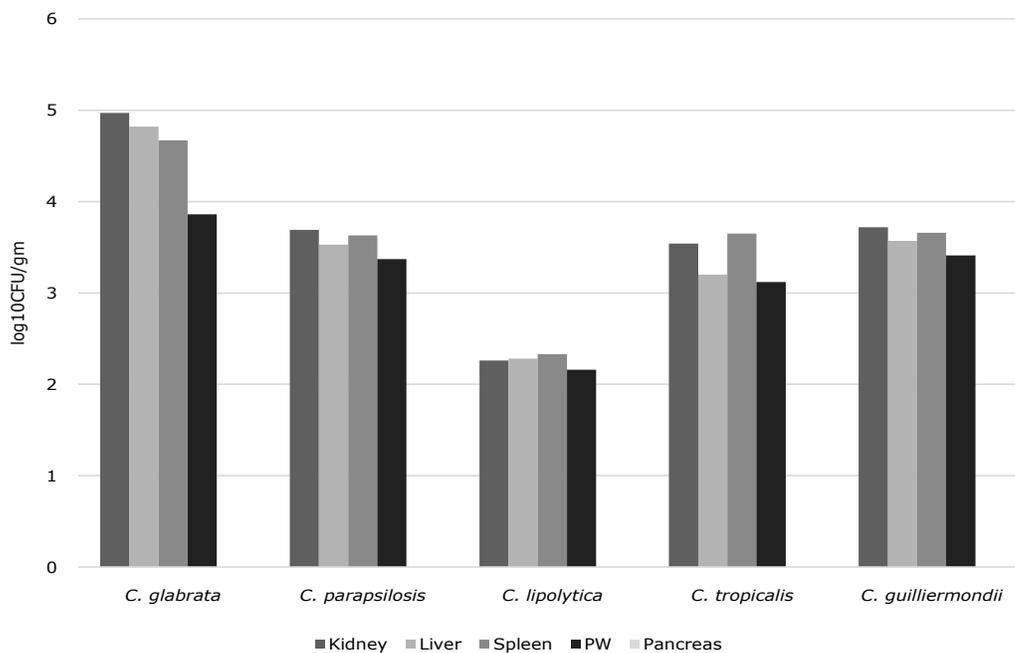


Fig. 7: Fungal Burden among immunocompetent groups

Comparatively, kidneys, liver and spleen, of *C. glabrata* and *C. lipolytica* infected animals had the highest and lowest fungal tissue burden respectively among immunocompetent mice (Fig. 7). The peritoneal effluent burdens of *C. glabrata*, *C. parapsilosis* and *C. guilliermondii* were similar statistically but much higher than those of *C. tropicalis* and *C. lipolytica*. None of the tested organisms was able to initiate infection in the pancreas in immunocompetent mice. Generally, immunosuppressed mice had significantly higher tissue burdens than immunocompetent ones ($p < 0.001$) except for liver burdens in *C. glabrata* infected groups and peritoneal effluent counts in *C. glabrata*, *C. parapsilosis* and *C. guilliermondii* where no significant difference was recorded.

Liver and spleen represented the most affected intra-abdominal site of infection with no significant difference between their tissue burdens in immunosuppressed mice infected with *C. glabrata*, *C. parapsilosis* and *C. lipolytica*. Similarly, in immunocompetent models, *C. glabrata*, *C. parapsilosis* and *C. lipolytica* had statistically similar tissue burdens in the liver, spleen and peritoneal effluent. In case of *C. tropicalis*, spleen had the highest fungal burden followed by liver and peritoneal fluid respectively in both immunosuppressed and immunocompetent models. Similarly, in *C. guilliermondii* infected neutropenic mice, spleen was the most affected organ, while there was no significant difference between all organs among immunocompetent mice. Of note, the least fungal burdens were observed

peritoneal effluent in almost all tested *Candida* species. The fungal disease progressed to the kidneys in both immunosuppressed and immunocompetent models of all *Candida* species. The Kidneys shared statistically similar fungal burdens with infected intra-abdominal organs in all *Candida* species and higher burdens than the liver in *C. tropicalis* and *C. guilliermondii* infected immunosuppressed models and the spleen in *C. glabrata* immunocompetent models.

Treatment was started for both immunosuppressed and immunocompetent models, using voriconazole for groups infected with *C. parapsilosis*, *C. lipolytica* and *C. guilliermondii* and micafungin for *C. tropicalis* and *C. glabrata* groups from the Vitek 2 system results (Table 4). All *Candida* species caused relatively high mortality (20 - 60%) in spite of the significant increase in the mean survival time after receiving treatment in all immunosuppressed mice. No significant difference in the mean survival time between immunosuppressed mice infected with different *Candida* strains after treatment.

In the treated immunocompetent model, all the tested animals survived the entire duration of the experiment, however, the increase in survival time compared to untreated groups was significant only for *C. glabrata* and *C. tropicalis*. Also, the difference in the mean survival time between treated immunosuppressed and treated immunocompetent mice was significant in case of *C. glabrata* only.

Table 4: Antifungal susceptibility of the tested strains using Vitek2 System

	FLU	VOR	CAS	MIC	AB	AFC
<i>C. glabrata</i>	≥64	≥8	≤0.25	≤0.06	0.5	≤1
<i>C. parapsilosis</i>	2	≤0.12	1	1	≤0.25	≤1
<i>C. lipolytica</i>	2	≤0.12	0.5	0.5	≤0.25	≤1
<i>C. tropicalis</i>	≤1	≤0.12	≤0.25	≤0.06	≤0.25	≤1
<i>C. guilliermondii</i>	16	0.25	1	2	8	≤1

FLU: Fluconazole; VOR: Voriconazole; CAS: Caspofungin; MIC: Micafungin; AB: Amphotericin B; AFC: flucytosine

Interestingly, despite the significant reduction of fungal burden among immunosuppressed animals to voriconazole and micafungin, their fungicidal effect (i.e., organ sterilization) was never observed. Also, groups of immunosuppressed mice infected with different *Candida* species had statistically similar fungal burdens in all organs after receiving treatment. For each *Candida* strain, there was no significant difference between tissue burdens of different organs, except for *C. glabrata* where kidneys had the highest burden of all organs. In contrast, organ homogenates from immunocompetent mice showed no detectable growth after treatment except the kidneys, liver and spleen of *C. glabrata* infected mice.

Discussion:

This model aims to study the pathogenicity and *in vivo* susceptibility of non-albicans *Candida* species associated with IAC in human to predict the frequency of infections, the outcome of clinical disease and response to antifungal therapy. By mimicking clinical situations in humans as closely as possible, experimental non-albicans *Candida* infections were induced in both immunocompetent and immunosuppressed mice intraperitoneally with *Candida* strains associated with human disease isolated from cases of abdominal surgeries, liver transplantation and continuous ambulatory peritoneal dialysis (CAPD).

Our results showed persistent infection in the peritoneal cavity with peritonitis, hepatosplenomegaly and abscess formation upon infection with non-albicans *Candida* species, especially in immunosuppressed animals. Liver and spleen were the main intra-abdominal sites of infection and pancreas was affected only in case of *C. tropicalis*-infected immunosuppressed mice. This result is at variance with a previous intra-abdominal model of infection (3), where abscess formation on intra-abdominal organs was the main manifestation and pancreas being the most predominant site of involvement in the case of intraperitoneal injection of *C. glabrata* along with sterile feces. Cheng et al., (3) also reported that neutropenic mice injected with *C. glabrata* alone did not develop abscess which was not the case in our model, where abscesses developed upon challenging mice with different *Candida* species using the same inoculum (5×10^8 CFU/ml) without feces. In spite of its retroperitoneal position and the inconsistency reported in previous studies (3),

the kidney was included in our study as an indicator to assess progression of infection following intraperitoneal introduction of the fungal pathogen. The disease progressed to the kidneys with similar or higher burdens of fungal pathogens detected in the kidneys than intra-abdominal organs.

When comparing the virulence of the non-albicans *Candida* species tested in the present study in terms of mortality rates, survival time and tissue burden at predetermined points after infection, it was shown that among both naïve and immunosuppressed mice, *C. glabrata* was the most virulent species followed by *C. tropicalis* which had similar survival rates and mean survival time but less tissue burden than *C. glabrata*. *C. parapsilosis* and *C. guilliermondii* shared almost similar virulence, while *C. lipolytica* was the least virulent of all. Our results are at variance with the study conducted by Arendrup et al., (21), where *C. tropicalis* was reported to be more virulent than *C. glabrata* in an experimental model that compared different medically important *Candida* species

Previous animal model studies reported low virulence of *C. glabrata* with inability to cause severe illness or mortalities even with high intravenous inoculum (21-23). Our study reports the ability of *C. glabrata* to cause acute infection with high mortality in both immunosuppressed and immunocompetent animals. *C. tropicalis* was previously reported to be highly virulent even in immunocompetent mice (21, 24). These findings agree with our results where 100% mortality rate in both immunocompetent and immunosuppressed mice models and high tissue burdens were recorded. Many studies have reported the low virulence of *C. parapsilosis* with the fungi failing to initiate infection even in immunosuppressed mice (21,24,25). However, de Bernardis et al., (26) reported that *C. parapsilosis* could initiate infection when high inoculum is administered. Similarly, *C. guilliermondii* and *C. lipolytica* was reported to be weak, low-virulent fungi pathogens causing no mortalities among infected animals (21, 27-29). Interestingly, our study showed high virulence of *C. parapsilosis* and *C. guilliermondii* with mortalities in immunosuppressed and immunocompetent mice which explained their high incidence in our last survey on IAC, where they represented the most prevalent non-albicans *Candida* species (30). Although *C. lipolytica* was the weakest pathogen in terms of mortality rates, survival time and tissue burdens in both

immunocompetent and immunosuppressed mice in our study, it caused mortality in immunosuppressed mice only, and lower than those caused by other tested species.

An increasing concern is being expressed about the emergence of antifungal resistance with accompanying increase in the prevalence of non-*albicans* *Candida* species among critically ill patients (9). Amphotericin B, fluconazole and echinocandins are the most commonly used antifungals in treating IAC (14). In our study, amphotericin B and fluconazole were excluded from treatment options according to the *invitro* susceptibility results. Micafungin was used *invivo* to treat mice infected with *C. tropicalis*, which reduced the fungal burden in all organs, and prolonged the survival time to some degree, however, it was not able to significantly reduce mortality rates or sterilize all organs in our immunosuppressed model. This finding agrees with the results of other studies, where micafungin did not completely resolve infections with *C. glabrata* (19, 31) and *C. tropicalis* (32).

Voriconazole was used in our study to treat *C. parapsilosis*, *C. lipolytica* and *C. guilliermondii*, which lowered the mortality rate and tissue burden in the mice organs. Zhao et al., (33) reported that fluconazole, itraconazole and posaconazole should not be used to treat *C. lipolytica*, but voriconazole may be useful for treatment. Our results agree with a previous experimental murine infection by *C. guilliermondii* where voriconazole was reported to be experimentally effective (34). Previous studies also reported that *C. parapsilosis* exhibited good susceptibility to voriconazole *in vitro* (35,36). However, voriconazole failed to completely eradicate the infection in immunosuppressed mice in our study.

In conclusion, IAC in Egypt is witnessing predominance of non-*albicans* *Candida* with species distribution and pathogenicity different from those reported worldwide. Further studies involving larger number of strains, tested with different inocula and several antifungal agents against each strain should be conducted to better understand the pathogenesis of the disease and predict treatment outcomes in clinical practice.

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Conflict of interest:

Authors declare no conflict of interest.

Compliance with ethical standards:

All applicable international, national, and/or institutional guidelines and ethical standards for the care and use of animals were followed.

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Original Article

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Correlation of methicillin resistance and virulence genes of *Staphylococcus aureus* with infection types and mode of acquisition in Sofia, Bulgaria

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Abstract:

Background: Infections due to methicillin resistant *Staphylococcus aureus* (MRSA) which is the most virulent species among the staphylococci have become a global health challenge. The aim of this study was to assess the correlation of genes encoding virulence and methicillin resistance in invasive and non-invasive isolates from inpatients/outpatients with staphylococcal infections in Sofia, Bulgaria.

Materials and methods: Non-duplicate *S. aureus* isolates were recovered from clinical samples obtained from a total of 368 in-patients with healthcare-associated infections and outpatients with community acquired infections, following overnight cultures of samples on Columbia agar with 5% sheep blood at 35°C. The isolates were presumptively identified by colony and Gram stain morphology, positive catalase reaction and plasma-coagulase test. Isolates were screened for methicillin resistance by the cefoxitin disk method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocol. The *mecA* and *mecC*, and 12 staphylococcal virulence genes were detected by a combination of monoplex and multiplex polymerase chain reaction (PCR) assays.

Results: The prevalence of MRSA based on carriage of *mecA* gene was 12%; 7.7% for outpatients and 16.2% for inpatients ($p < 0.05$). The frequency of toxin genes detection in the staphylococcal isolates were as follows; *sei* (72.6%), *seb* (59.8%), *seh* (41.3%), *sec* (38.3%), *seg* (37.5%), *sej* (32.3%), *sea* (26.6%), *sed* (10.3%), *tst* (6.5%), and *see* (4.3%). The virulence genes, *tst*, *sea*, *seb*, *sec*, *seg*, *seh* and *sei* were more frequently associated with MRSA than methicillin sensitive (MSSA) strains ($p < 0.05$). About one-third of the clinical *S. aureus* isolates harbored seven virulence genes; *sea*, *seb*, *sec*, *see*, *seg*, *seh* and *sei*, that were detected significantly more among the invasive isolates ($p < 0.05$).

Conclusions: This study shows the occurrence of highly virulent staphylococcal isolates in our geographical region.

Key words: *Staphylococcus aureus*, virulence, methicillin resistance

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Corrélation des gènes de résistance à la méthicilline et de virulence de *Staphylococcus aureus* avec les types d'infection et le mode d'acquisition à Sofia, Bulgarie

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Abstrait:

Contexte: Les infections dues à *Staphylococcus aureus* résistant à la méthicilline (SARM) l'espèce la plus virulente parmi les staphylocoques, sont devenues un problème de santé mondial. Le but de cette étude était

d'évaluer la corrélation des gènes qui codent pour la virulence et la résistance à la méthicilline avec des isolats invasifs ou non invasifs de patients hospitalisés/ambulateurs infectés par le staphylocoque dans Sofia, Bulgarie.

Matériels et méthodes: Des isolats de *S. aureus* non dupliqués ont été récupérés à partir d'échantillons cliniques prélevés chez 368 patients atteints d'infections associées aux soins de santé et de patients ambulatoires présentant des infections acquises en communauté, après avoir effectué des cultures pendant la nuit d'échantillons sur de la gélose Columbia contenant 5% de sang de mouton à 35°C. Les isolats ont été présumés identifiés par la morphologie de la colonie et de la coloration de Gram, la réaction positive à la catalase et le test plasma-coagulase. Les isolats ont été criblés pour la résistance à la méthicilline par la méthode de la céfoxitine selon le protocole du Comité européen sur les tests de sensibilité aux antimicrobiens (EUCAST). Les gènes *mecA* et *mecC*, ainsi que 12 gènes de virulence staphylococcique ont été détectés par une combinaison de tests de réaction en chaîne de la polymérase (PCR) monoplex et multiplex.

Résultats: La prévalence de SARM basée sur le portage du gène *mecA* était de 12%; 7,7% pour les patients ambulatoires et 16,2% pour les patients hospitalisés ($p < 0,05$). La fréquence de détection des gènes de toxines dans les isolats de staphylocoques était la suivante: *sei* (72,6%), *seb* (59,8%), *seh* (41,3%), *sec* (38,3%), *seg* (37,5%), *sej* (32,3%), *sea* (26,6%), *sed* (10,3%), *tst* (6,5%) et *see* (4,3%). Les gènes de virulence, *tst*, *sea*, *seb*, *sec*, *seg*, *seh* et *sei* étaient plus fréquemment associés à SARM que les souches sensibles à la méthicilline (MSSA) ($p < 0,05$). Environ un tiers des isolats cliniques de *S. aureus* portaient sept gènes de virulence; *sea*, *seb*, *sec*, *see*, *seg*, *seh* et *sei*, qui ont été détectés significativement plus parmi les isolats invasifs ($p < 0,05$).

Conclusion: Cela crée un risque de propagation d'isolats très virulents dans la région géographique

Mots-clés: *Staphylococcus aureus*, virulence, résistance à la méthicilline

Introduction:

Staphylococcus aureus is a facultative anaerobic gram-positive coccus, with grape-like arrangement. This bacterial species is a clinically relevant human and animal pathogen. In humans, *S. aureus* is a major cause of both community acquired (CA) and nosocomial or healthcare-associated (HA) infections such as skin, soft tissue and bone infections, postoperative wound infections, upper respiratory tract infections, pneumonia, bacteraemia and endocarditis (1-5). *S. aureus* is also among the most common pathogens causing food-borne diseases (6-10) and is associated with suppurative diseases in animals such as mastitis, septic arthritis, endocarditis, osteomyelitis, urinary tract infections and sepsis (7, 11, 12).

In many cases of staphylococcal infections, the clinical manifestations are associated with production of toxins, of which the most notable ones are toxic shock syndrome toxin 1 (TSST-1), heat-stable pyrogenic enterotoxins (SEs) responsible for food poisoning with 20 serologically distinct types, and epidermolytic (exfoliative) toxins (ET) responsible for scalded skin syndrome (SSS), occurring in many forms as ETA, ATB and ATD (6, 13-16). The most severe invasive and fatal infections caused by *S. aureus* are due to the cumulative effects of various extracellular products such as enzymes (thermonuclease, hyaluronidase, lipase), haemolysins/cytolysins, and other toxins, especially those exhibiting super-antigen activity (3, 6, 13).

The super-antigens (SAGs), TSST-1 and SEs, which stimulate polyclonal proliferation of T lymphocytes and non-effective immune responses are the most important toxigenic determinants and

virulence mediators in *S. aureus* infections (6, 10, 17, 18).

Methicillin resistant *S. aureus* (MRSA) can form a strong biofilm and easily colonize mucosa, endovascular catheters and/or implants, which is one reason why it causes chronic, recurrent or invasive nosocomial infections (4, 19, 20). This aim of this study is to assess the correlation between methicillin resistance, and virulence genes encoding predominantly SAGs, with invasive or non-invasive and CA or HA types of infection due to *S. aureus*.

Materials and methods:

Study setting and isolation of *S. aureus*

This study was conducted in three University Hospitals in Sofia, Bulgaria during the period September 2016 and November 2018. A total of 368 non-duplicate *S. aureus* were isolated from various human clinical samples obtained from in-patients with HA infections (185 isolates) and outpatients with CA infections (183 isolates), after overnight cultures of clinical samples on Columbia agar with 5% sheep blood (Himedia, India) at 35°C in aerobic atmosphere.

S. aureus was presumptively identified by colony and Gram stain morphology, positive catalase reaction and plasma-coagulase test (Rabbit Plasma, Himedia, India). Detailed biochemical identification, where necessary, was done with Crystal GP (Beckton Dickinson, Germany). Isolates were screened for methicillin resistance by the cefoxitin disk method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (<http://www.eucast.org>) (21). The isolates were stored in skim milk at -70°C until use.

Prior to the commencement of genotypic characterization, the isolates were sub-cultured three times on Brain Heart Infusion (BHI) agar (Himedia, India).

DNA extraction

Genomic DNA of pure cultures of isolates and control strains grown overnight on BHI agar (Himedia, India) at 35°C in O₂ containing atmosphere, were extracted using a DNAso₂-AM nucleic acid extraction kit (AmpliSens) in accordance with the manufacturer's instructions.

Polymerase chain reaction (PCR) assay

All phenotypically identified *S. aureus* strains were confirmed by PCR assay using species-specific primers targeting *Sau* 327 and *Sau* 1645 23S rRNA genes (8, 12). All confirmed *S. aureus* strains were then screened for carriage of *mecA* and *mecC* genes which encode abnormal penicillin binding proteins of the staphylococcal cell wall of MRSA, and for 12 virulence genes using primers as shown in Table 1. The primer sequences were verified for specificity using the Basic Local Alignment Search Tool (BLAST) program available at the NCBI website, Bethesda, Maryland, USA (<http://www.ncbi.nlm.nih.gov/BLAST>).

PCR was performed in a 25 µl reaction mix. DNA was amplified after optimization of the reaction using the following protocol; initial denaturation at 95°C for 3 minutes followed by denaturation at 95°C for 35 s; annealing at 54-63°C depending on primers (Table 1), extension at

72°C for 1 minute 30 s; and final extension at 72°C for 7 minutes. Prior to multiplex PCR assay, each gene was individually amplified (monoplex PCR). After successful reaction and visualization of amplicons in the monoplex PCR, mixes with two or more pairs of primers were prepared and amplifications were repeated by multiplex PCR.

Four sets of multiplex PCR were used to detect the genes of interest with the following mixes; Mix I contain primers for *hlg*, *sea*, *sed* and *tst* genes; Mix II contains primers, *Sau* 327 and *Sau* 1645 for specific *S. aureus* 23S rRNA gene, and primers for enterotoxin *seg*; Mix III contains primers for *seh*, *see* and *sej* genes; and Mix IV contains primers for *cna*, *seb*, *sec*, and *sei* genes. Monoplex PCR was used for Mix V and Mix VI which contains primers for *mecA* and *mecC* respectively, according to the criteria and protocols (4, 22) presented in Table 1.

S. aureus ATCC 29213 was used as a negative control strain for methicillin resistance (MSSA), and *S. aureus* ATCC 43300 and ATCC BAA2312 were used as positive control strains (MRSA) for *mecA* and *mecC* respectively. The amplified genes were separated on 1.5% agarose gel for 25-35 minutes at 140V, stained with ethidium bromide (0.5µg/mL) and visualized by UV trans-illumination (wavelength 312 nm). The PCR products were identified on the basis of their expected fragment size as presented in Table 1.

Table 1: The primers sequences and amplification conditions for detection of virulence genes of Bulgarian human isolates of *Staphylococcus aureus*

Primers	Oligonucleotide sequence (5'-3')	Annealing	Product (bp)	Reference
<i>Sau</i> 327	GGACGACATTAGACGAATCA-			
<i>Sau</i> 1645	CGGGCACCTATTTTCTATCT-	63°C	1318	12
<i>hlg</i> F	GCCAAATCCGTTATTAGAAAATGC			
<i>hlg</i> R	CCATAGACGTAGCAACGGAT	54°C	937	18
<i>cna</i> F	AGTGGTTACTAATACTG			
<i>cna</i> R	CAGGATAGATTGGTTTA	55°C	744	18
<i>tst</i> F	GTTGCTTGCGACAACCTGCTACAG			
<i>tst</i> R	TCAAGCTGATGCTGCCATCTGTG	54°C	209	18
<i>sea</i> F	ATGGTTATCAATGTGCGGGTG			
<i>sea</i> R	TGAATACTGTCTTGTGAGCACCA	54°C	344	18
<i>seb</i> F	TGGTATGACATGATGCCTGCAC			
<i>seb</i> R	AGGTAATCTATAAGTGCCTGCCT	55°C	196	18
<i>sec</i> F	GATGAAGTAGTTGATGTGTATGGATC			
<i>sec</i> R	AGATTGGTCAAACCTTATCGCCTGG	55°C	399	18
<i>sed</i> F	CTGAATTAAGTACCCGCGCT			
<i>sed</i> R	TCCTTTTGCAAATAGCGCCTTG	54°C	451	18
<i>see</i> F	AGGTTTTTTCACAGGTCATCC			
<i>see</i> R	CITTTTTTCTCGGTCAATC	55°C	209	8
<i>seg</i> F	AATTATGTGAATGCTCAACCCGATC			
<i>seg</i> R	CTTATATGGAACAAAAGGTAAGTTT	63°C	642	8
<i>seh</i> F	CAATCACATCATATGCGAAAGCAG			
<i>seh</i> R	CATCTACCCAAAACATTAGCACC	55°C	376	8
<i>sei</i> F	CTCAAGGTGATATTGGTGTAGG			
<i>sei</i> R	AAAAAACTTACAGGCAAGTCCATCTC	55°C	577	8
<i>sej</i> F	CATCAGAAGTGTGTTCCGCTAG			
<i>sej</i> R	CTGAATTTTACCATCAAAGGTAC	55°C	142	8
<i>mecA-F</i>	TCC AGA TTA CAA CTT CAC CAG G			
<i>mecA-R</i>	CCA CTT CAT ATC TTG TAA CG	57°C	162	4
<i>mec C-F</i>	TGTTGTAGCAATGTTTCACAC			
<i>mec C-R</i>	CAAGCACCTTAATATCAACGC	59°C	138	22

Statistical analysis

All statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, United States). Results were considered statistically significant at $p < 0.05$.

Results:

Of the 368 Bulgarian *S. aureus* isolates, 168 (45.7%) invasive isolates were obtained from sterile body sites; 34 from blood cultures, 88 from abscesses or soft tissue infections, 14 from middle ear fluids, 13 from sinus punctures, 12 from tracheal aspirates, 4 from joint aspirates, and 3 from bones in cases with osteomyelitis. Two hundred (54.3%) non-invasive isolates were recovered from 109 nasal/nasopharyngeal secretions, 58 from skin lesions (impetigo, furunculosis), 24 from uro-genital tract, and 9 from eye secretions (Table 2).

A total of 44 out of the 368 *S. aureus* were methicillin resistant, giving a MRSA prevalence rate of 12%. All the phenotypic MRSA strains were positive for *mecA* gene but none contained *mecC* gene. Only 7.7% (14 of 368) MRSA were isolated from outpatients, which was significantly less ($p < 0.05$) compared to those from inpatients (16.2%, 30 of 368) (Table 3). The distribution of virulence genes in both MRSA and MSSA isolates as well as among inpatients with HA infection, and outpatients with CA infections, is presented in Table 3.

The most prevalent virulence gene in all the groups was *hlg*, which encodes a pore-forming gamma-toxin with a wide-spectrum cytolytic effect and found in approximately 100% of the isolates. There was no

significant difference in the frequency of *hlg* gene among patients with CA and HA infections (Table 3), and among invasive and non-invasive infections (Tables 4), but it was dominant in MSSA isolates ($p < 0.05$) (Table 3). The virulence genes; *tst*, *sea*, *seb*, *sec*, *seg*, *seh* and *sei* (except *hlg*) were detected more frequently in MRSA than MSSA isolates ($p < 0.05$) (Table 3). Only two genes, *seb* and *sec*, were predominantly observed in relation to staphylococcal inpatient infections ($p < 0.05$).

MRSA strains were dominant (with 63.6%) among the invasive isolates compared to 36.4% among the non-invasive ones ($p < 0.05$) (Table 4). The SAGs encoding genes; *sea*, *seb*, *sec*, *see*, *seg*, *seh*, and *sei* were detected more frequently among the invasive isolates ($p < 0.05$) (Table 4). In addition, the *cna* gene, encoding a bacterial collagen adhesin with an inhibitory effect on complement activation, was detected in 14.2% of the *S. aureus* isolates, predominantly among in-patients and in invasive infections ($p < 0.05$).

The distribution of the staphylococcal toxin genes detected in this study compared with data from other countries is shown in Table 5. The frequency of virulence genes detected in this study are; *sei* (72.6%), *seg* (67.5%), *seb* (60.3%), *seh* (41.3%), *sec* (38.3%), *sej* (32.3%), *sea* (26.6%), *sed* (10.3%), *tst* (6.5%), and *see* (4.3%). Fig 1 shows the gel electrophoresis picture of the amplified products of *S. aureus mec* and virulence genes in the 4 multiplex PCR reactions (Mixes I-IV) and the two monoplex PCR reactions (Mixes V and VI).

Table 2: Distribution of *Staphylococcus aureus* isolates by clinical specimen types

Specimen types/isolates	No of isolates (%)
Invasive isolates	168 (45.7)
Blood culture	34 (9.2)
Abscess/Soft tissue	88 (23.9)
Middle ear fluid	14 (3.8)
Sinus punctures	13 (3.5)
Trachea aspirates	12 (3.3)
Joint aspirates	4 (1.1)
Bone samples	3 (0.8)
Non-invasive isolates	200 (54.3)
Nasopharyngeal secretion	109 (29.6)
Skin lesion	58 (15.8)
Urogenital tract	24 (6.5)
Eye secretion	9 (2.4)
Total	368 (100)

Table 3: The frequency of virulence genes among MRSA and MSSA isolates from inpatients and outpatients

Genes	MRSA (n=44)			MSSA (n=324)			p value (between MRSA and MSSA)	Total number of <i>S. aureus</i> isolates (n=368)			
	Inpatient % (n=30)	Outpatient % (n=14)	Total (%)	Inpatient (%) (n=155)	Outpatient % (n=169)	Total (%)		Inpatient % (n=183)	Out-patient (%) (n=185)	p value (between Inpatient and Out-patient)	Total (%)
<i>hlg</i>	93.3	100	88.6	98.1	98.8	98.5	0.0031*	95.7	98.9	0.1046	97.3
<i>cna</i>	33.3	0	22.7	23.2	3.6	13.0	0.1035	24.8	3.2	0.0001	14.2
<i>tst</i>	20.0	28.6	22.7	4.5	4.1	4.3	0.0001	7.0	6.0	0.8334	6.5
<i>sea</i>	53.3	85.7	63.6	23.9	19.5	21.6	0.0001	28.6	24.6	0.1257	26.6
<i>seb</i>	86.7	100	90.9	66.5	46.7	56.2	0.0001	69.7	50.8	0.0003	60.3
<i>sec</i>	66.6	57.1	63.6	49.7	21.3	34.9	0.0004	52.4	29.5	0.0004	38.3
<i>sed</i>	10.0	7.1	9.1	12.3	8.3	10.5	1.0000	12.4	8.2	0.2304	10.3
<i>see</i>	0	0	0	7.7	2.4	4.9	0.2350	6.5	2.2	0.0706	4.3
<i>seg</i>	86.7	100	90.9	66.5	69.8	64.2	0.0002	69.7	65.0	0.3741	67.4
<i>she</i>	53.3	85.7	63.6	43.2	33.7	38.3	0.0018	44.9	37.7	0.1703	41.3
<i>sei</i>	90.0	100	93.2	69.7	69.8	69.8	0.0005	73.0	72.1	0.9072	72.6
<i>sej</i>	33.3	50.0	38.6	34.2	29.0	31.5	0.3908	34.0	30.6	0.5049	32.3

*Results were considered statistically significant at $p < 0.05$ - in dark field

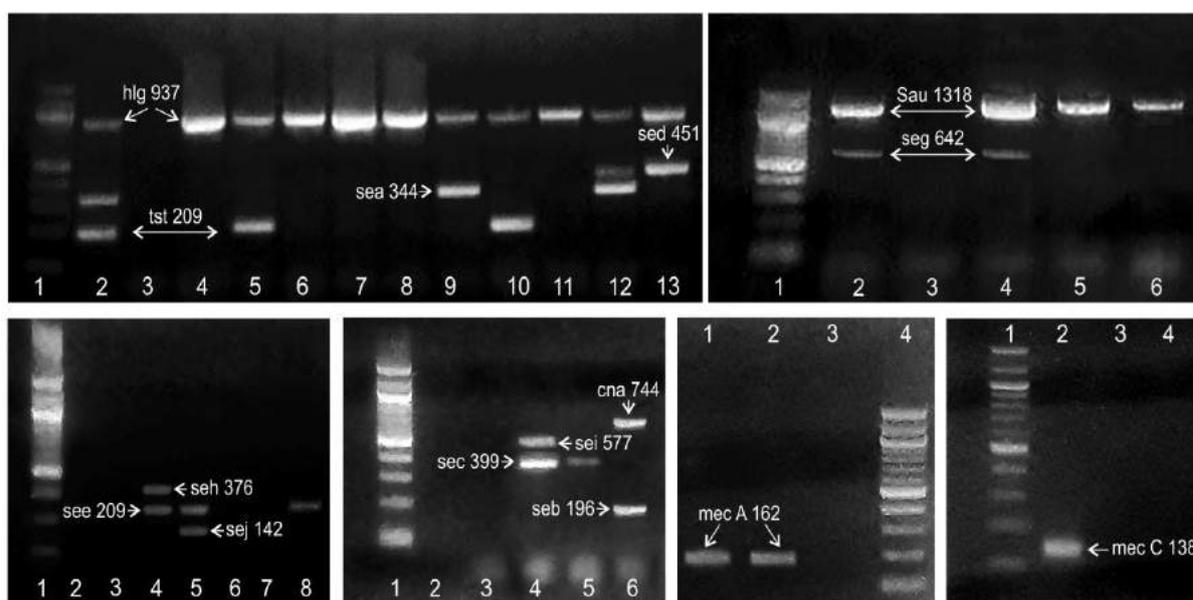
Table 4: The frequency of virulence genes among MRSA and MSSA invasive and noninvasive isolates

Genes	MRSA (n=44)			MSSA (n=324)			p value (between invasive and non-invasive)	Total number of <i>S. aureus</i> isolates (n=368)		
	Invasive isolates % (n=28)	Non-invasive isolates % (n=16)	Total (%)	Invasive isolates (%) (n=140)	Non-invasive isolates % (n=184)	Total (%)		Invasive isolates % (n=168)	Non-invasive isolates % (n=200)	Total (%)
<i>hlg</i>	92.9	81.25	88.6	100.0	78.3	98.5	98.8	96.0	0.1179	97.3
<i>cna</i>	35.7	0	22.7	25.7	3.3	13.0	24.8	3.0	0.0001*	14.1
<i>tst</i>	28.6	12.5	22.7	5.7	3.3	4.3	9.5	4.0	0.0358	6.5
<i>sea</i>	60.7	68.75	63.6	24.3	19.6	21.6	30.4	23.5	0.1159	26.6
<i>seb</i>	96.4	81.25	90.9	72.1	44.0	56.2	76.2	47.0	0.0001	60.3
<i>sec</i>	71.4	50.0	63.6	48.6	24.5	34.9	52.4	26.5	0.0001	38.3
<i>sed</i>	7.1	12.5	9.1	16.4	6.0	10.5	14.9	6.5	0.0098	10.3
<i>see</i>	0	0	0	8.6	2.2	4.9	7.1	2.0	0.0201	4.3
<i>seg</i>	85.7	100.0	90.9	77.9	53.8	64.2	79.2	57.5	0.0001	67.4
<i>seh</i>	57.1	85.0	63.6	43.2	31.0	38.3	49.4	34.5	0.0042	41.3
<i>sei</i>	100.0	81.25	93.2	47.9	64.1	69.8	81.0	65.5	0.0010	72.6
<i>sej</i>	34.1	50.0	38.6	37.9	26.6	31.5	36.9	59.5	0.0940	32.3

*Results were considered statistically significant at $p < 0.05$ - in dark field

Table 5: Distribution of staphylococcal virulence genes (SAGs) among *S. aureus* isolates from Bulgaria and other countries

Virulence genes (SAGs)	Bulgaria (this study) %	Turkey ^{3,23} %	Czech Republic ²¹ %	France ³² %	Colombia ¹⁶ %	Canada ³⁵ %	China ³⁰ %	India ²⁰ %	Korea ³³ %	Iran ^{25,26} %
<i>tst</i>	6.5	27.9	2	0-38	6.2	24.3	48.1	24.3	52.6	25.0
<i>sea</i>	26.6	4.6-13.5	12	6-34	11.1	19.6	44.4	38.6	47.4	40.6
<i>seb</i>	59.8	8.7	3	0-31	9.9	5.6	6.48	26.8	0	25.0
<i>sec</i>	38.3	4.0-52.9	2	0-19	16.0	7.5	9.26	0		0
<i>sed</i>	10.3	4.8	17	0-14	3.7	1.9	5.56	0	0	0
<i>see</i>	4.3	0	0	0	3.2	0	0	0	0	0
<i>seg</i>	67.4	24.6-89.4	77	75	51.9		40.7		61.1	27.8
<i>seh</i>	41.3	35.6	0	0-20	35.8		1.85			
<i>sei</i>	72.6	51.3-65.4	77	75	44.4		14.8		70.5	
<i>sej</i>	32.3	0.66-22.1	17		3.7		13.9			

Fig 1: Gel electrophoresis of the PCR products of *Staphylococcus aureus* virulence and *mec* genes (Amplicons from mixes I, II, III, IV, V, VI)

Discussion:

There are wide differences in the reported geographical distribution of MRSA, from 2.9% to 8.7 % in Turkish nasal carriers and respiratory tracts of ill persons (3, 23), up to 38.7% in Colombia (16), 39.6% in India (24), 41-48% in Iran (25, 26) and 44% in Nigeria from animal samples (27). The methicillin resistance rate observed in this

study was 12%, with 7.7% from outpatients and 16.2% from inpatients. The prevalence of MRSA is known to vary in relation to where the infection was acquired, whether in the community (CA) or in healthcare (HA) setting (3, 4, 5, 19, 20).

In almost all the *S. aureus* isolates in our study, we found the *hlg* gene in the genome of the organism in both MSSA and MRSA (though more frequent in MSSA),

invasive and non-invasive, and CA and HA isolates. This gene is responsible for the synthesis of extracellular toxic polypeptide (gamma-haemolysin) with strong cytolytic activity against a broad spectrum of eukaryotic cells (erythrocytes, neutrophils, monocytes, lymphocytes, macrophages) and non-specific activity against intracellular membrane structures in various mammalian cells with detergent-like action (28). Available data suggest that *hlg* extracellular product contributes to immune evasion and potential staphylococcal survival in biological fluids. In high concentration, this bi-component pore-forming toxin has dermo-necrotic and lethal activity in experimental animal model and is being studied as a potential component of a multivalent vaccine (28). Our results are similar to data from the USA and Iran reporting high prevalence of *hlg* among MRSA and MSSA isolates (25, 29).

In contrast, the *cna* gene was detected in only about 14% of the staphylococcal isolates. *S. aureus* can produce collagen adhesin (CNA) to interact with collagen in humans and animals. This adhesin plays a leading role in colonization, prolongation and persistence of *S. aureus* infection in human host. Another mechanism of CNA action is inhibition of complement activation. In experimental animal models, progression of bacterial infections as a result of staphylococcal CNA or other adhesins such as YadA of *Yersinia enterocolitica*, *Enterococcus faecalis* adhesin ACE, *E. faecium* adhesin ACM, and *Streptococcus mutans* adhesin CNM, have been established (30). The frequent correlation of *cna* with invasive isolates predominantly in inpatients suggests *cna* encodes a product with high and specific virulent potential.

The combinations of three genes, *sei*, *seg* and *seb*, which codes for strong super-antigens (SAGs) were detected in more than 60% of the Bulgarian staphylococcal isolates tested in this study, predominantly among invasive isolates. About one-third of the clinical *S. aureus* isolates harbored seven genes, *sei*, *seb*, *seh*, *sec*, *seg*, *sej*, and *sea*, encoding super-antigenic toxins, and as many as 25% of the isolates carried at least nine genes while nearly 40% carried at least six virulence genes. This high carriage rate put the populace at risk of infections from highly virulent *S. aureus* strains in our geographical region.

The frequency of SAGs gene detection reported varies from countries to countries as shown in Table 5. The high prevalence of *sei* and *seg* genes in our study is similar to what has been reported in Turkish paediatric patients and healthy students (3, 23), and in Czech Republic, France, Colombia, Mexico and Korea (16, 20, 31, 32, 33) but data from

India, China, Iran and Canada present *sea* as the most frequent SAGs gene (24, 25, 34, 35).

Among the MRSA isolates in our study, the genes encoding SEs that were dominant with high frequency (63.6% to 93.2%) and significantly associated with methicillin resistance ($p < 0.05$) were *sea*, *seb*, *sec*, *seg*, *seh* and *sei*. This is similar to the study of Ortega et al., (5) who reported *sea*, *seb*, *sed*, *seg*, *sei* and *sej* genes as the most prevalent in their MRSA isolates. However, they detected *tst-1* gene more frequently among their MSSA isolates, whereas in our study, *tst-1* gene was more frequently detected in the MRSA isolates. The prevalence of *tst-1* gene in the *S. aureus* isolates in the present study and from other European countries as shown in Table 5 is low compared to the prevalence in China and Korea (about 50% of isolates). The *tst-1* gene was detected equally in isolates from inpatients and outpatients ($p > 0.05$) but was more frequently detected in the invasive staphylococcal isolates ($p < 0.05$). The prevalence of both *seb* and *sec* genes, which are the most frequently detected genetic elements in patients with HA *S. aureus* infections and in invasive disease, was significantly higher ($p < 0.05$) in our study than from other countries.

The genetic profile of the invasive *S. aureus* isolates was different in the present study with more virulence genes detected compared to non-invasive isolates ($p < 0.05$). The genes mainly detected in the invasive staphylococcal isolates were *cna*, *tst-1*, *seb*, *seh*, *sec*, *sed*, *see*, *seg*, *seh* and *sei* in various combinations. Some of these genes have been reported to correlate with nosocomial and invasive infections (16, 20, 24). In a Colombia study (16), five SAG genes out of 22 tested genetic elements were found in invasive isolates. Some of the genes, *sei*, *sea* and *seg*, most frequently detected in our study were reported to be harbored by more than 33% of *S. aureus* isolates in patients who died from staphylococcal bacteraemia (24).

Conclusion:

The prevalence of MRSA in the Bulgarian human staphylococcal isolates in our study was 12%, and the strains were recovered mainly from patients with HA infections. More than 60% of the staphylococcal isolates carried *sei*, *seg* and *seb* genes which encode strong Sags predominantly among the invasive isolates, in addition to the *hlg* gene which encode a pore-forming toxin. Furthermore, approximately one-third of the clinical *S. aureus* isolates

harbored seven (*sei*, *seb*, *seh*, *sec*, *seg*, *sej* and *sea*) out of the twelve virulence genes encoding super-antigen toxins, in addition to the *hlg* gene. The results from the present study showed that the genome of clinical *S. aureus* isolates in Bulgaria contain wide array of genes encoding immuno-modulatory molecules. This may put the populace at risk of acquisition and spread of highly virulent *S. aureus* strains in our geographical region.

Conflict of interest:

No conflict of interest is declared

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Characterization of antibiotic resistance and species diversity of staphylococci isolated from apparently healthy farm animals

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Department of Microbiology, Faculty of Life Sciences, University of Benin, PMB 1154, Benin City, Nigeria
*Correspondence to: eigbinosa@gmail.com**Abstract:**

Background: *Staphylococcus* species are adaptable commensals usually involved in a diverse multiplicity of ailments in animals and humans. This study surveyed the occurrence, antibiotic-resistance profile and putative resistant genetic elements of staphylococci isolates from apparently healthy farm animals

Methodology: Nasal and rectal samples were collected from a total of 400 cows and pigs in Benin City between May and December 2017. Staphylococci were isolated following aerobic cultures of samples using standard microbiological methods. Susceptibility profiles of the isolates to eighteen selected antimicrobials were determined using the Kirby-Bauer disk diffusion test. Species of staphylococci were established and antibiotic resistance genes detected by the polymerase chain reaction using species-specific and antibiotic-resistant primers respectively

Result: A total of 139 staphylococci isolates were phenotypically and genotypically identified from the food-producing animals; 87 (62.6%) from pigs and 52 (37.4%) from cows. The most frequent *Staphylococcus* species were *Staphylococcus haemolyticus* 38 (27.3%), *Staphylococcus aureus* 27 (19.4%) and *Staphylococcus capitis* 21 (15.1%). Antibiotic resistance profile showed 120 (86.3%) isolates to be resistant to penicillin G, 100 (71.9%) to nalidixic acid and 99 (71.2%) to minocycline. The prevalence of antibiotic resistance genes assessed were *mecA* 78 (56.1%), *mphC* 23 (16.6%), and *ermA* 20 (14.4%).

Conclusion: Our finding indicates that food animals are potential reservoirs of antibiotic resistant staphylococci which pose a significant threat to food security and public health.

Keywords: food animals; antibiotic-resistant; foodborne pathogen; staphylococci; resistance elements

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Caractérisation de la résistance aux antibiotiques et de la diversité des espèces de staphylocoques isolés d'animaux de ferme apparemment en bonne santé

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*Correspondance à: eigbinosa@gmail.com**Abstrait:**

Contexte: Les espèces de *Staphylococcus* sont des agents commensaux adaptables généralement impliqués dans une grande diversité de maladies chez les animaux et les humains. Cette étude a examiné l'occurrence, le profil de résistance aux antibiotiques et les éléments génétiques potentiellement résistants d'isolats de staphylocoques provenant d'animaux d'élevage apparemment en bonne santé.

Méthodologie: Des échantillons nasaux et rectaux ont été prélevés chez 400 vaches et porcs au total dans la ville de Benin City entre mai et décembre 2017. Les staphylocoques ont été isolés suite à des cultures aérobies d'échantillons à l'aide de méthodes microbiologiques standard. Les profils de sensibilité des isolats à dix-huit antimicrobiens sélectionnés ont été déterminés à l'aide du test de diffusion sur disque Kirby-Bauer. Les espèces

de staphylocoques ont été établies et les gènes de résistance aux antibiotiques ont été détectés par réaction en chaîne de la polymérase en utilisant respectivement des amorces spécifiques à l'espèce et des bactéries résistantes aux

Résultat: Un total de 139 isolats de staphylocoques ont été identifiés phénotypiquement et génotypiquement à partir des animaux producteurs d'aliments. 87 (62,6%) de porcs et 52 (37,4%) de vaches. Les espèces de *Staphylococcus* les plus fréquentes étaient *Staphylococcus haemolyticus* 38 (27,3%), *Staphylococcus aureus* 27 (19,4%) et *Staphylococcus capitis* 21 (15,1%). Le profil de résistance aux antibiotiques a montré que 120 (86,3%) des isolats étaient résistants à la pénicilline G, 100 (71,9%) à l'acide nalidixique et 99 (71,2%) à la minocycline. La prévalence des gènes de résistance aux antibiotiques évalués était *mecA* 78 (56,1%), *mphC23* (16,6%) et *ermA* 20 (14,4%).

Conclusion: nos résultats indiquent que les animaux destinés à l'alimentation sont des réservoirs potentiels de staphylocoques résistants aux antibiotiques qui constituent une menace importante pour la sécurité alimentaire et la santé publique

Mots-clés: animaux d'élevage; résistant aux antibiotiques; agent pathogène d'origine alimentaire; staphylocoques, éléments de résistance

Introduction:

Staphylococcus species are adaptable commensals usually involved in a diverse multiplicity of ailments in animals and humans with their pathogenicity associated with invasive capacity, antibiotic resistance, and toxin-mediated virulence (1, 2). In livestock, *Staphylococcus aureus* has been described as a significant cause of skin and soft tissue infections, mastitis and systemic infections (3) and is considered a key foodborne pathogen (4).

The demand for animal proteins is increasing globally at a relatively high rate for human consumption. Concern about the threat of antibiotic-resistant strains of *Staphylococcus* species has increased in recent years (5). The emergence of antibiotic resistance has been recognized to be the result of extensive prophylactic and therapeutic use of antimicrobials as growth promoters in food-producing animals (6, 7). Such antimicrobials are frequently used in human medicine for therapy of infections and prophylaxis during medical procedures such as surgeries, chemotherapy and organ transplantation (8). The widespread use of antimicrobials in food animals contribute to the development of antimicrobial-resistant bacteria (ARB) by means of natural selection and thus constitute a significant risk to public health.

Antibiotic resistance from animals can be disseminated to humans through food products (9), environment (10) and by direct contact to agricultural workers (11). Although it is difficult to establish a direct connection due to the organic character of antibiotic selection pressure, reports have shown a close relationship between the occurrence of livestock-associated antibiotic-resistant bacteria in humans and animals (12). Similarly, the rate of antimicrobial use in food-producing animals and the prevalence of antibiotic-resistant bacteria in humans (13) and animals (14) have been documented. *Staphylococcus* species from

food-producing animals frequently harbour resistance elements. *S. aureus* are now generally resistant to methicillin and most other β -lactam antimicrobials. Methicillin resistance in staphylococci is mediated usually by *mecA* gene carried on staphylococcal chromosomal cassette (SCC*mec*) (15) which codes for altered penicillin-binding protein 2a or 2' (PBP2a or 2') with low binding affinity to beta-lactamase resistant penicillins such as oxacillin and methicillin, and other beta-lactam antimicrobials (16).

The genotypic characterization of *Staphylococcus* species is essential to assess the risk of dissemination of resistant staphylococcal isolates between humans, environment and animals. There are enormous concern regarding the public health implication of methicillin-resistant *S. aureus* (MRSA) connected with livestock since MRSA and their resistance genes can spread from humans to animals via the food chain or through direct contact (17). Diversity of MRSA strains have been recovered from small ruminants or cow milk as well as different dairy products in different countries (18, 19).

In 2009, the European Food Safety Authority (EFSA) expressed growing concerns for public health orchestrated by the occurrence of MRSA in food animal production. The authority therefore suggested that additional studies be conducted on sampling, identification and characterization of MRSA carriage in animals and humans, and the environment coupled with food contamination (20). The current study aimed to characterize antibiotic resistant *Staphylococcus* species from food animals in Benin City, Nigeria.

Materials and methods:

Sample collection

A total of 400 samples (200 nasal and 200 rectal) samples were collected from cows and pigs in Benin City between May and

December 2017. Samples were collected with sterile swabs by first moistening in sterile normal saline and gently swabbing the nasal and rectal cavities of the food-producing animals. Informed consent was obtained from the farm owners prior to sampling. Samples were immediately transported on ice packs to the Applied Microbial Processes and Environmental Health Research Group Laboratory, Department of Microbiology, University of Benin, Nigeria for analysis within 24 hours of collection.

Culture isolation and biochemical identification of staphylococci

Swab samples were immediately agitated on 5 mL tryptone soy broth (Lab M, Lancashire, United Kingdom) and incubated aerobically for 18-24 hours at 37°C. After 18 hours, an aliquot of 100 µL was inoculated on mannitol salt agar (Lab M, Lancashire, United Kingdom) and further incubated aerobically for 18-24 hours at 37°C. After incubation, 'golden yellow' and other related colonies were Gram stained and identified by biochemical tests such as coagulase, DNase, slide agglutination (BBL™ Staphyloslide™), and mannitol and sugar fermentation tests (21, 22). All tests were performed in triplicates with *S. aureus* ATCC 12600 used as control strain in each test procedure. The staphylococci isolates were confirmed with analytical profile index (API) Staph (BioMerieux, France). Identified staphylococci were colony purified on nutrient agar (Lab M, Lancashire, United Kingdom) and stored on nutrient agar slants at 4°C until further use.

Susceptibility profile of staphylococci isolates

Susceptibility profile of the *Staphylococcus* species to antimicrobials was carried out using Kirby-Bauer/CLSI disk diffusion method (23). Briefly, the purified isolates were inoculated into 5.0 mL Mueller-Hinton broth (MHB) (Lab M, Lancashire, United Kingdom) and incubated overnight. The optical density (OD) of the turbidity of the broth was adjusted to OD of 0.5 McFarland standards which gives equivalence of 1×10^8 CFU/mL. Using a sterile swab, broth cultures were aseptically swabbed on Mueller Hinton agar (Lab M, Lancashire, United Kingdom). Antibiotic disks were aseptically placed on the agar plates with sterile forcep. Plates were incubated at 37°C for 24 hours and diameter of zone of inhibition for each isolate was measured with a ruler. Susceptibility or resistance of each isolate was determined by comparing the diameter of zone of inhibition with the interpretative chart of the Clinical and Laboratory Standards Institute (23).

The antibiotic disks (Mast Diagnostics, Merseyside, United Kingdom)

used were; meropenem (10 µg), penicillin G (10 units), ceftazidime (30 µg, surrogate for testing *S. aureus* against oxacillin), ceftazidime (30 µg), cefotaxime (30 µg), tetracycline (30 µg), doxycycline (30 µg), minocycline (30 µg), clindamycin (2 µg), erythromycin (10 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), nalidixic acid (30 µg), sulfamethoxazole-trimethoprim (23.75 µg/1.25 µg), chloramphenicol (30 µg), kanamycin (30 µg), and gentamicin (10 µg).

Multiple antibiotic resistance index of isolates

The multiple antibiotic resistance index (MARI) for each isolate was calculated as number of antibiotics to which resistance occurred divided by the total number of antibiotics to which the isolate was tested (24). Multidrug resistance was defined as resistance to three or more antimicrobial classes (25).

Genomic DNA extraction

Genomic DNA from *Staphylococcus* isolates was extracted using the boiling method. Briefly, the *Staphylococcus* isolates were re-inoculated in 5.0 mL of tryptone soy broth and incubated at 37 °C for 18-24 hours. Thereafter, 150 µL of the cell suspension was dispensed into 2.0 mL Eppendorf tube, and the mixture was heated in a dry bath (MK200-2, Shanghai, China) for 15 minutes at 100 °C for cell lysis. The lysed cell mixture was centrifuged with the aid of a mini centrifuge (Mini 14 k, Zhuhai, Guangdong, China) at 14, 500 r/minute, for 5 minutes. The supernatant was carefully separated from the cell residues and stored at -20°C as template target gDNA.

PCR identification of *Staphylococcus* species

PCR was performed for all staphylococcal isolates using genus-specific and species-specific primers (Table 1). For genus specific amplification, the simplex PCR conditions used included denaturation at 96 °C for 3 minutes, followed by 40 cycles at 95 °C for 30 s, annealing at 55 °C for 60 s, extension at 72 °C for 30 s, with a final extension at 72 °C for 3 minutes (26) using a Peltier-based Thermal Cycler (MG96p/Y, Hangzhou, Zhejiang China). *S. aureus* ATCC 12600 served as positive control and nuclease-free water as negative control. The PCR products were electrophoresed on 1.5 % agarose gel which was stained with ethidium bromide and visualized under the UV transilluminator (Vilber Lourmat, EBOX VX5, France).

Species-specific identification was carried out using multiplex PCR primers targeting *S. epidermidis*, *S. saprophyticus*,

S. aureus and *S. xylosus* (at respective base-pair size in Table 1) and the PCR conditions included denaturation at 94 °C for 3 minutes followed by 40 cycles at 95 °C for 1 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, with a final extension at 72 °C for 3 minutes (27). The PCR products were electrophoresed using 1.5 % agarose gel (CLSAG100, Warwickshire, United Kingdom).

For other species, the multiplex PCR program conditions were denaturation at 94 °C for 10 minutes followed by 35 cycles at 94 °C for 15 s, 30 s at respective annealing temperature regimen for *S. warneri* (60 °C), *S. haemolyticus* (50 °C) and *S. capitis* (59 °C) respectively, and extension at 72 °C for 30 s (28).

PCR detection of antibiotic resistance genes

PCR detection of macrolide-resistant genes (*ermA*, *ermB*, *ermC*, *mphC*) was done in accordance with multiplex PCR procedure

of Sauer et al., (29) using primers presented in Table 2. PCR program conditions included an initial denaturation step for 5 minutes at 94 °C followed by 30 cycles of denaturation for 60 s at 94 °C, with the following respective annealing temperature regimen; *ermA* (51 °C), *ermB* (51 °C), *ermC* (51 °C), *mphC* (55 °C) for 60 s, and extension for 60 s at 72 °C with a final extension for 5 minutes at 72 °C (30, 31, 32).

PCR conditions for *vanA* and *vanB* genes included an initial denaturation for 5 minutes at 94 °C, followed by 10 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 64 °C, and extension for 45 s at 72 °C (33, 34). PCR condition for *mecA* included an initial denaturation for 5 minutes at 94 °C, followed by 25 cycles, denaturation for 30 s at 94 °C, annealing for 45 s at 50 °C, and extension for 2 minutes at 72 °C, with a final extension for 10 minutes at 72 °C (35).

Table 1: Primers used for staphylococci identification

Microorganisms	Primers	Primer sequence (5'-3')	Size (bp)	References
<i>Staphylococcus</i> genus	TStaG422 TStag765	GGCCGTGTTGAACGTGGTCAAATCA TIACCATTTCAGTACCTTCTGGTA	370	Martineau et al. (26)
<i>S. haemolyticus</i>	ShaeF ShaeR	GTTGAGGGAACAGAT CAGCTGTTTGAATATCTT	85	Iwase et al. (28)
<i>S. capitis</i>	ScapF ScapR	GCTAATTTAGATAGCGTACCTTCA CAGATCCAAAGCGTGCA	208	Iwase et al. (28)
<i>S. xylosus</i>	XylF XylR	AACGCGCAACGTGATAAAATTAATG AACGCGCAACAGCAATTACG	539	Morot-Bizot et al. (55)
<i>S. warneri</i>	SwarF SwarR	TGTAGCTAACTTAGATAGTGTTCCTTCT CCGCCACCGTTATTCTT	63	Iwase et al. (28)
<i>S. aureus</i>	Sa442-1 Sa442-2	AATCTTTGTCCGGTACACGATATTCTTCACG CGTAATGAGATTTTCAGTAGATAATACAACA	1108	Morot-Bizot et al. (55)
<i>S. saprophyticus</i>	Sap1 Sap2	TCAAAAAGTTTTCTAAAAAATTTAC ACGGGCGTCCACAAAATCAATAGGA	221	Morot-Bizot et al. (55)
<i>S. epidermidis</i>	Se705-1 Se705-2	ATCAAAAAGTTGGCGAACCTTTTCA AAAAGAGCGTGGAGAAAAGTATCA	1,124	Morot-Bizot et al. (55)

Table 2: Primers used for amplification of antibiotic resistance genes in staphylococci isolates

Genes	Primers	Primer sequence (5'-3')	Size (bp)	References
<i>mecA</i>	mecA1 mecA2	GTAG AAAT GACT GAAC GTCC GATAA CCAA TTCC ACAT TGTT TCGG TCTAA	310	Geha et al. (35)
<i>vanA</i>	van A1 van A2	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	Dutka-Malen et al. (33)
<i>vanB</i>	van B1 van B2	GTGC TGCG AGAT ACCA CAGA CGAACACCATGCAACATTTT	1145	Ramos-Trujillo et al. (34)
<i>mphC</i>	mph (C)-1 mph (C)-2	GAGA CTAC CAAG AAGA CCTGACG CATA CGCC GATT CTCC TGAT	722	Lüthje and Schwarz (30)
<i>ermA</i>	erm(A)-1 erm(A)-2	GCGGTAAACCCCTCTGAG GCCTGTCCGGAATTGG	434	Werckenthin and Schwarz (31)
<i>ermB</i>	erm(B)-1 erm(B)-2	CATT TAAC GACG AAAC TGGC GGAA CATC TGTG GTAT GGCG	425	Jensen et al. (32)
<i>ermC</i>	erm(C)-1 erm(C)-2	ATCT TTGA AATC GGCT CAGG CAAA CCCG TATT CCAC GATT	295	Jensen et al. (32)

Results:

Frequency of staphylococci isolation from mannitol salt agar

The frequency of *Staphylococcus* isolates recovered from the food producing animals in Table 3 shows an overall isolation rate from mannitol salt agar of 64.3% (257 of 400), with 98 (24.5%) from cows (47 from nasal and 51 from rectal samples), and 159 (39.8%) from pigs (83 from nasal and 76 from rectal samples).

Table 3: The frequency of isolation of *Staphylococcus* from culture of samples on mannitol salt agar

Number and source of samples examined		Number of samples positive for staphylococci
Cow	nasal sample (n=100)	47 (47)
	rectal sample (n=100)	51 (51)
Pig	nasal sample (n=100)	83 (83)
	rectal sample (n=100)	76 (76)
Total (n=400)		247 (64.3)

Distribution of the *Staphylococcus* species in cows and pigs

The frequency distribution of *Staphylococcus* species identified by both phenotypic and genotypic methods from cow and pigs is presented in Table 4. A total of 139 *Staphylococcus* species were identified from the 400 samples, giving a 34.8% recovery from these food animals, with 87 (62.6%) from pigs (51 from nasal and 36 from rectal samples) and 52 (37.4%) from cows (33 from nasal and 19 from rectal samples). The frequency distribution of the *Staphylococcus* species in descending order are; *S. haemolyticus* (27.3%), *S. aureus* (19.4%), *S. capitis* (15.1%), *S. epidermidis* (9.4%), *S. saprophyticus* (7.2%), *S. xylosus* (5.0%) and *S. warneri* (2.9%). Other staphylococci species constituted 13.7%.

Table 4: Frequency distribution of *Staphylococcus* species identified by phenotypic and genotypic methods from nasal and rectal samples of cow and pigs

<i>Staphylococcus</i> species	Cow			Pig			Total (%)
	nasal	rectal	Subtotal (%)	nasal	rectal	Subtotal (%)	
<i>S. aureus</i>	11	3	14 (26.9)	8	5	13 (14.9)	27 (19.4)
<i>S. epidermidis</i>	3	3	6 (11.5)	4	3	7 (8.0)	13 (9.4)
<i>S. capitis</i>	5	-	5 (9.6)	13	3	16 (18.4)	21 (15.1)
<i>S. xylosus</i>	3	-	3 (5.8)	3	1	4 (4.6)	7 (5.0)
<i>S. haemolyticus</i>	5	5	10 (19.2)	10	18	28 (32.2)	38 (27.3)
<i>S. saprophyticus</i>	-	3	3 (5.8)	7	-	7 (8.0)	10 (7.2)
<i>S. warneri</i>	2	2	4 (7.7)	-	-	-	4 (2.9)
Other <i>S.</i> species	4	3	7 (13.5)	6	6	12 (13.8)	19 (13.7)
Total	33	19	52 (37.4)	51	36	87 (62.6)	139 (100)

Table 5: Antimicrobial susceptibility profile of the *Staphylococcus* species

Antimicrobial class	Antibiotics	<i>Staphylococcus</i> species (n=139)		
		Resistant	Intermediate	Sensitive
Carbapenems	Meropenem	18 (12.9)	0 (0)	121 (87.1)
	Penicillin G	120 (86.3)	0 (0)	19 (13.7)
Cephalosporins	Cefoxitin	78 (56.1)	0 (0)	61 (43.9)
	Ceftazidime	72 (51.8)	43 (30.9)	24 (17.3)
	Cefotaxime	36 (25.9)	11 (7.9)	92 (66.2)
Tetracyclines	Tetracycline	102 (73.4)	0 (0)	37 (26.6)
	Doxycycline	91 (65.5)	12 (8.6)	36 (25.9)
	Minocycline	99 (71.2)	17 (12.2)	23 (16.5)
Lincosamides	Clindamycin	46 (33.1)	24 (17.3)	69 (49.6)
Macrolides	Erythromycin	36 (25.9)	16 (11.5)	87 (62.6)
Quinolones	Ofloxacin	14 (10.1)	18 (12.9)	107 (76.9)
	Ciprofloxacin	9 (6.5)	13 (9.4)	117 (84.2)
	Levofloxacin	3 (2.2)	0 (0)	136 (97.8)
	Nalidixic Acid	100 (71.9)	0 (0)	0 (0)
Folate inhibitors	Sulfamethoxazole-trimethoprim	97 (69.8)	23 (16.5)	19 (13.7)
Phenicol	Chloramphenicol	0 (0)	7 (5.0)	132 (94.9)
Aminoglycosides	Kanamycin	0 (0)	6 (4.3)	133 (95.8)
	Gentamycin	0 (0)	11 (7.9)	128 (92.1)

Antimicrobial susceptibility profile of the staphylococci isolates

The resistant profile of the *Staphylococcus* species is presented in Table 5 which shows resistance rate to penicillin G of 86.3% (120 of 139), 71.9% to nalidixic acid, 71.2% to minocycline, 69.8% to trimethoprim-sulfamethoxazole, 65.5% to doxycycline, 56.1% to ceftaxime (oxacillin), 51.8% to ceftazidime, and 33.1% to clindamycin. The *Staphylococcus* species were sensitive to levofloxacin 97.8% (136 of 139), 95.8% to kanamycin, 94.9% to chloramphenicol, 92.1% to gentamycin, 87.1% to meropenem, 84.2% to ciprofloxacin, 76.9% to ofloxacin, 66.2% to cefotaxime and 62.6% to erythromycin.

Multidrug-resistance and multiple antibiotic-resistant index of *Staphylococcus* species

The multidrug resistance profile of the *Staphylococcus* species as presented in Table 6 shows 100 (71.9%) isolates resistant to three different antibiotic classes (NAL^R, TET^R and PEN^R), 67 (48.2%) isolates

resistant to eight antibiotics in five different classes (TMP^R, NAL^R, MIN^R, DOX^R, TET^R, CAZ^R, OXA^R and PEN^R), and three (2.2%) isolates resistant to fifteen antibiotics in eight different classes (TMP^R, NAL^R, LEV^R, CIP^R, ERY^R, CLI^R, MIN^R, DOX^R, TET^R, CTX^R, CAZ^R, OXA^R, PEN^R and MEM^R). The multiple antibiotic resistant index (MARI) ranged from 0.17 to 0.83 (Table 6).

Distribution of antibiotic-resistant genes in staphylococci isolates

The distribution of antibiotic-resistant genes shows that all 78 staphylococci isolates resistant to ceftaxime (i.e. phenotypic MRSA) carried the *mecA* gene. Of the 36 isolates resistant to the erythromycin (macrolide), 23 (63.8%) harboured the *mphC* gene, 20 (55.6%) had the *ermA* gene, 4 (11.1%) had the *ermB* gene, and 11 (30.6%) had the *ermC* gene (Table 7). However, 12 multidrug resistant (MDR) isolates harboured *vanA* gene but none contained *vanB* gene.

Table 6: Multidrug-resistant profile of the *Staphylococcus* species

Number of antimicrobial class	Number of antibiotics	Resistance phenotype	Number of isolates (n=139)	MARI
3	3	NAL ^R , TET ^R , PEN ^R	100 (71.9)	0.17
4	5	TMP ^R , NAL ^R , MIN ^R , TET ^R , PEN ^R	96 (69)	0.27
4	6	TMP ^R , NAL ^R , MIN ^R , DOX ^R , TET ^R , PEN ^R	90 (64.7)	0.33
5	7	TMP ^R , NAL ^R , MIN ^R , DOX ^R , TET ^R , CAZ ^R , PEN ^R	70 (50.4)	0.35
5	8	TMP ^R , NAL ^R , MIN ^R , DOX ^R , TET ^R , CAZ ^R , OXA ^R , PEN ^R	67 (48.2)	0.39
6	9	TMP ^R , NAL ^R , CLI ^R , MIN ^R , DOX ^R , TET ^R , CAZ ^R , OXA ^R , PEN ^R	45 (32.4)	0.50
7	11	TMP ^R , NAL ^R , ERY ^R , CLI ^R , MIN ^R , DOX ^R , TET ^R , CTX ^R , CAZ ^R , OXA ^R , PEN ^R	35 (25.2)	0.61
8	12	TMP ^R , NAL ^R , ERY ^R , CLI ^R , MIN ^R , DOX ^R , TET ^R , CTX ^R , CAZ ^R , OXA ^R , PEN ^R , MEM ^R	16 (11.5)	0.67
8	13	TMP ^R , NAL ^R , OFX ^R , ERY ^R , CLI ^R , MIN ^R , DOX ^R , TET ^R , CTX ^R , CAZ ^R , OXA ^R , PEN ^R , MEM ^R	13 (9.4)	0.72
8	14	TMP ^R , NAL ^R , CIP ^R , ERY ^R , CLI ^R , MIN ^R , DOX ^R , TET ^R , CTX ^R , CAZ ^R , OXA ^R , PEN ^R , MEM ^R	9 (6.5)	0.78
8	15	TMP ^R , NAL ^R , LEV ^R , CIP ^R , ERY ^R , CLI ^R , MIN ^R , DOX ^R , TET ^R , CTX ^R , CAZ ^R , OXA ^R , PEN ^R , MEM ^R	3 (2.2)	0.83

MEM: Meropenem, PEN: Penicillin G, OXA: Oxacillin, CAZ: Ceftazidime, CTX: Cefotaxime, TET: Tetracycline, DOX: Doxycycline, MIN: Minocycline, CLI: Clindamycin, ERY: Erythromycin, OFX: Ofloxacin, CIP: Ciprofloxacin, LEV: Levofloxacin, NAL: Nalidixic Acid, TMP: Trimethoprim-sulfamethoxazole, CHL: Chloramphenicol, KAN: Kanamycin, GEN: Gentamycin, Values in parenthesis denote percentage. MARI: multiple antibiotic resistance index

Table 7: Distribution of antibiotic-resistant genes

Antibiotic-resistant genes	No of phenotypically resistant isolates to the antibiotics used	Frequency of resistance genes screened (%)
<i>mecA</i>	78	78 (100)
<i>vanA</i>	ND	12
<i>vanB</i>	ND	0
<i>mphC</i>	36	23 (63.8)
<i>ermA</i>	36	20 (55.6)
<i>ermB</i>	36	4 (11.1)
<i>ermC</i>	36	11 (30.6)

ND = Not Determined

Discussion:

This study characterized staphylococci isolates from two food animals, cow and pigs, which are common source of animal proteins consumed in our environment. The most frequently identified staphylococci in descending order from the food animals in our study are *S. haemolyticus* (27.3%, n=38), *S. aureus* (19.4%, n=27), *S. capitis* (15.1%, n=21), *S. epidermidis* (9.4%, n=13), *S. saprophyticus* (7.2%, n=10), *S. xylosus* (5.0%, n=7) and *S. warneri* (2.9%, n=4). This is different from the pattern in a similar study by Chajęcka-Wierżchowska et al., (36), where the most frequently identified staphylococci were *S. xylosus* (n=29, 50%), *S. epidermidis* (n=16, 27.6%), *S. lentus* (n=7, 12.1%), *S. saprophyticus* (n=4, 6.9%), *S. hyicus* (n=1 1.7%) and *S. simulans* (n=1 1.7%). Although *S. xylosus*, *S. epidermidis* and *S. saprophyticus* were identified in both studies, *S. haemolyticus*, *S. aureus* and *S. capitis*, the three most frequently isolated staphylococci in our study were absent in Chajęcka-Wierżchowska et al., study while *S. hyicus*, *S. simulans* and *S. lentus* isolated in their study were completely absent in our study.

In the study by Taponen et al., (37) on bovine mastitic milk, the most common coagulase negative staphylococci species identified were *S. simulans*, *S. epidermidis*, *S. chromogenes* and *S. haemolyticus* which are similar to the ones from our study on food animals with respect to *S. epidermidis* and *S. haemolyticus*. Also, in the study by Beyene et al., (38) on 193 samples collected from abattoir and dairy farms, 92 (47.7%) were positive for *Staphylococcus* species with *S. aureus* (n=31; 16.1%), *S. intermedius* (n=21; 10.9%), *S. hyicus* (n=16; 8.3%), and other coagulase negative staphylococci (n=24; 12.4%). The differences in the species of staphylococci identified in different studies may be related to geographical distribution and methods employed in identification of the species from the animals.

There have been reports of alarming high levels of *S. aureus* resistance to commonly used antimicrobials such as tetracycline and penicillins (including amoxicillin) in cows (39, 40). The high resistance of staphylococci isolates in our study to penicillin (86%), tetracycline (73%), sulfamethoxazole-trimethoprim (72%), cefoxitin (surrogate for oxacillin, 56%), and ceftazidime (52%) agrees with reports from earlier studies (39, 40), which suggest that antimicrobial resistance must have developed in the staphylococci isolates occasioned by indiscriminate and prolonged use of antimicrobials. Chajęcka-Wierżchowska et

al., (36) reported that most of the staphylococci isolates from ready-to-eat food of animal origin in their study were resistant to cefoxitin (41.3%), clindamycin (36.2%), tigecycline (24.1%), rifampicin (17.2%) and erythromycin (13.8%). Majority of the staphylococci isolates from Beyene et al., (38) study also demonstrated resistance to cefoxitin (55.8%), vancomycin (65.1%), cloxacillin (79.1%), nalidixic acid (88.4%) and penicillin G (95.3%). These largely agree with some of the findings in our study.

The staphylococci isolates in Beyene et al., (38) study were multidrug resistant, exhibiting resistance to more than three antibiotic classes, which agrees with findings of the present study, with about 72% of the staphylococci isolates showing resistance to three or more classes of antibiotics. The multidrug resistance rate in our study is however higher than the 32.2% reported by Chajęcka-Wierżchowska et al., (36). The predominant multidrug resistance phenotype reported from 46 isolates reported by Li et al., (41) was penicillin-ampicillin-kanamycin-gentamicin-tetracycline but this differs from the commonest phenotype, penicillin-tetracycline-nalidixic acid, reported in the current study.

Globally, livestock farming has improved food production at a reduced cost per unit produced with several pitfalls from increased antimicrobial resistance. This present study has further strengthened the fact that food animals can act as reservoir for antimicrobial resistant *Staphylococcus* species. Linking antimicrobial ingestion in food animals to drug-resistant infections of humans is intrinsically complex due to the environmental nature of the selection pressure for antibiotic-resistant pathogens as well as the occurrence of non-specific routes of transmission throughout the environment. An increasing body of evidence has emerged to strengthen the fact that repeated usage of antimicrobials in intensive livestock farming systems lead to antimicrobial resistance, which is of clinical importance in human medicine (42, 43).

The resistance of *S. aureus* and other staphylococci isolates to beta-lactams such as penicillin G and oxacillin is very evident. Resistance to Penicillin G is a significant concern since this antibiotic is the major antibiotic group that is recommended for staphylococcal mastitic infection. The frequent therapeutic usage of antibiotics in cows may lead to selection and dissemination of resistant strains even as Jaims et al., (44) reported that the development of antimicrobial resistance occurs from repeated therapeutic and/or indiscriminate use of antimicrobials. Resistance to antibiotics is

usually expressed on mobile genetic elements such as plasmids and transposons that can be disseminated from one staphylococcal species to another (45). *S. aureus* resistance to penicillin G is due to the production of beta lactamase enzyme carried on transmissible plasmids, which inactivates penicillin and other beta-lactam antimicrobials. This study also demonstrated the occurrence of macrolide resistance both phenotypically and genotypically. Resistance to macrolide and lincosamide has previously been reported in coagulase negative staphylococci (CoNS) including *S. epidermidis* recovered from cows with mastitis (46).

In this study, all phenotypically methicillin resistant staphylococci (cefoxitin resistance) carried the *mecA* gene while 64%, 56%, 11% and 31% of the isolates that were phenotypically resistant to erythromycin respectively carried the macrolide resistance genes; *mphC*, *ermA*, *ermB* and *ermC*. This is similar to the findings of Chajęcka-Wierżchowska et al., (36) where all the MRSA strains in their study also harboured *mecA* gene but the erythromycin resistant isolates carried only the *ermC* gene. However, 84% of *mecA*-positive strains reported by Vyletelova et al., (47) expressed resistance to cefoxitin in the disk diffusion test. In the study by Couto et al., (48) conducted on animals over a 16 year period, the *mecA* gene was identified in 11.6% of the staphylococcal isolates which included MRSA (40.7%), methicillin resistant *Staphylococcus pseudintermedius* (8.7%) and methicillin-resistant coagulase negative staphylococci (26.7%). The prevalence of *mecA* gene in their study was low compared to our study and this difference could be related to differences in the food animals studied. Saputra et al., (49) also reported an overall low frequency of *mecA* gene among *S. pseudintermedius* and *S. aureus* as 11.8% and 12.8% respectively from animals but Ruzauskas et al., (50) reported 20 of 21 *mecA* gene in methicillin resistant staphylococci obtained from 395 clinical samples of diseased animals while the remaining one (1) isolate was positive for *mecC* gene.

The *mecA* gene encodes abnormal penicillin-binding protein 2a or 2' (PBP2a or PBB2') which has a low binding affinity for β -lactam antibiotics. Therefore, this group of antibiotics is not effective against bacteria expressing *mecA* gene. Expression of *mecA* gene however depends on a number of factors such as media type, pH, incubation temperature and presence of beta-lactam agents in the medium (51). The gene may therefore remain silent and unexpressed if these optimum conditions are not met. Other

possibility includes mutations in the promoter or coding region of the gene. In addition, staphylococcal isolate may carry another *mec* gene types such as *mecB*, *mecC* or *mecD*, which may also express abnormal PBPs that can cause methicillin resistance (50).

Vancomycin has often been regarded as the last line of antibiotic for staphylococci infections as most isolates have been reported to be sensitive to the antibiotic (52). However, findings from our study revealed that some staphylococci carried *vanA*, the gene that has been reported to be responsible for high level resistance to vancomycin in *S. aureus* (53). We could not test our isolates against vancomycin with the CLSI recommended broth dilution or E-test method (23) because this was not available in our centre at the time of this study. This resistant strain (vancomycin resistant *S. aureus*, VRSA) could constitute another important challenge to public health in the near future.

Conclusion:

Antibiotic resistance in pathogens is usually associated with mobile genetic elements such as plasmids, conjugative transposons and integrons (54). Selection and proliferation of antibiotic-resistant strains can occur, and these can be spread to the environment through animal wastes leading to increase in the resistance reservoir pool in the environmental microbiome (55). Findings from our study revealed a high prevalence of antibiotic-resistant *Staphylococcus* species in food-producing animals in Benin City, Nigeria, which could have resulted from overuse of antibiotics which acts as selection pressure and from poor hygiene practices of the animal handlers which is responsible for spread of the resistant pathogens. Improving hygienic measures in handling of food-producing animals and stopping the routine use of antibiotics as prophylactic, therapeutic or growth promoters in animal feeds or water are crucial public health measures.

Conflicts of Interest:

Authors declare no conflict of interest

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**Original Article****Open Access****Biochemical and bacteriological profiles of asymptomatic bacteriuria among school children in Ago-Iwoye, Nigeria**

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Background: Asymptomatic bacteriuria (ASB) in children is a predisposing factor to symptomatic urinary tract infection (UTI) that may be complicated by blood stream infections if not appropriately treated with resultant mortality or morbidity. The objectives of this study are to determine the prevalence of ASB, and evaluate both biochemical and bacteriological characteristics of urine samples of primary school pupils in Ago-Iwoye, Ijebu North Local Government Area (LGA), Ogun State, Nigeria.

Methodology: Three hundred and seventy-two (186 males and 186 females) apparently healthy (asymptomatic) pupils aged 2-16 years from four randomly selected primary schools in the LGA were screened for ASB. Clean catch specimen of midstream urine was collected from each subject. Biochemical analysis of the urine was performed with Combi 10 reagent strip. MacConkey and Cysteine Lactose Electrolyte Deficient (CLED) agar plates were inoculated with calibrated wireloop delivering 0.01 ml of urine for aerobic culture at 37°C for 24 hours. Identification of significant bacteria on culture plates was done using conventional biochemical tests.

Results: The frequency of clear, slightly turbid and turbid urine were 31 (8.3%), 99 (26.6%) and 56 (15.1%) respectively. All analyzed urine samples were alkaline and negative for ketone, glucose and blood, but contained protein in 230 (61.8%), bilirubin in 184 (49.5%), nitrites in 64 (17.2%) and urobilinogen in 14 (3.7%) subjects. The prevalence of significant bacteriuria was 11.8% (44 of 372) with 7.0% in males and 16.7% in females ($p = 0.0063$). The frequency of bacteria isolated in descending order were *Escherichia coli* 61.4%, *Staphylococcus saprophyticus* 61.4%, *Staphylococcus aureus* 45.5%, *Bacillus subtilis* 45.5%, *Enterococcus faecalis* 43.2%, *Enterobacter* spp 36.4%, *Serratia marscencen* 31.8%, *Klebsiella pneumoniae* 22.7%, *Proteus mirabilis* 22.7% and *Pseudomonas aeruginosa* 20.5%.

Conclusion: This result highlights the presence of significant bacteriuria among apparently healthy pupils in the study area, with higher prevalence in the female pupils. The apparent risk of developing symptomatic UTI with the attendant complications in these pupils should spur preventive education of parents/guardians and the general populace about this entity.

Keywords: Asymptomatic bacteriuria, *S. saprophyticus*, morbidity, prevalence, primary school pupils

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Profils biochimiques et bactériologiques de la bactériurie asymptomatique chez des écoliers à Ago-Iwoye, Nigéria

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Contexte: La bactériurie asymptomatique chez l'enfant est un facteur prédisposant à l'infection symptomatique des voies urinaires qui peut se compliquer d'infections du flux sanguin s'il n'est pas traité correctement avec la

mortalité ou la morbidité qui en résulte. Les objectifs de cette étude sont de déterminer la prévalence d'ASB et d'évaluer les caractéristiques biochimiques et bactériologiques d'échantillons d'urine d'élèves du primaire à Ago-Iwoye, région du gouvernement local d'Ijebu North, État d'Ogun, au Nigéria.

Méthodologie: Trois cent soixante douze (186 garçons et 186 filles) des élèves apparemment sains (asymptomatiques) âgés de 2 à 16 ans de quatre écoles primaires sélectionnées au hasard dans la LGA ont été soumis à un dépistage du PNA. Des échantillons de capture d'urine à mi-parcours ont été recueillis chez chaque sujet. L'analyse biochimique de l'urine a été réalisée avec une bandelette de réactif Combi 10. Des plaques de gélose MacConkey et Cysteine Lactose Déficiant en électrolyte (CLED) ont été inoculées avec du fil électrolytique calibré, délivrant 0,01 ml d'urine pour une culture aérobie à 37 ° C pendant 24 heures. L'identification des bactéries significatives sur des plaques de culture a été réalisée à l'aide de tests biochimiques classiques

Résultats: La fréquence des urines claires, légèrement troubles et troubles était respectivement de 31 (8,3%), 99 (26,6%) et 56 (15,1%). Tous les échantillons d'urine analysés étaient alcalins et négatifs pour la cétone, le glucose et le sang, mais contenaient des protéines chez 230 (61,8%), de la bilirubine chez 184 (49,5%), des nitrites chez 64 (17,2%) et de l'urobilinogène chez 14 (3,7%). La prévalence de la bactériurie significative était de 11,8% (44 sur 372) avec 7,0% chez les hommes et 16,7% chez les femmes ($p = 0,0063$). La fréquence des bactéries isolées par ordre décroissant était *Escherichia coli* 61,4%, *Staphylococcus saprophyticus* 61,4%, *Staphylococcus aureus* 45,5%, *Bacillus subtilis* 45,5%, *Enterococcus faecalis* 43,2%, *Enterobacter* spp 36,4%, *Serratia marcescens* 31,8%, *Klebsiella pneumoniae*, *Proteus mirabilis* 22,7% et *Pseudomonas aeruginosa* 20,5%.

Conclusion: Ce résultat met en évidence la présence d'une bactériurie significative chez les élèves apparemment en bonne santé dans la zone d'étude, avec une prévalence plus élevée chez les élèves de sexe féminin. Le risque apparent de développer une infection urinaire symptomatique accompagnée des complications associées chez ces élèves devrait inciter à l'éducation préventive des parents / tuteurs et de la population en général sur cette entité.

Mots-clés: bactériurie asymptomatique, *S. saprophyticus*, morbidité, prévalence, élèves du primaire

Introduction:

The prevalence of significant bacteriuria and the consequences in children especially primary school pupils have been well documented (1,2,3). Traditionally, the presence of bacteria in urine is said to be significant when the bacterial count is more than 10^5 colony forming units (CFU) per millilitre of urine and such condition is not uncommon among public primary school pupils in Ogun State (4), considering their low socio-economic status and prevailing poverty that prevents access to health care service and education.

The prevalence of significant bacteriuria is known to vary from place to place. In India, Kaushik and Chaudhary reported a low prevalence of 5% significant bacteriuria among primary school pupils (5) while Mzaki *et al.*, and Ocoru *et al.*, in Tanzania and Uganda respectively reported high rates of 20.3% and 26.8% among primary school children (6, 7). In Nigeria, Aiyegoro *et al.*, reported 11.9% rate among children in Ile-Ife (8) while Ogbukagu *et al.*, reported a high prevalence rate of 17.6% among children in Anambra State (9). A previous study by Ayoade *et al.*, among University students in Redemption camp in Ogun State, Nigeria, reported 25% rate among sampled subjects (10). Due to paucity of information on this entity in our environment, this study was conducted to determine the prevalence of ASB and evaluate biochemical and bacteriological characteristics

of urine samples of apparently healthy primary school pupils in Ago-Iwoye, Ijebu-North Local Government area of Ogun State, Nigeria.

Materials and methods:

Study setting, design and subjects

This cross-sectional study was conducted in Ago Iwoye, Ijebu North Local Government Area (LGA) of Ogun State, Nigeria from March 2012 to March 2014. A total of 372 apparently healthy primary school pupils (186 males, 186 females) aged 2 to 16 years were enrolled into the study from 4 randomly selected public primary schools in the LGA. Informed consent of the parents/guardians of each pupil and the permission of each school head teacher were obtained. The study was approved by the Ethics Committee of the Ijebu North Local Government Area Council.

Children with fever and urinary symptoms such as dysuria, passage of dark urine, frequency, suprapubic or renal pain, facial or leg swellings, and those who have received antibiotics during 48 hours prior to sample collection were excluded.

Sample collection

A labeled, sterile wide mouth, screw cap plastic container of 20 ml capacity was given to each parent/guardian with verbal and written instructions on the procedure for collecting mid-stream urine samples from the pupils. The samples were collected and immediately transported to the Microbiology

Laboratory of the Olabisi Onabanjo University, Ago Iwoye, Ogun State, Nigeria for analysis.

Macroscopy and biochemical analysis

The urine samples were examined visually for turbidity (clear, slightly turbid or turbid) and appearance (clear, pale yellow, yellowish, amber or blood stained). The biochemical analysis was carried out using Combi 10 reagent strip (BHL Pharmaceuticals, United Kingdom) according to manufacturer's instructions. The parameters examined were pH, specific gravity, ketones, protein, bilirubin, urobilinogen, glucose, nitrite, blood and leucocytes.

Culture and isolation of bacteria

MacConkey and Cysteine Lactose Electrolyte Deficient (CLED) agar plates (Merck, Germany) were inoculated using a calibrated sterile wire loop delivering 0.01 ml of urine sample. The plates were incubated aerobically at 37°C for 24 hours. The number of colonies counted on each plate was multiplied by a factor of 100 to estimate the colony forming units (CFU) per millilitre of urine, and significant bacteriuria was taken as 10⁵ CFU/ml.

Identification of bacteria

Colonies from culture plates with significant bacteriuria were first Gram stained and bacteria identified to species level using conventional biochemical tests (11, 12). The Gram positive cocci were tested for catalase, coagulase, deoxyribonuclease, mannitol fermentation, aesculin hydrolysis, and sodium chloride utilization while the Gram positive bacilli were identified by spore staining, mannitol egg yolk fermentation and sugar fermentation tests. The Gram negative bacteria were biochemically identified using oxidase, indole, citrate utilization, motility, methyl red, voges proskaeur, and triple sugar iron tests scheme (11, 12).

Antibiotic susceptibility testing

Antibiotic susceptibility testing was done by the modified Kirby Bauer disk diffusion method (13) using the following antibiotic

disks; cotrimoxazole (25µg), erythromycin (5µg), gentamicin (10µg), augmentin (30µg), streptomycin (10µg), tetracycline (10µg) and chloramphenicol (10µg) for Gram positive bacteria while augmentin (30µg), ofloxacin (5µg), gentamicin (10µg), nalixidic acid (30µg), nitrofuratoin (200µg), cotrimoxazole (25µg), amoxicillin (25µg) and tetracycline (25µg) were used for Gram negative bacteria. The diameter of zone of inhibition was interpreted as sensitive or resistance using the guidelines of CLSI (14).

Statistical analysis

Data were analysed with GraphPad InStat (GraphPad Software, Inc., San Diego). Association between variables was established using Chisquare test, with level of significance set at $p < 0.05$

Results:

A high percentage of urine samples 93 (25%) were pale yellow in colour, 54 (14.5%) deep yellow, 32 (8.6%) amber and 7 (1.8%) clear, while none of the urine sample contained visible blood. In terms of consistency, 99 (26.6%) urine samples were slightly turbid, 56 (15.1%) were turbid while 31 (8.3%) were clear. The chemical analysis of the urine samples showed 230 (61.8%) were positive for protein, 184 (49.5%) for bilirubin, 64 (17.2%) for nitrite and 14 (3.7%) for urobilinogen. Leucocyte was present in 158 (42.5%) urine samples (Table 1).

Table 2 shows the prevalence of significant bacteriuria to be 11.8% (44 of 372) with 7.0% (13 of 186) in males and 16.7% (31 of 186) in females ($p = 0.0063$). The frequency of each bacteria species isolated from urine in descending order as presented in Table 3 are *Escherichia coli* (61.4%), *Staphylococcus saprophyticus* (61.4%), *Staphylococcus aureus* (45.5%), *Bacillus subtilis* (45.5%), *Enterococcus faecalis* (43.2%), *Enterobacter* spp (36.4%), *Serratia marscencen* (31.8%), *Klebsiella pneumoniae* (22.7%), *Proteus mirabilis* (22.7%) and *Pseudomonas aeruginosa* (20.5%).

Table 1: Macroscopic and biochemical analysis of urine samples from school children in Ago-Iwoye, Ogun State, Nigeria

Parameters	Number	Percentage
Appearance		
Clear	5	1.3
Pale yellow	93	25.0
Deep yellow	52	14.0
Amber	222	59.7
Blood stained	0	0
Consistency		
Turbid	146	39.2
Hazy	199	53.5
Clear	27	7.3
pH		
acidic	0	0
neutral	0	0
alkaline	372	100
Specific gravity		
1.000	240	64.5
1.005	60	16.1
1.010	20	5.3
1.015	24	6.4
1.020	28	7.5
Ketones		
Positive	0	0
Negative	372	100
Protein		
Present	230	61.8
Negative	142	38.2
Bilirubin		
Positive	184	49.5
Negative	188	50.5
Urobilinogen		
Positive	14	3.7
Negative	358	96.3
Glucose		
Positive	0	0
Negative	372	100
Nitrite		
Positive	64	17.2
Negative	308	82.8
Blood		
Positive	0	0
Negative	372	100
Leukocytes		
Positive	158	42.5
Negative	214	57.5

Table 2: Significant bacteriuria among primary school children in Ago Iwoye in relation to gender

Significant bacteriuria	Gender		Total (%)
	Male (%)	Female (%)	
Positive	13 (7.0)	31 (16.7)	44 (11.8)
Negative	173 (93.0)	155 (83.3)	328 (88.2)
Total	186	186	372

$\chi^2 = 7.449, p = 0.0063$

Table 3: Distribution of bacterial uropathogens among primary school pupils in Ago-Iwoye in relation to gender

Bacteria	Male	Female	Total (%)
Gram positive			
<i>Staphylococcus saprophyticus</i>	9	18	27
<i>Staphylococcus aureus</i>	7	13	20
<i>Enterococcus faecalis</i>	7	12	19
<i>Bacillus subtilis</i>	6	14	20
Gram negative			
<i>Escherichia coli</i>	9	18	27
<i>Enterobacter</i> spp	5	11	16
<i>Serratia marcescens</i>	5	9	14
<i>Klebsiella pneumoniae</i>	2	8	10
<i>Proteus mirabilis</i>	4	6	10
<i>Pseudomonas aeruginosa</i>	3	6	9
Total	57	115	172

The isolates were counted per urine sample and all urine yielded polymicrobial growth. The total number of isolates was calculated by adding up the number of time each isolate was recovered from the 44 subjects with significant bacteriuria

Discussion:

The prevalence rate of 11.8% for significant bacteriuria in our study compares favorably with the 11.9% rate reported by Aiyegoro et al., among children in Ile-Ife (8) which is a region in the same southwestern geographical zone as our study area. In addition, the macroscopic features of urine reported in our study are similar to those reported by Dada and Aruwa in Akure, southwest Nigeria (3). The rate in our study is however less than 17.6% rate reported by Ogbukagu et al., in southeastern zone of Nigeria (9) and also less than 25% rate reported in a previous study by Ayoade et al., among university students in Ogun State (10). This is not unexpected as rates of ASB tend to vary from region to region, and are generally higher among young adults in reproductive age group like the university students in the study by Ayoade et al., (10). The significantly higher prevalence of ASB in females in our study is in line with what has been generally established. The female urinary system is in close proximity with the anus and vaginal, and coupled with the shorter female urethra, this encourages colonization and ascending infection from normal vaginal and anal flora.

The findings on chemical analysis of the urine may reveal underlying pathology when certain analytes such as glucose, ketones or blood, which are usually not present in urine of normal persons, are found. In this study however, none of the urine samples from the children contained glucose, ketone or blood. While some authors have reported presence of blood in urine (haematuria) as a common occurrence without associated disease process within the urinary tract, others have emphasized haematuria as a sign of urinary tract pathology from infections, stones or

cancer (15, 16). The presence of blood in urine samples (terminal haematuria) is however an important marker for the diagnosis of urinary schistosomiasis in an endemic region (17). The fact that none of the urine samples in our study contained blood is an indication that the studied population had no underlying pathology especially related to schistosomiasis (18, 19, 20).

The finding of *E. coli* as the most prevalent uropathogen in significant bacteriuria in our study agrees with reports of previous studies (21, 22), and the higher prevalence in females is similar to other reports (23). This observation is also in agreement with the study of Abdul et al., who reported *E. coli* (a human faecal flora that inhabits the colon as an innocuous commensal) to be the most prevalent facultative gram negative bacillus causing UTI in their study (24). The isolation of other members of the family enterobacteriaceae such as *Enterobacter* spp, *S. marcescens*, *K. pneumoniae* and *P. mirabilis* in urine in our study is also due to the fact that these organisms are frequently present in small number as normal flora of the intestine from where they can colonize anterior urethra and be present in significant number in voided urine (25). Similarly, *Ps. aeruginosa* may be present as normal flora of the intestine and anterior urethra, however it is usually a cause of hospital acquired UTI particularly in patients with immunosuppression, malignancy, cystic fibrosis, burns and traumatic wound injuries (26).

Staphylococcus saprophyticus was the most prevalent Gram positive uropathogen in ASB occurring with same frequency as *E. coli* in our study. This finding agrees with the study by Ranjbar et al., (23) who reported *S. saprophyticus* as the most prevalent, but differs from those of Forouzan et al., who reported *S. aureus* as the most prevalent Gram

positive uropathogen in their study (27). *S. saprophyticus* has long been recovered as normal flora of urogenital tract of women (28) but it has also been described as the second most common cause of uncomplicated acute UTI after *E. coli* in young sexually active women in the western world (29). The high frequency of *S. saprophyticus* isolation in both male and female pupils with ASB in this study implies that this Gram positive pathogen with others such as *S. aureus* and *Enterococcus faecalis*, should be considered as significant pathogens when investigating bacteria agents of UTI in our region.

Conclusion:

Results of the study highlights the presence of significant bacteriuria, predominantly caused by *E. coli* and *S. saprophyticus*, among apparently healthy pupils in the study area, with higher prevalence in the female pupils. The apparent risk of developing symptomatic UTI with its attendant complications in these pupils should spur preventive education of the parents/guardians and the general populace of the possible menace of this entity.

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**Original Article****Open Access****Bacteria urinary tract infection in HIV-infected children and adolescents in Abuja, Nigeria: a cross-sectional study**¹Okechukwu, A. A., and ²Thairu, Y.Departments of ¹Paediatrics and ²Microbiology, University of Abuja Teaching Hospital, Gwagwalada, Abuja, Nigeria
Correspondence to: nebokest@yahoo.com; +2348036719906**Abstract:****Background:** Urinary tract infection (UTI) remains the second commonest opportunistic infections among HIV infected children. This study was conducted to determine the prevalence and causative bacteria of UTI in HIV infected children and adolescents on antiretroviral medications in our health institution.**Method:** The study was a cross sectional design conducted between October 2017 and March 2018 among HIV infected children and adolescents aged 2 months to 18 years on follow up attendance at the Paediatric Outpatient Special Treatment Clinic (POSTC) of University of Abuja Teaching Hospital (UATH). Early morning midstream urine was collected from each participant for urinalysis, microscopy and aerobic bacterial culture. Bacteria were identified from culture by standard microbiological methods and antibiogram of the isolates was determined by the disk diffusion method.**Result:** Of 166 HIV infected children and adolescents studied, 106 (63.9%) were males, 82 (49.4%) were in age group 5-10 years, and 110 (66.3%) were from lower socio-economic class. Significant bacteria (UTI) were isolated in 54 (32.5%) subjects, with 38 (70.4%) from females, and 51 (94.4%) from those on first line antiretroviral therapy. Isolates recovered were *Escherichia coli* 20 (37.0%), *Klebsiella pneumoniae* 16 (29.6%), *Staphylococcus aureus* 8 (14.8%), *Pseudomonas aeruginosa* 6 (11.1%), and *Proteus mirabilis* 4 (7.4%). Leucocyturia in 19 (35.2%), nitrituria in 10 (18.5%), and haematuria in 15 (27.8%) subjects with significant bacteriuria were also recorded. Isolates were sensitive to ofloxacin (81.5%), nalidixic acid (74.1%) and cefuroxime (61.1%), while they were resistant to cotrimoxazole (100%), ampicillin (98.1%) and piperacillin (94.4%). Significant difference was observed in the mean CD4 cell count and viral load of subjects with significant bacteriuria compared to those without; 838.6 ± 177.8 versus 1009.9 ± 234.7 cells/μL ($p=0.02$), and 10, 360.5 ± 471.0 versus 5, 840.8 ± 563.8 copies/ml ($p=0.003$) for CD4 cell count and viral load respectively.**Conclusion:** This study reported a high prevalence of UTI among HIV infected children and adolescents, especially in those with high viral load. Routine screening for UTI should be offered to HIV infected children and adolescents with high viral load.**Keywords:** HIV, urinary tract infection, children, adolescents

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Copyright 2019 AJCEM Open Access. This article is licensed and distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution and reproduction in any medium, provided credit is given to the original author(s) and the source.**Infection des voies urinaires par des bactéries chez des enfants et des adolescents infectés par le VIH à Abuja, au Nigeria: étude transversale**¹Okechukwu, A. A., et ²Thairu, Y.Départements de ¹pédiatrie et ²microbiologie, Hôpital universitaire de Abuja, Gwagwalada, Abuja, Nigeria
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Abstrait:

Contexte: L'infection des voies urinaires (UTI) reste la deuxième infection opportuniste la plus répandue chez les enfants infectés par le VIH. Cette étude a été menée pour déterminer la prévalence et la bactérie causale des infections urinaires chez les enfants et les adolescents infectés par le VIH prenant des antirétroviraux dans notre établissement de santé.

Méthode: L'étude était une conception transversale menée entre octobre 2017 et mars 2018 chez des enfants et des adolescents infectés par le VIH âgés de 2 mois à 18 ans et suivis à la Clinique de traitement spécial pour enfants ambulatoires (POSTC) de l'Hôpital universitaire de Abuja (UATH). Des échantillons d'urine, de microscopie et de cultures bactériennes aérobies ont été recueillis chez chaque participant. Les bactéries ont été identifiées à partir de cultures par des méthodes microbiologiques standard et l'antibiogramme des isolats a été déterminé par la méthode de diffusion sur disque.

Résultat: Sur 166 enfants et adolescents infectés par le VIH étudiés, 106 (63,9%) étaient des hommes, 82 (49,4%) étaient âgés de 5 à 10 ans et 110 (66,3%) appartenaient à la classe socio-économique inférieure. Des bactéries significatives (UTI) ont été isolées chez 54 sujets (32,5%), dont 38 (70,4%) de femmes et 51 (94,4%) de celles sous traitement antirétroviral de première intention. Les isolats récupérés étaient *Escherichia coli* 20 (37,0%), *Klebsiella pneumoniae* 16 (29,6%), *Staphylococcus aureus* 8 (14,8%), *Pseudomonas aeruginosa* 6 (11,1%) et *Proteus mirabilis* 4 (7,4%). Une leucocyturie chez 19 sujets (35,2%), une nitriturie chez 10 (18,5%) et une hématurie chez 15 sujets (27,8%) présentant une bactériurie importante ont également été enregistrés. Les isolats étaient sensibles à l'ofloxacine (81,5%), à l'acide nalidixique (74,1%) et au céfuroxime (61,1%), tandis qu'ils étaient résistants au cotrimoxazole (100%), à l'ampicilline (98,1%) et à la pipéracilline (94,4%). Une différence significative a été observée entre le nombre moyen de cellules CD4 et la charge virale des sujets présentant une bactériurie significative par rapport à ceux ne présentant pas; $838,6 \pm 177,8$ par rapport à $1009,9 \pm 234,7$ cellules/ μL ($p = 0,02$) et $10,360,5 \pm 471,0$ par rapport à $5\,840,8 \pm 563,8$ copies/ml ($p = 0,003$) pour le nombre de cellules CD4 et la charge virale, respectivement.

Conclusion: Cette étude a révélé une prévalence élevée d'UTI chez les enfants et les adolescents infectés par le VIH, en particulier chez ceux ayant une charge virale élevée. Un dépistage systématique des infections urinaires doit être proposé aux enfants et aux adolescents à charge virale élevée infectés par le VIH

Mots-clés: VIH, infection des voies urinaires, enfants, adolescents

Introduction:

Globally over 3.4 million children are living with human immunodeficiency virus (HIV) infection at the end of 2011, 91% of which are in sub-Saharan Africa, which harbors only 10% of the world's population (1, 2). Kidney disease is a widely recognized frequent complication of HIV infection and often manifests as HIV associated nephropathy (HIVAN), urinary tract infections (UTI), electrolyte abnormalities, among others (3, 4). UTI is defined as the presence of bacterial growth of greater than 10^5 colony forming units per ml in a clean-catch, mid-stream voided urine sample, or greater than 10^3 organisms per ml in a catheter or supra-pubic aspirated urine. UTI is the second most common infection in children (5).

Urinary tract is a unique sterile space lined with transitional mucosa cells and usually impermeable to microorganisms. However, infection occurs when pathogenic organisms enter the space and subsequently adhere to its mucosal lining (5). The mechanisms of entrance are either through retrograde ascent of fecal-perineal bacteria from the host bowel or nosocomial following instrumentation, or as part of a systemic infection (5, 6). Gender, age, race, circumcision, and immune status of

individuals are host factors that affect the incidence and bacterial adherence in UTI (5, 6).

Notable urinary pathogens causing UTI include Gram-negative rods (*Escherichia coli*, *Citrobacter* spp, *Enterobacter* spp), Gram-negative cocci (*Neisseria gonorrhoeae*), Gram-positive cocci (*Staphylococcus aureus*, *Staphylococcus saprophyticus*), and other pathogens such as *Candida albicans*, and *Chlamydia trachomatis* (5, 6). This risk of UTI is high in women, infants, elderly, and people with spinal cord injuries, urinary catheters, diabetes, immunodeficiency, and underlying urologic abnormalities (7, 8, 9, 10).

The hallmark of HIV infection is the profound immunosuppression that results from continued depletion of CD4 cells. This immune deficient state predisposes the individual to a wide variety of opportunistic infections (OIs) and renal syndromes that causes neurologic complications leading to urinary stasis and ultimately UTI (11,12). The incidence of UTI in HIV population is clearly related to infection and immune function, as determined by lymphocytes CD4+ cells count. UTI is one of the most common bacterial infections associated with morbidity and hospitalization in HIV infected individuals (13).

Available literature on UTI and HIV/AIDS however showed conflicting reports,

with some authors reporting higher frequency of UTI among HIV cohorts especially adult population, (14,15) while others reported no significant impact of the disease on the incidence of UTI (16,17). Asharam et al., (16) found no significant impact of HIV/AIDS on bacterial UTI among infected children aged 0–12 years in South Africa, which was similar to the report of O'Regan et al., (17) in Canada. However in Nigeria, Ibadin et al., reported high frequency of UTI in adolescent and young adult patients with advanced WHO clinical stages (18). Kaplan et al., (15) also reported high incidence of UTI (20.0%) among his AIDS cohort.

Because of paucity of information on incidence of UTI among HIV children in this part of the country, this study was conducted to determine the prevalence and aetiological agents of UTI among HIV-infected children and adolescents on highly active antiretroviral therapy (HAART) at a tertiary health institution in Abuja, Nigeria, with the aim that these findings will provide insight on the magnitude of the problem among infected children and adolescents in our environment.

Methodology:

Study setting and design

A cross sectional study was carried out at the Paediatric Outpatient Special Treatment Clinic (POSTC) of the University of Abuja Teaching Hospital (UATH) between October 2017 to March 2018. POSTC is an out-patient clinical service area where HIV infected children and exposed babies are seen and followed up for treatment/monitoring. It has consulting rooms for the doctors, nurses, and adherence counselors. Record clerks, Pharmacists and nutritionists are also at their disposal on week days (Monday-Friday, from 7.30 am to 4 pm.).

UATH is a 350 bed capacity referral hospital, sub-serving the people of Federal Capital Territory, Abuja and five neighbouring states. It is one of the first centers to start offering free HIV/AIDS services in the country in 2005, through the President Emergency Plan for AIDS Relief (PEPFAR).

Subjects

The subjects were paediatric HIV infected patients between the ages 2 months and 18 years diagnosed either by serological method or by polymerase chain reaction (PCR) test, and started on anti-retroviral (ARV) therapy. Consecutive eligible children attending

the POSTC were recruited and subsequently enrolled into the study after parents/caregivers provided written informed consent, and children 7 years and above provided written informed assent.

Inclusion criteria for the study were; HIV infected children and adolescents from 2 months to 18 years of age on ARV therapy, patients/caregivers/children residing within FCT Abuja for easy collection of early morning urine, parents/caregivers acceptance to be part of the study, and older children who gave assent for the study. Exclusion criteria include those unwilling to participate in the study, exposed babies, those residing outside FCT, and patients with other forms of nephropathy such as nephrotic syndrome, acute glomerulonephritis and others. Ethical clearance was obtained from the Ethics Committee of the health institution before the commencement of the study.

Specimen collection

Clinical and physical examinations were carried out on each subject after enrollment by the attending physician. All the subjects were then given plastic universal sterile transparent container with screw cap, and oral and written instructions were provided for each subject or their parents/guardians on the method of urine collection.

Early morning clean catch specimens of midstream voided urine were collected. For the female subjects, they were instructed to clean their genitalia from anterior to posterior in a unidirectional manner with the labial held apart, and for the infants, the parent/caregiver were advised to hold the child on the laps with the genitals exposed prior to collection. The urine samples were delivered to the laboratory within one hour of collection.

Blood sample was also collected for CD4⁺ T-cell (CD4) and viral load (VL) estimation from those subjects who have not had these estimated in the last 3 months preceding the study.

Bacterial cultures and identification

Each urine specimen was first examined macroscopically and then divided into two equal volumes. The first was used for urinalysis and Gram's staining, while the second volume was centrifuged at 3000 rpm for 5 minutes, and used for urine microscopy and inoculation of Blood and Cysteine Lactose Electrolyte Deficient (CLED) agar plates. Calibrated wire loop delivering 0.002 ml of the centrifuged urine was used to inoculate the

culture media. The cultures were incubated at 37°C for 18-24 hours aerobically. After overnight incubation, colony number and characteristics on the agar plates were determined, and then Gram stained. Colonies that were Gram positive on CLED were further characterized using conventional biochemical scheme of catalase, coagulase and novobiocin disc tests, while those with Gram negative reaction were characterized using the indole, motility, methyl red, Voges-Proskauer and citrate utilization (IMMVPC) tests.

The presence of at least one Gram positive or negative bacteria per oil-immersion field in the uncentrifuged urine or colony count of greater than 10^5 CFU/ml of urine from the overnight culture on Blood agar plate was considered significant bacteriuria. Positive nitrite on urinalysis and presence of pus cells were considered features suggestive of UTI.

Antibiotic susceptibility testing

Antibiotic susceptibility of each isolate along with control strains (*Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* ATCC 29212) was determined using the Muller-Hinton agar media by the disk diffusion method. Three to five pure colonies of similar appearance of each isolate and the control strains were emulsified in 4 ml sterile saline. The inoculum suspension was standardized by comparing the turbidity with 0.5 McFarland standards. Muller-Hinton agar plate was streaked with the inoculum suspension using sterile swab, and antibiotic discs were placed on each agar plate after 5 minutes using sterile forcep. Within 30 minutes of applying the discs, the plates were incubated at 35°C for 18 to 24 hours, following which the test and the control agar plates were examined, and the diameters of zone of inhibition (in mm) were measured with a ruler.

The zone diameters of inhibition of the antibiotics for the control strain were first compared with the CLSI standards and if within the acceptable limits, the zone diameters for the test isolates were then compared with CLSI zone diameter breakpoints, and result interpreted as sensitive, intermediate or resistance.

CD4 cell count & viral load estimation

The CD4 cell count was measured

using automated Partec Cyflow easy count kit (Partec code no. 05-8401, Western Germany), and viral load (VL) measurement was done with Roche Smp/prep/cobs Taqman 96, USA.

Data analysis

Data were analysed using SPSS version 21.0 and results presented in frequency tables, percentages, means and standard deviations. The test for association between variables was done with the student t-test. A p value < 0.05 was considered significant.

Results:

Table 1 shows the demographic distribution of the 166 subjects, comprising 106 (63.9%) males, 82 (49.4%) in age group 5-<10 years, 114 (68.7%) were Christian, 110 (66.3%) from low socio-economic class, and 142 (85.5%) on 1st line ART. The mean CD4 cell count was 894.5 ± 449.1 cells/ μ l, and mean VL was $9,136.1 \pm 306.0$ copies/ml.

The characteristic of the study population with and without isolates is shown in Table 2. Bacteria were isolated from urine of 54 (32.5%) subjects; *Escherichia coli* in 20 (37.0%), *Klebsiella pneumoniae* 16 (29.6%), *Staphylococcus aureus* 8 (14.8%), *Pseudomonas aeruginosa* 6 (11.1%), and *Proteus mirabilis* 4 (7.4%). Leucocyturia, nitrituria, and haematuria were reported in 19 (35.2%), 10 (18.5%), and 15 (27.8%) subjects with urinary bacterial isolates respectively.

Sixteen (29.6%) isolates were recovered from subjects in age group 10-15 years, 38 (70.4%) from females, 36 (66.7%) from Christians, and 31 (57.4%) from subjects in low socio economic class. There was no significant difference in socio-economic status ($p=0.303$), age group ($p=0.744$), religion ($p=0.699$), and leucocyturia, nitrituria or haematuria ($p=0.64$) between subjects with urinary isolates and those without isolates. There was however a significant difference in the CD4 cell count (838.6 ± 177.8 vs 1009.9 ± 234.7 cells/ μ l, $p=0.02$) and viral load ($10,360.5 \pm 471.0$ vs $5,840.8 \pm 563.8$ copies/ml, $p=0.003$) between subjects with urinary isolates compared to those without isolates, and subjects on 1st and 2nd line medication ($p=0.024$), and gender ($p=0.03$).

Table 1: Demographic and clinical characteristics of the study population

Characteristics	Male (%)	Female (%)	Total (%)
Number of Subjects	106 (63.9)	60 (36.1)	166 (100)
Age group (years)			
<5	7 (53.8)	6 (46.2)	13 (7.8)
5-<10	55 (67.1)	27 (32.9)	82 (49.4)
10-15	34 (61.8)	21 (38.2)	55 (33.1)
>15	10 (62.5)	6 (37.5)	16 (9.6)
Religion			
Christianity	78 (68.4)	36 (31.6)	114 (68.7)
Islam	28 (53.8)	24 (46.2)	52 (31.3)
Socioeconomic class			
High	15 (68.2)	7 (31.8)	22 (13.3)
Middle	24 (70.6)	10 (29.4)	34 (20.4)
Low	67 (60.9)	43 (39.1)	110 (66.3)
ARVT			
1 st line	92 (64.8)	50 (35.2)	142 (85.5)
2 nd line	14 (58.3)	10 (41.7)	24 (14.5)
Weight, CD4 and Viral Load			
Weight (kg)	*30.1±11.5	*31.52±13.7	30.6±12.3
CD4 (cells/μl)	*882.0±435.0	*916.6±475.9	894.5±449.1
Viral Load (copies/ml)	*10,158.75±332.6	*7,360.5±257.6	9,136.1±306.0

ARVT = Anti-retroviral therapy; *Values are mean ±SD

Table 2: Characteristics of subjects with and without urinary isolates

Characteristics	With isolates (%)	Without isolates (%)	Total (%)	p value
Total Number	54 (32.5)	112 (67.5)	166 (100)	
Age group (years)				
<5	4 (7.4)	9 (8.0)	13 (7.8)	0.744
5-<10	13 (24.1)	55 (49.1)	82 (49.4)	
10-15	16 (29.6)	39 (34.8)	55 (33.1)	
> 15	7 (13.0)	9 (8.0)	16 (9.6)	
Gender				
Male	16 (29.6)	90 (80.4)	106 (63.8)	0.031
Female	38 (70.4)	22 (19.6)	60 (36.2)	
Religion				
Christianity	36 (66.7)	78 (69.6)	114 (68.7)	0.699
Islam	18 (33.3)	34 (30.4)	50 (31.3)	
Socioeconomic status				
High	9 (16.7)	13 (11.6)	22 (13.3)	0.303
Middle	14 (25.9)	20 (17.9)	34 (20.4)	
Low	31 (57.4)	79 (70.5)	110 (66.3)	
ARVT				
1 st line	51 (94.4)	91 (81.3)	142 (85.5)	0.024
2 nd line	3 (5.6)	21 (18.8)	24 (14.5)	
Types of isolates				
<i>Escherichia coli</i>	20 (37.0)	-	20 (12.1)	
<i>Klebsiella pneumoniae</i>	16 (29.6)	-	16 (9.6)	
<i>Staphylococcus aureus</i>	8 (14.8)	-	8 (4.8)	
<i>Pseudomonas aeruginosa</i>	6 (11.1)	-	6 (3.6)	
<i>Proteus mirabilis</i>	4 (7.4)	-	4 (2.4)	
Leucocyturia	19 (35.2)	3 (2.7)	22 (13.3)	0.64
Nitrituria	10 (18.5)	1 (0.9)	11 (6.6)	
Haematuria	15 (27.8)	4 (3.6)	19 (11.4)	
Weight (kg)	*31.4±8.6	*30.2±7.3	30.6±12.3	0.54
CD4 (cells/μl)	*838.6±177.8	*1009.9±234.7	894±449.1	0.02
Viral Load (copies/ml)	*10,360.5± 471.0	*5,840.8±563.8	9,136±306.1	0.003

Table 3: Antibiotic susceptibility of isolates from subjects with urinary tract infection

Antibiotic sensitivity	<i>E. coli</i> (%) (n = 20)	<i>K. pneumoniae</i> (%) (n=16)	<i>S. aureus</i> (%) (n = 8)	<i>Ps. aeruginosa</i> (%) (n=6)	<i>P. mirabilis</i> (%) (n=4)	Total (%) (n = 54)
Ofloxacin	18 (90)	14 (87.5)	7 (87.5)	5 (83.3)	3 (75)	44 (81.5)
Augmentin	3 (15)	7 (43.8)	2 (25)	3 (50.0)	3 (50)	19 (35.2)
Cefuroxime	12 (60)	11 (68.8)	5 (62.5)	4 (66.7)	1 (25)	33 (61.1)
Nalidixic acid	15 (83.3)	12 (75)	6 (75.5)	5 (83.3)	2 (50)	40 (74.1)
Gentamicin	9 (45)	7 (43.8)	3 (37.5)	2 (33.3)	2 (50)	23 (42.6)
Tetracycline	0	0	0	0	0	0
Antibiotic resistance						
Cotrimoxazole	20 (100)	16 (100)	8 (100)	6 (100)	4 (100)	54 (100)
Ampicillin	19 (95)	16 (100)	8 (100)	6 (100)	4 (100)	53 (98.1)
Amoxycillin	18 (90)	15 (93.8)	8 (100)	6 (100)	3 (75)	50 (92.6)
Erythromycin	16 (80)	12 (75.0)	7 (87.6)	5 (83.3)	3 (75)	43 (79.6)
Piperacillin	20 (100)	15 (93.8)	8 (100)	4 (66.7)	4 (100)	51 (94.4)

The susceptibility patterns of the isolates are shown in Table 3. While ofloxacin, nalidixic acid and cefuroxime were the most effective antibiotics *invitro* with 44 (81.5%), 40 (74.1%), and 33 (61.1%) of the isolates respectively sensitive to them, augmentin and tetracycline were the least effective with 19 (35.2%) and 0 (100%) isolates respectively, sensitive to them. The isolates were largely resistant to cotrimoxazole 54 (100%), ampicillin 53 (98.1%), and piperacillin 51 (94.4%).

Ofloxacin was the most effective antibiotic *invitro* with 18 (90.0%) *E. coli*, 14 (87.5%) *K. pneumoniae*, and 7 (87.5%) *S. aureus* isolates sensitive to it. Tetracycline was the least effective with no isolate sensitive to it, while 9 (45.0%) *E. coli*, 7 (43.8%) *K. pneumoniae*, 3 (37.5%) *S. aureus*, 2 (33.3%) *Ps. aeruginosa*, and 2 (50.0%) *P. mirabilis* were sensitive to gentamicin.

Discussion:

This study shows a high prevalence rate of 32.5% for UTI among HIV-infected children and adolescents on follow up visit to the Paediatric Outpatient Special Treatment Clinic of University of Abuja Teaching Hospital, Abuja, Nigeria. This was far lower than 96.6% reported among HIV infected children aged 0-12 years in South Africa by Asharam et al., (16). The difference between the two studies might be due to the study design and nature of the patients in the two groups. While the South African study was a retrospective one carried out among symptomatic hospital patients with culture-proven UTI, the present study was a cross sectional survey of HIV patients on outpatient routine clinic visit. The prevalence in our study was however comparable to figures obtained from HIV infected children and adults

in other centers and other countries; 24.7% in children from Lagos (19), 22.2% among HIV children (20), 23.2% among adults in Poland (21), 48.7% among adults from South Africa (11), 40.4% among adults in Nigeria (22), and 57.3% from another Nigerian study among adult population (23). The rate in our study was also higher than the rates reported from other studies among HIV infected children; 9.5% from Zimbabwe (24), 16.8% from Jamaica (3), 13.2% from Romania (25), 6.3%, 10.3% and 6.8% from other studies from Nigeria (18,26,27).

The high prevalence of UTI among HIV infected children and adolescents in this study require further evaluation for clinical significance. This is important because of attendant risk of developing renal scarring from pyelonephritis and long term complication of hypertension and chronic kidney disease. The reason for the high prevalence of UTI in this study might be due to the practice of discontinuation of chemoprophylaxis with cotrimoxazole in HIV patients whose immunity has remarkably improved. This practice is common in other centers, and appeared logical as suggested by Imamura from Japan (28). Co-trimoxazole is generally recommended in HIV infected patients for prevention of opportunistic infections (OIs). HAART is equally the most effective approach in prevention of OIs however, OIs are still occurring in patients on HAART, especially those who accessed care late (28). The late access to care might be the case of patients in this study which though showed a good immunological response (mean CD4 cell counts of 894.5±449.1 cells/μl) but still had evidence of UTI.

Subjects with isolates in their urine had significantly lower CD4 cell count and higher VL than those without isolates; 838.6±177.8 vs 1009.9±234.7 cells/μl ($p=0.02$) for CD4 cell

count, and $10,360.5 \pm 471.0$ vs $5,840.8 \pm 563.8$ copies/ml, ($p=0.003$) for VL. This is similar to findings of other studies where significantly lower CD4 cell count and WHO stage 3 and 4 clinical disease consistent with high VL, was observed in infected children and adults with UTI (21, 29, 30). Iduoriyekemwen et al., (26) however did not make such observations among their study cohort in Nigeria. The reason for the significant difference in CD4 cell count and VL of those with and those without isolates in their urine might be related to late recognition of virological failure that may have occurred in those on 1st line ARV medications as subjects on 1st line drugs were observed to have significant isolates in their urine. Okechukwu and Amajuoyi (31) in their 9 year review of ART failure among HIV infected children in the same study center observed as much as 15.9% to have failed 1st line ART. This virological failure may go on for several years before immunological and clinical failures become evident with risk of OIs.

VL monitoring is the state-of-the-art monitoring of patients on ART. This test is to be done at least every 3-4 months after the initial test at diagnosis. Due to high cost, VL and resistant testing are not usually done in many HIV centers in resource limited settings like ours hence patients who fail virologically may be missed for a long time before they are switched to 2nd line medication. Recently though, regular VL monitoring has been institutionalized in our center.

Urinary pathogens were significantly isolated more in females than males (70.4% vs 29.6%, $p=0.031$) in the present study. This is consistent with findings of other studies in both children and adults; 17.7% vs 7.0% among HIV children from Lagos (19), 75.0% vs 25.0% by Iduoriyekemwen et al., (26) in children from Benin, 42.8% vs 10.2% among uninfected children from Lagos (32), 89.5% vs 10.4% by Iweriobor et al., (12) among adults from South Africa, 90% vs 10% by Ibadin et al., (18) among adolescents and young adults in Nigeria, and 6.6% vs 4.8% among infected children and adults in Jos, Nigeria by Sheyin et al., (33). The reasons for higher prevalence of UTI in female subjects as previously established are related to short length of female urethra and the close proximity of vagina to anal orifice. This anatomical profile of female gender enhances retrograde ascent of fecal-perineal bacteria, increase peri-urethral colonization and easy contamination of enteric organisms from rectum to the urinary tract (5, 6). Also absence of prostatic fluid in females which tend to decrease colonization in male

because of its bacteriostatic property may also contribute to higher prevalence of UTI in females (6, 34). Decreased incidence of UTI in males could also be due to wide practice of circumcision in male especially among the Christian which formed 68.7% of the subjects in this study. This singular practice in males does not only reduce colonization of the urethral opening with enteric organisms, but also reduce the risk of UTI (25).

The frequency of isolated bacteria from urine in our study compared favourably with those from other HIV studies in both children and adults across the globe (12, 16-27). These uropathogens easily get access to the urinary space through retrograde ascent from the host's bowel (5,6) but could also result from nosocomial cause or from haematogenous seeding from distant sites. While ofloxacin, nalidixic acid and cefuroxime were the most effective antibiotics *in vitro* for the uropathogens in the present study, co-trimoxazole, ampicillin and piperacillin were the least effective. Similar patterns have been observed among isolates in different studies on HIV infected children and adults (12, 16-27).

Leucocyturia is referred to as the presence of white blood cells (WBC) in urine, which can easily be detected using a dipstick urinalysis in a reaction that is based on the presence of leucocyte esterase, an enzyme found in azurophilic granules of monocytes and granulocytes. Bacteria and epithelial cells of the urinary tract do not contain esterase hence a positive leucocyte esterase test in dipstick urinalysis implies an inflammation and not necessarily an infection. Leucocyturia is abnormal, although some experts believe that observation of more than 5 WBC per high power field (HPF) is indicative of pathological leucocyturia. However, UTI is more likely when leucocyturia is associated with bacteriuria. In the present study, 35.2% of the subjects with isolates in their urine had leucocyturia, an association that is strongly in support of UTI. This is similar to 33.3% leucocyturia reported by Dondo et al (24) among their cohorts.

Nitrituria is the presence of nitrite in the urine, which is usually absent in normal urine. Nitrite is produced by the reductive effect of enzyme reductase on nitrate in the urine. This enzyme is produced by nitrite producing organisms such as *Enterobacter*, *Citrobacter*, *Klebsiella*, *E. coli*, *Ps. aeruginosa* and *P mirabilis* when present in the urine (35). In the present study, nitrituria was seen in 18.5% of subjects with positive isolates in the urine signifying presence of nitrite producing organisms which constituted 85.2% of isolates

in urine of subjects in this study. Dondo et al (24) in their study however observed only one among 21 subjects with isolates in their urine to have positive nitrite test, though nitrate producing organisms constituted 78.8% of isolates in their study.

Haematuria is defined as the presence of 5 or more red blood cells (RBC) per high power field (HPF) in two consecutive fresh, centrifuged specimens obtained at least one week apart (36). The dipstick urinalysis method is the commonest method for detection of haematuria in urine and has been reported to be 100% sensitive and 99% specific in detecting 5-10 RBC/HPF (5-10 RBC/ μ l of urine or 0.015 mg/dl of haemoglobin). Haematuria can originate from any part of the kidney as injury to the uro-epithelium such as irritation, inflammation or invasion by organism can result in haematuria. In the present study, 27.8% of subjects with isolates in their urine had positive dipstick urinalysis. However microscopy was not conducted for the presence of red cell cast, dysmorphic RBC or acanthocytes to determine if the haematuria is of glomerular or tubular origin or from other parts of the urinary tract.

Conclusion

There is high prevalence of UTI among HIV infected children and adolescents on outpatient follow up at the University of Abuja Teaching Hospital, Abuja, Nigeria. This is more likely to occur in subjects with high VL. Routine urine screening with dipstick urinalysis and culture for evidence of UTI are recommended for HIV infected children and adolescents with high viral load for early diagnosis.

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**Original Article****Open Access****Effect of physical stresses on survivability and post-exposure antibiotic susceptibility of coliforms in environmental waters and wastewaters**

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*Correspondence to: oo.adebisi@outlook.com; soji-olusegun@unilorin.edu.ng**Abstract:****Background:** Coliform bacteria are majorly introduced into water bodies (river and wastewater) as a result of faecal pollution, agricultural run-offs and several anthropogenic activities. Despite the effectiveness of water treatment methods, pathogens still persist in water; hence the relevance of assessing the ability of these pathogens to survive the lethal actions of physical stresses and the possible impact on antibiotic susceptibility pattern of the organisms.**Methodology:** The survivability of *Escherichia coli* strains (NCM3722, FAP1 and ST2747), *Enterobacter cloacae* GGT036 and *Shigella sonnei* 53G was assessed in environmental and waste waters for 21 days. The effect of three treatment regimens (UV radiation, solar radiation and boiling) on the survival of the coliforms was evaluated. Also, the antibiogram of the isolates post-UV exposure was assayed.**Results:** Although there was significant reduction (≥ 3 -log) in the population of the bacteria overtime, all the coliforms survived in the waters for 21 days. The effect of UV radiation was significant on all organisms (> 3 log reductions). Solar radiation for 60 minutes had significantly lesser effect than boiling for 15 minutes. Surviving cells of all isolates demonstrated multiple drug-resistance post exposure to UV radiation.**Conclusion:** This study revealed the ability of coliforms to persist in waters after treatment and proves that UV radiation may not be effective in attenuation of antibiotic resistance.**Keywords:** Survivability; Coliforms; Antibiotic susceptibility; Water; Wastewater

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Copyright 2019 AJCEM Open Access. This article is licensed and distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution and reproduction in any medium, provided credit is given to the original author(s) and the source.**Effet des contraintes physiques sur la capacité de survie et la susceptibilité aux antibiotiques post-exposition aux coliformes dans les eaux de surface et les eaux usées**

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*Correspondance à: oo.adebisi@outlook.com; soji-olusegun@unilorin.edu.ng**Abstrait:****Contexte:** Les bactéries coliformes sont principalement introduites dans les eaux (rivières et eaux usées) en raison de la pollution fécale, des écoulements agricoles et de plusieurs activités anthropiques. Malgré l'efficacité des méthodes de traitement de l'eau, les agents pathogènes persistent dans l'eau; D'où la pertinence d'évaluer la capacité de ces agents pathogènes à survivre aux actions létales des stress physiques et à l'impact possible sur le profil de sensibilité des organismes aux antibiotiques.**Méthodologie:** La capacité de survie des souches d'*Escherichia coli* (NCM3722, FAP1 et ST2747), de *Enterobacter cloacae* GGT036 et de *Shigella sonnei* 53G a été évaluée pendant 21 jours dans des eaux environnementales et des eaux usées. L'effet de trois schémas thérapeutiques (rayonnement UV, rayonnement solaire et ébullition) sur la survie des coliformes a été évalué. En outre, l'antibiogramme des isolats après l'exposition aux UV a été testé.**Résultats:** Bien qu'il y ait eu une réduction significative (≥ 3 log) de la population de bactéries en heures supplémentaires, tous les coliformes ont survécu dans les eaux pendant 21 jours. L'effet du rayonnement UV était significatif sur tous les organismes (réductions de > 3 log). Le rayonnement solaire pendant 60 minutes a

eu un effet significativement moindre que celui d'ébullition pendant 15 minutes. Les cellules survivantes de tous les isolats ont démontré une résistance multiple aux médicaments après une exposition aux rayons UV.

Conclusion: cette étude a révélé la capacité des coliformes à persister dans les eaux après traitement et prouve que le rayonnement UV peut ne pas atténuer efficacement la résistance aux antibiotiques.

Mots-clés: Survivabilité; Les coliformes; Sensibilité aux antibiotiques; Eau; Eaux usées

Introduction:

Coliforms serve as the conciliation between the demand for safe water and the tedious process of isolation and culture of pathogens in water. Positive results of coliforms in water samples usually suggest the presence of disease-causing pathogens which may be life threatening. Basically, coliforms are unlikely to cause diseases but there are exceptions with some strains at specific physiological conditions. *Escherichia coli*, *Enterobacter* sp., *Shigella* sp., *Salmonella* sp. and *Enterococci* sp. are commonly isolated coliform bacteria in environmental waters and their occurrence or survival are dependent on various physiological processes (1).

Escherichia coli NCM3722 is a wild-type prototrophic strain that is capable of optimal growth in minimal medium- it can survive on inorganic salts only with simple energy sources such as sugar and water (2). This strain exhibits unique physiological properties including galactose metabolism. *Escherichia coli* FAP1 is a strain that has been reported to harbour the CTX-M ESBL gene that codes for resistance against cephalosporins (3). *Escherichia coli* ST2747 is a pathogenic intestinal strain unique for its virulence in intestinal infections (4). *Enterobacter cloacae* GGT036 is a furfural-tolerant strain with ability to persist in the environment for a long period (5). *Enterobacter* species are pathogens that can cause fatal infections especially in hospital settings. *Shigella sonnei* 53G is a pathogenic strain that carries plasmid-encoded resistance genes and also a potential aetiology of bacillary dysentery (6).

Coliform bacteria are majorly introduced into water bodies (river and wastewater) as a result of faecal pollution, agricultural run-offs and several anthropogenic activities. Presence of pathogens in rivers and wastewater could serve as a channel for community-acquired infections through consumption or use in agricultural or domestic purposes. There are a couple of primary methods in use for reducing pathogens and coliforms in water to an acceptable standard for potability.

Water decontamination procedures include boiling, filtration, ultraviolet, solar and chemical disinfection (7). Boiling water to a 'rolling-boil' level for 1 minute at temperature of 212°F or 100°C has been

reported to inactivate bacteria, viruses, protozoa and other waterborne pathogens (8). The heat involved in boiling damages the structural composition of organisms thereby interfering with basic metabolic processes required for survival. Some studies have reported that *Shigella*, *Salmonella*, *E. coli* (ETEC) and *Vibrio cholerae* can be inactivated by boiling water at 65°C for 20 minutes (9). However, the effectiveness of boiling on the varying waterborne pathogens is dependent on temperature and time.

Solar disinfection involves the use of energy from sunlight for pathogen reduction in water and wastewater. The effect of this low-cost technique on pathogens has been reported to be detrimental through denaturation of cells resulting in death. The effectiveness of solar radiation in waterborne pathogen elimination however depends on the intensity of the sunlight, atmospheric temperature, water depth, turbidity of the water as well as duration of exposure (10, 11). Ultra violet (UV) rays form part of the natural sunlight but they have a higher spectrum frequency than visible light and lower than x-ray. UV light is effective against bacteria, viruses and also *Giardia lamblia* cysts and *Cryptosporidium* oocysts. The extent of effectiveness of UV disinfection however depends on the dose/power of the light, delivery of the light, time of exposure and the turbidity of the water (7, 12).

Despite the effectiveness of these water treatment methods, pathogens still persist in some water samples, hence the relevance of assessing the ability of these pathogens to survive the lethal actions of physical stresses and the possible impact on antibiotic susceptibility pattern of the organisms. In this research, the survivability of *E. coli* ST2747, *E. coli* NCM3722 *E. coli* FAP1, *Enterobacter cloacae* strain GGT036 and *Shigella sonnei* 53G after exposures to boiling, solar disinfection and ultraviolet treatment was assessed. The research also evaluated the effect of these treatment methods on the antibiotic susceptibility of the organisms.

Materials and Methods:

Collection and analysis of water and wastewater samples

The river water was collected from Oyun River dam at the University of Ilorin

main campus and borehole water from private borehole located at Jalala village, Ilorin, Nigeria. Agricultural wastewater was collected from a fish farm at Oke-Odo community, Ilorin while domestic wastewater was composited from samples collected from outlets of kitchen and bathroom of a flat at a private estate in Tanke, Ilorin. Temperature and pH of samples were measured at the site of collection using a mercury-in-glass thermometer and hand-held pH meter. Turbidity of water and wastewater samples was determined using a spectrophotometer measured at 460nm wavelength.

Preparation of pure bacterial cell suspension

The isolates (*Escherichia coli* NCM3722, *Escherichia coli* FAP1, *Escherichia coli* ST2747, *Enterobacter cloacae* GGT036 and *Shigella sonnei* 53G) used in this study were previously recovered from river water, characterised and identified using 16S rRNA sequencing. The antibiogram profiles of the isolates have also been previously determined and they were kept in the laboratory of Department of Microbiology, University of Ilorin. The isolates were resuscitated from their stocks on CLED agar (CM-CLED096, pH 7.5; Rapid Lab, UK). The purity of the culture was validated by repeated sub-culturing.

To obtain large cell culture at exponential growth phase for the survival experiment, the isolates were first grown in nutrient broth in a shaker incubator (37°C, 100 rpm) for 48–72 hours. The cell pellets were harvested by centrifugation at 3500 rpm for 10 minutes (Axiom Medical). After each run of centrifugation, the supernatant was carefully decanted and cell pellets re-suspended in a phosphate buffer solution (PBS). The process was repeated several times until pure cell pellets were obtained. Finally, the cells were suspended in PBS and kept in the refrigerator at about 4°C until use but not for more than four days.

Inoculum standardization

McFarland standards (barium chloride and sulphuric acid) were used to standardize the inoculum to a cell concentration in the range of 10^6 to 10^8 cells as most frequently found in polluted water and wastewater in nature. Using the spectrophotometer (600nm), the absorbance of the cell suspension was adjusted by dilution with sterile distilled water to achieve bacteria number of approximately $1-2 \times 10^8$ CFU/ml (13).

Survival experiment

Prior to the experiment, the water and wastewater samples were sterilised by repeated autoclaving at 121°C for 15 minutes for three consecutive times for three

alternating days (14). This was to ensure that all microbial spores were totally destroyed and bacterial re-growth is prevented. The effectiveness of this sterilisation technique was initially verified by plating out 1 ml of the sterilized wastewater on nutrient agar and potato dextrose agar and incubating at 37°C for 24 hours and 25°C for 7-10 days, respectively. Previous studies have shown that this process does not have significant effect on the properties of the environmental media (14, 15, 16). Aliquots (1 ml) of cell suspensions were added to 99 ml of sterilised water or wastewater in 250 ml capacity glass flasks to give a final cell concentration of approximately 1.2×10^6 CFU/ml.

Static state incubation of the seeded environmental media was performed at room temperature ($25 \pm 2^\circ\text{C}$) and sampling was carried out on days 0, 3, 6, 9, 12, 15, 18 and 21 for assessment of bacteria survival. At the times of sampling, the flasks were gently swirled for about 1 minute to dislodge any biofilm or settled microbial mass on the walls and surfaces of the flasks. Sub-samples were aseptically withdrawn from the flasks and appropriate serial dilutions made. From the last three diluents, 0.5 ml aliquots were taken and inoculated on appropriate culture agar media. MacConkey agar was used to culture the three strains of *E. coli*, *Shigella sonnei* 53G was cultured on Salmonella Shigella Agar (SSA) while *Enterobacter cloacae* GGT036 was cultured on Cysteine Lactose Electrolyte Deficient (CLED) agar (15, 16).

Disinfection treatment of water and wastewater

Before introducing the standardized inocula of isolates (1.2×10^6 CFU/ml), the water and wastewater were first sterilized following the procedure described earlier. The seeded water and waste water (100 ml in 250 ml capacity glass flasks) were treated to assess the effectiveness of selected treatment/disinfection techniques for the water and wastewater. Three different treatment regimens were studied: (1) Solar disinfection was carried out by exposing to sunlight (at $38 \pm 2^\circ\text{C}$) for 60 minutes with the flasks placed on a height of 120 m above ground level and without any structure obstructing; (2) Moist heat disinfection was performed by heating the flasks placed in a water bath to 100°C for 15 minutes; (3) Ultraviolet disinfection was done by exposing the water and wastewater (20 ml) in 90mm glass Petri dishes placed at approximately 50 cm to a UV light source for 15 and 30 minutes. After exposure, an aliquot (1ml) of each treated sample was plated on appropriate culture media using the pour-

plate technique. The plates were incubated at 37°C for *Enterobacter cloacae* GGT036 and *Shigella sonnei* 53G and at 45°C for the strains of *Escherichia coli*. Enumeration was done after 48 hours of incubation. While a single flask/Petri dish was used for each treatment set-up, the inoculation on culture plates was done in replicates (15, 16).

Antibiotic susceptibility testing

The surviving cells of the isolates after exposure to UV radiation for 30 minutes were tested against eight antibiotics in five different classes (penicillins, cephalosporins, nitrofurans, fluoroquinolones and aminoglycosides) using the Kirby-Bauer disk diffusion method (13). Prior to susceptibility assay, the cells were re-cultured into a sterile nutrient broth (48 hours inoculation in a shaker incubator at 37°C and 100 rpm) to obtain enough cell mass up to a concentration of $1-2 \times 10^8$ CFU/ml.

Mueller Hinton agar was inoculated with the test isolates (obtained from the various treated water and wastewater samples after exposure). Thereafter, the

following eight antibiotic disks were placed on the agar surface; ceftazidime (30µg), cefuroxime (30µg), gentamicin (10µg), ciprofloxacin (5µg), ofloxacin (5µg), amoxicillin/clavulanate (30µg), nitrofurantoin (300µg), and ampicillin (10µg). The plates were incubated for 16-24 hours at 37°C. To evaluate possible effect of prolonged exposure, the plates were further incubated for 72 hours, following which the diameter of zone of inhibition around each antibiotic disk was measured with a ruler.

Results:

Physicochemical characteristics of the environmental waters and wastewaters

Results presented in Table 1 show that physicochemical parameters of the borehole and river water samples were not different. However, the domestic and agricultural (fishery) wastewaters differed in their pH and turbidity. The domestic wastewater had the highest turbidity and lowest pH.

Table 1: Physicochemical properties of water and wastewater samples

Type	Sample	Colour/appearance	Temp. (°C)	pH	Turbidity (Å)
Wastewater	Fishery	Greenish, with lots of suspended particles	31	9.7	0.455
	Domestic	Cloudy, with suspended particles	32	5.0	1.737
Water	Borehole	Colourless and clear	32	8.7	0.014
	River	Colourless, with few suspended particles	32	8.9	0.016

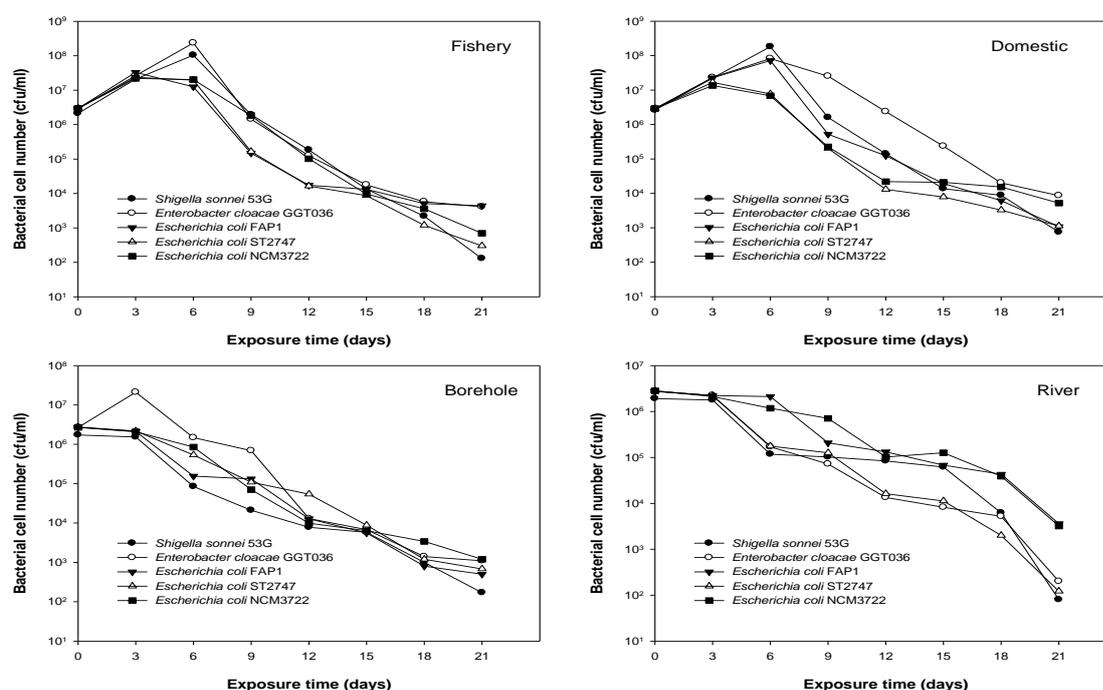


Fig 1: Survival of *Shigella sonnei* 53G (●), *Enterobacter cloacae* GGT036 (○), *Escherichia coli* FAP1 (▼), *Escherichia coli* ST2747 (▲) and *Escherichia coli* NCM3722 (■) in environmental waters and wastewaters

Survivability of pathogens in environmental waters and wastewaters

Survivability of the bacteria in different environmental waters and wastewaters are presented in Figure 1. All the five bacteria demonstrated similar survival trends in the wastewaters, with an appreciable increase in cell populations in the first 6 days before a gradual decline thereafter. The decline became significant after 18 days of incubation for all organisms in fishery wastewater (>3 log reduction from 1.2×10^6 to $1.2\text{--}5.8 \times 10^3$ CFU/ml) (Fig. 1a) and for *Shigella sonnei* 53G, *E. coli* strains FAP1 and ST2747 in domestic wastewater (>3 log reduction from 1.2×10^6 to $3.3\text{--}8.8 \times 10^3$ CFU/ml) (Fig. 1b). While *Shigella sonnei* 53G appeared to propagate better in the initial days, it did not survive as much as the *E. coli* strains in the latter days in the wastewaters. There was no difference in the pattern of growth or extent of survival of the three strains of *E. coli* investigated. *Enterobacter cloacae* GGT036 survived better than both *Shigella sonnei* 53G and *E. coli* ST2747 in the wastewaters (Fig. 1a–b).

Unlike the wastewaters, neither borehole nor river water seemed to support the proliferation of bacteria nonetheless the organisms survived for over 21 days in them (Fig. 1a–d). There were differences in the pattern and extent of bacteria survival in the borehole and river water (Fig. 1c–d). *Enterobacter cloacae* GGT036, *E. coli* strains ST2747 and NCM3722 maintained their populations for the first 6 days while *Shigella sonnei* 53G and *E. coli* FAP1 numbers declined after 3 days in borehole water (Fig. 1c). In the river water, the population of *E. coli* FAP1 was maintained up to 6 days but that of *Enterobacter cloacae* GGT036 or *E. coli* ST2747 reduced after 3 days, unlike what was observed in the borehole water

(Fig. 1d). In the borehole water, decline in population of all bacterial species was significant after 15 days of incubation (>3 log reduction from 1.2×10^6 to $5.6\text{--}8.8 \times 10^3$ CFU/ml) (Fig. 1c). In river water, the decline in bacterial populations became significant for *Shigella sonnei* 53G, *Enterobacter cloacae* GGT036 and *E. coli* ST2747 after 18 days (>3 log reduction from 1.2×10^6 to $2.0\text{--}6.2 \times 10^3$ CFU/ml) and for *E. coli* strains FAP1 and NCM3722 only by 21 days of incubation (>3 log reduction from 1.2×10^6 to $3.2\text{--}3.6 \times 10^3$ CFU/ml) (Fig. 1d).

Effects of treatment regimens on survivability of isolates in environmental and wastewaters

Results of the effect of UV radiation on bacterial survival in environmental waters and wastewaters are shown in Table 2. The effect of UV radiation was significant on all organisms (> 3 log reductions in cell numbers); this being greater at longer exposure duration. Although not statistically significant, there were greater reductions in cell numbers of *Shigella sonnei* 53G than for *Enterobacter cloacae* GGT036 at the two exposure times and for the three strains of *E. coli* after 30 minutes of exposure, in all waters and wastewater investigated (Table 2). Solar radiation for 60 minutes had significantly less effect than wet heat treatment for 15 minutes on bacterial survival (Table 3). While solar radiation for 60 minutes achieved only 2–3 log reductions in cell numbers, boiling for 15 minutes resulted in >4 log reductions in cell numbers or caused completed elimination of the organisms, particularly in the environmental waters (Table 3). In comparison, UV radiation for at least 30 minutes was nearly as effective as boiling for 15 minutes at drastically reducing ($P < 0.05$) the pathogen populations in the wastewaters.

Table 2: Effect of ultraviolet radiation on bacterial survival in environmental waters and wastewaters

Bacteria ¹	Bacterial Counts (cfu/ml)							
	Fishery		Domestic		Borehole		River	
	Exposure Duration (minutes)							
	15	30	15	30	15	30	15	30
<i>Shigella sonnei</i> 53G	2.94×10^3	1.62×10^4	5.40×10^2	4.00×10^4	1.00×10^2	8.00	2.00×10^2	1.30×10^4
<i>Enterobacter cloacae</i> GGT036	2.17×10^3	1.26×10^2	2.29×10^3	9.20×10^4	2.10×10^3	1.80×10^4	9.80×10^2	8.80×10^4
<i>Escherichia coli</i> FAP1	8.60×10^2	7.20×10^4	5.20×10^2	6.30×10^4	9.00×10^2	2.90×10^4	2.60×10^2	5.80×10^4
<i>Escherichia coli</i> ST2747	1.18×10^2	1.02×10^4	6.80×10^2	5.80×10^4	2.80×10^2	6.40×10^4	3.40×10^3	9.90×10^4
<i>Escherichia coli</i> NCM3722	6.80×10^3	6.40×10^4	9.50×10^2	5.80×10^4	5.40×10^2	4.80×10^4	2.80×10^3	2.40×10^4

¹Initial inoculum size was estimated by McFarland standard to approximately 1.2×10^6 CFU/ml

Table 3: Effect of sunlight heat and boiling on bacterial survival in environmental waters and wastewaters

Bacteria ¹	Bacterial Counts (CFU/ml)							
	Fishery		Domestic		Borehole		River	
	Sunlight (60 mins)	Boiling (15 mins)	Sunlight (60 mins)	Boiling (15 mins)	Sunlight (60 mins)	Boiling (15 mins)	Sunlight (60 mins)	Boiling (15 mins)
<i>Shigella sonnei</i> 53G	2.20×10^3	1.32×10^4	1.82×10^0	1.28×10^2	1.57×10^3	<1	1.73×10^3	<1
<i>Enterobacter cloacae</i> GGT036	1.70×10^0	1.54×10^2	1.24×10^0	9.40×10^4	1.82×10^3	3.20×10^4	1.87×10^3	1.00×10^4
<i>Escherichia coli</i> FAP1	2.53×10^0	1.90×10^4	2.46×10^3	2.20×10^4	1.99×10^0	<1	2.26×10^0	<1
<i>Escherichia coli</i> ST2747	2.37×10^3	1.38×10^2	1.86×10^3	1.20×10^4	1.65×10^3	<1	1.48×10^3	1.30×10^4
<i>Escherichia coli</i> NCM3722	2.14×10^3	2.02×10^4	2.68×10^3	2.60×10^4	2.08×10^2	2.00	1.97×10^3	<1

¹ Initial inoculum size was estimated by McFarland standard to approximately 1.2×10^6 CFU/ml

Antibiotic susceptibility of test isolates after exposure to UV radiation for 30 minutes

Antibiotic susceptibility profiles of the five bacteria recovered from the various environmental waters and wastewaters after exposure to UV radiation for 30 minutes are presented in Figure 2. In general, the effect of UV radiation on antibiotic sensitivity of the organisms varied depending on the environmental waters or wastewaters. However, all the isolates exhibited multi-drug resistance with resistance to at least six of the eight antibiotics used. In the fishery wastewater, all the isolates recovered showed resistance to the antibiotics except *Escherichia coli* ST2747 and *Enterobacter cloacae* GGT036 which both showed intermediate susceptibility to ofloxacin and ciprofloxacin (Fig 2a).

From domestic wastewater, *Enterobacter cloacae* GGT036, *Escherichia coli* NCM3722 and *Shigella sonnei* 53G exhibited intermediate susceptibility to ofloxacin, ciprofloxacin and gentamicin (Fig 2b). In borehole water, all the isolates recovered showed intermediate susceptibility to ciprofloxacin and ofloxacin except *Enterobacter cloacae* GGT036 which was resistant to the latter; while *Escherichia coli* FAP1 alone showed intermediate susceptibility to gentamicin (Fig 2c). Among the isolates recovered from the river water, *Escherichia coli* FAP1 and *Enterobacter cloacae* GGT036 were both sensitive to ofloxacin while *Shigella sonnei* 53G showed intermediate susceptibility to it. *Escherichia coli* ST2747, *Enterobacter cloacae* GGT036 and *Shigella sonnei* 53G were intermediately susceptible to ciprofloxacin while other isolates were resistant (Fig 2d). Ofloxacin, gentamicin and ciprofloxacin displayed better efficacy against the isolates compared to other antibiotics.

Discussion:

The temperature of the environmental waters and wastewaters used in this study were within the optimum temperature range for the survival of most mesophiles but the temperature recorded for borehole water is higher than the permissible standard of 25°C (14). Generally, the temperature range of the waters recorded in this study is higher than previous studies where temperature ranges within $25 \pm 2^\circ\text{C}$ were reported (17, 18, 19). The variation in the temperatures may be due to seasonal and geographical influence on the samples collected. The pH values of the water and wastewater samples varied from slightly acidic to slightly alkaline (5.0-9.7) which are however slightly higher than the recommended range for wastewaters (6.5-9.0) and surface/ground waters (6.0-8.0). The acidic pH and high turbidity observed in the domestic wastewater could be influenced by the nature of the organic and inorganic contents such as sewage, detergents and toxic elements (19). Fish wastes and fish feeds contain carbonate salts that can make water more basic, hence the fairly higher alkalinity observed in fishery wastewater compared to the environmental waters. Comparatively, the turbidity of the wastewaters was higher than environmental waters which may be due to the presence and high amounts of suspended and dissolved solids.

All the coliforms persisted in the river and borehole waters for over 21 days with an estimated 4-log and 5-log reduction respectively in the bacterial counts over an exposure time of 21 days. The survivorship of the coliforms in these environmental waters despite the low content of suspended solids or organic matter may be due to their capability to grow optimally in minimal

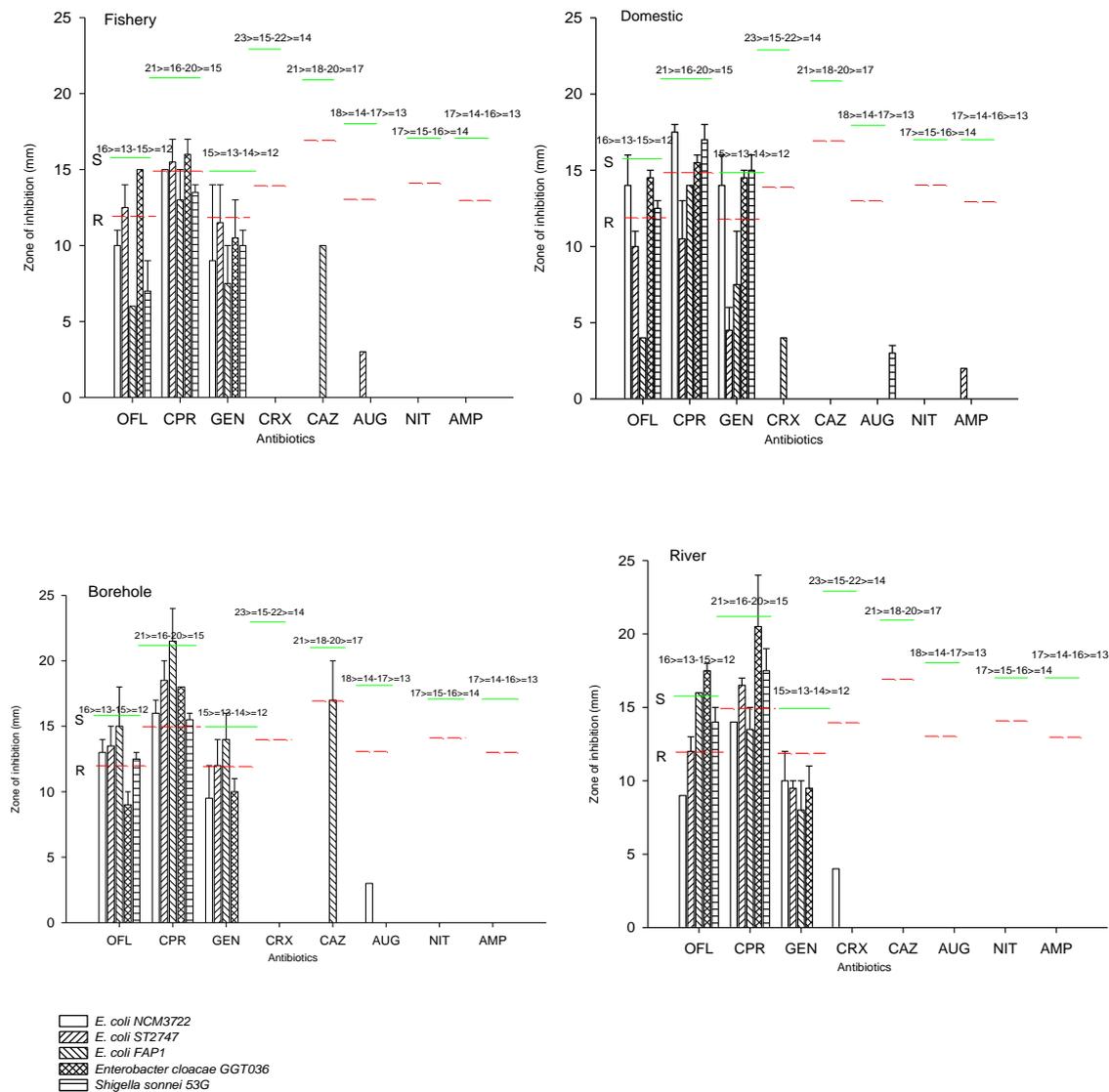


Fig 2: Susceptibility of the isolates from the waters to antibiotics after UV radiation exposure
S represents 'sensitive'; R represents 'resistant'

medium and utilize simple energy sources (2). The survival pattern of all the bacterial isolates in the wastewaters was similar with a significant decline in bacterial counts from days 6 to 12; while fluctuations in growth were observed in the environmental waters until bacterial counts significantly declined from days 15 to 21.

In all the water samples, *Escherichia coli* strains showed higher survivability compared to *Shigella sonnei* and *Enterobacter cloacae*; thus reiterating the capability of these strains to switch to a state of dormancy and persist in the environment for a long period of time (20, 21). The survival rate of these coliforms in these waters especially borehole water is intriguing and this may present a health risk in the

discharge, use and consumption of these waters.

Ultraviolet radiation has gained widespread use as a means of inactivating microorganisms in water bodies. Its general acceptance has been favoured by its efficacy at low contact time, no formation of harmful by-products and no chemical involvement (22, 23, 24). In this study, all bacterial counts decreased with increase in contact time to UV radiation with at least 4-log reduction. In fishery wastewater, over 2-log reduction was observed in the bacterial counts from exposure for 15 to 30 minutes. A similar trend was observed in the domestic wastewater with bacterial counts after 30 minutes being almost negligible. In borehole water, bacterial counts declined drastically to

<65 cfu/ml after 30 minutes while in river water, bacterial counts were less than 100 cfu/ml after 30 minutes of UV exposure. *Shigella sonnei* 53G showed a notable inactivation rate to UV disinfection with < 20 cfu/ml in all the water samples after 30 minutes of UV exposure.

Solar irradiation has been used extensively in disinfection of chemically and biologically contaminated water. Bacteria inactivation by solar disinfection in the wastewaters and environmental waters was significantly different. The coliforms survived better in the wastewaters after 60 minutes of sunlight exposure compared to the environmental waters. This observation could be attributed to the effect of turbidity on light penetration into the water samples. Bacterial inactivation by solar irradiation has been studied to be proportional to the intensity of sunlight while presence of suspended solids in water reduces the intensity of light (9).

Boiling water at 100°C can reportedly inactivate a wide range of microorganisms. Similar to the survivability rate to solar irradiation, the coliforms survived better in the wastewaters than the environmental waters after 15 minutes of boiling. In borehole and river waters, bacterial counts were less than 1 cfu/ml for three of the coliforms in each of the waters. *Enterobacter cloacae* GT036 survived better than other coliforms in the borehole water after boiling with bacterial count of 3.20×10^1 . In the wastewaters, at least 4-log reduction was observed in the bacterial counts after 15 minutes of boiling but the counts were still considerably higher than the environmental waters. The survival rate of this study conforms to existing investigations that killing effect against bacteria by boiling is achievable at temperatures above 65°C with less than 1 minute per log reduction (24). However, the efficacy of boiling is dependent on clarity of water and contact time (25); thus longer exposure time or pre-clarification may be required to completely inactivate the coliforms in wastewater due to its high turbidity.

Studies have reported that antibiotic sensitivity of bacteria increases after UV radiation, although some bacteria exhibit increased resistance due to R-factor mediated resistance to UV light (26, 27, 28). In this study, all the coliforms remained multi-drug resistant after UV exposure for 30 minutes but there was no significant difference in the resistance profiles of the isolates pre and post exposure. The persistence of these MDR enteric bacteria in water and wastewater post-disinfection further affirms these sources as reservoir and transmission routes for resistant pathogens.

Wastewaters provide an environment for potential exchange of antibiotic resistance genes between pathogens, hence the need for effective treatment (29). Incessant discharges of domestic and veterinary effluents into the environment thus presage extensive dissemination of antibiotic resistant pathogens. Also, the increased and intensive use of antibiotics in food animal production in the last decades has greatly influenced antibiotic residue concentrations in their wastewaters, and consequently the threat to public health (30). These results therefore suggest that UV radiation may inactivate coliform growth but may not effectively attenuate antibiotic resistance.

Conclusion:

This study emphasises the influence of different treatment regimens on coliform survival in wastewaters and environmental waters. Thus, boiling exhibited the highest efficacy with about 5-log reduction in survival rate of the coliforms while the least efficacy was seen in solar irradiation. Also, the effect of the stress by UV radiation did not increase the sensitivity of the coliforms to antibiotics. Consequently, the quality of waters and wastewaters should be duly monitored to prevent the spread of resistant pathogens that could pose great risks to public health.

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**Original Article****Open Access****Phenotypic characterization of mycobacteria isolates from tuberculosis patients in Kaduna State, Nigeria**^{1,2}Ahmadu, I., ¹Olonitola, O. S., ¹Suleiman, A. B., ³Lawan, M. K., and ⁴Makolo, D.¹Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Nigeria²National Tuberculosis Reference Laboratory, Zaria, Nigeria³Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria⁴Department of Sciences, School of Preliminary Studies, Kogi State Polytechnic, Lokoja, NigeriaCorrespondence to: isiyakuadabka@yahoo.com**Abstract:****Background:** Tuberculosis (TB) remains one of the leading public health challenges in Nigeria and the burden is still high. There is hence a need for continuous characterization of mycobacteria to obtain current data that will aid the ongoing TB prevention and control programme. The aim of this study was to phenotypically characterize mycobacteria isolates recovered from clinical specimens of patients with tuberculosis in Kaduna State, Nigeria.**Methods:** Two thousand, two hundred and twelve (2212) sputum samples were collected from patients clinically suspected to have TB in three different zones of Kaduna State, Nigeria, between May 2017 and October, 2018. Samples were processed by decontaminating with NaOH-Citrate N-acetyl-L-Cysteine method for Ziehl Neelsen (ZN) AFB microscopy and culture on Lowenstein Jensen (LJ) slants which were incubated at 37°C for 8 weeks. Positive LJ cultures were further analyzed with a rapid TB antigen assay (SD-Bioline) to differentiate *Mycobacterium tuberculosis* complex (MTBC) from Non Tuberculous Mycobacteria (NTM).**Results:** Out of the 2212 patients with suspected TB, 300 (13.6%) were positive for AFB by microscopy with Zone A (Kaduna North) having the highest AFB positive cases of 169 (15.2%). Of the 300 AFB positive samples, 272 (91.0%) were culture positive on LJ medium, 18 (6.0%) were culture negative and 10 (3.0%) were culture contaminated. Result of the distribution of mycobacteria among infected patients within the study area revealed that 219 (80.5%) were infected with MTBC, 42 (15.4%) with NTM and 11 (4.0%) with both MTBC and NTM.**Conclusion:** A relatively high number of TB in the study area was caused by NTM. There is need for advanced diagnostic tools that can differentiate MTBC and NTM strains among TB patients in all TB Reference Laboratories in Nigeria.**Keywords:** Phenotypic, Characterization, Tuberculosis, Mycobacteria

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Copyright 2019 AJCEM Open Access. This article is licensed and distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution and reproduction in any medium, provided credit is given to the original author(s) and the source.**Caractérisation phénotypique d'isolats de mycobactéries provenant de patients atteints de tuberculose dans l'État de Kaduna, au Nigéria**^{1,2}Ahmadu, I., ¹Olonitola, O. S., ¹Suleiman, A. B., ³Lawan, M. K., et ⁴Makolo, D.¹Département de microbiologie, Faculté des sciences de la vie, Université Ahmadu Bello, Zaria, Nigeria²Laboratoire national de référence sur la tuberculose, Zaria, Nigéria³Département de santé publique vétérinaire et de médecine préventive, Faculté de médecine vétérinaire, Université Ahmadu Bello, Zaria, Nigéria⁴Département des sciences, École d'études préliminaires, École polytechnique d'État de Kogi, Lokoja, NigériaCorrespondance à: isiyakuadabka@yahoo.com

Abstract:

Contexte: La tuberculose reste l'un des principaux problèmes de santé publique au Nigéria et le fardeau est encore lourd. Il est donc nécessaire de caractériser en permanence les mycobactéries pour obtenir les données actuelles qui aideront le programme de prévention et de contrôle de la tuberculose en cours. Le but de cette étude était de caractériser phénotypiquement les isolats de mycobactéries récupérés à partir d'échantillons cliniques de patients atteints de tuberculose dans l'État de Kaduna, au Nigéria.

Méthodes: Deux mille douze cent douze (2212) échantillons d'expectorations ont été prélevés chez des patients cliniquement suspects de tuberculose dans trois zones différentes de l'État de Kaduna, au Nigéria, entre mai 2017 et octobre 2018. Les échantillons ont été traités par décontamination au NaOH-citrate. Méthode N-acétyl-L-Cystéine pour la microscopie AFB de Ziehl Neelsen (ZN) et culture sur des pentes de Lowenstein Jensen (LJ) qui ont été incubées à 37 ° C pendant 8 semaines. Les cultures de LJ positives ont ensuite été analysées avec un dosage rapide de l'antigène de la tuberculose (SD-Bioline) afin de différencier le complexe *Mycobacterium tuberculosis* (MTBC) des mycobactéries non tuberculeuses (NTM).

Résultats: Sur les 2212 patients suspects de tuberculose, 300 (13,6%) étaient positifs pour AFB par microscopie, la zone A (Kaduna North) présentant le plus grand nombre de cas positifs avec 169 (15,2%). Sur les 300 échantillons AFB positifs, 272 (91,0%) étaient positifs en culture sur le milieu LJ, 18 (6,0%) étaient négatifs en culture et 10 (3,0%) étaient contaminés par la culture. Le résultat de la distribution des mycobactéries parmi les patients infectés dans la zone d'étude a révélé que 219 (80,5%) étaient infectés par le MTBC, 42 (15,4%) avec les NTM et 11 (4,0%) avec les deux types de MTBC.

Conclusion: Un nombre relativement élevé de tuberculose dans la zone d'étude a été causée par les MNT. Il existe un besoin d'outils de diagnostic avancés permettant de différencier les souches de MTBC et de MNT parmi les patients atteints de tuberculose dans tous les laboratoires de référence pour la tuberculose au Nigeria.

Mots-clés: Phénotypique, Caractérisation, Tuberculose, Mycobactéries

Introduction:

Mycobacterium tuberculosis also known as the tubercle bacilli is a pathogenic bacterium of the genus *Mycobacterium* and the causative agent of most cases of tuberculosis (1, 2). First discovered in 1882 by Robert Koch, *M. tuberculosis* has an uncommon waxy layer on its cell surface (primarily mycolic acid) which makes the cell impervious to Gram staining, hence acid-fast detection techniques are used for its identification in the laboratory (3, 4). *Mycobacterium tuberculosis* is a non-motile and non sporulating rod. In smears stained with carbol fuschin or auramine and examined under the light microscope, the tubercle bacilli typically appear as straight or slightly curved rods but depending on growth conditions and age of the culture, bacilli may vary in size and shape from short coccobacilli to long rods. The dimensions of the bacilli have been reported to be 1-10µm in length (usually 3-5µm), and 0.2-0.6 µm in width (5, 6).

Tuberculosis (TB) has a long history. It had existed before the establishment of recorded history and has left its mark on human creativity, music, art, and literature. It has also influenced the advancement of biomedical sciences and healthcare and may have killed more persons than any other microbial pathogen (7, 8). This disease known in the past as the "White Plague" is an ancient disease. Recent genetic evidence suggests that even our remote hominid ancestors, who lived three million years ago, may have suffered from TB (6, 9).

Mycobacterium tuberculosis forms a complex that comprises members implicated in human tuberculosis. The complex comprises seven members; *M. tuberculosis*, *Mycobacterium*

bovis, *Mycobacterium africanum*, *Mycobacterium canettii*, *Mycobacterium microti*, *Mycobacterium caprae* and *Mycobacterium pinnipedii*. *Mycobacterium tuberculosis* is the primary causative agent of human TB; *M. bovis* is responsible for bovine TB and includes the vaccine strain *M. bovis* BCG; *M. africanum* is the main causative agent of TB in West Africa (10) while *M. canettii* is a rare TB strain which produces smooth and glossy colonies, with all cases so far isolated from people who have been to the horn of Africa (5, 11); *M. pinnipedii* has been shown to be responsible for TB in marine host while *M. caprae* is responsible for TB in goats and *M. microti* infects larger mammals. *Mycobacterium tuberculosis* as the predominant cause of human TB is the most successful of human bacterial pathogens, and is sometimes referred to as Koch's bacillus after the discoverer (12).

While the disease is preventable and curable, TB has remained a significant cause of morbidity and mortality in resource limited nations (13). Currently it has re-emerged in developed nations as well due to its synergy with human immunodeficiency virus/acquired immune deficiency syndrome, demographic changes and human migrations (14, 15). On the basis of tuberculin reactivity, one third of the world's population is believed to be infected with latent TB. These infected individuals are thus at risk of developing TB later in life as their immunity wanes from aging or HIV co-infection (2, 16) even as TB is a major opportunistic infection in HIV infected persons (15, 17). This study was thus carried out to characterize mycobacteria among presumptive TB patients at the three major TB reference hospitals in Kaduna State, Nigeria.

Materials and Methods:

Study area

Kaduna State is located in the Northwest geo-political zone of Nigeria, lying between latitude 6° and 11° North and longitude 7° and 44° East, and is 608 meters above sea level. The study locations were Ahmadu Bello University Teaching Hospital Shika, National TB and Leprosy Training Centre Zaria and General Hospital Kafanchan in Giwa, Zaria and Jama'a Local Government Areas respectively (Fig.1).

Study design and population

The study is cross sectional, involving TB patients attending the Directly Observed Treatment Short Course (DOTS) clinics in the three senatorial districts of Kaduna State, Nigeria. Samples collected were from presumptive TB patients attending the DOTS clinics in the selected hospitals (Ahmadu Bello University Teaching

Hospital Shika; National TB Referral Hospital, Zaria, and General Hospital, Kafanchan).

Sample size determination

Sample size was determined by the formula, $N = t^2 pq / d^2$ (18), where N=minimum sample size, t=standard normal distribution at 95% confidence interval (1.96), d=allowable error taken as 0.05, p=known prevalence rate of the infection, and q=1-p. The prevalence rate of NTM infection (p) used was 15.5% (19), which gives a sample size of 201. To adjust for expected losses due to contaminated cultures, non-growth and other factors, the calculated sample size was increased by 20% (20) to 240. However due to differences in the study locations, the sample size was increased to 300. The proportion to size (PPS) sampling as recommended by the World Health Organization (21) was used to allocate sample size across the three selected hospitals.

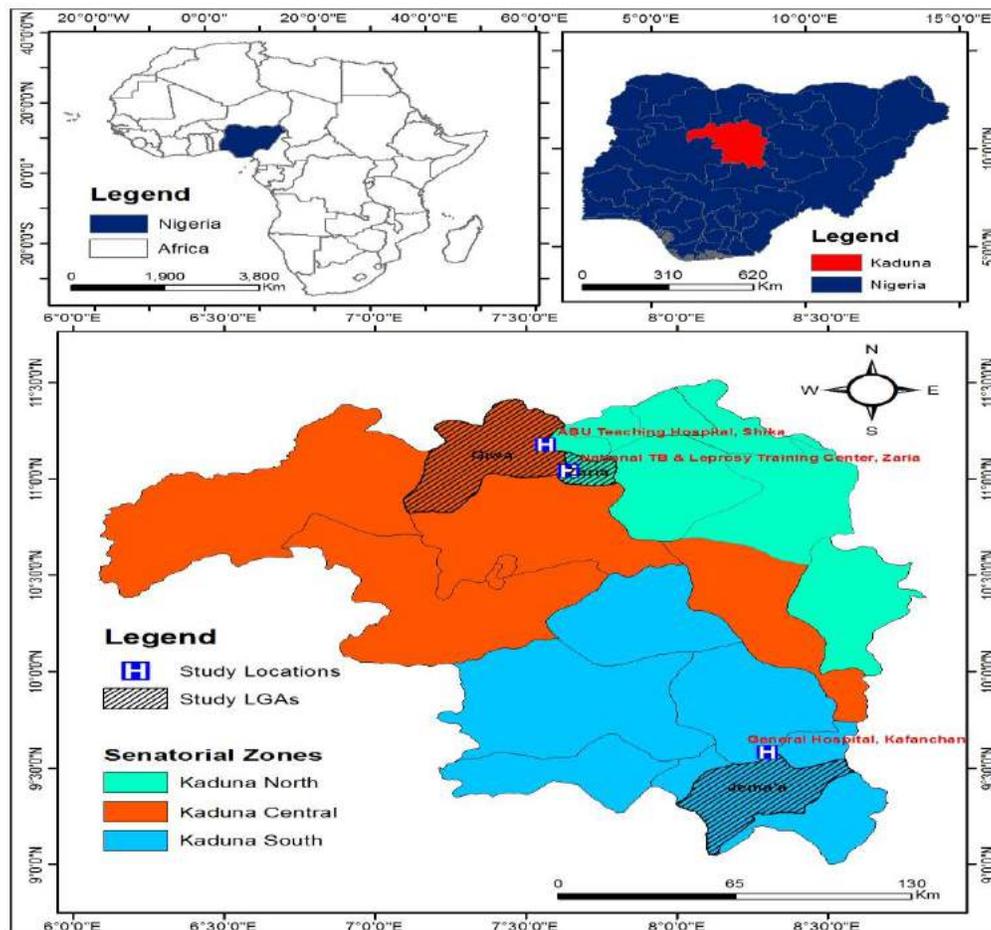


Fig. 1: Map of Africa, Nigeria and Kaduna State showing the study locations

Inclusion and exclusion criteria

The inclusion criteria for the study are; new smear positive TB patients, willingness to participate through informed consent, and patients aged 15 years and above. Patients who were smear negative, those with confirmed drug resistant TB, those on re-treatment TB therapy, and those who did not give informed consent were excluded

Ethical approval

Ethical approval for this study was obtained from the State Ministry of Health, Kaduna State, Nigeria.

Collection of demographic and clinical data

A structured questionnaire was used to collect demographic characteristics (age, sex, ethnicity, education, and marital status), health/behavioral factors (HIV, diabetes, smoking, alcohol use), environmental exposures (farming, animal contact, and dust season) and clinical variables (site, smear results) from each subject.

Sputum sample collection

One spot sputum sample was collected in a labeled standard screw-capped leak-proof sputum container with specific clinic identification and study numbers according to the National TB programme guidelines.

Sputum transportation

Sputum smear microscopy was performed at the study sites, and AFB smear positive specimens were transported in cold boxes packed with ice and thermometer (to monitor temperature) within 4 days of collection to the National TB Reference Laboratory by a courier company. Before transportation, the specimens were kept in a refrigerator at 2-8°C.

Culture of specimens

All clinical specimens were processed in a Biological Safety Cabinet (BSC) using the NALC-NaOH method as described by Steingart et al., (22). Equal volume (5ml) of NALC-NaOH and sputum were mixed. The mixture was vortexed and incubated for 15 minutes, and 35 ml Phosphate buffer was added to the NALC-NaOH-

sputum mixture and the tubes centrifuged at 4°C for 15 minutes at 3000 x g. The supernatant was carefully discarded and the sediment was re-suspended in 2 ml buffer. Exactly 0.1 ml of the sediment was inoculated into Lowenstein Jensen's (LJ) slants and incubated in 37°C incubator as described by Cadmus et al., (23). Smears were prepared from the portion of the sediment as described by Angra (24).

Sputum microscopy

The heat fixed smear was allowed to cool and stained with 1% carbol fuschin solution and heated from beneath by using spirit lamp until steam comes off from the stain. The stain was allowed to act on the smear for 10 minutes and the procedure was repeated 3 times. The slides were tilted to remove excess stain and washed gently with running tap water. Decolorization was done after rinsing the slides with water and flooded with 3% acid alcohol for 2-3 minutes. The slides were then rinsed with water and flooded with 0.1% methylene blue for 1 minute. The slides were rinsed with water and allowed to air dry (25). The acid fast bacilli (AFB) load was recorded according to the International Union against Tuberculosis and Lung Disease (IUATLD) standard (26).

Results:

Prevalence of mycobacteria by microscopy

Of the 2212 patients with suspected TB whose sputum were tested from the three Senatorial zones, 300 (13.6%) samples were positive by AFB microscopy (Fig. 2)

Distribution of mycobacteria by microscopy in the study area

The result obtained showed that samples collected from Zone A (Kaduna North senatorial zone) National TB Reference Hospital, Saye-Zaria, had the highest number of TB positive cases of 169 (15.2%) followed by Zone B (Kaduna Central Senatorial zone) ABUTH, Shika with 94 (13.4%) and Zone C (Kaduna South Senatorial zone) General Hospital, Kafanchan with the lowest 37 (9.3%) (Table 1)

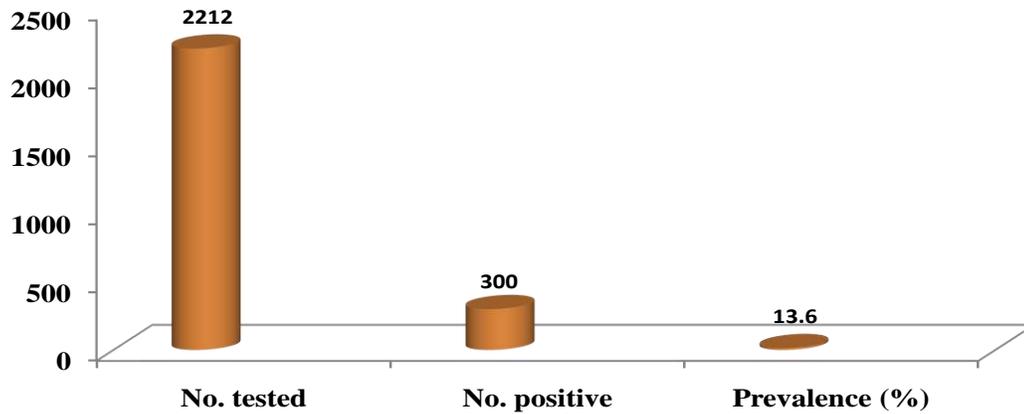


Fig.2: Prevalence of TB by microscopy among the study population

Table 1: Frequency distribution of TB by microscopy in the study area

Study site	Number of samples collected	Number of positive samples	Prevalence (%)
Zone A (North)	1112	169	15.2
Zone B (Central)	703	94	13.4
Zone C (South)	397	37	9.3
Total	2212	300	13.6

$\chi^2 = 8.654, p = 0.0132$

Table 2: Distribution of mycobacteria by culture on Lowenstein Jensen medium

Culture result	Number of patient samples	Percentage (%)
Positive	272	91.0
Negative	18	6.0
Contaminants	10	3.0
Total	300	100

Distribution of positive culture samples

The percentage distribution of the 300 AFB positive samples based on culture on Lowenstein Jensen's (L J) medium is shown in Table 2, with 272 (91.0%) culture positive, 18 (6.0%) culture negative and 10 (3.0%) culture contaminated.

Distribution of mycobacteria among culture positive participants

The distribution of mycobacteria among the 272 culture positive participants is shown in Table 3 which revealed that MTBC were isolated in 219 (80.5%), NTM in 42 (15.4%) and co-infection of MTBC/NTM in 11 (4.0%) participants.

Table 3: Distribution of mycobacteria among the culture positive participants

Mycobacteria	Number positive	Percentage (%)
MTBC	219	80.5
NTM	42	15.4
MTBC + NTM	11	4.0
Total	272	100

MTBC = *Mycobacterium tuberculosis* Complex, NTM = Non tuberculous Mycobacteria

Discussion:

In this study, the overall prevalence of TB by AFB microscopy among patients in Kaduna State was 13.6%, and 12.3% by culture. The 13.6% rate is lower than the 18.6% reported by Aliyu (25) and 16.7% reported by Mamuda et al., (27). This lower prevalence rate may be due to the category of studied subjects but may also not be unconnected with the synergistic efforts of the stakeholders involved in the control of TB in Nigeria. The high (91%) culture positive rate in AFB smear positive sputum obtained in this study may be due to the fact that samples were transported in cold chain and processed within the acceptable time limit in a high quality laboratory. Our culture positive rate is higher than the 85% rate reported by Selvakumar et al., (28) in India and close to the 100% rate reported by Simeon et al., (29).

Clinical specimens from non-sterile body sites such as sputum for TB culture are usually subjected to pre-treatment process involving digestion, homogenization, decontamination and concentration to eradicate more rapidly growing contaminants such as normal flora (other bacteria and fungi) without affecting the viability of the mycobacterial organisms that grow slowly (30). The contamination rate observed in our study was 3% and this low rate could be attributed to the fact that samples were processed and analyzed in high quality standard laboratory. This rate is within the acceptable range of WHO established standard of 3–5% for solid LJ culture method (31) and similar to those of Addo et al., (32) who reported 4.3% contamination rate. The contamination rate in our study is lower than Kassidy et al., (33) study in Uganda who reported high contamination rate of 31% for solid LJ medium, which is far above the recommended threshold of 5% for laboratories that receive freshly produced sputum samples. Our rate is also lower than those from studies in Nigeria and Zambia where reported contamination rates were 14.7% and 10.8% respectively (19, 34).

Smear positive but culture negative TB rate obtained from this study was 6%. This could be attributed to dead acid fast bacilli or low number of viable bacilli undetected by LJ media or the killing action of NaOH used for

decontamination. This rate is lower than that obtained by Mamuda et al., (26) in Nigeria with 8% and Addo et al., (35) in Ghana with 9.8%.

The predominance of *M. tuberculosis* strains in our study is in agreement with studies from other African countries (36, 37). However, studies from other West African countries have reported a range of 9% to 28% of MTBC isolates to be *M. africanum*. Studies by Addo et al., (32), Niobe-Eyangoh et al., (36), Traore et al., (38) and Gomgnimbou et al., (39) have reported low prevalence of *M. bovis* in the African countries of Mali (0.8%), Ghana (3%), Burkina Faso (0%) and Cameroun (0.2%). *Mycobacterium bovis* infection mostly results from spread of livestock and or their products which may be transmitted through other routes than the respiratory system.

The MTBC prevalence of 80.5% in our study is less than 91% reported by Addo et al., (32) in Ghana but similar to the study by Aliyu et al., (19) with prevalence rate of 85%. The studies by Ani et al., (40) and Cadmus et al., (41) in Nigeria and USA reported lower rates of 69% and 48% respectively. The high prevalence of MTBC (80.5%) in our study when compared to the prevalence of NTM (15.4%) disagrees with the study of Muyoyeta et al., (34) who reported that in prevalence surveys, where mycobacterial speciation has been carried out, NTM may be three times more commonly found than MTBC in humans. This contradiction may be due to differences in environmental factors and control policies of animal movements and grazing activities in Nigeria.

The prevalence of 15.4% for NTM in this study is lower than the 39% reported in Ibadan by Cadmus et al., (41). The reason for this difference could be attributed to the lower sample size of only 23 patients used in the Ibadan study compared to 300 in the present study. Furthermore, our NTM prevalence is lower compared to the 23.1-26.6% rate reported in Northern Nigeria by Mawak et al., (42) and much lower than the prevalence of 56.9% reported in a study carried out in Taiwan by Chien et al., (43), but our rate is similar and comparable to the 16.5% recently reported in the south-south region of the country by Pokam and Asuquo (44) and 15% reported in a similar study by Kim et al., (45). The NTM prevalence in our study is however

higher than the 11% previously reported by Idigbe et al., (46) in Lagos, a city in South-western Nigeria, and also higher than 11% recently reported by Borroni et al., (47) in Burkina Faso (a country in West Africa), Machado et al., (48) in Ontario Canada, and 6.4% reported by Wang et al., (49) in China, another vastly developing country but with an improved health care system compared to Nigeria.

The relatively high prevalence of NTM in our study may also be attributed to the fact that the study was conducted during both wet (rainy) and dry (harmattan) seasons. Harmattan is a West African trade wind that occurs during the winter and is characterized by heavy amount of dust in the air, low humidity, and reduced visibility (50). High risks for environmentally acquired pulmonary mycobacterial infections have been previously reported for individuals with occupational exposures to dust (51, 52, 53). The prevalence of MTBC/NTM in this study was 4% which is lower than the report of Simeon et al., (29) who reported 13% from Ibadan. This might be due to the differences in sample size used in the studies but could also be due to varied sociodemographic and environmental factors.

Conclusion:

Based on the findings of this study, we conclude that an overall TB prevalence of 13.6% by AFB microscopy was obtained, with the highest rate of 15.2% in Zone A (Kaduna South). This study also reported a high MTBC prevalence rate of 80.5% among patients seeking TB treatment but confirmed the occurrence of NTM in 15.3% among the TB patients. Even though AFB microscopy permits a rapid identification of mycobacteria, it is not capable of distinguishing MTBC from NTM. This poses a great challenge to the TB control programme in Nigeria. Hence, inclusion of molecular screening assays that is capable of rapid detection of NTM infections in high burden resource limited settings should be a priority.

Acknowledgments:

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Short Communication

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Prevalence and antibiotic resistance profiles of extended spectrum β -lactamase-producing *Escherichia coli* among paediatric patients with urinary tract infection in St. Patricks' Hospital, Mile Four, Abakaliki, Ebonyi State, Nigeria

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Abstract:

Background: The extended spectrum beta-lactamase (ESBL)-producing *Escherichia coli* strains which have been implicated in septicaemia among hospitalized children is a serious concern due to their high resistance rates to commonly used antimicrobial agents. The objective of this study was to determine the prevalence and antibiotic susceptibility of urinary ESBL-producing *E. coli* in paediatric patients who had clinical evidence of urinary tract infections (UTI).

Methodology: Clean catch specimens of urine collected from 100 eligible paediatric patients with clinical evidence of UTI in St. Patricks' Hospital, Mile Four, Abakaliki, Ebonyi State, were cultured for isolation of *E. coli* using standard bacteriological techniques. Isolates were confirmed for ESBL production by double disk synergy test (DDST), and antibiotic susceptibility of the ESBL-producing ones was determined by the modified Kirby Bauer disk diffusion method.

Results: Twenty one (21%) *E. coli* were isolated out of which 11 (52 %) were ESBL producers, all of which were totally resistant (100%) to cefotaxime, ticarcillin and sulfamethoxazole-trimethoprim, 85% to aztreonam and 83% to ceftazidime. The multiple antibiotic resistance index (MARI) values ranged from 0.4 to 0.9, which implies high usage of antimicrobials

Conclusion: The high prevalence of multi-drug resistant ESBL-producing *E. coli* obtained in this study shows that there has been overuse (abuse or misuse) of antibiotics in the study area. There is need for antimicrobial stewardship programme that will ensure prudent use of antimicrobial agents to forestall the emergence and spread of multi-drug resistant bacteria.

Keywords: Paediatrics, *Escherichia coli*, ESBL, urine, multi-drug resistance

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Profils de prévalence et de résistance aux antibiotiques d'*Escherichia coli* produisant des β -lactamases à spectre étendu chez des patients pédiatriques présentant une infection des voies urinaires à l'hôpital St. Patricks, Mile Four, Abakaliki, État d'Ebonyi, Nigéria

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Abstrait:

Contexte: Les souches d'*Escherichia coli* productrices de bêta-lactamase à spectre étendu (BLSE) qui ont été impliquées dans la septicémie chez les enfants hospitalisés constituent un grave problème en raison de leur taux de résistance élevé aux agents antimicrobiens couramment utilisés. L'objectif de cette étude était de déterminer la prévalence et la sensibilité aux antibiotiques d'*E. coli* producteurs de BLSE dans l'urine chez les patients pédiatriques présentant des signes cliniques d'infections des voies urinaires (UTI).

Méthodologie: Des échantillons d'urine prélevés chez 100 patients pédiatriques éligibles présentant des signes cliniques d'UTI à l'hôpital St. Patricks, à Mile Four, à Abakaliki, dans l'État d'Ebonyi, ont été cultivés pour l'isolement de *E. coli* à l'aide de techniques bactériologiques classiques. Les isolats ont été confirmés pour la production de BLSE par un test de synergie à double disque (DDST) et la sensibilité aux antibiotiques des producteurs de BLSE a été déterminée par la méthode de diffusion sur disque de Kirby Bauer modifiée.

Résultats: Vingt et un (21%) *E. coli* ont été isolés, dont 11 (52%) étaient des producteurs de BLSE, qui étaient tous totalement résistants (100%) au céfotaxime, à la ticarcilline et au sulfaméthoxazole-triméthoprim, 85% à l'aztréonam et au 83 % en ceftazidime. Les valeurs de l'indice de résistance multiple aux antibiotiques (MARI) allaient de 0,4 à 0,9, ce qui implique une utilisation élevée d'antimicrobiens

Conclusion: La prévalence élevée d'*E. coli* productrice de BLSE résistante à plusieurs médicaments obtenue dans cette étude montre qu'il y a eu surutilisation (abus ou abus) d'antibiotiques dans la zone d'étude. Un programme de gestion des antimicrobiens est nécessaire pour garantir une utilisation prudente des agents antimicrobiens afin de prévenir l'émergence et la propagation de bactéries multirésistantes aux médicaments

Mots-clés: pédiatrie, *Escherichia coli*, BLSE, urine, multirésistance

Introduction:

Beta-lactam antibiotics are the most commonly used therapeutic agents, accounting for over 50% of antibiotics used for treatment of bacterial infections, due to their broad antibacterial spectrum and excellent safety profile (1). The use of β -lactam antibiotics had however been hampered by β -lactamases which are enzymes that inactivate the β -lactam ring of the antibiotics thereby rendering them ineffective. The extended spectrum β -lactamases (ESBLs) produced by Gram-negative bacilli such as *Escherichia coli* have the ability to hydrolyse β -lactams including the third generation cephalosporins and aztreonam and yet are inhibited by clavulanic acid. ESBLs are mostly plasmid-mediated and can be transferred from one strain to another among bacterial species.

The prevalence of antibiotic resistance in bacterial species such as *E. coli*, *Klebsiella* and *Proteus* species isolated from clinical patients is increasing worldwide (2). In particular, strains of *E. coli* and *Klebsiella* species producing ESBL enzymes have become a worldwide problem with serious negative impact on the efficacy of β -lactam therapy. Thus, there is need for efficient infection control practices for containment of outbreaks of these strains.

The presence of ESBLs is associated with multi-drug resistance to various other classes of antibiotics such as monobactam, carbapenems, amino-glycosides, ciprofloxacin and erythromycin (1). The detection of these enzymes is therefore important in preventing treatment failures from infections caused by

these pathogens. The objective of this study therefore is to determine the prevalence and antibiotic resistance patterns of urinary ESBL-producing *E. coli* isolates from paediatric patients in St. Patricks' Hospital, Mile Four, Abakaliki, Ebonyi State.

Materials and methods:

Study area

St. Patrick's hospital, Mile Four, Abakaliki is a missionary hospital located in Ebonyi State. The hospital specializes majorly in maternal and child care, and has an outpatient department (OPD), nursery ward, children's ward, maternity ward, and child's welfare clinic.

Study population

The study population consists of children (15 years and below) with clinical evidence of UTI who were seen at the outpatient department (OPD), and those hospitalized in the nursery and children's wards.

Ethical clearance

Ethical clearance was obtained from the Research and Ethics committee of the hospital before the commencement of sample collection. Informed consent was also obtained from the parents/guardians of the children.

Sample collection, culture isolation and biochemical identification

Urine sample was collected as clean-catch early morning urine from each child into a sterile container free of preservatives. Each

specimen was labelled accordingly with the date and time of collection, and identification number. Samples were transported immediately to the Laboratory unit of the Department of Applied Microbiology, Ebonyi State University, for bacteriological analysis.

The urine samples were cultured on eosin methylene blue (EMB) and MacConkey agar plates, and incubated aerobically at 37°C for 24 hours. *E. coli* was presumptively identified as green metallic sheen colonies on EMB agar and as non-mucoid pinkish colonies on MacConkey agar. The isolates were confirmed as *E. coli* using standard conventional biochemical identification tests scheme (3).

Antibiotic susceptibility test of isolates

Antimicrobial susceptibility test of the isolates was done using the modified Kirby-Bauer disk diffusion technique (4) on Mueller-Hinton (MH) agar. Inoculum of each isolate prepared by suspending pure colonies of the bacteria from nutrient agar subculture plate in normal saline was standardized by comparing with 0.5 McFarland turbidity standards. This was inoculated on the surface of the MH agar plates with sterile swab using the following antibiotic disks (Oxoid, UK); cefotaxime (CTX, 30µg), ceftazidime (CAZ, 30µg), tobramycin (TOB, 15µg), aztreonam (AZ, 30 µg), gentamicin (GEN, 10µg), sulfamethoxazole-trimethoprim (SXT, 75µg), ciprofloxacin (CIP, 5µg), ertapenem (ERT, 10µg), ticarcillin (TIC, 75µg), amikacin (AMK, 30µg), and amoxicillin/clavulanic acid (AMC, 25µg/10 µg).

The plates were incubated aerobically at 37°C for 18-24 hours. The zones of inhibition were measured with a meter rule and the results interpreted according to the Clinical and Laboratory Standards Institute guidelines (5).

Phenotypic confirmatory test for ESBL production

Escherichia coli isolates exhibiting reduced susceptibility to any of the 3rd generation cephalosporins (suspected ESBL producers) were phenotypically confirmed for ESBL production using the double disc synergy test (DDST) (6). This was performed as a standard disc diffusion assay on Mueller-Hinton agar (Oxoid, UK) plates in line with CLSI guideline (5). Sterile swabs were dipped into

standard bacterial suspension standardized to 0.5 McFarland turbidity standards and inoculated on the MH agar plates. Amoxicillin/clavulanic acid (20/10 µg) disk was placed at the centre of the plate and cefotaxime (30µg) and ceftazidime (30µg) were placed at a distance of 15 mm (centre to centre) from the central disk.

The plates were incubated aerobically at 37°C for 24 hours. ESBL production was confirmed when the zones of inhibition of the cephalosporins (cefotaxime and ceftazidime) increased in the presence of amoxicillin-clavulanic acid disk. A ≥ 5mm increase in the inhibition zone diameter for either of the cephalosporins tested in combination with amoxicillin-clavulanic acid over the zone when tested alone, confirmed ESBL production (6). *E. coli* ATCC 25922 was used as quality control strain.

Determination of multiple antibiotic resistance index (MARI)

Multiple antibiotic resistance index (MDRI) was calculated as MDRI = a/b where 'a' is the number of antibiotics to which the isolate was resistant to, while 'b' is the total number of antibiotics to which the isolate was tested against (7).

Results:

Twenty one *E. coli* were isolated from urine samples of 100 children studied; 9 isolates were from those in age group < 5 years, 4 (44.4%) of which were ESBL producing; 4 isolates were from those in age group 5-10 years, 2 (50%) of which were ESBL producer; and 8 isolates were from age group 11-15 years, 5 (62.5%) of which were ESBL producers ($X^2 = 0.1740$, $p = 0.9167$) (Table 1)

ESBL-producing *E. coli* isolates were totally resistant to cefotaxime, aztreonam, ticarcillin, sulfamethoxazole-trimethoprim and ciprofloxacin among children aged <5 years. Isolates also exhibited total resistance to cefotaxime, ceftazidime, ticarcillin and sulfamethoxazole-trimethoprim among children aged 5-10 years, and similarly, there was total resistance of isolates to cefotaxime, aztreonam, ticarcillin and sulfamethoxazole-trimethoprim from children aged 11- 15 years (Table 2)

Table 1: Frequency of *Escherichia coli* and ESBL producing isolates from urine samples of paediatrics patients in St Patricks Hospital, Mile Four, Abakaliki, Ebonyi State, Nigeria

Age group (years)	No of urine samples	No of <i>Escherichia coli</i> isolates (%)	No of ESBL-producing <i>E. coli</i> isolates (%)
< 5	34	9 (26.5)	4 (44.4)
5 - 10	39	4 (10.3)	2 (50.0)
11 - 15	27	8 (29.6)	5 (62.5)
Total	100	21 (21.0)	11 (52.4)

$\chi^2 = 0.1740, p = 0.9167$ (no significance difference)

Table 2: Antibiotic susceptibility of ESBL-producing *Escherichia coli* urinary isolates from children at St. Patricks Hospital, Abakaliki, Mile Four, Ebonyi State, Nigeria

Age group (years)/ Antibiotics(μ g)	< 5 (n = 4)		5 - 10 (n = 2)		11 - 15 (n = 5)	
	Susceptible	Resistance	Susceptible	Resistance	Susceptible	Resistance
Cefotaxime (30)	0	4	0	2	0	5
Ceftazidime (30)	1	3	0	2	1	4
Aztreonam (30)	0	4	1	1	0	5
Amikacin (30)	2	2	2	0	4	1
Ticarcillin (75)	0	4	0	2	0	5
Ertapenem (10)	1	3	1	1	2	3
Gentamicin (10)	1	3	1	1	4	1
Sulfamethoxazole-trimethoprim (75)	0	4	0	2	0	5
Ciprofloxacin (5)	0	4	1	1	1	4
Tobramycin (15)	1	3	1	1	2	3

n = no of *Escherichia coli* isolates

Table 3: Multiple antibiotic resistance index (MARI) of the ESBL-producing *Escherichia coli* isolates from paediatric patients in St Patrick Hospital, Abakaliki, Ebonyi State, Nigeria

ESBL producing <i>Escherichia coli</i> isolate	MARI
< 5 years	
E7	1.0
E10	0.7
E14	0.9
E20	0.8
	Average MARI = 0.9
5 - 10 years	
E17	0.9
E18	0.4
	Average MARI = 0.7
11 - 15 years	
E2	0.8
E4	0.7
E11	0.7
E13	0.6
E21	0.8
	Average MARI = 0.7

The average multiple antibiotic resistance index (MARI) values of the ESBL-producing *E. coli* isolates in age groups < 5 years, 5-10 years and 11-15 years are respectively 0.9, 0.7, and 0.7 (Table 3). There was no statistically significant difference in the average MARI values between the age groups ($p > 0.05$)

Discussion:

In our study, the prevalence of *E. coli* in 100 children with urinary tract infection at St. Patricks Hospital, Abakaliki, Ebonyi State, Nigeria is 21%. In a related study by Sani *et al.*, (8), the prevalence of 45% for *E. coli*

isolates was reported in 222 urinary samples, and in another similar one by Sabrina *et al.*, (9), a prevalence of 37.5% for *E. coli* was reported in 280 urinary samples of children with urinary tract infection. Although these studies are similar to the present one conducted by us, the prevalence of *E. coli* in urinary tract infection in our study is lower than the prevalence in these other studies, probably because of the smaller sample size in our study.

In our study, 52% (11 of 21) of the *E. coli* isolates were phenotypically confirmed ESBL-producer, which compared with a related study by Amita and Rajesh who reported 58% *E. coli* to be ESBL producer in their study (10). In the study by Babak *et al.*, (11), a high prevalence of ESBL producer in *E. coli* of 68.2% in children aged 9-12 years was reported, which agrees with 62.5% reported among comparative age group (11-15 years) in our study. This may suggest a higher prevalence of ESBL producers among urinary *E. coli* isolates in older children.

The ESBL-producing *E. coli* isolates in our study exhibited total resistance to cefotaxime, ticarcillin and sulfamethoxazole/trimethoprim among the children across age strata, and to aztreonam, ceftazidime and ciprofloxacin in specific age groups. This is similar to the study of Rajesh *et al.*, (12) who reported high resistance rates of ESBL-producing *E. coli* isolates to ticarcillin, ampicillin, monobactam (aztreonam) and cephalosporins. The average multiple antibiotic resistance index (MARI) value of 0.8 reported for the ESBL-producing *E. coli* isolates in this study is an indication that antibiotics are overused in our environment.

Conclusion:

The high prevalence of multi-drug resistant ESBL-producing *E. coli* obtained from children with urinary tract infection in St. Patrick Hospital, Abakaliki, Ebonyi State, shows that there has been overuse (abuse or misuse) of antibiotics in the area. There is need for antimicrobial stewardship programme that will ensure prudent use of antimicrobial agents to forestall the emergence and spread of multi-drug resistant bacteria

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**Short Communication****Open Access****Prevalence of HIV infection among newly admitted students in Ebonyi State University, Abakaliki, Nigeria**

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*Correspondence to: nworieamos@gmail.com; Mobile: +2348100226465**Abstract:**

Background: Human immunodeficiency virus (HIV) and the associated acquired immune deficiency syndrome (AIDS) have remained a serious scourge and a major public health concern, affecting millions in sub-Saharan Africa despite awareness campaigns, preventive measures and promotion of antiretroviral regimens. This study determined the prevalence of HIV among newly admitted students of Ebonyi State University as a measure of the impact of awareness campaign towards prevention of HIV transmission.

Methods: Newly admitted students of Ebonyi State University totalling 2,736 who voluntarily enrolled for the study were screened for HIV infection using the national HIV testing algorithm after information relating to their personal lifestyle, knowledge of safer sex and preventive measures have been obtained with the use of a client intake form.

Results: Of the 2,736 subjects screened, 6 were positive for HIV, giving a prevalence rate of 0.22%, with prevalence rate of 0.29% (4 of 1344) in females and 0.14% (2 of 1392) in males ($X^2=0.2041$, $p=0.6514$). The positive subjects were spread across age groups 15-19 years (1), 20-24 years (4) and 25-29 years (1). Males and females who have had sex were 801 and 579 out of which 239 and 209 respectively acknowledged to have had unprotected sex within three months of the study.

Conclusion: The low HIV prevalence rate of 0.22% among school age and young adults in this study may indicate that awareness and safe sex campaigns in Ebonyi State have positive impact in HIV prevention amongst these groups of people.

Keywords: HIV, students, Ebonyi State University, Nigeria, prevalence, campaign

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Copyright 2019 AJCEM Open Access. This article is licensed and distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution and reproduction in any medium, provided credit is given to the original author(s) and the source.**Prévalence de l'infection à VIH chez les étudiants nouvellement admis à l'Université d'Ebonyi, Abakaliki, Nigéria**

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Contexte: le virus de l'immunodéficience humaine (VIH) et le syndrome d'immunodéficience acquise (SIDA) associé restent un grave fléau et un grave problème de santé publique, touchant des millions de personnes en Afrique subsaharienne en dépit des campagnes de sensibilisation, des mesures préventives et de la promotion des schémas thérapeutiques antirétroviraux. Cette étude a déterminé la prévalence du VIH parmi les étudiants nouvellement admis à l'Université d'Ebonyi en tant que mesure de l'impact de la campagne de sensibilisation sur la prévention de la transmission du VIH.

Méthodes: Les étudiants nouvellement admis à l'Université d'Ebonyi, sur un total de 2 736 inscrits volontairement à l'étude, ont été dépistés pour l'infection à VIH à l'aide de l'algorithme national de dépistage du VIH, après que des informations relatives à leur mode de vie personnel, à leur connaissance du sexe sans risque et à des un formulaire d'admission du client.

Résultats: Sur les 2 736 sujets dépistés, 6 étaient séropositifs, soit un taux de prévalence de 0,22%, avec un taux de prévalence de 0,29% (4 sur 1344) chez les femmes et de 0,14% (2 sur 1392) chez les hommes ($X^2 = 0,2041$, $p = 0,6514$). Les sujets positifs étaient répartis dans les groupes d'âge 15-19 ans (1), 20-24 ans (4) et 25-29 ans (1). Les hommes et les femmes ayant eu des rapports sexuels comptaient 801 et 579 personnes, dont

239 et 209 respectivement ont reconnu avoir eu des rapports sexuels non protégés dans les trois mois suivant l'étude.

Conclusion: Le faible taux de prévalence du VIH de 0,22% chez les enfants d'âge scolaire et les jeunes adultes dans cette étude peut indiquer que les campagnes de sensibilisation et de promotion du sexe sans risque dans l'État d'Ebonyi ont un impact positif sur la prévention du VIH parmi ces groupes de personnes

Mots-clés: VIH, étudiants, université d'Ebonyi, Nigéria, prévalence, campagne

Introduction:

Globally, HIV infection has continued to ravage the world for nearly three decades (1). In 2015, an estimated 36.9 million people were reported to be living with HIV worldwide (2). In developing countries, HIV epidemic has increased health challenges and eroded development. It has also facilitated the re-emergence of disease conditions such as tuberculosis (TB) and other opportunistic infections thought to have been controlled (3).

The incidence of HIV/AIDS particularly in Nigeria has been a major source of concern to health professionals, healthcare givers and public health institutions. After the emergence of HIV in Nigeria, the seroprevalence rate increased from 1.8% in 1991 to 5.5% in 2003 but reduced to 3.1% in 2013 (4). Research has shown that Nigeria has the second highest number of people living with HIV in the world after South Africa with about 3.2 million people infected as at 2014 (5). In Nigeria, the HIV epidemic has been described as heterogeneous because prevalence is declining in some communities and states while it is increasing in others (5). The latest report showed that it has indeed declined in most states of Nigeria (6)

HIV has been reported to affect more females than males in Nigeria (7) and to be more prevalent in adolescents than children and old adults (8). The adolescents who exhibit rapid physical and emotional development as well as sexual maturation are more exposed to HIV infection and other sexually transmitted diseases due to their increasing sexual adventure, risky behavioral practices and other factors (9). In 2015, the National Agency for Control of AIDS (NACA) reported a HIV prevalence of 4.2% among adolescents aged 15-24 years who constitute 40% of all reported new cases of HIV in the country (10).

The knowledge of HIV and its mode of transmission are important in its control and eradication. Although several studies have suggested that the level of awareness among Nigerian adolescents is high, especially with regards to prevention and care (11,12,13), the use of condom as a means of preventing the spread of this infection is low among this group. It was reported that 56.4% and 39.6% of adolescent boys and girls respectively had unprotected sex with their partners (14). According to available data from household surveys (2006–2013) in selected countries

where adolescent girls were at higher risk of HIV infection, condom usage at last higher-risk sex, ranges from as low as 8.5% in the Democratic Republic of Congo to as high as 52% in Cameroon with 38% rate in Nigeria (15). Increased use of condom could reduce the transmission amongst this age group.

This study determined the prevalence of HIV amongst newly admitted students in Ebonyi State University, Abakaliki and their level of awareness based on campaign efforts of critical stake holders in HIV control and management in the country.

Materials and Methods:

Study setting

The cross sectional study was carried out at Ebonyi State University Ultramodern Diagnostic Laboratory and Research Centre located at the Department of Medical Laboratory Science, Faculty of Health Sciences and Technology, Abakaliki, Ebonyi State, Southeast Nigeria. The laboratory is a research centre of the University, offering special services in all specialties of laboratory medicine. It also serves as a counselling and screening centre of Ebonyi State Agency for the Control of HIV/AIDS with about 4000 clients including newly admitted students of the University counselled and screened for HIV yearly.

Study population

The study population consisted of newly admitted students of Ebonyi State University in the 2017/2018 academic session who consented to and had counselling during their medical screening. The annual students' intake in the University is between 4000-5000 who are usually screened to document their health status. All the subjects were in the age range of 15 - 35 years. Informed consent of the students and appropriate ethical clearance were obtained.

Method for HIV screening

Alere Determine (Alere Medical Co. Ltd. Matsuhidai, Matsudo-Shi, Chiba, Japan), Unigold (Trinity Biotech Plc, Bray, Co. Wicklow, Ireland), and Stat-Pak (Chembio Diagnostics Systems, Inc. Medford, New York, USA) were used for the determination of HIV status. All the reagents were supplied by Ebonyi State Agency for the Control of HIV/AIDS (EBOSACA), Abakaliki. The test was carried out

in line with the approved national algorithm for Nigeria where Alere Determine was used as the first step screening, Unigold was used for confirmation only when Alere Determine was positive and Stat-Pak was used as tie breaker when there was disparity in the results between Alere Determine and Unigold. Tests were carried out following manufacturer's instructions and results were interpreted and recorded as shown in Table 1. The client intake form (HIV counselling and testing form) provided by Ebonyi State Agency for the Control of HIV/AIDS, was used to collect socio-demographic data, knowledge of risk factors, transmission and prevention of HIV/AIDS.

Table 1: National HIV test algorithm in Nigeria

Test 1 (Determine)	Test 2 (Unigold)	Test 3 (Stat-Pak)	HIV status
Negative			Negative
Positive	Positive		Positive
Positive	Negative	Negative	Negative
Positive	Negative	Positive	Positive
Positive	Invalid	Positive	Positive
Positive	Invalid	Negative	Negative

Data analysis

Data were analysed using the Statistical Package for Social Sciences (SPSS) software version 20.0 and results presented in frequency tables and simple percentages. Association between categorical variables was tested using Chi-square (X²) test and the level of statistical significance was set at *p* < 0.05.

Results:

Table 2 showed the gender and age group distribution of subjects. Out of the 2736 subjects screened (1392 males and 1344 females), 6 (2 males and 4 females) were positive giving a prevalence rate of 0.22%. The prevalence was 0.14% in males and

0.29% in females. The association between gender and HIV infection was not statistically significant (*p*=0.6514).

There were 1108 subjects in age group 15-19 years, 1357 in age group 20-24 years, 236 in age group 25-29 years, and only 35 were 30 years and above. The percentage of subjects in age group 15-24 years was 90.1% while the percentage for age group 25 years and above was 9.9%. The prevalence of HIV among subjects in age group 15-24 years was 0.2% while for age group 25 years and above, the prevalence was 0.37% (X²= 0.3081, 95% CI 0.06442-4.690, *p*=0.5789). Only 1 of 1108 (0.09%) subjects in the age group 15-19 years, 4 of 1357 (0.29%) in age group 20-24 years, and 1 of 236 (0.42%) in age group 25-29 years were HIV positive, while none of the 35 (0%) who were 30 years and above was HIV positive. The association between age group and HIV infection was not statistically significant (*p*=0.6316).

Table 3 showed the frequency of HIV infection in relation to sexual behaviour of subjects and the use of condom. No positive case was detected among 562 males and 370 females who had sex with use of condom prior to the study. Out of 239 males and 209 females who had sex without the use of condom within the time period, 2 and 3 positives cases respectively, were detected (X²=7.577, *p*=0.0059).

Table 4 showed the subjects awareness of their HIV status. Subjects who knew their HIV status before the study were 553 out of which 1 tested positive. Subjects who did not know their status before the study were 2,183, out of which 5 tested positive. The association between prior knowledge of subject's HIV status and HIV infection was not statistically significant (X²=0.047, *p*=0.8286).

Table 2: Gender and age group distribution of subjects in relation to HIV infection

Characteristics	Number of subjects tested	Number of subjects positive for HIV (%)	X ² value	95% CI	<i>p</i> -value
Gender					
Male	1392	2 (0.14)	0.2041	0.2110-2.032	0.6514*
Female	1344	4 (0.29)			
Age group (years)					
15 - 19	1108	1 (0.09)	1.724		0.6316*
20 - 24	1357	4 (0.29)			
25 - 29	236	1 (0.42)			
30 & above	35	0			

* Not statistically significant at *p* > 0.05

Table 3: Frequency of HIV infection in relation to sexual intercourse and the use of condom among subjects

Subjects	Sexual intercourse with condom use		Sexual intercourse without condom use		Total	X ²	p value
	Male	Female	Male	Female			
HIV positive	0	0	2	3	5	7.577	0.0059*
HIV negative	562	370	237	206	1375		
Total	562	370	239	209	1380		

*Statistically significant at $p < 0.05$

Table 4: Subject awareness of their HIV status in relation to HIV infection

Subjects	Number tested (%) (n=2736)	Number positive (%) (n=6)	X ² value	95% CI	p value
Subjects who knew their HIV status	553 (20.2)	1 (0.18)	0.04687	0.1375-4.943	0.8286*
Subjects who do not know their HIV status	2183 (79.8)	5 (0.23)			

* Not statistically significant at $p > 0.05$

Discussion:

HIV is an endemic disease in Nigeria. The findings of this study indicated an overall low prevalence (0.22%) of HIV among newly admitted students of Ebonyi State University. This prevalence is low when compared to the National HIV & AIDS and Reproductive Health Survey (NARHS) (16) which reported a prevalence of 1.1% in the Southeast. The low prevalence could be as a result of increasing awareness of school age children about safe sex and HIV prevention measures. Government, non-governmental (NGOs) and community based organisations (CBOs) have over the years mounted vigorous HIV awareness campaign to stem the tide of HIV scourge. Reports elsewhere have indicated a higher prevalence among these groups (17). The higher prevalence of HIV among females (0.29%) compared to their male counterparts (0.14%) in this study is supported by reports elsewhere (18). Several reasons have been adduced for the higher prevalence among females, including their vulnerabilities, often associated with poor economic and socio-cultural status, shy attitude towards sex education, and abhorrence of sex outside marriage culminating in restriction of young girls from access to sexual health and HIV services.

There was no significant association between age of subjects and predisposition to HIV infection, although the prevalence of HIV infection of 0.3% among age group 20-24 years and 0.42% among age group 25-29 years are higher than 0.09% among the age group 15-19 years, which is consistent with the Nnewi study which reported age group 25-29 years to have the highest prevalence (19), and also with the 2010 HIV survey report of a

higher prevalence among age group 20-30 years (20).

Sexual intercourse is a major route of transmission of HIV. Our study showed that 5 of the 448 subjects who had unprotected sex prior to the study were HIV positive and all cases of HIV infection were among those who had unprotected sex. This supports the use of condom as an effective way of preventing HIV transmission. In a review of 14 studies on the use of condom, the incidence of HIV infection among those who always use condoms was 1.14% while it was 5.75% among those who never used them (21).

Awareness of HIV and its mode of transmission are important in its eradication. In this study, the percentage of subjects who had knowledge of their HIV status was low at 20.2% compared to 79.8% for those who were not aware of their HIV status, although the HIV infection rate was not significantly different in the two groups ($p=0.8286$). Nevertheless, the low level of awareness could be attributed to the fact that Ebonyi State is an agrarian state with majority of the families living in rural areas with their children who constitute over 80% of student intake in the university. Although Egbo and Chukwu (22) had reported an appreciable awareness among women farmers of Ebonyi State, our findings suggests otherwise.

Furthermore for fear of death and stigmatization, most of these rural dwellers could not consent to HIV screening test or were completely oblivious of the reality of HIV. Our assertion was reinforced by Adekeye (23), who in his study reported that more than half of the respondents studied did not consent to screening for HIV because of the risk of stigmatization if tested positive, with tendency to suicide as a consequence.

Conclusion:

There was a very low prevalence of HIV amongst newly admitted students of Ebonyi State University, Abakaliki in this study. Majority of the students however had no knowledge of their HIV status, which portends serious challenge to HIV prevention effort. Subjects in age group 20-29 years had a higher prevalence of HIV infection than those in age group 15-19 years, and those above 30 years of age. The use of condom is very important in the control of HIV as no case was recorded amongst those who used condoms. Further studies are recommended to ascertain whether the demographic prevalence observed is wide spread or limited to newly admitted students of Ebonyi State University. Sensitization to increase HIV awareness is advocated to curb further spread of the virus.

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**Short Communication****Open Access****Bacteriological profiles of acute suppurative otitis media in children in Brazzaville, Congo**¹Otuouana Dzon, H. B., ¹Ngouoni, G. C., ²Diembi, S., ¹Tsierie-Tsoba, A., ³Kambourou, J., ¹Itiere-Odzili, F. A., and ¹Ondzotto, G.¹Otolaryngology Department, Brazzaville University Hospital Center, BP 13356, Congo²Otolaryngology Department, General Hospital, Adolph Sicé, Congo³Paediatric Intensive Care Department, Brazzaville University Hospital Center, CongoCorrespondence to: hb.otouana@gmail.com; (00242) 069422411**Abstract:****Background:** Acute suppurative otitis media (ASOM) is one of the main indications for antibiotic prescription in children. The close proximity of the middle ear to the brain and the increasing resistance of microbial organisms involved in otitis media make this pathology of great concern in children. The objective of this study is to determine the bacteriological profile of acute otitis media in Congo as a guide to the choice of antibiotics for empirical therapy.**Methodology:** A cross sectional study of children less than 17 years old with acute suppurative otitis media in the otorhinolaryngology service of the Brazzaville University Hospital, Congo, was conducted over a 14 month period. All subjects whose samples were sterile or contaminated (poly-microbial culture) and those who received antibiotic-corticosteroid therapy were excluded. The identification of bacteria to species level was done using conventional biochemical identification tests scheme. Antibiotic sensitivity was performed on isolates using the modified Bauer Kirby disk diffusion test on plain Mueller Hinton (MH) agar and MH agar with 5% horse blood.**Results:** Four bacteria families/species were identified; *Staphylococcus aureus* (32.7%), family Enterobacteriaceae (28.6%), *Streptococcus pneumoniae* (26.5%) and *Pseudomonas aeruginosa* (12.2%). *Ps. aeruginosa* was associated with greenish otorrhea while *S. aureus*, Enterobacteriaceae and *S. pneumoniae* were associated with yellowish otorrhea ($p = 0.001$).**Conclusion:** The bacterial aetiology of acute suppurative otitis media varies from country to country. In Congo, this study reports four main bacteria families/species involved in acute otitis media with high resistance to β -lactam antibiotics but high sensitivity to macrolides and fluoroquinolones.**Key words:** otitis; child; antibiogram; bacteria; Brazzaville

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Copyright 2019 AJCEM Open Access. This article is licensed and distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution and reproduction in any medium, provided credit is given to the original author(s) and the source.**Profils bactériologiques de l'otite moyenne suppurative aiguë chez les enfants à Brazzaville, Congo**¹Otuouana Dzon, H. B., ¹Ngouoni, G. C., ²Diembi, S., ¹Since-Tsoba, A., ³Kambourou, J., ¹Itiere-Odzili, F. A., et ¹Ondzotto, G.

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Correspondance à: hb.otouana@gmail.com; (00242) 069422411**Abstrait:****Contexte:** L'otite moyenne suppurative aiguë est l'une des principales indications de la prescription d'antibiotiques chez les enfants. La proximité de l'oreille moyenne au cerveau et la résistance croissante des organismes microbiens impliqués dans l'otite moyenne rendent cette pathologie extrêmement préoccupante

chez les enfants. L'objectif de cette étude est de déterminer le profil bactériologique de l'otite moyenne aiguë au Congo en tant que guide pour le choix des antibiotiques pour un traitement empirique.

Méthodologie: Une étude transversale sur les enfants de moins de 17 ans atteints d'otite moyenne suppurée aiguë dans le service d'otorhinolaryngologie de l'hôpital universitaire de Brazzaville au Congo a été menée sur une période de 14 mois. Tous les sujets dont les échantillons étaient stériles ou contaminés (culture polymicrobienne) et ceux ayant reçu un traitement antibiotique-corticostéroïde ont été exclus. L'identification des bactéries au niveau de l'espèce a été réalisée à l'aide d'un programme d'essais d'identification biochimique conventionnel. La sensibilité aux antibiotiques a été réalisée sur des isolats en utilisant les tests de diffusion sur disque Bauer Kirby modifiés sur gélose Mueller Hinton (MH) et gélose MH avec 5% de sang de cheval.

Résultats: Quatre familles/espèces de bactéries ont été identifiées. *Staphylococcus aureus* (32,7%), la famille des Enterobacteriaceae (28,6%), *Streptococcus pneumoniae* (26,5%) et *Pseudomonas aeruginosa* (12,2%). *Ps. aeruginosa* était associé à une otorrhée verdâtre, alors que *S. aureus*, Enterobacteriaceae et *S. pneumoniae* étaient associés à une otorrhée jaunâtre ($p = 0,001$).

Conclusion: L'étiologie bactérienne de l'otite moyenne suppurée aiguë varie d'un pays à l'autre. Au Congo, cette étude fait état de quatre familles / espèces de bactéries principales impliquées dans les otites moyennes aiguës présentant une résistance élevée aux antibiotiques β -lactames mais une sensibilité élevée aux macrolides et aux fluoroquinolones.

Mots-clés: otite; enfant; antibiogramme; les bactéries; Congo Brazzaville

Introduction:

Acute suppurative otitis media is bacterial infection of the middle ear cavities with purulent effusion that collects in the middle ear and become externalized as ear discharge (1, 2). It is the usually the first bacterial infection in children under two years of age and one of the leading indication for antibiotic prescription (3, 4). However, the proximity of the middle ear to the brain on the one hand and the increasing resistance of microorganisms to antibiotics on the other hand make otitis media of serious concern in young children (1-3).

The causative pathogens of otitis media vary depending on geographical location and pathogen distributions. In France, the causative organisms such as *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* are well established for otitis media thus the choice of antibiotics for empirical therapy is guided (2, 5, 6). This however is not the case in Congo, where no study on bacterial ecology of acute suppurative otitis media has been performed. In order to guide empirical antibiotic therapy, this study aimed to assess the bacteriological profile of microbial pathogens responsible for acute suppurative otitis media in Congo.

Material and methods:

Study setting and design

This was a cross-sectional study performed in the otorhinolaryngology service of the Brazzaville University Hospital and the National Public Health Laboratory over a period of 14 months (01 October 2013 to 30 November 2014).

Subjects, inclusion and exclusion criteria

The subjects were children less than 17 years old with acute suppurative otitis media whose otorrhea had occurred less

than 6 weeks. All subjects whose samples were sterile or contaminated (poly-microbial culture) and those who received antibiotic-corticosteroid therapy were excluded.

Sample collection and culture isolation of bacteria

Ear discharge was collected with sterile swabs after careful disinfection of the auricle. Two samples were taken per patient, which were sent immediately to the National Laboratory of Public Health in thermostable containers for processing. The first sample was used for direct examination by Gram stain microscopy and the second for aerobic culture and isolation of bacteria. The culture media inoculated according to the Gram reaction were Mueller Hinton (MH) and Eosin Methylene Blue (EMB) agar. The identification of bacteria to species level was done using conventional biochemical identification tests scheme.

Antibiotic sensitivity testing

Antibiotic sensitivity was performed on isolates using the modified Bauer Kirby disk diffusion test on plain MH agar and MH agar with 5% horse blood.

Data and statistical analysis

Data for analysis entered into Microsoft Excel spreadsheet included age and gender of subjects, macroscopic appearance of otorrhea, Gram reactions, frequency of isolation and susceptibility profiles of bacterial species. Data were analyzed with EPIINFO3.5.4 software Chi square test was used to measure association between types of otorrhea and isolated bacteria, with $p < 0.05$ taken as significant.

Results:

A total of 63 patients had clinical features of acute suppurative otitis media out of 716 patients who received pediatric

consultation during the period of study, representing 8.7% or 1.2 cases of acute suppurative otitis media per week. The mean age of subjects was 3 years with 40 (63%) males and 23 (37%) females (a ratio of 1.7:1). The difference in occurrence of ASOM between male and female gender was not statistically significant ($p > 0.05$). Specimens were positive for bacteria in 49 of the 63

patients (78%); Gram-positive cocci in 29 (59%) and Gram-negative bacilli in 20 (41%). Table 1 shows the different species of bacteria isolated, and this differ according to the macroscopic appearance of ear discharge ($p = 0.001$) (Table 2). The susceptibility of bacteria isolates to antibiotics is shown in Table 3.

Table 1: Isolated bacteria from acute suppurative otitis media in Brazzaville, Congo

Bacteria family	Isolated species	Frequency (%)
Micrococcaceae	<i>Staphylococcus aureus</i>	16 (33)
Enterobacteriaceae	<i>Enterobacter species</i>	6 (12)
	<i>Escherichia coli</i>	3 (6)
	<i>Klebsiella pneumoniae</i>	2 (4)
	<i>Proteus mirabilis</i>	1 (2)
	<i>Proteus vulgaris</i>	1 (2)
	<i>Acinetobacter species</i>	1 (2)
Streptococcaceae	<i>Streptococcus pneumoniae</i>	13 (27)
Pseudomonaceae	<i>Pseudomonas aeruginosa</i>	6 (12)
	Total	49 (100)

Table 2: Isolated bacteria and appearance of otorrhea in acute suppurative otitis media in Brazzaville, Congo

Bacteria	Otorrhoea		Total (%)
	Yellowish (%)	Greenish (%)	
<i>Staphylococcus aureus</i>	10 (21)	6 (12)	16 (33)
Enterobacteriaceae	12 (24)	1 (2)	13 (26)
<i>Streptococcus pneumoniae</i>	10 (21)	4 (8)	14 (29)
<i>Pseudomonas aeruginosa</i>	0	6 (12)	6 (12)
Total	32 (65)	17 (35)	49 (100)

Table 3: Susceptibility of bacteria isolates to antibiotics in acute suppurative otitis media in Brazzaville, Congo

Antibiotics	<i>S. aureus</i> (%)	Enterobacteriaceae (%)	<i>S. pneumoniae</i> (%)	<i>P. aeruginosa</i> (%)
Oxacillin	9 (56.3)	8 (57.1)	5 (38.5)	0
AMC	0	0	0	0
Cefalexin	11 (68.8)	12 (85.7)	9 (69.2)	0
Ceftriaxone	5 (31.3)	3 (21.4)	5 (38.5)	4 (66.7)
Erythromycin	0	7 (50)	0	6 (100)
Spiramycin	0	4 (28.6)	0	3 (50)
Ciprofloxacin	14 (87.5)	3 (21.4)	6 (46.2)	6 (100)
Norfloxacin	10 (34.5)	7 (24.1)	12 (41.4)	0
Ofloxacin	2 (12.5)	7 (50)	9 (69.2)	3 (50)

AMC = amoxicillin-clavulanic acid

Discussion:

In this study, acute suppurative otitis media accounted for 8.7% of paediatric consultations in Brazzaville University Hospital (approximately 1.2 cases per week). This incidence seems low compared to the reports of other authors, in particular Levy et al., who reported a higher incidence of 6 cases per week (7). In contrast, Asse et al., reported a much lower incidence of 5 cases per 1000 admissions (8).

We found 49 positive cultures, which represented 77.8% of the total patients sampled. The high proportion of positive cultures is explained by the mode of transport of the sterile swabs in thermostable containers. It is indeed recommended to transport the samples in preservative tubes to prevent the degradation of fragile bacteria (9). The groups of Gram positive and negative cocci reported in this study were also reported by Hounkpatin et al., in Benin (10) but the causative organisms of otitis media are known to vary from country to country.

In France, *H. influenzae* and *M. catarrhalis* are predominant pathogens involved in acute otitis media (11-14) but these organisms were not involved in bacterial ecology of otitis media in our study. Rather, *S. aureus*, Enterobacteriaceae, *S. pneumoniae* and *Ps. aeruginosa* were isolated. In Benin and Cote d'Ivoire, the main isolated organisms were also *S. aureus* and *Ps. aeruginosa* (10, 15), which agrees with our study. In Tunisia however, the predominant organisms were *H. influenzae*, *S. pneumoniae* and *Streptococcus pyogenes* (16).

The appearance of otorrhea may correlate with the bacterial species involved in acute suppurative otitis media. We found that yellowish otorrhea was associated with *S. aureus*, Enterobacteriaceae and *S. pneumoniae* while *Ps. aeruginosa* produced greenish otorrhea ($X^2=15.76$, $p=0.001$). Tanon-Anoh et al., in Cote d'Ivoire reported that yellowish and greenish otorrhea were the two commonly encountered discharges in ASOM though the authors did not report any possible correlation with the causative organisms (15). While some bacteria produce enzymes that enable them break down host cells to produce purulent secretions, others are able to produce pigments responsible for the appearance of discharge. This is the case of pyoverdine which is a pigment produced only by certain strains of family Pseudomonaceae such as *Ps. aeruginosa* responsible for greenish otorrhea (17).

All isolated organisms in this study were resistant to the amoxicillin-clavulanic

acid combination. This high resistance rate in our study can be explained by the fact that there has been inappropriate and over use of this antibiotic combination in Congo (10). Among the beta-lactams tested, only cefalexin was effective on Enterobacteriaceae, but the use of this antibiotic in Congo must be judiciously monitored.

The *S. aureus* isolates were sensitive to ciprofloxacin while the *S. pneumoniae* isolates were susceptible to ofloxacin. Although, the efficacy of the quinolones in this study is encouraging, they must be used with caution in children under 10 years of age because of their potential deleterious effects on growth cartilages (18). The high sensitivity of *S. pneumoniae* to ofloxacin and cefalexin in our study is contrary to the observations of other authors (10, 19, 20). *Ps. aeruginosa* which has been reputed to be resistant to several antibiotics (10), was however 100% sensitive to ciprofloxacin and erythromycin in our study.

Conclusion:

Acute suppurative otitis media in children is a relatively common condition depending on whether it is paediatric or otolaryngological consultation. Contrary to what obtains in the Western countries where *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* are predominant pathogens of ASOM, in Congo the predominant pathogens are *S. aureus*, family Enterobacteriaceae, *S. pneumoniae* and *Ps. aeruginosa*. Thus empirical antibiotic therapy of ASOM in Congo should cover for these pathogens for effective treatment in order to prevent complications and development of resistance.

Conflict of interest:

No conflict of interest is declared

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