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Review Article **Open Access**

Yellow fever in Nigeria: a review of the current situation

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Abstract:

Several African countries including Nigeria have been battling with public health challenges for decades. Nigeria is currently facing several public health emergencies including cholera, circulating vaccine-derived poliovirus infection, cerebrospinal meningitis, monkey pox, measles, Lassa fever, and Yellow fever outbreaks in some states, as well as a humanitarian crisis in the northeast region of the country. Sporadic outbreaks of Yellow fever have been occurring in the country since September 2017 involving all thirty six states of the Federation, resulting in about 90 deaths (case fatality rate of 2.2%) and 31 deaths among confirmed cases (case fatality rate of 19.0%). Although, there is currently no specific treatment for Yellow fever, vaccination with the Yellow fever vaccine provides life-long protection, and is the most important means of preventing the disease. Despite the availability of an effective vaccine, the re-emergence of Yellow fever is directly correlated with its continuous dissemination in several countries to date. Timely detection of Yellow fever and rapid response through emergency vaccination campaigns are essential for controlling outbreaks. Vector surveillance and control are important components of reducing transmission in epidemic situations. This review attempts to provide update information on the current situation of Yellow fever in Nigeria with highlights on the history, pathogenesis and diagnosis of the disease.

Key words: Yellow fever, Nigeria, Outbreaks, Mosquitoes

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Fièvre jaune au Nigéria: état des lieux

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Abstrait:

Plusieurs pays africains, dont le Nigéria, luttent contre des problèmes de santé publique depuis des décennies. Le Nigéria est actuellement confronté à plusieurs urgences de santé publique, y compris le choléra, une infection à poliovirus en circulation, une méningite cérébro-spinale, la variole du singe, la rougeole, la fièvre de Lassa et la fièvre jaune dans certains États, ainsi qu'une crise humanitaire dans le nord-est du pays. Des épidémies sporadiques de fièvre jaune se sont produites dans le pays depuis septembre 2017 dans les trente-six États de la Fédération, entraînant environ 90 décès (taux de létalité de 2,2%) et 31 décès parmi les cas confirmés (taux de létalité de 19,0%). Bien qu"il n"existe actuellement aucun traitement spécifique contre la fièvre jaune, la vaccination avec le vaccin contre la fièvre jaune offre une protection à vie et constitue le principal moyen de prévention de la maladie. Malgré la disponibilité d'un vaccin efficace, la réémergence de la fièvre jaune est directement corrélée à sa diffusion continue dans plusieurs pays à ce jour. La détection rapide de la fièvre jaune et une réponse rapide au moyen de campagnes de vaccination d'urgence sont essentielles pour contrôler les épidémies. La surveillance et le contrôle des vecteurs sont des éléments importants de la réduction de la transmission en situation épidémique. Cette revue tente de fournir des informations actualisées

sur la situation actuelle de la fièvre jaune au Nigéria, en mettant en évidence l'histoire, la pathogenèse et le diagnostic de la maladie

Mots-clés: fièvre jaune, Nigéria, épidémies, moustiques

Introduction:

Yellow fever is an acute viral haemorrhagic disease that is vaccine preventable, yet it is widely distributed in the tropics of Latin America and Africa where infections cause an estimated 29,000 to 60,000 deaths annually (1). Yellow fever virus is transmitted to humans through the bites of infected mosquitoes (primarily in the genus *Aedes*), and is principally maintained by a sylvatic (jungle) transmission cycle involving non-human primate reservoirs. Urban Yellow fever outbreaks occur when infected people introduce the virus into heavily populated areas with competent vector populations and insufficient vaccination coverage.

The spectrum of human clinical disease caused by the virus is broad, ranging from asymptomatic infections and mild febrile illness to severe disease and death (2). Although most patients with Yellow fever are asymptomatic, approximately 15-25% of infected symptomatic persons develop severe disease and the case fatality rate is 20%–60% (3).

A safe and effective vaccine against Yellow fever exists, and some countries require vaccinations for travellers. Other efforts to prevent infection include reducing the population of the transmitting mosquitoes (2). Despite the availability of an effective vaccine, the re-emergence of Yellow fever is directly linked with its continuous dissemination in several countries. Brazil, Angola, Democratic Republic of Congo, and Nigeria accounted for thousands of documented cases between 2016 and 2018 (4), which can be interpreted as a probable indicator of what will occur if no action is taken. Therefore, this review highlights the epidemiological features and the current outbreak of Yellow fever in Nigeria.

Methodology:

A review of the current situation of Yellow fever in Nigeria was conducted by searching for relevant published materials and bibliographic citations including original and review articles, personal communications, libraries, books, and conference papers through the period 1985 to 2019 with the use of Google scholar engine.

The keywords employed for the search were Yellow fever, history of Yellow fever, Yellow fever in Nigeria, diagnosis of Yellow fever, pathogenesis of Yellow fever, epidemiology of Yellow fever, and current situation of Yellow fever in Nigeria. A total of 205 reference materials were identified but following assessment of the relevance of the materials, only 40 reference materials were selected for the review. The process is depicted in Fig 1.

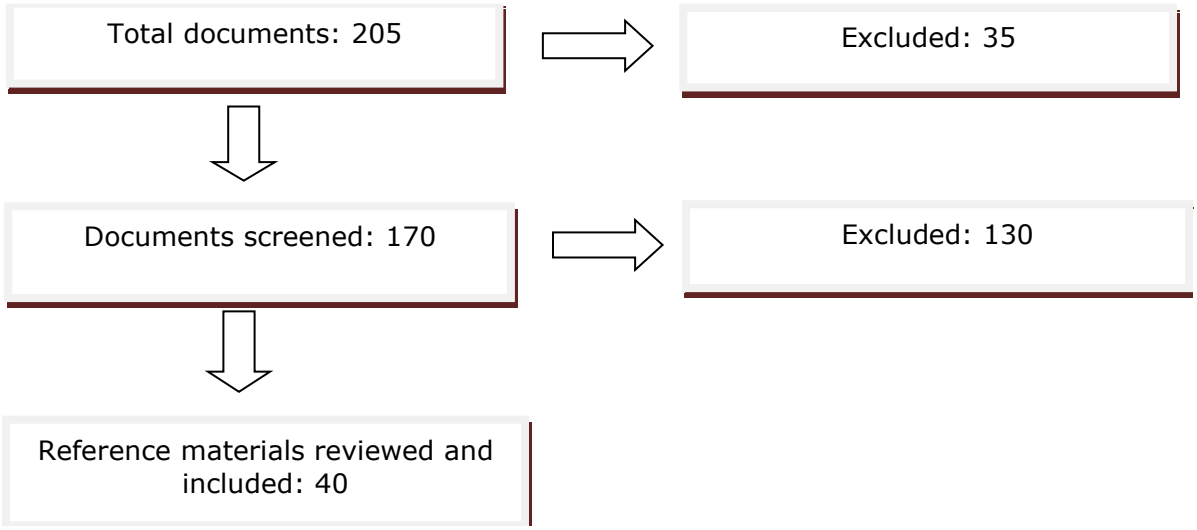

Fig. 1: Flowchart for selection of relevant documents used for the review

History of Yellow fever

Yellow fever has had an important role in the history of Africa, America, Europe, and the Caribbean. The evolutionary origins of Yellow fever most likely lie in Africa and scientists believe that Yellow fever evolved in Africa about 3,000 years ago (5). Phylo-genetic analyses indicate that the virus originated from East or Central Africa, with transmission between primates and humans, and spread from there to West Africa (6). The virus as well as the vector *Aedes aegypti*, a mosquito specie, were probably brought to the western hemisphere and the Americas by slave trade ships from Africa after the first European exploration in 1492 (7).

Although it was realized early that the disease was not contagious, the source was wrongly attributed to environmental miasmas. In 1848, Josiah C. Nott suggested that Yellow fever was spread by insects. In 1881, Carlos Finlay proposed that mosq-uitoes were responsible for disseminating the disease rather than direct human contact (8, 9). Due to the extremely high loss of lives to Yellow fever in the Spanish–American War in the 1890s, the United States Government dispatched a team of doctors in the Army led by Walter Reed to Cuba to investigate the cause of Yellow fever. The team successfully proved Finlay's ”mosquito hypothesis” for Yellow fever transmission and confirmed that *Aedes aegypti* mosquito was the primary vector for Yellow fever virus transmission to humans. In ground breaking virologic studies, the team demonstrated that the disease was caused by an agent that could be filtered from the blood of infected individuals (10). Yellow fever virus was the first virus shown to be transmitted by mosquitoes (11).

Structure of Yellow fever virus

The Yellow fever virus belongs to the family Flaviviridae and genus Flavivirus. The viral morphology is spherical, enveloped, with particle size ranging from 40-50 nm in diameter with icosahedral nucleocapsid symmetry and surface projections of 5 to 10 nm. Its nucleic acid is linear, positive-sense, single-stranded RNA and 11 kilo base long. Mature Yellow fever virions are icosahedral and comprise a nucleocapsid (made of capsid protein subunits) surrounded by a lipid bilayer derived from host membranes. The viral envelope is studded with dimers of envelope (E) glycoprotein and membrane (M) protein (Fig 2). The E glycoprotein is the major component of the virion surface and possesses most of the biologic activity, including cell-surface receptor binding, virion assembly and fusion activity at low pH, and immunogenicity (12). The virions are stable at slightly alkaline pH (pH 8.0) and low temperatures, but are readily inactivated at acidic pH (pH<4.0), temperatures of ≥ 56°C for 30 minutes, organic solvents, detergents, ultraviolet light, gamma radiation and various disinfectants.

Seven genotypes of the virus have been described; five in Africa and two in South America, and these include; (i) West Africa genotype I which has been associated with Yellow fever outbreaks in Nigeria, Cameroon and Gabon; (ii) West Africa genotype II has been associated with outbreaks in Senegal, Guinea, Ivory Coast and Ghana; (iii) East and Central African genotype is found in Sudan, Ethiopia, Democratic Republic of Congo and Central Africa Republic; (iv) East African genotype circulates in Uganda and Kenya; (v) Angola genotype is found in Angola; (vi) South American genotype I was isolated from Brazil, Bolivia, Colombia, Ecuador, Panama and Venezuela, and (vii) South American genotype II was isolated in Peru, Bolivia and Western Brazil (13).

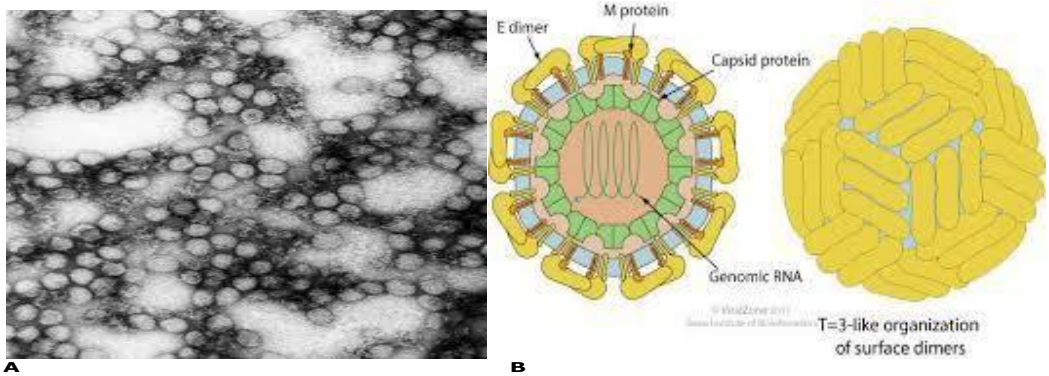

Fig 2: (A) Photomicrograph showing multiple Yellow fever virions; (B) The immature (intracellular) and mature (extracellular) infectious virion. *Source Public Health Image Library, Centers for Disease Control.

Mode of transmission

Yellow fever virus is mainly transmitted through the bite of the Yellow fever mosquito *Aedes aegypti*, but other *Aedes* mosquitoes such as the tiger mosquito *Aedes albopictus* can also serve as a vector for this virus. Like other arboviruses which are transmitted by mosquitoes, Yellow fever virus is taken up by a female mosquito when it ingests the blood of an infected human or another primate. The virus reaches the stomach of the mosquito, and if the concentration is high, the virions can infect epithelial cells and replicate within the cell, from where they reach the haemocoel (the blood system of mosquitoes) and then the salivary glands. Transovarial and transtadial transmissions of the virus within *A. aegypti* are indicated. This refers to the transmission from a female mosquito to her eggs and then larvae. This infection of vectors without a previous blood meal seems to play a role in single, sudden breakouts of the disease (14).

When next the infected mosquito sucks blood, it injects its saliva into the wound. An infected female mosquito inoculates approximately 1,000 to 100,000 virus particles intradermally during blood feeding. Virus replication begins at the site of inoculation (in dendritic cells of the epidermis) and spreads through lymphatic channels to regional lymph nodes and then the bloodstream.

Three epidemiologically distinct infectious cycles occur, in which the virus is transmitted from mosquitoes to humans or other primates, and this includes jungle or forest (sylvatic), intermediate (savannah), and urban cycle (Fig 3). The sylvatic cycle involves transmission of the virus between non-human primates (e.g. monkeys) and mosquito species such as *Aedes africanus* (in Africa) or mosquitoes of the genera *Haemagogus* and *Sabethes* (in South America) which serve as vectors found in the forest canopy. The virus is transmitted by mosquitoes from monkeys to humans when humans are visiting or working in the jungle. In Africa, an intermediate (savannah) cycle exists that involves transmission of virus from mosquitoes to humans living or working in jungle border areas. In this cycle, the virus can be transmitted from monkey to human or from human to human via mosquitoes of the genus *Aedes*. The urban cycle involves transmission of the virus between humans and urban mosquitoes, primarily *Aedes aegypti.* It is well adapted to urban areas, and can also transmit other viruses, including Zika, Dengue and Chikungunya. The virus is usually brought to the urban setting by a viraemic human who was infected in the jungle or savannah (15).

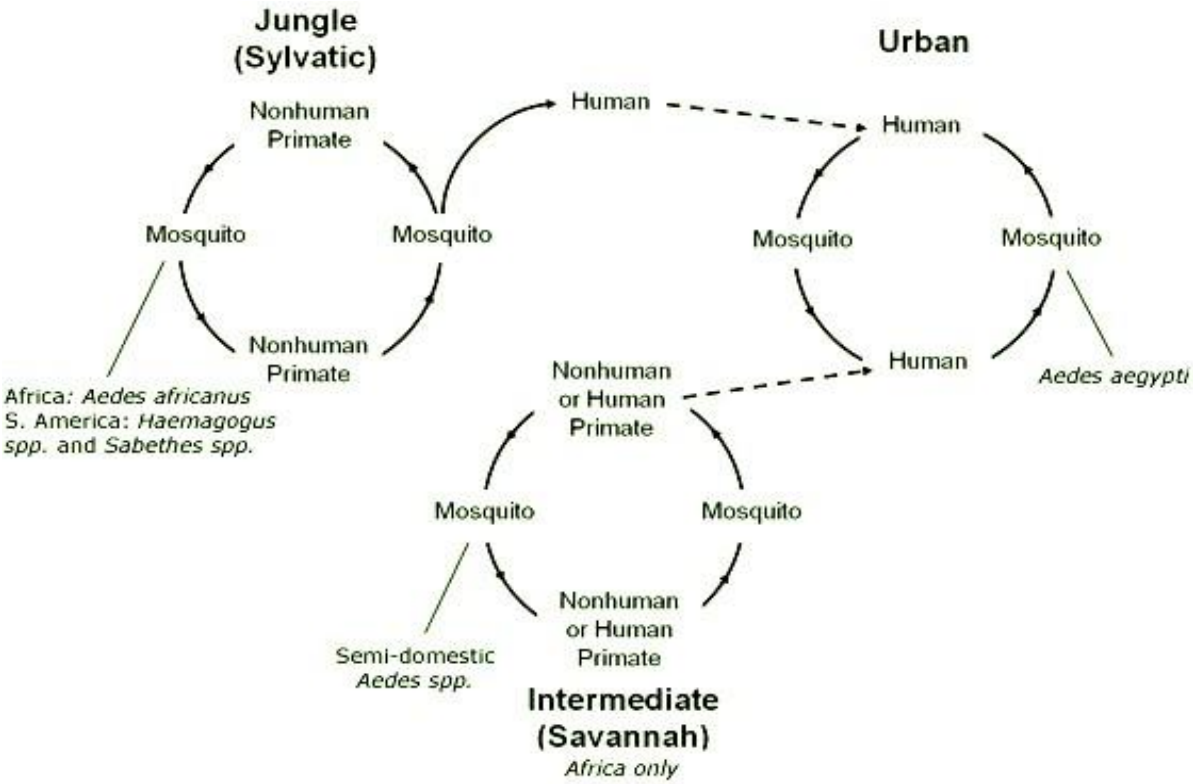

Fig 3: Transmission cycles of Yellow fever virus. *Source: CDC, 2019

Pathogenesis of Yellow fever:

Virus replication

The virus gains entrance through receptor-mediated endocytosis. A capsid protein facilitates viral binding while a membrane protein (E protein), which is a minor glycoprotein, initiates infection and mediates viral entry. The interaction between Yellow fever virus and cell is extremely complex, as the virus has adapted to exploit the host"s machinery for macromolecular synthesis for its own propagation and to antagonize or circumvent antiviral responses. The virus modulates pattern recognition receptors (PRR), stress granules, and membranous structures to promote crucial steps in its life cycle (16).

The Yellow fever virus like other extracellular flavivirus particles bind to target cells by interaction with cell-surface receptors which are yet to be identified, and are internalized by receptor-mediated endocytosis. A conformational rearrangement of the E glycoprotein occurs in the lower pH environment of the endosome, which facilitates fusion of the viral lipid envelope with the endosomal membrane and release of the nucleocapsid into the cell"s cytoplasm (17). After the nucleocapsid disassembles, replication proceeds with the immediate translation of the genome. The newly translated and processed viral non-structural (NS) proteins associate to form the replicase.

The non-structural protein 1 (NS1) may play a role in RNA replication, NS2A protein is involved in RNA replication and packaging, NS2B and NS3 form a complex and are involved in polyprotein processing and replication of RNA (18, 19). The replicase recognizes secondary structure in the 3′ terminus of the genomic RNA and the RNA-dependent RNA polymerase. NS5 initiates the synthesis of full-length negative-sense RNA copies from the genome template. These negative-sense RNAs are rapidly transcribed to produce progeny positive-sense RNA genomes.

Flavivirus replication occurs in association with host cell membranes (20). RNA synthesis occurs in the cytoplasm and protein synthesis takes place in the endoplasmic reticulum (ER). Infection causes dramatic proliferation of spherical invaginations known as vesicle packets (VP) in the perinuclear region of the ER, at least in part through activity of the NS4A protein (21, 22, 23). The localization of several viral NS proteins and dsRNA replicative intermediates in VPs suggests they are the site of viral replication (24).

Depending on the virus strain and cell type, the synthesis of Yellow fever virus RNA is detectable within three to six hours after infection, and progeny virions are released by about 12 hours. Immature, non infectious virions assemble within the ER, where viral RNA complexes with the C protein and is packaged into an ER-derived lipid bilayer containing heterodimers of the prM and E proteins, indicating that budding through the host cell membrane occurs intracellularly (25). Subcellular transport of immature flavivirus particles to the cell surface is thought to occur by the translocation of immature virion-containing vesicles from membranous components of the cell to the plasma membrane. Fusion of these vesicles with the plasma membrane then releases the vesicle contents including virions into the extracellular environment.

During assembly and transport of immature virions, the precursor to structural proteins (C, prM, and E) protects these proteins from undergoing irreversible conformational changes in acidic compartments of the secretory pathway. Virion maturation occurs in the transgolgi network by a delayed furin-mediated cleavage of the prM to M, triggering rearrangements in the E protein that promote infectivity (26). Infectious, mature virus particles are released by exocytosis into the extracellular medium. The mean time between being bitten by the infected mosquito and the onset of the first symptoms is 3-6 days but may reach 10-15 days (27, 28).

Pathological changes in organs

After invasion in the host, Kupfer cells (fixed liver macrophages) are infected within 24hours. The infection quickly disseminates to the kidneys, lymph nodes, spleen, and bone marrow. Renal failure occurs as renal tubules undergo fatty change and eosinophilic degeneration, likely due to direct viral effect, hypotension, and hepatic involvement. The liver is the most important organ affected in Yellow fever. Hepatocellular damage is characterized by lobular steatosis, necrosis, and apoptosis with subsequent formation of „Councilman bodies", which are degenerative eosinophilic hepatocytes. The kidneys also undergo significant pathologic changes. Central nervous system (CNS) findings can be attributed to cerebral edema and hemorrhages compounded on metabolic disturbances.

The bleeding diathesis of this disease is secondary to reduced hepatic synthesis of clotting factors, thrombocytopenia and platelet dysfunction. The terminal event of shock can be attributed to a combination of direct parenchymal damage and a systemic inflammatory response. Encephalopathy is also a common feature of Yellow fever (29,

30). Finally, circulatory shock develops secondary to cytokine storm, with evidence of increased levels of interleukin (IL)-6, IL-1 receptor antagonist, interferon-inducible protein-10, and tumor necrosis factor–alpha.

Clinical features of Yellow fever

The clinical manifestations of Yellow fever vary from an asymptomatic, abortive infection in which symptoms abate rapidly after the first phase to an invariably fatal, fulminating disease with symptoms following a biphasic course. Yellow fever shares clinical features with other viral haemorrhagic fevers (VHFs) such as Dengue haemorrhagic fever, Lassa fever, and Crimean-Congo haemorrhagic fever. The clinical manifestation of Yellow fever is biphasic with viraemic (mild to moderate) and toxaemic (severe/malignant) phases.

The viraemic phase is characterized by mild infection with fever, headache, chills, back pain, fatigue, loss of appetite, muscle pain, nausea, and vomiting. Mostly, these symptoms subside within 2 to 4 days, characterizing mild and moderate cases, which are estimated to account for 20-30% of infected patients.

The toxemic phase takes place in approximately 15% of patients and begins after a period of clinical improvement that follows the first phase, lasting 24 hours on average (25). It is characterized by recurdescence of high fever, chills, worsening of headache and myalgia, and involvement of various organs and systems. Hepatic induced coagulopathy produces severe haemorrhagic manifestations with petechiae, ecchymoses, epistaxis, and the characteristic "black vomit" (haematemesis), and gastrointestinal haemorrhage. Yellow fever is distinguished from other viral haemorrhagic fevers by the characteristic severity of liver damage and appearance of jaundice. Moreover, damage to the kidneys frequently leads to extreme albuminuria and acute renal failure.

Antibodies can be detected at this stage while viremia is usually absent. Cardiovascular dysfunction and neurological impairment with seizures usually occurs (3). Often, the unusual pairing of fever with bradycardia (Faget sign) is observed. Late CNS manifestations such as confusion, seizure and coma are also observed. Up to half of these patients progress to death in 10 to 14 days, and the rest recover without significant sequelae (31, 26).

Diagnosis of Yellow fever

Presumptive diagnosis is based on the patient's clinical features, vaccination status, and travel history, including destination, time of year, and activities. Clinical diagnosis requires that Complete blood count, urinalysis, liver function tests, coagulation tests, viral blood culture, and serologic tests should be obtained. Yellow fever is easily confused with Dengue, Lassa, Ebola, malaria, typhoid, hepatitis, and other diseases, as well as poisoning, hence these diseases should be excluded, and laboratory confirmation of Yellow fever should be done.

Laboratory diagnosis of Yellow fever is generally accomplished by molecular biology studies such as reverse transcription-polymerase chain reaction (RT-PCR), immuno histochemistry/histopathological analysis and serological tests (32, 33).

Treatment of Yellow fever

There is no specific antiviral drug available for the treatment of Yellow fever and there has not been any successful treatment of the disease with interferon-gamma, ribavirin and EICAR drugs (34). Studies (35,36) revealed that Yellow fever virus is susceptible to sofosbuvir both *invitro* and *invivo* suggesting that this drug may represent a novel therapeutic option for the treatment of Yellow fever.

However, severely ill patients are usually admitted to the intensive care unit and provided with vasoactive medications, fluid resuscitation, and ventilator support. In patients with moderate or severe clinical status, hospitalization with clinical and laboratory follow-up are crucial. In mild cases, outpatient care with daily visits is considered, with guidance on the risk of rapid aggravation. In this case it is necessary the prescription of hydration (60 mL/kg/day) and drugs dependent on hepatic metabolism should be avoided. Fresh frozen plasma and vitamin K have been administered to replenish clotting factors.

Prevention & control of Yellow fever

A safe, reasonably priced and extremely effective vaccine is available for the prevention of Yellow fever. Sustained immunity and life-long protection against Yellow fever disease is conferred adequately with a single dose of Yellow fever vaccine. The elimination of prospective mosquito breeding sites, application of larvicides to water storage containers and other places where standing water collects can reduce the threat of Yellow fever transmission in urban areas.

Both vector surveillance and control are components of the prevention and control of vector-borne diseases. Personal

protective measures such as clothing minimizing skin exposure and repellents are recommended to avoid mosquito bites. Health information related to the diseases should be made available to the entire populace.

Epidemiology of Yellow fever

Global

Vaccination has decreased worldwide epidemics of Yellow fever, but the infection has re-emerged in many parts of Africa and South America. No one is immune from Yellow fever, and it occurs in people of all ages and races. The highest mortality rates are reported in infants and elderly, who often have depressed immune systems. Most cases are diagnosed in unvaccinated travellers to sub-Saharan Africa or South America. Occasionally, travellers from endemic countries may carry the disease to uninfected regions. To avoid the importation of the disease, many countries require proof of Yellow fever vaccination prior to issuing a visa, especially if travellers are going to, or have visited, Yellow fever endemic areas (37).

Yellow fever is endemic in tropical and subtropical areas in Africa and South America (38). According to the World Health Organization, 47 countries in Africa and 13 countries in South America are endemic for Yellow fever (39, 40). The World Health Organization estimates from the early 1990s indicated that 200,000 cases of Yellow fever, with 30,000 deaths, were expected globally each year, with 90% occurring in Africa (41). Fig 4 shows areas with risk of Yellow fever virus transmission in Africa and hence the great need for vaccination. The recent situation of Yellow fever in some continents with major outbreaks is described below.

The Americas

Between January 2017 and December 2018, six countries and territories in the region of the Americas reported confirmed cases of Yellow fever; Bolivia, Brazil, Colombia, Ecuador, French Guiana, and Peru. The number of cases reported during this period in the region of the Americas exceeded the number reported in several decades. Yellow fever outbreak in the State of Minas Gerais, Brazil was reported in 2002–2003, when 63 confirmed cases, including 23 deaths (case fatality rate, 37%) were detected (42).

In December 2016, cases of Yellow fever were reported in Minas Gerais again and the outbreak extended to areas located in proximity of the State. As of mid-April 2017, a total of 2,422 cases (including 623 confirmed, 1128 discarded and 671 suspected cases under investigation) were reported (43). These included 326 deaths (209 confirmed, 53 discarded and 64 cases under investigation). The case fatality rate among confirmed cases was 34% (43). In 2019, Brazil and Peru reported confirmed cases that occurred between December 2018 and January 2019.

Fig 4: Areas with Risk of Yellow Fever Virus Transmission in Africa *Source: CDC, 2019

In Brazil, it is currently the period which has historically been recognized as having the highest transmission (seasonal period), which occurs between December and May. The expansion of the historical area of Yellow fever transmission to areas previously considered risk-free led to two waves of transmission, one during the 2016/2017 season, with 778 human cases including 262 deaths, and another during the 2017/2018 season, with 1,376 human cases, including 483 deaths (43). In the current season (2018/2019), 12 confirmed human cases, including 6 deaths, have been reported in the municipalities of Eldorado (9 cases), Jacupiranga (1 case), Iporanga (1 case), and Cananeia (1 case), all of which are located in the southern part of São Paulo State (33). Among these confirmed cases, 83% (10/12) are male, the median age is 45 years, and 83% (10/12) are rural workers.

Additionally, the Paraná Secretariat of Health reported that tests performed on dead monkeys in Antonina, on the Paraná coast, were positive for Yellow fever. Human cases reported during the current 2018/2019 season in four municipalities of São Paulo State, as well as the confirmation of epizootics in the State of Paraná, mark the beginning of what could be a third cycle and a progression of the outbreak towards the Southeast and South regions of the country (43).

Africa

An estimated 90% of Yellow fever cases occur on the African continent (44). In 2008, the largest number of recorded cases was in Togo. In late 2015 to December 2016, the largest outbreak of Yellow fever originated from Angola and spread to neighbouring countries (45). The outbreaks started in a crowded urban environment from Luanda and spread quickly to the rest of the country and beyond borders. Cases of Yellow fever in Angola had been exported to countries including Democratic Republic of Congo (DRC), Kenya and Mainland China (46). From early December 2015 to late July 2016, a total of 3818 cases were reported. Among these, 3294 cases had laboratory tests and 879 were confirmed. The majority of the confirmed cases were in males aged 15-19 years, followed by 20-24 years (47).

As of mid-June 2016, almost half of the country"s population of 10,641,209 people have been vaccinated. This response exhausted the global stockpile of Yellow fever vaccines several times (41). The last case in Angola was detected on 23 June 2016. In late December 2016, Angola declared the end of the Yellow fever outbreak (40). Overall, Angola reported a total of 4,306 cases and 376 deaths, of which 884 cases and 121 deaths were laboratory confirmed (47).

The DRC is located in a geographical area known to be Yellow fever endemic, and autochthonous cases are regularly reported. On 22 March 2016, the National International Health Regulations (IHR) Focal Point of the DRC notified WHO of cases of Yellow fever in connection with the outbreak occurring in Angola. The last case in DRC was detected on 12 July 2016. In mid-February 2017, DRC declared the end of the Yellow fever outbreak (47). A total of 2987 cases of were reported from all 26 provinces of DRC during the outbreak, of which 81 cases were laboratory confirmed, with 16 deaths (case fatality rate among confirmed cases 20%). Most of the confirmed cases acquired the infection from Angola (48).

Yellow fever in Nigeria

Historical perspectives

The earliest outbreak of Yellow fever in Nigeria was reported in Lagos in 1864, with subsequent regular outbreaks. Two epidemics occurred in Ogbomosho in 1946 and in Eastern Nigeria in 1951-1953 (49). The 1969 Yellow fever outbreak in Jos, Plateau State was the first recognized occurrence of epidemic Yellow fever in Nigeria in 17 years at that period. The first laboratory confirmation of Yellow fever was in a patient whose illness began on 7th September 1969.

The Virus Research Laboratory of the University of Ibadan, Nigeria, was informed that suspected cases of Yellow fever had occurred in Jos on 23 October 1969. The diagnosis was confirmed by virus isolation and the existence of a widespread outbreak on the Jos Plateau and adjacent areas was established. This was the first recognized epidemic of Yellow fever in Nigeria since 1953 (50). Approximately 252 patients with Yellow fever were hospitalized between September and December, 1969. The case fatality rate for hospitalized patients was approximately 40%. It was estimated that up to 100,000 cases of Yellow fever may have occurred during the epidemic (50).

Current situation in Nigeria

For a space of 21 years, no further confirmed cases of Yellow fever was reported until September 2017 when Yellow fever was established in a seven-year old child in Ifelodun Local Government Area of Kwara State. Since then, Nigeria has been experiencing periodic outbreaks of the disease. On 22 November 2018, a cluster of suspected Yellow fever cases and deaths in

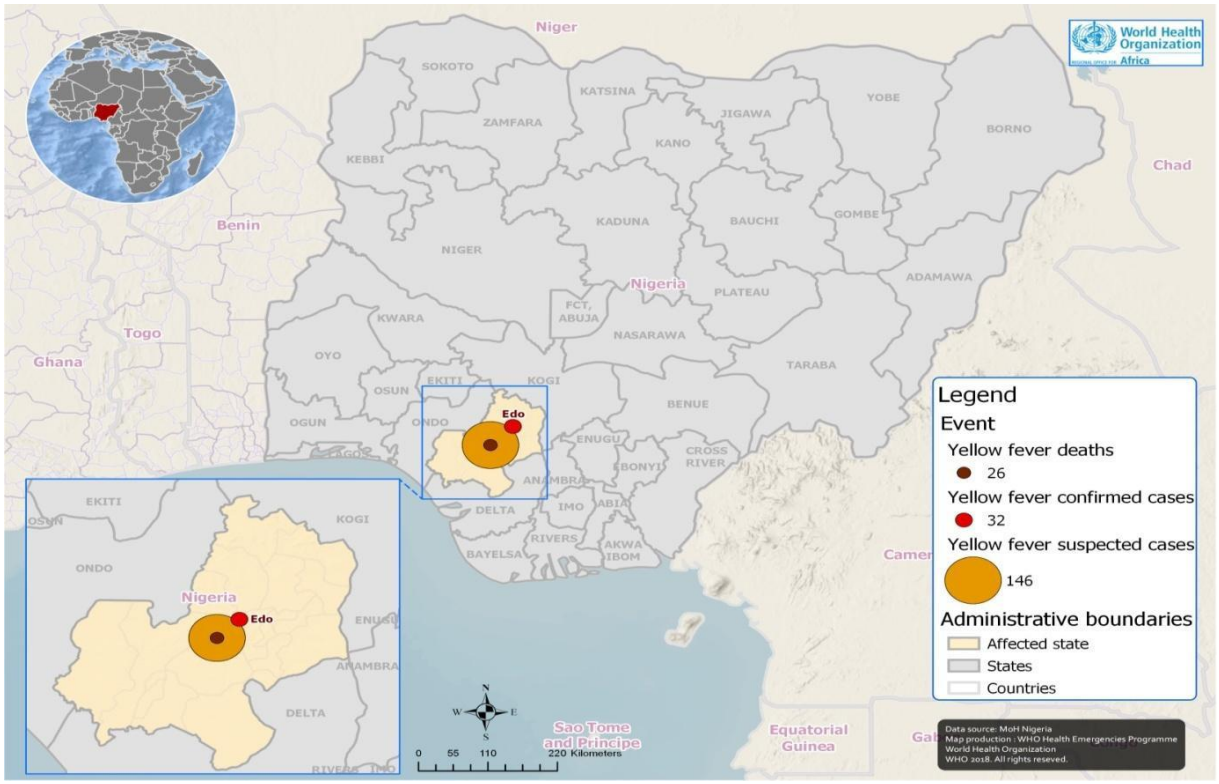

Fig 5: Confirmed and suspected Yellow fever cases in Edo State outbreak, Nigeria, data as of 31 December 2018 *Source; WHO, 2019

Edo State, Nigeria was reported to the World Health Organization. Edo State is also a known endemic area for Lassa fever which was initially suspected as causing the outbreak. From 22 September through 31 December 2018, a total of 146 suspected, 42 presumptive positive, and 32 confirmed cases, including 26 deaths, have been reported across 15 of 18 Local Government Areas of the State (51) (Fig 5). The most affected age group was 10–19 years with 48 cases (33%), followed by the age group 20–29 with 36 cases (25%). Primarily, the affected LGAs were in the rural areas however, suspected cases were reported from three urban LGAs at the end of November 2018. There was also a report of two presumptive positive and one confirmed case in Oredo LGA, which included the densely populated State capital, Benin City, of about 1.5 million inhabitants.

From 2017 to 2018, 163 cases were confirmed in 17 States; Kwara (8), Kogi (12), Kano (1), Zamfara (19), Kebbi (7), Nasarawa (3), Niger (1), Katsina (2), Edo (90), Ekiti (2), Rivers (1), Anambra (1), FCT (11), Benue (1), Delta (1), Ondo (2) and Abia (1). From September 2017 to December 2018, 283 samples (presumptive positive, 246 and inconclusive, 37) were sent to Institute Pasteur (IP) Dakar for reconfirmation. All the States in Nigeria (including the Federal Capital Territory) reported 4,132 suspected cases in 616 LGAs. Of all suspected, probable and confirmed cases, 90 deaths were recorded with 31 deaths among confirmed cases. The case fatality rate for all cases (suspected, probable and confirmed) was 2.2% and 19.0% for confirmed cases. Fig 6 shows the map of Nigeria revealing States with suspected, probable and confirmed cases of Yellow fever as at December 2018 (51).

In May 2019, 332 suspected cases of Yellow fever were reported, with 1 presumptive positive and 1 inconclusive case. The Nigerian Center for Disease and Control (NCDC) Central Public Health Laboratory (CPHL) reported 1 presumptive positive case from Akoko South East LGA in Ondo State, and 1 inconclusive case from Gokona in Rivers State. Three new confirmed cases from IP Dakar were received from Onitsha North LGA in Anambra State, 1 from Arewa Danladi LGA in Kebbi State and 1 from Kware LGA in Sokoto State. These were the last IP Dakar confirmed cases received on 24 May 2019. Sokoto is the latest State to report a confirmed case since the onset of the outbreak in 2017 (51).

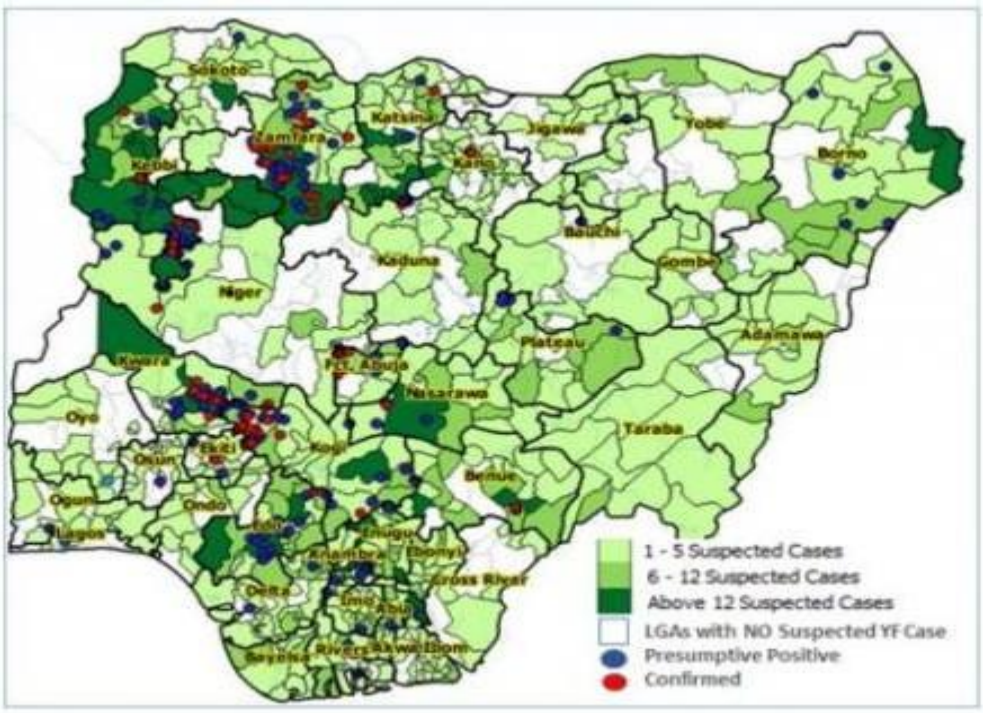

Fig 6: Map of Nigeria showing States with suspected, probable and confirmed cases of yellow fever as at 30th December, 2018. Source* NCDC, 2019

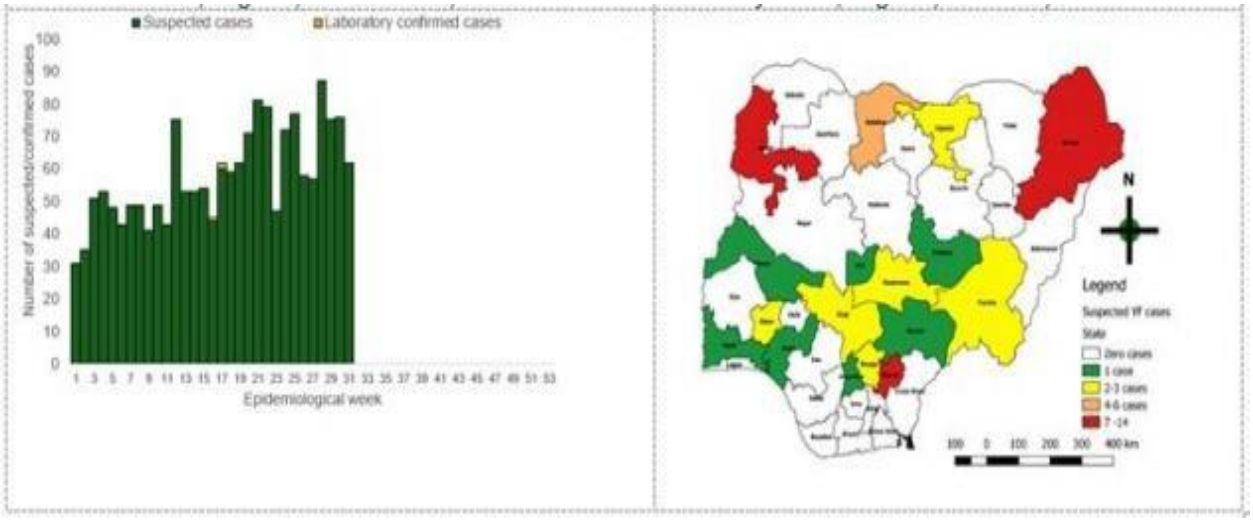

Fig 7: Number of suspected and confirmed cases of Yellow fever, week 1-31, 2019 (left) and Map of Nigeria showing the location of suspected cases by State, week 31, 2019 (right). Source* NCDC, 2019

Between 1 January and 31 May 2019, 1,255 suspected cases were reported in 424 LGAs (Fig 7). Laboratories in Nigeria have recorded 37 presumptive positive and 12 inconclusive cases from 12 States in 38 LGAs. From the 37 presumptive and 12 inconclusive samples sent to Dakar, 15 were confirmed and distributed as follows; Edo (7 and 1 inconclusive), Ondo (2), Imo (1), Osun (1), Anambra (1), Kebbe (1) and Sokoto (1). One death from suspected Yellow fever case has been recorded from Adamawa State with a case fatality Rate of 0.08% (51).

In Izzi Local Government Area of Ebonyi State, new cases of infected persons and deaths from fever of unknown origin prompted the State Public Health team to commence investigations. As at 31 July 2019, 3 cases had tested positive for Yellow fever at NCDC"s National Reference Laboratory, which triggered an immediate response. The Ebonyi State Epidemiology

Team is currently guiding the response team and is in collaboration with the NCDC, National Primary Health Care Development Agency (NPHCDA) and the World Health Organisation. Between May 1 and August 7, 2019, Yellow fever cases and 20 deaths were discovered in the study area which was an indication that disease transmission was ongoing and concealed to health authorities in Izzi Local Government Area. However, on 10 August 2019, the NCDC stated in a press release that the agency is aware of a suspected outbreak of Yellow fever in Ebonyi State and has had a Rapid Response Team in partnership with World Health Organization, supporting Ebonyi State"s response since 30 July 2019 (52).

Nigeria Public Health response

A comprehensive and long term strategy to eliminate Yellow fever epidemics globally is built on lessons learned, with the intention of "Eliminating Yellow fever Epidemics" (EYE) by 2026, and its three strategic objectives include; (i) protection of populations at risk; (ii) prevention of intern-ational spread, and (iii) rapid containment of outbreaks.

The EYE Strategy places Nigeria as a priority country and over 60 million people are expected to be protected against Yellow fever in the country by the end of 2021. During the 67th Session of the World Health Organization Regional Committee meeting, Health Ministers of African countries including Nigeria agreed on 10 priority actions to guide countries on the eradication of the Yellow fever epidemics by 2026.

The Government of Nigeria launched the Yellow fever reactive immunization campaign with support from the World Health Organization and other collaborators, funded by GAVI, the Vaccine Alliance, in December 2018. The campaign was implemented in 13 LGAs of Edo State, where cases have been confirmed. Due to the incomparable Yellow fever outbreak recently identified in the State, the immunization drive force was enacted. National preventive and reactive mass vaccination (~33 million doses) campaigns have been conducted in the country since 2017. A proposal has been submitted to GAVI, the Vaccine Alliance, to vaccinate (~23 million doses) people in 12 States over the next 3 years (51).

The International Coordination Group (ICG) on Vaccine Provision, funded by GAVI, the Vaccine Alliance, approved release of 3.1 million doses of Yellow fever vaccines. In the same vein, the World Health Organization pledged to donate 12 million doses of Yellow fever vaccines in 2018, and 19 million in 2019 to support Nigeria"s campaign against the disease. A rapid response team under the leadership of National Primary Health Care Development Agency (NPHCDA) and Nigeria Centre for Disease Control, composed of National and State Health authorities, and other WHO collaborators, was set up to examine the cases, fortify efforts to reduce virus circulation, and sensitize health workers and communities on methods of preventing Yellow fever.

To ensure that Yellow fever is eradicated from Nigeria, the following measures are recommended; (i) vaccine coverage of over 80% with a 60-80% security threshold, are necessary to interrupt local transmission (human-mosquito-human) of Yellow fever virus within a community and to ensure that sporadic unvaccinated cases do not generate additional cases hence, any person 9 months and older should be vaccinated against Yellow fever. Travellers going to Yellow fever endemic countries should receive vaccination against the virus at least 10 days before travel and should take steps to prevent mosquito bites while in the country; (ii) there should be a strong disease surveillance system and diagnostic capacities that will allow for early detection of outbreaks and rapid implementation of control measures that can help mitigate the risk of spread and the use of extensive resources; and (iii) vector surveillance that identifies prevalence of *Aedes aegypti* and other *Aedes stegomyia* species should be greatly enhances as this will provide information on the risk of an urban outbreak in the country.

Conclusion:

Yellow fever is often represented as a neglected disease, yet it still remains a considerable threat to human health and economy, as exhibited by the outbreaks of 2016-2018 in areas with historically low or no Yellow fever activity. Nigeria is one of 50 global partners battling to eliminate Yellow fever within the next 10 years. However, the current report of positive cases and mortality recorded in the year 2019 reveals ongoing transmission in the country. Hence, a holistic approach by relevant stakeholders and the entire populace is required to eliminate Yellow fever in Nigeria.

References:

1. Garske, T., Van Kerkhove, M. D., Yactayo, S., et al. Yellow fever in Africa: estimating the burden of disease and impact of mass vaccination from outbreak and serological data. PLoS Med. 2014; 6; 11 (5): e1001638.
2. World Health Organization. "Yellow fever Fact sheet N°100". 2013a.

3. Center for Disease Control. Yellow fever. Atlanta, GA: US Department of Health and Human Services, Center for Disease Control 2018. https://www.cdc.gov/yellowfever/symptoms/ind ex.html
4. Pan American Health Organization. Yellow Fever – Epidemiological Update. Domingo, C., Charrel, R. N., Schmidt-Chanasit, J., Zeller, H., Reusken, C. Yellow fever in the diagnostics laboratory. Emerg Microbes Infect. 2018; 7 (1): 129.
5. Gould, E. A., de Lamballerie, X., Zanotto, P. M., et al. "Origins, evolution, and vector/host co-adaptations within the genus Flavivirus". Adv Virus Res. 2003; 59: 277–314
6. Bryant, J. E., Holmes, E. C., and Barrett, A. D. "Out of Africa: A Molecular Perspective on the Introduction of Yellow Fever Virus into the Americas". PLoS Pathog. 2007; 3 (5): e75.
7. Haddow, E. "The natural history of Yellow fever in Africa". Proceedings of the Royal Society of Edinburgh B. 2012; 191–227.
8. Charles, F. "The mosquito hypothetically considered as an agent in the transmission of Yellow fever poison". New Orleans Med and Sur J. 1881; 601–616.
9. Chaves-Carballo, E. "Carlos Finlay and yellow fever: triumph over adversity". Mil Med. 2005; 881–885.
10. Staples, J. E., and Monath, T. P. Yellow fever: 100 years of discovery. JAMA. 2008; 9: 60–962
11. Barrett, A. D., and Higgs, S. "Yellow fever: a disease that has yet to be conquered". Annu Rev Entomol. 2007; 52: 209–29.
12. Burke, D. S., and Monath, T. P. Flaviviruses. In: Knipe D. M., and Howley, P. M (eds). Fields Virology. Lippincott, Williams & Wilkins; Philadelphia: 2001: 1025–1043.
13. Nina, K. S., Hewad, L., Ousmane, F., et al. Biological and Phylogenetic Characteristics of Yellow Fever Virus Lineages from West Africa. J Virol. 2013; 2895–2907
14. Fontenille, D., Diallo, M., Mondo, M., et al. "First evidence of natural vertical transmission of Yellow fever virus in *Aedes aegypti,* its epidemic vector". Trans Roy Soc Trop Med and Hyg. 1997; 533–535
15. Center for Disease Control and Prevention. National Centre for Emerging and Zoonotic Infectious Diseases (NCEZID), Division of Vector-Borne Diseases (DVBD), 2019
16. Fernandez-Garcia, M. D., Mazzon, M., Jacobs, M., et al. Pathogenesis of flavivirus infections: using and abusing the host cell. Cell Host Microbe. 2009; 318–328
17. Bressanelli, S., Stiasny, K., and Allison, S. L. Structure of a flavivirus envelope glycoprotein in its low pH induced membrane fusion conformation. EMBO J. 2004; 728–738
18. Chung, K.M., Liszewski, M. K., Nybakken, G. West Nile virus non-structural protein NS1 inhibits complement activation by binding the regulatory protein factor H. Proc Natl Acad Sci USA. 2006; 103 (50): 1911–1916.
19. Issur, M., Geiss, B. J., and Bougie, I. The flavivirus NS5 protein is a true RNA guanylyltransferase that catalyzes a two-step reaction to form the RNA cap structure. RNA. 2009; 15 (12): 2340–2350.
20. Miller, S., and Krijnse-Locker, J. Modification of intracellular membrane structures for virus replication. Nat Rev Microbiol. 2008; 6 (5): 363–374
21. Mackenzie, J. M., Jones, M. K., and Westaway, E. G. Markers for trans-Golgi membranes and the intermediate compartment localize to induced membranes with distinct replication functions in flavivirus-infected cells. J Virol. 1999; 73: 9555–9567
22. Roosendaal, J., Westaway, E. G., and Khromykh, A. Regulated cleavages at the West Nile virus NS4A-2K-NS4B junctions play a major role in rearranging cytoplasmic membranes and Golgi trafficking of the NS4A protein. J Virol. 2006; 80 (9):4623–4632.
23. Welsch, S., Miller, S., Romero-Brey, I., et al. Composition and three-dimensional architecture of the dengue virus replication and assembly sites. Cell Host Microbe. 2009; 5 (4): 365–375.
24. Mackenzie, J. M., Kenney, M. T., and Westaway, E. G. West Nile virus strain Kunjin NS5 polymerase is a phosphoprotein localized at the cytoplasmic site of viral RNA synthesis. J Gen Virol. 2007; 88: 1163–1168.
25. Lorenz, I. C., Kartenbeck, J., Mezzacasa, A., et al. Intracellular assembly and secretion of recombinant subviral particles from tick-borne encephalitis virus. J Virol. 2003; 77 (7): 4370–4382.
26. Li, L., Lok, S. M., Yu, I. M., et al. The flavivirus precursor membrane-envelope protein complex: structure and maturation. Science. 2008; 319 (5871): 1830–1844.
27. Paules, C. I., and Fauci, A. S. Yellow fever - Once again on the radar screen in the Americas. N Engl J Med. 2017; 376 (15): 1397-1399.
28. World Health Organization. Yellow fever. http://www.who.int/csr/disease/yellowfev/en/ 2018.
29. Barrett, A. D. T. The re-emergence of Yellow fever. Science. 2018; 361:847-848
30. Faria, N. R., Kraemer, M. U. G., Hill, S. C., et al. Genomic and epidemiological monitoring of Yellow fever virus transmission potential. Science. 2018; 361: 894-899
31. Monath, T. P. Yellow fever: an update. Lancet Infect Dis. 2001; 1: 11-20
32. Domingo, C., Charrel, R. N., Schmidt-Chanasit, J., et al. Yellow fever in the diagnostics laboratory. Emerg Microbes Infect. 2018; 7 (1): 129.
33. Figueiredo, P. O., Silva, A. T. S., Oliveira, J. S., et al. Detection and molecular characterization of Yellow fever virus, 2017, Brazil. EcoHealth. 2018; 15 (4): 864-870.
34. Possas, C., Lourenço-de-Oliveira, R., Tauil, P. L. et al. Yellow fever outbreak in Brazil: the puzzle of rapid viral spread and challenges for immunization. Mem Inst Oswaldo Cruz. 2018; 10: e180278.
35. Freire, F. D., Gomes, C. H. M., Cardoso, P. S., et al. Yellow fever: An old disease, but with new challenges. Conexão Ci. 2018; 13: 79-86.
36. De Freitas, C. S., Higa, L. M., Sacramento, C. Q., et al. Yellow fever virus is susceptible to sofosbuvir both *in vitro* and *in vivo*. PLoS Negl Trop Dis. 2019; 13 (1): e0007072.
37. Tolle, M. A."Mosquito-borne diseases". Curr Probl Pediatr Adolesc Health Care. 2009; 39: 97–140
38. Woodall, J. P., and Yuill, T. M. "Why is the Yellow fever outbreak in Angola a 'threat to the entire world'?". Int J Infect Dis. 2016; 48: 96–97.
39. World Health Organization. Eliminate Yellow fever Epidemics (EYE): a global strategy, 2017-2026. Wkly Epidemiol Rec. 2017; 92 (16): 193-204.
40. World Health Organizations. Vaccines and vaccination against Yellow fever. WHO position paper. Wkly Epidemiol Rec. 2013; 88: 269-284
41. World Health Organization. Disease outbreak news: Yellow fever – Brazil 2017. http://www.who.int/csr/don/13-january-2017-yellow-fever-brazil/en/.
42. Pan American Health Organization / World Health Organization. Epidemiological Update: Yellow Fever. 25 January 2019, Washington, D.C.: PAHO/WHO, 2019.
43. Chippaux, J.P., and Chippaux, A. Yellow fever in Africa and the Americas: a historical and epidemiological perspective. J Venom Anim Toxins Incl Trop Dis. 2018; 24:20.
44. World Health Organization. Yellow fever Fact sheet. 2016a.

http://www.who.int/mediacentre/factsheets/fs100/en/. Accessed 21 Apr 2017
45. Jentes, E. S., Poumerol, G., and Gershman, M. D. The revised global yellow fever risk map and recommendations for vaccination, 2010: consensus of the Informal WHO Working Group on Geographic Risk for Yellow Fever. Lancet Infect Dis. 2011; 11: 622-632.
46. World Health Organization. The yellow fever outbreak in Angola and Democratic Republic of the Congo ends 2017. http://www.afro.who.int/en/media-centre/pressreleases/item/9377-the-yellow-fever-outbreak-in-angola-and-democratic-republic-of-the-congo-ends.html. Accessed 24 Apr 2017
47. World Health Organization. Yellow fever situation report - Angola 2016 http://www.who.int/emergencies/yellow-fever/situation-reports/29-july-2016/en/. Accessed 21 Apr 2017
48. MacNamara, F. N. World Health Organization Bulletin. 1954; 11: 391401.
49. Carey, D. E., Kemp, G. E., Troup, J. M., et al. Epidemiological aspects of the 1969 Yellow fever epidemic in Nigeria. Bull World Health Organ. 1972; 46 (5): 645–651.
50. World Health Organization. Emergencies preparedness response. Yellow fever – Nigeria, 2019. Disease outbreak news.
51. Nigeria Centre for Disease Control. Yellow Fever Monthly Situation Report in Nigeria. May, 2019 Report. www.ncdc.gov.ng
52. Nigeria Centre for Disease Control. The NCDC is Aware of a Suspected Outbreak of Yellow Fever in Ebonyi State. 2019. www.ncdc.gov.ng

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Original Article **Open Access**

Fungal neonatal and infantile sepsis in Egypt: risk factors and identification of fungal isolates

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Abstract:

Background: Invasive fungal diseases (IFDs) are opportunistic infections associated with significant mortality in paediatric patients, especially in those with compromised immune system and neonates with very low birth weight (VLBW). The objectives of this study are to determine the prevalence, clinical features and fungi isolates of neonatal sepsis in three hospitals in Egypt.
Methodology: The study is a cross sectional survey of 176 neonates with clinical sepsis admitted to the neonatal intensive care units (NICU) of the three hospitals over a period of one year (February 2015 to January 2016). A minimum of two blood samples (collected within 24 hours) from each neonate were cultured for bacteria in automated BacT/AlerT and conventional culture bottles, while Saboraud-Brain Heart Infusion broth was inoculated for fungi culture. Positive growths from the broth were sub-cultured on Sabouraud Dextrose Agar (SDA) plates for aerobic incubation at 25°C and 37°C for 2 weeks. Identification of fungi colonies on SDA was by conventional morphology and confirmation on chromogenic agar media. Phylogenetic analysis of representative fungi isolates was done by partial nucleotide sequencing of D1-D2 domain of the large subunit rRNA gene.
Results: Of the 176 neonates, blood culture was positive for pathogens in 55 (31.3 %) samples and fungi were isolated in 26 (14.8 %); yeast (25) and mould (1). The commonly isolated yeasts were *Candida albicans, Candida tropicalis,* and *Candida krusei* representing 34.6%, 30.8% and 23.1%, respectively of the total fungi isolated. The phylogenetic analysis in comparison to Genbank data showed defined clades for *Candida tropicalis*, *Candida parapsilosis*, *Candida albicans* and *Pichia kudriavzevii*
Conclusion: This current study highlights the changing pattern of neonatal infections in Egypt caused by *Candida*, with increasing incidence of infections caused by non-*albicans Candida* species.

Key words: fungal infection, neonatal, risk factors, PCR, yeast

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Infection fongique néonatale et infantile en Égypte: facteurs de risque et identification des isolats fongiques

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Abstrait:

Contexte: Les maladies fongiques invasives (IFD) sont des infections opportunistes associées à une mortalité significative chez les patients pédiatriques, en particulier ceux dont le système immunitaire est compromis et les nouveau-nés de très faible poids à la naissance (VLBW). Les objectifs de cette étude sont de déterminer la

prévalence, les caractéristiques cliniques et les isolements fongiques de la sepsie néonatale dans trois hôpitaux en Égypte.
Méthodologie: L'étude est une enquête transversale menée auprès de 176 nouveau-nés présentant une septicémie clinique et admis dans les unités de soins intensifs néonatals des trois hôpitaux sur une période d'un an (de février 2015 à janvier 2016). Un minimum de deux échantillons de sang (recueillis dans les 24 heures) de chaque nouveau-né ont été cultivés pour la bactérie dans des flacons de culture automatisés BacT/AlerT et conventionnels, tandis que le bouillon Saboraud-Brain Heart Infusion a été inoculé pour la culture de champignons. Les croissances positives du bouillon ont été sous-cultivées sur des plaques de gélose Sabouraud Dextrose Agar (SDA) pour une incubation aérobie à 25°C et à 37°C pendant 2 semaines. L'identification des colonies de champignons sur la SDA a été réalisée par la morphologie conventionnelle et confirmée sur un milieu chromogène en gélose. L'analyse phylogénétique d'isolats de champignons représentatifs a été réalisée par séquençage partiel de nucléotides du domaine D1-D2 du gène de l'ARNr de grande sous-unité.
Résultats: Sur les 176 nouveau-nés, la culture de sang était positive pour les agents pathogènes dans 55 échantillons (31,3%) et les champignons ont été isolés dans 26 (14,8%); levure (25) et moisissure (1). Les levures communément isolées étaient *Candida albicans*, *Candida tropicalis* et *Candida krusei*, représentant respectivement 34,6%, 30,8% et 23,1% du total des champignons isolés. L'analyse phylogénétique comparée aux données de Genbank a montré des clades définis pour *Candida tropicalis*, *Candida parapsilosis*, *Candida albicans* et *Pichia kudriavzevii*
Conclusion: La présente étude met en évidence l'évolution du schéma des infections néonatales causées par *Candida* en Égypte, avec une incidence croissante des infections causées par des espèces de *Candida* non albicans.

Mots-clés: infection fongique, néonatale, facteurs de risque, PCR, levure

Introduction:

Generally, neonatal infections continue to be an important cause of morbidity and mortality worldwide. This is due to lack of adequate preventive and therapeutic strategies in low income settings and increased survival of preterm and low weight newborns with lengthy stays in intensive care units (NICUs) in high income countries (1)**.**

Neonatal sepsis refers to infection in newborn less than 28 days old with presence of microorganisms (bacteria or fungi) in the blood stream in the setting of fever. Preterm babies are more commonly affected than term babies (2). Two types of neonatal sepsis can be defined according to time and mode of infection; early onset sepsis (EOS) results from maternal intrapartum transmission of invasive organisms and can be diagnosed when there is positive microbial cultures during the first 7 days of life or during the first 72 hours of life in case of very low birth weight (VLBW) infants (3). Late-onset sepsis (LOS) referred to infection in blood and cerebrospinal fluid occurring after 7 days of delivery, which occurs from postnatal acquisition (nosocomial or community sources) of pathogens (4)**.**

It has been reported that the incidence of sepsis during the neonatal period is higher than any other period of life and varies from one place to another (5), and from nursery to nursery depending on predisposing factors to infections (6, 7). Several predisposing factors for fungal infections have been identified, some of which are related to host factor and others to prolonged hospitalization. Innate characteristics of preterm infants that predispose them to invasive fungal infections include relative immunodeficiency resulting from decreased number of T-cells and neutrophils, immature skin structure, disruption of cutaneous barriers, prolonged use of antimicrobials, indwelling central venous catheters, hyper alimentation, incubation, steroids and other uncommon risk factors (8).

Candida species are important hospital acquired pathogens in infants admitted to the neonatal intensive care units (NICU). In VLBW infants (birth weight less than 1500g), *Candida albicans* is the third most common cause of neonatal LOS, which occurs after the first 72 hours of life (9). *Candida albicans* represents 60% of neonatal fungi infections (10, 11) but non–*albicans Candida* species are also associated with neonatal infections and includes *Candida tropicalis, Candida parapsilosis, Candida krusi* and *Candida glabrata,* which are considered emerging pathogens (12).

The symptoms and signs of neonatal sepsis are subtle and overlap with other life threatening infectious diseases, thereby making early diagnosis difficult (13, 14). Blood culture remains the gold standard for definitive diagnosis of neonatal sepsis (15). Mortality from invasive candidiasis (IC) is influenced by variables such as drugs, timing, and duration of treatment. Prompt treatment and appropriate dosing for neonatal IC are important strategies to halt progression of disease. The objectives of this study are to determine the prevalence of fungal neonatal sepsis, characterize the aetiological agents and compare the pattern with that of our previous study conducted over a decade ago.

Materials and method:

Study design and setting

This was a cross sectional study conducted in neonatal intensive care units (NICUs) of three Egyptian hospitals; Al-Azhar University Hospital, Ain-Shams University Hospital and Wadi El-Neel Hospital, between February 2015 and January 2016.

Subjects

A total of 176 neonates and infants less than 6 months of age with clinical sepsis admitted to the hospitals during the period of study were recruited for the study. Full clinical history and physical examination were conducted on each subject and data such as gestational age, birth weight, mode of delivery, and risk factors for sepsis were collected into a design form.

Specimen culture and microbial isolation

A minimum of two blood samples (collected within 24 hours) from each neonate were inoculated into BacT/Alert bottles for incubation in automated bacteria BacT/Alert blood culture machine (BioMérieux France) or into Thioglycollate broths for conventional aerobic and anaerobic bacteria incubation for 14 days. For fungi isolation, blood samples were inoculated into Saboraud-Brain Heart Infusion broth and positive growth sub-cultured on Saboraud Dextrose Agar (SDA) plates for aerobic incubation at 25°C and 37°C for two weeks.

Biochemical identification of fungi isolates

For mould, identification was carried out on the basis of macroscopic and microscopic morphology using the universal mycology manual (16). Identification of yeast species was carried out according to macroscopic characteristics on Wickerham agar medium, color production on chromogenic agar media (Sigma Chemicals, USA), germ tube test, chlamydospores formation, and carbohydrates fermentation and assimilation (17).

Partial sequencing and phylogenetic analysis of fungi isolates

DNA was extracted using the protocol of GeneJET™ genomic DNA purification kit (Thermo #k0721). PCR was carried out using Maxima Hot Start PCR Master Mix (Thermo, #k1061) with amplification of the divergent domain at the 5′ end of the large subunit (LSU) rRNA gene using primers; NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3'). The PCR conditions set were 35 cycles of initial denaturation at 94°C for 3 min followed by denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and extension at 74°C for 1.5 min, with a final extension at 74°C for 5 min. The PCR product was purified using GeneJET™ PCR purification Kit (Thermo, k0701), and stored at -20°C prior to sequencing.

The PCR products of five representative isolates were sequenced on GATC Company using ABI 3730xl DNA sequencer with the forward and reverse primers that target the D1-D2 domain of the large subunit rRNA gene. The forward and reverse DNA sequence reads for each isolate were assembled using codon code aligner software and submitted to Genbank with assigned accession numbers.

A phylogram was constructed for the 5 clinical isolates and their closely related strains according to Genbank data based on nucleotide divergence in the D1-D2 domain. The evolutionary history was inferred using the Neighbor-Joining method (18), and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) was shown next to the branches (19). The tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method (20) and were in the units of the number of base differences per sequence. The analysis involved 56 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 594 positions in the final dataset. Evolutionary analyses were conducted in MEGA X software (21).

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 20.0. Quantitative data were expressed as mean ± standard deviation (SD) while qualitative data were expressed as frequency and percentages. The Chi- square (X^2) test was used to compare proportions between two qualitative para- meters. The confidence interval was set to 95% and the accepted margin of error was set to 5%. The significance of probability (p value) was evaluated as follow; $p<0.05$ was considered significant, $p<0.001$ was consi- dered highly significant, and $p>0.05$ was considered not significant.

Ethical issues

The ethical committees of the participating hospitals approved the study. All patients' information and test results were kept confidential.

Results:

A total of 176 neonates and infants less than 6 months old with clinical sepsis were enrolled, 97 males and 79 females (M: F ratio of 1.2). The age range of subjects is 1-210 days with mean age of 39.7 days (Table 1). Blood culture was positive for pathogens in 55 subjects (31.3%) and for fungi in 26 (14.7%), with 21.5% in females, which was significantly higher than 9.3% in males (p=0.0391).

Twenty one (18.4%) of the 114 early onset cases had fungi isolated compared to 5 of 62 (8.1%) late onset cases but there was no significant difference between the two cases (p=0.1037). Twenty three of 136 (16.9%) LBW babies had fungi sepsis compared to 3 of 40 (7.5%) NBW babies but the difference was not significant (p=0.220). Nine of 87 (10.3%) babies who had normal vaginal birth had fungi sepsis while 17 of 89 (19.1%) babies with cesarean section (CS) birth had fungi sepsis but no significant difference was observed (p=0.1543).

The frequency of isolation of the fungi species is shown in Table 2 with 24 isolates being *Candida* species, one isolate was *Pichia* (yeast) and one isolate was a mould (*Penicillium*). Out of the 24 *Candida* species, non-*albicans* species accounted for 15 (62.5%). However the most frequent species in descending order were *C. albicans* (n=9, 34.6%), *C. tropicalis* (n=8, 30.8%) and *C. krusei* (n=6, 23.1%).

The Phylogenetic tree (Fig 1) shows selected 5 pathogenic yeast isolates and their closely related strains according to Genbank data. The phylogenetic tree showed defined clades for *C. tropicalis* (isolate 3, MK686027 and isolate 5, MK686029), *C. parapsilosis* (isolate 4, MK686028), *C. albicans (*isolate 2, MK686026), and *P. kudriavzevii* (isolate 1, MK686025).

Table 1: Demographic data and of risk factors for fungi sepsis among neonates and infants in Egypt

Demographic data and risk factors	No of subjects (%) (n=176)	No of subjects with fungi isolates (%) (n=26)	X^2	95% CI	*p* value
Gender					
Male	97(55.1)	9(9.3)	4.255	0.2034-0.9142	0.0391*
Female	79(44.9)	17 (21.5)			
Age (days)					
Mean ± SD	39.7				
Range	1–210				
Weight (gm)					
LBW (<2500)	136 (77.3)	23 (16.9)	1.491	0.7136-7.126	0.2220
NBW (≥2500)	40 (22.7)	3 (7.5)			
Sepsis onset					
EOS	114 (67.8)	21 (18.4)	2.648	0.9056-5.762	0.1037
LOS	62 (35.3)	5 (8.1)			
Mode of delivery					
NVD	87 (49.4)	9 (10.3)	2.029	0.2553-1.149	0.1543
CS	89 (50.6)	17 (19.1)			

LBW = low birth weight; NBW = normal birth weight; SD = standard deviation; EOS = early onset; LOS = late onset; NVD = normal vaginal delivery; CS = cesarean section; X2 = Chi square; CI = confidence interval; * = significant difference

Table 2: Frequency of isolated fungi in neonates and infants with sepsis in Egypt

Fungi pathogen	Frequency of isolation	Percentage
Candida albicans	9	34.6
Candida tropicalis	8	30.7
Candida krusei	6	23.1
Candida parapsilosis	1	3.8
Pichia kudriavzevii	1	3.8
Penicillium oxalicum	1	3.8
Total	26	100

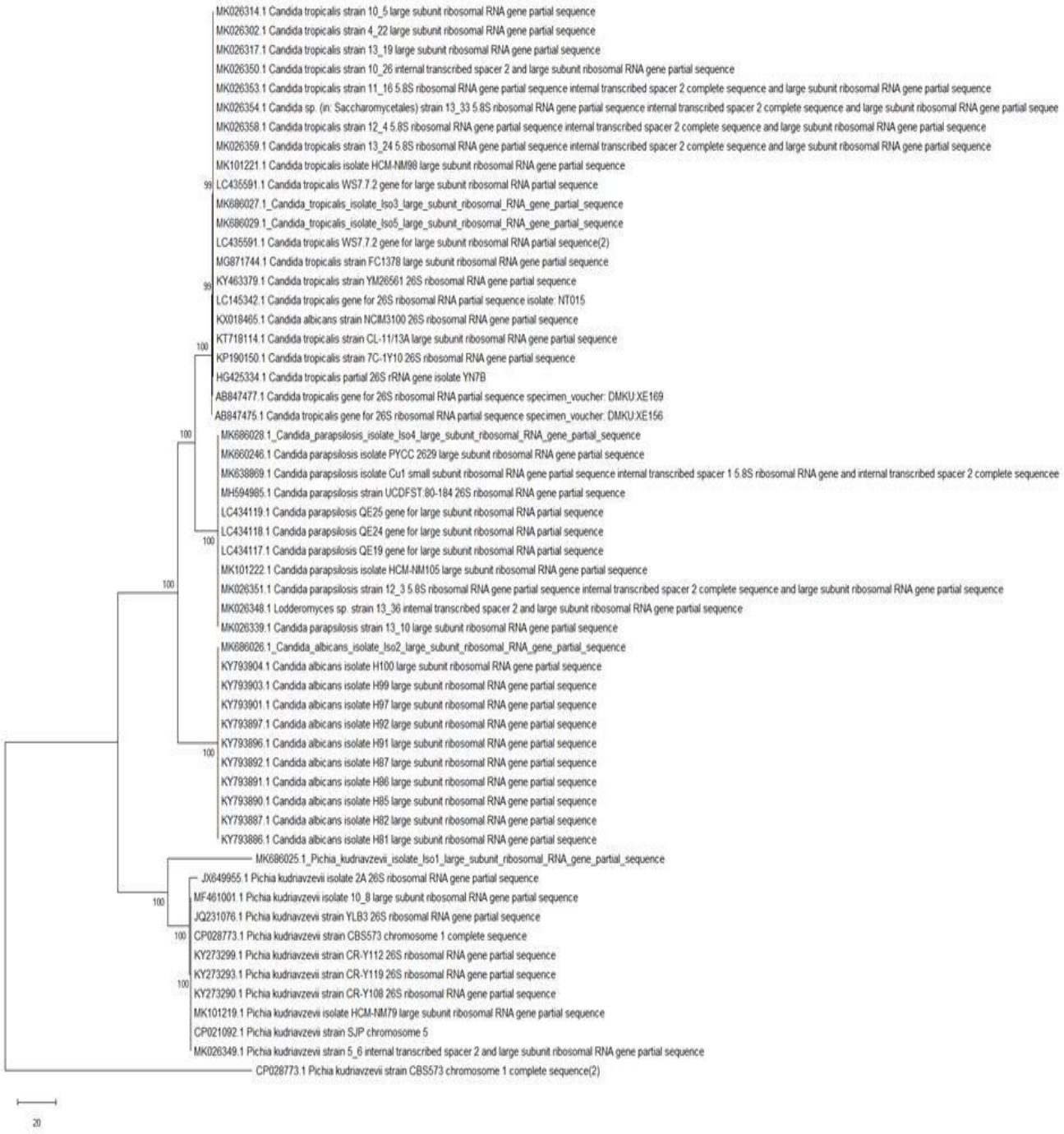

Fig 1: Phylogenetic tree of the pathogenic yeast isolates and closely related strains according to Genbank data

Discussion:

Invasive candidiasis is an emerging cause of LOS, especially among infants who receive broad-spectrum antimicrobial agents (22). Globally, neonatal sepsis is still one of the major causes of morbidity and mortality in spite of recent advances in health care (23). Differing estimates of disease burden have been reported from high-income countries compared with reports from low-and-middle-income countries (24). More than 40% of under-five deaths globally occur in the neonatal period resulting in 3.1 million newborn deaths each year (25). The majority of these deaths usually occur in low income countries and almost 1 million of these deaths are attributed to infectious causes including neonatal sepsis, meningitis and pneumonia (26). The prevalence of fungal

sepsis in neonates admitted in NICU, ranges from 2.6 to 16.7% (27).

In the present study, the prevalence of 14.7% for fungal sepsis is similar to the rates of 11%, 13.6%, 13.9% and 11.97% reported by Rani et al., (28), Agarwal et al., (29), Chen et al., (30) and Chavhan et al., (27) respectively. In a previous study by Mokhtar *et al*., (11), the rate of fungal neonatal sepsis reported was 12% of the total diagnosed cases while bacterial sepsis represented 88%, but in the current study, fungal sepsis has increased to 14.7%. Advances in neonatal management have led to considerable improvement in newborn survival (31).

The results in this study showed that candidal sepsis commonly occurred in low weight and preterm neonates, similar results were reported in other studies by Schellack and Gous (32), Femitha and Joy (33), Kapila (34), and Koppad and Prakash (35). Low weight and preterm are risk factors for fungal sepsis because of immaturity of their immune system and invasive intervention. *Candida* species has become the third most common cause of late onset sepsis in NICU with mortality figures varying between 15 to 59% (36).

The present study shows the changing pattern of candidal species (from the last decade) among neonatal fungal sepsis with increasing prevalence of non-*albicans Candida* species which in this study constituted 62.5% of the 24 *Candida* isolates. These findings are consistent with other recent studies by Kapila (34), Koppad (35) and Chavhan (27) which reported prevalence rates of 86.4%, 71% and 65% respectively, for non-*albicans Candida* species. Although initial reports indicated that most cases were due to *C. albicans*, more recent studies showed the emergence of the non-*albicans* *C. parapsilosis*, by 54% and 30% in the study by Ballot et al., (37) and 30% in another study by Chavhan et al., (27).

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References:

1. Cortese, F., Scicchitano, P., Gesualdo, M., et al. Early and Late Infections in Newborns: Where Do We Stand? A Review, Pediatrics and Neonatology. 2016 :57, 265-273
2. Wynn, J. L. Defining neonatal sepsis. Curr Opin Pediatr. 2016; 28 (2): 135-140.
3. Mukhopadhyay, S., and Puopolo, K. M. Risk Assessment in Neonatal Early-Onset Sepsis, Semin Perinatol. 2012;
4. Camacho-Gonzalez, A., Spearman, P. W., and Stoll, B. J. Neonatal infectious diseases: evaluation of neonatal sepsis. Pediatr Clin North Am. 2013: 60 (2): 367-389.
5. Thaver, D., and Zaidi, A. K. M. Burden of Neonatal Infections in Developing Countries, A Review of Evidence From Community-Based Studies. Pediatr Infect Dis J. 2009; 28: 1.
6. Jumah, D. S., and Hassan, M. K. Predictor of mortality outcome in neonatal sepsis. Med J Basrah University. 2007: 25 (1):
7. Klein, O., and Remington, J. S. Current concepts of infection of the fetus and newborn infant. In: Remington J., and Klein, J. (eds). Infectious Diseases of the Fetus and Newborn. W B Saunders, Philadelphia, Pa, USA, 2000: 1 - 24
8. Manzoni, P., Mostert, M., and Castagnola, E. Update on the management of *Candida* infections in preterm Neonates. BMJ. 2014
9. Dessì, A. Neonatal fungal infections new strategies in diagnosis: J Pediatr Neonatal Individualized Med. 2014; 3 (2): e030222.
10. Kaufman, D. A. Fungal Infections in Preterm Infants: Medscape, 2014.
11. Mokhtar, E., El-Shereef, A., Abdel-Kader, A., et al. Early Diagnosis of Neonatal Sepsis Caused by Yeast Infection, Austin J Publ Hlth Epidemiol. 2014; 1 (2):
12. Pfaller, M. A., Pappas, G. P., and Wingard, J. R. Invasive Fungal Pathogens: Current Epidemiological Trends. Clin Infect Dis. 2006;
13. English, M., Ngama, M., Mwalekwa, L., et al. Signs of illness in Kenyan infants aged less than 60 days, Bulletin of the World Health Organization, 2004; 82 (5): 323–329.
14. Young Infants Clinical Signs Study Group: Clinical signs that predict severe illness in children under age 2 months: a multicentre study, Lancet. 2008
15. Utomo, M. T. Risk factors of neonatal sepsis: a preliminary study in Dr. Soetomo hospital. Indonesian J Trop Infect Dis, 2010; 1: 23–26
16. De Hoog, G. S. Atlas of Clinical Fungi, Second Edition, Amer Society for Microbiology. 2000 :1-1126
17. Lodder, J. The Yeasts: a taxonomic study, North-Holland Publishing Company, Amsterdam, second edition. 2000.
18. Saitou, N., and Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution. 1987; 4: 406-425.
19. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution. 1985. 39: 783-791.
20. Nei, M., and Kumar, S. Molecular Evolution and Phylogenetics. Oxford University Press, New York, 2000.
21. Kumar, S., Stecher, G., Li, M., et al. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution. 2018, 35: 1547-1549.
22. Shane, A.L., Stoll, B. J. Recent Developments and Current Issues in the Epidemiology, Diagnosis, and Management of Bacterial and Fungal Neonatal Sepsis, Am J Perinatol. 2013; 30 (02): 131-142

23. Wu, J. H., Chen, C. Y., Tsao, P. N., et al. Neonatal sepsis: a 6-year analysis in a neonatal care unit in Taiwan. Pediatrics and Neonatology, 2009; 50 (3): 88–95
24. Shane, A. L., Sánchez, P. J., and Stoll, B. J. Neonatal sepsis. Lancet. 2017; 390 (10104): 1770 - 1780.
25. UNICEF, WHO, The World Bank, and The United Nations, Levels and Trends in Child Mortality, UNICEF, New York, NY, USA, 2011
26. Black, R. E., Cousens, S., Johnson, H. L., et al., Global, regional, and national causes of child mortality in 2008: a systematic analysis. Lancet, 2010; 375 (9730): 1969 - 1987.
27. Chavhan, S. K., Meena, J. S., Meena, K. K., and Meena, K. J. Candidemia in Neonatal Intensive Care Units: Magnitude, Species Identification And Antifungal Susceptibility Pattern, IOSR Journal of Dental and Medical Sciences. 2017; 16 (6):
28. Rani, R., Mohapatra, N. P., Mehta, G., et al. Changing trends of *Candida* species in neonatal septicaemia in a tertiary North Indian hospital. Indian J Med Microbiol. 2002
29. Agarwal, J., Bansal, S., Malik, G. K., and Jain, A. Trends in neonatal septicaemia: Emergence of non-*albicans Candida*. Indian Pediatr. 2004; 41: 712-715
30. Chen, J., Jiang, Y., Wei, B., et al. Epidemiology of and risk factors for neonatal candidaemia at a tertiary care hospital in western China. BMC Infect Dis. 2016; 16: 700.
31. Kumar, A., Yadav, A., Gathwala, G., et al. Study of risk factors for candida species colonisation of neonatal intensive care unit patient. Int J Pharma and Biosci. 2012; 3 (2): 193-199
32. Schellack, N., and Gous, A. G. S. Amphotericin B in the management of fungal infections in a neonatal intensive care unit: experiences in a teaching hospital. South Afr J Epidemiol Infect. 2012;
33. Femitha, P., and Joy, R. Candidaemia in neonatal ICU-experience from a tertiary care hospital. Curr Pediatr Res. 2013; 17 (1): 44-48
34. Kapila, S., Goel, S. P., and Prakash, A. Identification of *Candida* species in neonatal septicemia. Int J Contemp Pediatr. 2016; 3(2): 601-605
35. Koppad, B., and Prakash, K. P. Neonatal candidiasis: clinical spectrum and epidemiology in a tertiary care centre, Int J Contemp Pediatr. 2017; 4 (2): 438-441
36. Montagna, M. T., Lovero, G., De Giglio, O., et al. Invasive fungal infections in neonatal intensive care units of Southern Italy: a multicentric regional active surveillance. J Prev Med Hyg. 2010; 51 (3): 125-
37. Ballot, D. E., Bosman, N., Nana, T., et al. Background changing patterns of neonatal fungal sepsis in a developing country. J Trop Pediatr. 2013; 59 (6): 460–464

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Original Article **Open Access**

Coagulase negative staphylococci in Anti-Cancer Center, Batna, Algeria: antibiotic resistance pattern, biofilm formation, and detection of *mecA* and *icaAD* genes

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Abstract:

Background: Coagulase-negative staphylococci (CoNS) are normal microbial flora found on the skin and mucous membranes of mammals. Considered for a long time as avirulent commensals, these bacteria are now recognized as opportunistic pathogens by virtue of their high resistance to multiple antibiotics and capacity for biofilm formations, which made them important agents of nosocomial and community-acquired infections. The objectives of this study are to determine the antibiotic resistance pattern and biofilm formation, and to detect *mecA* and *icaAD* genes in clinical CoNS isolates from Batna's Anti-Cancer Center (ACC) in Algeria.
Methods: A total of 66 CoNS were isolated from different samples and identified by API Staph system. *In vitro* antibiotic susceptibility testing (AST) of each isolate to selected antibiotics was determined by the disk diffusion method, and minimum inhibitory concentrations (MICs) of oxacillin and vancomycin were determined by E-test. Biofilm formation was assessed by Tissue Culture Plate (TCP) and Congo Red Agar (CRA) methods. The polymerase chain reaction (PCR) was used to amplify *mecA* gene in 9 oxacillin-resistant and 1 oxacillin-sensitive CoNS, and *icaAD* gene in 9 biofilm forming and 1 non-biofilm forming CoNS. Sequencing of the 16S rDNA of 1 *mecA* and 1 *icaAD* positive isolates was performed by the Sanger method.
Results: Nine species of CoNS were identified, with *Staphylococcus epidermidis* (n=29, 44%) and *Staphylococcus haemolyticus* (n=15, 22.7%) constituting the largest proportion, and isolated mainly from the onco-haematology service unit of the center. The isolates were resistant to penicillin G (98.5%), cefoxitin (80.3%) and oxacillin (72.2%). The TCP method was more sensitive (89.4%) than CRA method (31.8%) in detecting biofilm formation. The *mecA* gene was detected in 66.7% (6/9) of oxacillin resistant CoNS and the *icaAD* gene in 55.6% (5/9) of TCP positive CoNS isolates
Conclusion: *Invitro* resistance to methicillin (oxacillin) and biofilm formation were high among the CoNS isolates in this study, but the association of these with respective carriage of *mecA* and *icaAD* genes was low.

Keywords: Coagulase negative staphylococci, identification, antibiotic resistance, biofilm, PCR

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Staphylocoques à coagulase négative au Centre Anti-Cancer du Batna, Algérie: résistance aux antibiotiques, formation de biofilms et détection des gènes *mecA* et *icaAD*

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Abstrait :

Contexte: Les staphylocoques à coagulase négative (CoNS) sont une flore microbienne normale présente sur la peau et les muqueuses humaines des mammifères. Considérés depuis longtemps comme des commensales avirulentes, ces bactéries sont reconnues comme agents pathogènes opportunistes grâce à leurs multiples propriétés coexistantes de résistance aux antibiotiques et de formation de biofilms qui constituent des agents importants d'infections nosocomiales et communautaires. l'objectif de cette étude est de déterminer la résistance aux antibiotiques, la formation de biofilms et pour rechercher des gènes *mecA* et *icaAD* dans les isolats cliniques de staphylocoques à coagulase négative du Centre Anti-Cancer (AAC) de Batna en Algérie.
Méthodes: au total de 66 des SCN ont été isolés de différents prélèvements et identifiés par galerie API Staph. Le test de sensibilité aux antibiotiques *In vitro* de chaque isolat par rapport aux antibiotiques sélectionnés a été déterminé par la méthode de diffusion sur disque, et les concentrations minimales inhibitrices (MICs) de l'oxacilline et de la vancomycine ont été déterminées par E-test. La formation de biofilm a été évaluée par la méthode de culture de tissu en plaque (TCP) et la méthode de Rouge Congo Agar (CRA). La réaction en chaîne par polymérase (PCR) a été utilisée pour amplifier l'ADN du gène *mecA* dont 9 des SCN résistants à l'oxacilline et 1 sensible à l'oxacilline et le gène *icaAD* dont 9 des SCN formant biofilm et 1 non-formant biofilm. Le séquençage de l'ADNr 16S des isolats positifs, 1 *mecA* et 1 *icaAD* ont été réalisés par la méthode de Sanger.
Résultats: Neuf espèces des SCN ont été identifiées avec *Staphylococcus epidermidis* (n=29, 44%) et *Staphylococcus haemolyticus* (n=15, 22,7%) constituant la plus grande proportion, et isolées principalement de l'unité de service d'onco-hématologie du centre. Les isolats étaient résistants à la pénicilline G (98,5%), à la céfoxitine (80,3%) et à l'oxacilline (72,2%). La méthode TCP était plus sensible (89,4%) que la méthode CRA (31,8%) dans la détection de la formation de biofilm. Le gène *mecA* a été détecté dans 66,7% (6/9) des SCN résistants à l'oxacilline et le gène *icaAD* dans 55,6% (5/9) des isolats positifs des SCN pour CRA.
Conclusion: La résistance à la méthicilline (oxacilline) *in vitro* et la formation de biofilms étaient élevées chez les isolats des SCN de cette étude, mais leur corrélation avec le portage respectif des gènes *mecA* et *icaAD* était faible.

Mots-clés: Staphylocoque à coagulase négative, identification, résistance aux antibiotiques, biofilm, PCR

Introduction:

Coagulase negative staphylococci (CoNS) are normal microbial flora found on the skin and human mucous membranes of mammals (1). These bacteria, which have for a long time adjudged to be avirulent commensals, are currently considered the predominant pathogens (2) and major cause of nosocomial and community-acquired infections (3).

The most common species that cause disease in humans are *Staphylococcus epidermidis* which causes bacteremia in patients with implanted medical devices (such as prostheses and catheters), surgical wound infection, peritonitis in patients with continuous peritoneal dialysis, osteomyelitis, and endophthalmitis; *Staphylococcus haemolyticus* which causes endocarditis, peritonitis, sepsis and infections of the urinary tract, wounds, bones and joints; and *Staphylococcus saprophyticus* which causes urinary tract infections and septicemia. The other important opportunistic pathogenic species include *Staphylococcus hominis*, *Staphylococcus warneri*, *Staphylococcus capitis*, *Staphylococcus simulans*, *Staphylococcus cohnii*, *Staphylococcus xylosus* and *Staphylococcus saccharolyticus* (4).

The treatment of the infections by CoNS is difficult because many clinical isolates exhibit multiple and high resistance to antibiotics, leading to increased inefficiency of a wide range of antibiotics (5). There are several reports in literature showing high resistance to methicillin and other antibiotics among CoNS isolates (6, 7, 8). Methicillin resistance in CoNS is caused by the *mecA* gene that encodes penicillin-binding protein 2a (PBP2a) that has low binding affinity to β-lactams (2, 9**,** 10**,** 11).

The ability to form biofilm is the most important virulence factor in CoNS, which facilitates its adhesion to and colonization of artificial materials (8). Bacteria in biofilms can resist antibiotics at concentrations up to 1000 times higher than those active on the same bacteria in the planktonic state (12). The biofilm consists of layers of cellular clusters integrated in a matrix of extracellular polysaccharide, called polysaccharide intracellular adhesion (PIA). The enzymes implied in the synthesis of PIA are encoded by the *ica* operon including the *icaA*, *icaD*, *icaB* and *icaC* genes (13).

The two common phenotypic methods for detecting biofilm formation, Tissue Culture Plate (TCP) and Congo Red Agar (CRA), were described by Christensen et al., (14) and Freeman et al., (15) respectively. Molecular detection of the *icaAD* locus was initially described by Heilmann, et al., (16) in *S. epidermidis* but few years later, its presence was confirmed in many other species of staphylococci isolated from implant related-infections (12). The objectives of this study are to evaluate antibiotic resistance and biofilm formation in CoNS isolated from patients at the Anti-Cancer Center of Batna, Algeria, and to detect the responsible *mecA* and *icaAD* genes in these isolates.

Materials and method:

Study setting and bacterial isolates

This study was conducted at the Microbiology Laboratory of the Anti-Cancer Center (ACC), Batna, Algeria. Sixty six coagulase negative staphylococci (CoNS) were isolated from clinical samples (urinary probes, urine, blood culture, pus, wound, pleural fluid, cerebrospinal fluid, intravenous catheter, and drain) obtained from hospitalised patients in various service units (onco-haematology, onco-paediatric, intensive care and carcinological surgery) of the center and from outpatients, between 1st of January and 30th of June, 2017. Duplicate samples were excluded.

The samples were cultured on Mannitol Salt (Chapman) agar and incubated at 37°C for 48 hours. All strains were identified by colony morphology, Gram stain reaction, catalase production, coagulase assay, and the API Staph system (bioMérieux, France). The purified isolates were stored at 4°C.

Antibiotic susceptibility testing of isolates

In vitro antibiotic susceptibility testing (AST) was performed by the disk diffusion test on Mueller-Hinton (MH) agar as described by Bauer et al., (17) and the interpretation of the results was done according to Clinical and Laboratory Standards Institute (CLSI) guidelines (18). All isolates were tested with the following antibiotics: penicillin (10 UI), cefoxitin (30 µg), gentamicin (10 µg), amikacin (30 µg), kanamycin (30 µg), erythromycin (15 µg), tetracycline (30 µg), ofloxacin (5 µg), laevofloxacin (5 µg), ciprofloxacin (5 µg), clindamycin (2 µg), chloramphenicol (30 µg), rifampicin (5µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), fusidic acid (10 µg), pristinamycin (15 µg) and teicoplanin (30 µg). The minimum inhibitory concentrations (MICs) of oxacillin and vancomycin for each isolate were determined on MH using E-test method, and results interpreted according to CLSI guidelines (18).

Detection of biofilm formation by Congo Red Agar (CRA) method

The qualitative determination of the biofilm formation was carried out by culturing CoNS isolates on Congo Red Agar as proposed by Freeman et al., (15). The prepared medium was inoculated with CoNS isolates and incubated at 37°C for 24 to 48 hours. Biofilm-forming bacteria appear black with a crystalline lens of dry consistency where the Congo Red dye interacts directly with certain bacterial polysaccharides forming a slime while the non-biofilm forming colonies remained red.

Detection of biofilm formation by Tissue Culture Plate (TCP) method

Quantitative determination of biofilm formation on microplates was evaluated as described by Christensen et al., (14) with some modifications. The microplates used were made of polystyrene with 96 wells on which the bacteria adhere and form biofilm. The isolates were cultivated on nutrient agar for 18-24 hours at 37°C. A colony of each isolate was inoculated into 5 ml Trypticase Soy Broth (TSB) and incubated at 37°C for 24 hours, and the culture was then diluted 1:100 in the TSB (+1% glucose). Each well of the microplate was filled with 200µL of this dilution (three independent cultures for each isolate). A sterile broth of TSB (+1% glucose) was used as a negative control.

The microplates were sealed and incubated for 24 hours at 37°C. Thereafter, the contents of the wells were gently removed and washed four times with sterile physiological water and then dried at 60°C for 30 minutes. The cells adhering to the polystyrene support in each of the wells were stained with 200µl of 1% crystal violet (w/v) and incubated for 30 minutes following which excess crystal violet was removed by 5 successive washes with sterile distilled water and the plates dried at room temperature. The dye incorporated by the adhered cells was solubilized with 200µL of 95% ethanol (v/v).

The amount of dye solubilized was measured by reading the optical density (OD) at 550 nm using Bio-Rad ELISA reader (PR 3100 TSC) (19). The interpretation of the results was performed according to the criteria of Stepanovic et al., (20). The OD of the isolate was obtained by the arithmetic mean of three wells and this value was compared with the mean absorbance of negative control (ODc). The isolates were classified as non-biofilm producer (OD≤ ODc), weak biofilm producer (ODc<OD≤2 ODc), moderate biofilm producer (2ODc<OD≤ 4ODc) and strong biofilm producer (4ODc< OD).

Detection of *mecA* and *icaAD* genes by PCR and 16s rDNA sequencing

The PCR of *mecA* and *icaAD* genes and the sequencing of the 16S rRNA genes were performed in the Laboratory of DIAG-GENE, Angers, France. Nine MRCoNS strains (no 1 to 9) and 1 MSCoNS strain (no 10), were selected for *mecA* gene detection. Similarly, 9 biofilm-forming strains (no 11 to 19) and 1 non-biofilm forming strain (no. 20) were selected from the TCP results for *icaAD* gene detection.

PCR assay

The *mecA* and *icaAD* genes amplifications were performed by PCR as described by

Poulsen et al., (21) and Yazdani et al., (22) respectively, using the following specific primers; *mecA*-F:5'-GGGATCATAGCGTCATTA-TTC-3' and *mecA*-R:5'-AACGATTGTGACACGA-TAGCC-3', *icaAD*-F: 5'-TATTCAATTTACAGTCG-CAC-3' and *icaAD*-R: 5'-GATTCTCTCCCTCTCT GCCA-3'. The DNA amplification was done in a Mycycler thermal cycler (Bio-Rad, USA). The amplification products (10µL) were eletro-phoresed on 1.5% agarose gel followed by staining in an ethidium bromide bath (0.5µg/ml). The amplified DNA products were visualized under UV transillumination.

Sequencing of the 16S rRNA genes

Sequencing of amplified and purified 16S rDNA of strain number 3 (*mecA*+) and strain number 13 (*icaAD*+) was performed as described by Sanger et al., (23), using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) and two universal primers (27F and 1492R). The 16S sequences obtained were then compared with those of the GeneBank database using the BLAST programme (24).

Results:

Identification of CoNS isolates

Sixty six CoNS belonging to 9 different species were identified by the conventional methods; *S. epidermidis* 29, *S. haemolyticus* 15, *S. hominis* 8, *S. chromo-genes* 6, *S. xylosus* 4, *S. capitis* 1, *S. sapro-phyticus* 1, *S. cohnii* 1, and *S. simulans* 1 (Table 1).

Table 1: Species distribution of CoNS isolates in Anti-Cancer Center, Batna, Algeria

CoNS isolate	Frequency	Percentage
Staphylococcus epidermidis	29	43.9
Staphylococcus haemolyticus	15	22.7
Staphylococcus hominis	8	12.1
Staphylococcus chromogenes	6	9.1
Staphylococcus xylosus	4	6.1
Staphylococcus capitis	1	1.5
Staphylococcus cohnii	1	1.5
Staphylococcus saprophyticus	1	1.5
Staphylococcus simulans	1	1.5
Total	66	100

Table 2 shows the distribution of the CoNS according to the clinical samples; 28 (42.4%) were from blood cultures (*S. epidermidis* 11, *S. haemolyticus* 8, *S. hominis* 8 and *S. chromogenes* 2); 13 (19.7%) from catheter samples (*S. epidermidis* 8, *S. haemolyticus* 2, *S. chromogenes* 1, *S. xylosus* 1 and *S. capitis* 1); 8 (12.1%) from pus samples (*S. epidermidis* 2, *S. chromogene*s 2, *S. haemolyticus* 1, and *S. xylosus* 3); 6 (9.1%) from urine samples (*S. epidermidis* 3, *S. haemolyticus* 2, and *S. hominis* 1.

Table 2: Specimen distribution of CoNS isolates in Anti-Cancer Center, Batna, Algeria

Samples	Frequency	Percentage
Blood	28	42.4
Vascular catheter	13	19.7
Pus	8	12.1
Urine	6	9.1
CSF	5	7.6
Pleural fluid	3	4.5
Wound	1	1.5
Urinary probe	1	1.5
Drain	1	1.5
Total	66	100

CoNS = coagulase negative staphylococci

The CoNS isolates were found mainly in the onco-haematology service (n=30, 45.5%), out of which 13 were *S. epidermidis* (Table 3).

Table 3: Distribution of CoNS isolates by service unit in Anti-Cancer Center, Batna, Algeria

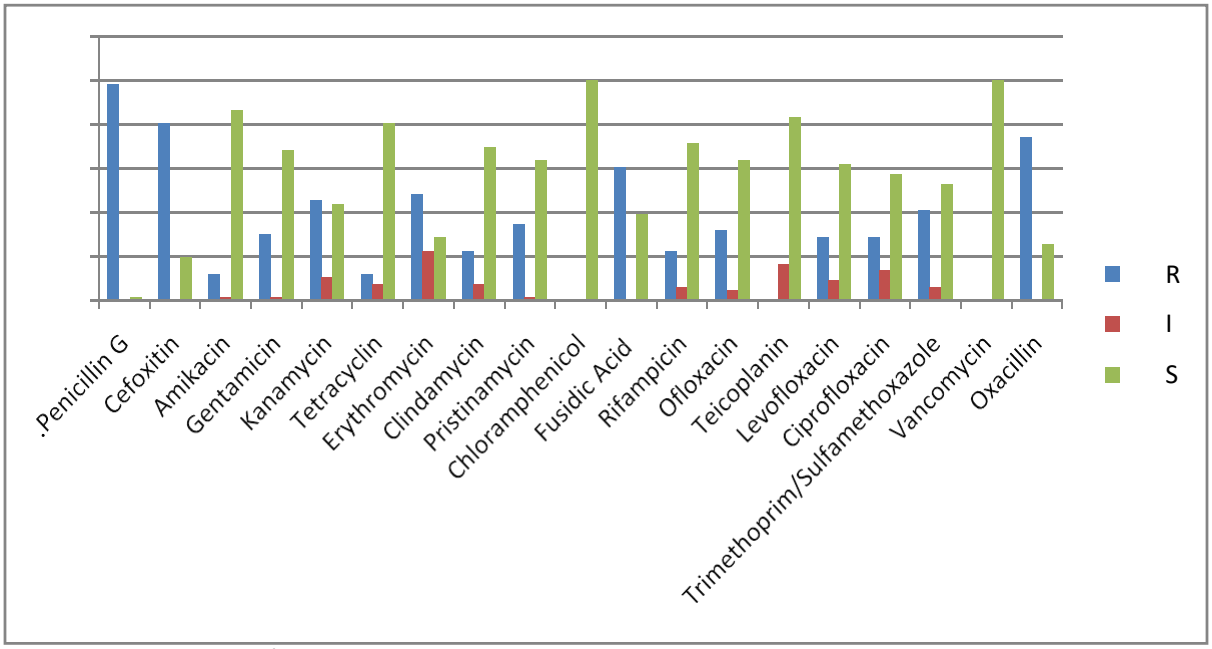
Service unit	No of CoNS	Percentage
Onco-haematology	30	45.5
Carcinological surgery	14	21.2
Onco-paediatric	10	15.2
Intensive care	7	10.6
Outpatient	5	7.6
Total	66	100

CoNS = coagulase negative staphylococci

Antimicrobial susceptibility profiles of CoNS isolates

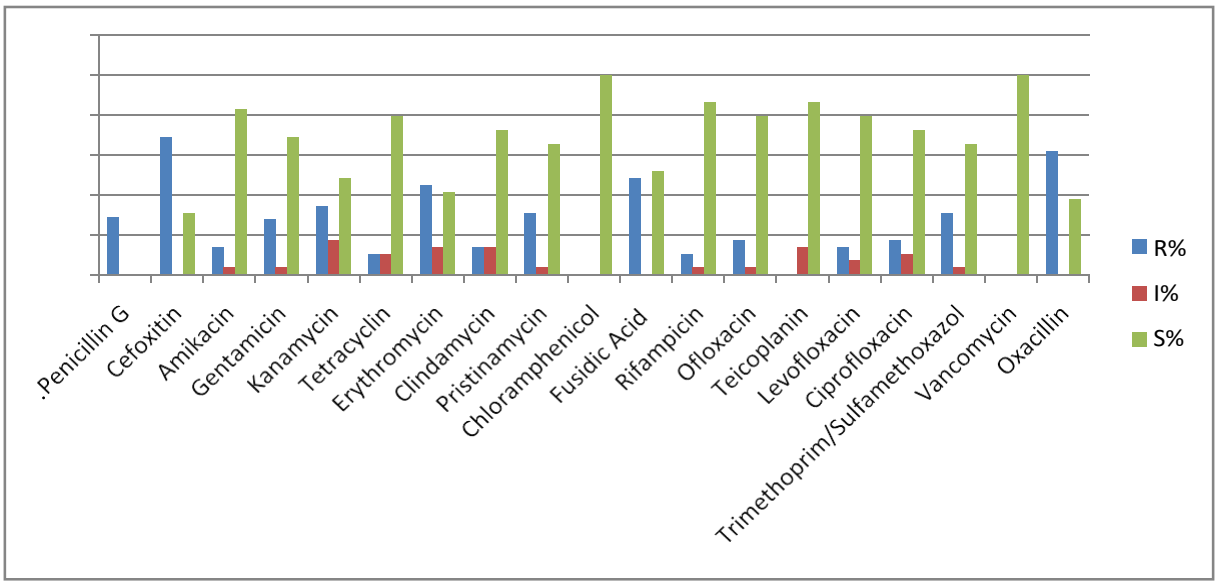
The AST profile of the isolates is detailed in Fig 1. The isolates exhibited high resistance to penicillin G (98.5%), cefoxitin (80.3%), oxacillin (74.2%), fusidic acid (60.6%), but were totally sensitive to chlora-mphenicol and vancomycin (by the E-test). The isolates were also largely sensitive to amikacin (86.4%), teicoplanin (83.3%) and tetracycline (80.3%) but susceptibility was reduced to rifampicin (71.2%), clindamycin (69.7%), gentamicin (68.2%), pristinamycin (63.6%), ofloxacin (63.6%), laevofloxacin (62.1%), erythromycin (50%) and kanamycin (45%).

The most frequent CoNS isolates, *S. epidermidis*, was totally resistant to penicillin G (100%), 69% to cefoxitin, and 60% to oxacillin by the E-test (Fig. 2).



R = resistance; I = intermediate; S = sensitive

Fig 1: Antimicrobial susceptibility profiles of coagulase negative staphylococci isolates



R = resistance; I = intermediate; S = sensitive

Fig 2: Antimicrobial susceptibility profiles of *Staphylococcus epidermidis*

Biofilm formation

Fig. 3 shows the result of biofilm formation by the CRA method with 21 (31.8%) CoNS isolates forming biofilm while 45 (68.2%) were negative. The results of biofilm formation by the TCP method showed that 59 (89.4%) of the 66 isolates formed biofilms with different intensities (Fig 4); 16 (24.2%) were strong biofilm forming, 23 (34.9%) were moderate, 20 (30.3%) were weak, and 7 (10.6%) were non-biofilm forming bacteria.

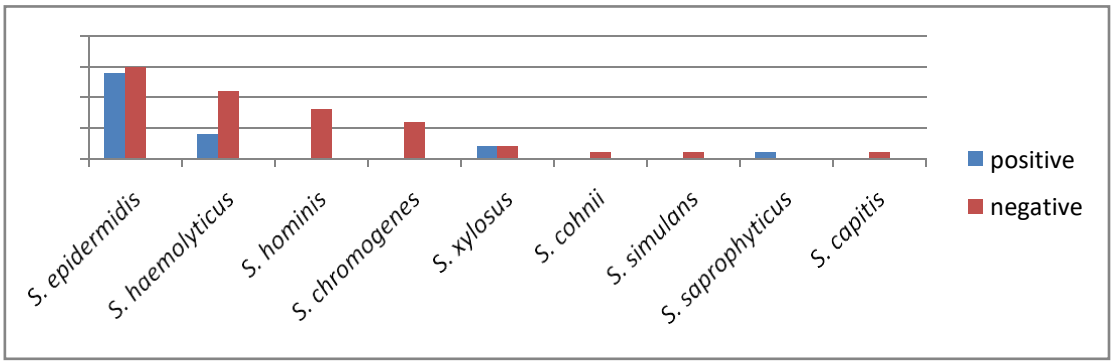


Fig 3: Biofilm formation tested by Congo Red Agar method

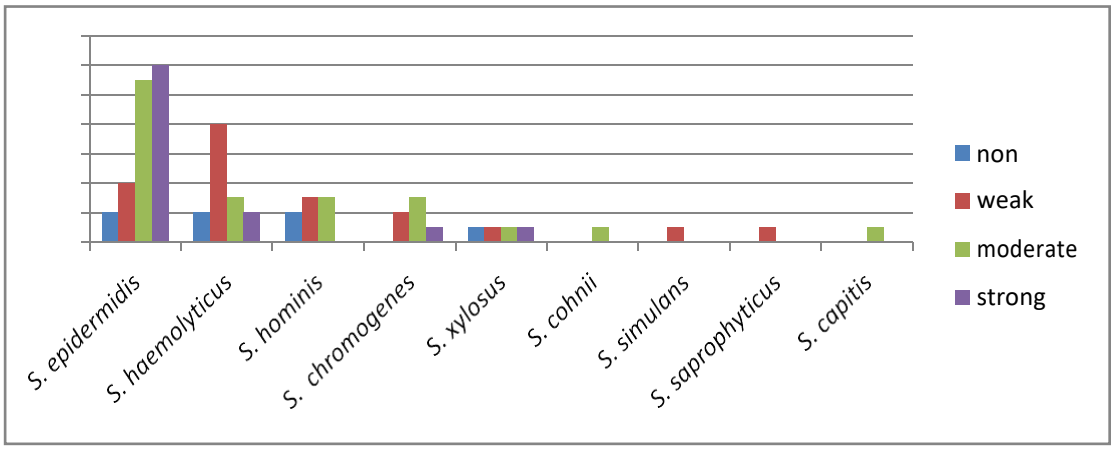
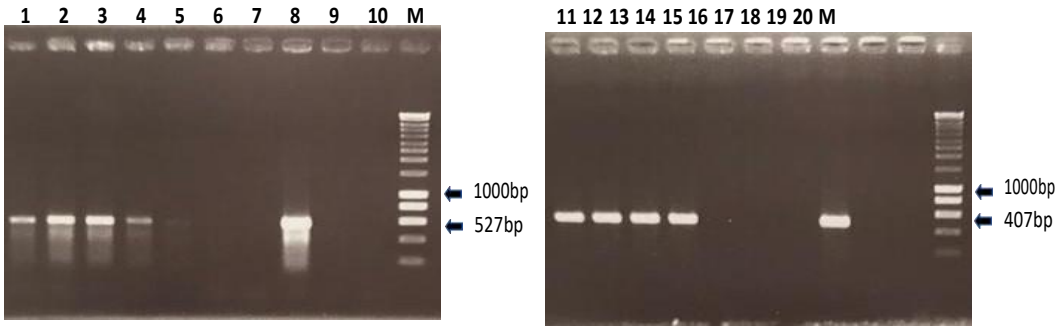


Fig 4: Biofilm formation tested by the Tissue Culture Plate method

Result of *mecA* and *icaAD* genes PCR

Of the 9 CoNS isolates resistant to oxacillin by the AST tested for carriage of *mecA* gene, only 6 amplified for the gene (Fig 5a). Similarly, only 5 of the 9 CoNS isolates positive for biofilm production by the CRA method amplified for the *icaAD* genes (Fig 5b). Sequencing of the 16S rRNA genes of in each isolate positive for *mecA* and *icaAD* shows percentage similarity to respective GenBank strains as shown in Table 4.



(a) *mecA* gene (b) *icaAD* gene

M: molecular size marker (Smart Ladder 200bp, Eurogentech); 1-9: MRCoNS; 10: MSCoNS; 11-19: TCP+ CoNS; TCP- CoNS

Fig 5: Agarose gel electrophoresis of *mecA* and *icaAD* genes

Table 4: Percentage similarity of two CoNS isolates on BLAST analysis

Identification number (N°) isolate	%similarity to strain in GenBan	Identification
3	100	*Staphylococcus haemolyticus* strain FC2950
13	100	*Staphylococcus epidermidis* strain K121

Discussion:

Our study identified 9 species out of the 66 CoNS characterized, with predominance of *S. epidermidis* (44%), *S. haemolyticus* (22.7%) and *S. hominis* (12.1%). This similar pattern has been reported by Shah et al., (25) who reported 35.1% for *S. epidermidis,* 33.1% for *S. saprophyticus* and 8.8% for *S. haemolyticus,* as well as by many other researchers (1,8,26,27). The CoNS in our study especially *S. epidermidis* were found in onco-haematology service unit mainly from blood cultures which is similar to what Marsik et al., reported in their study (28). CoNS is the most frequently isolated bacteria from blood cultures and is a serious health challenge in developing as well as in many developed countries (4). *S. epidermidis* is the most common species involved in blood stream infections, which is attributed to its ability to colonize central venous catheters and other implanted medical devices (29).

CoNS resistant to multiple antibiotics have become a great challenge in nosocomial infections. In this study, 80.3% of the CoNS isolates were methicillin resistant by the cefoxitin disk and 74.2% by the oxacillin E-test, and resistance to penicillin G was 98.5%. This high resistance rate to both methicillin and penicillin G have been reported by several other researchers (1,6,7,30,31,32). Methicillin resistance is usually associated with *mecA* gene that encodes abnormal penicillin binding protein PBP2a (PBP2a) with low binding affinity for β- lactam antibiotics (5). The *mecA* gene is located on a mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*) which can be transferred between and within the staphylococcal species (33). MRSA isolates are usually not reliably detected by phenotypic techniques, especially as the expression of *mecA* gene varies according to strains, some of which may possess heterogeneous resistance, hence the need for genotypic characterization (34).

In our study, no CoNS isolates was resistant to vancomycin by the E-test MIC, which agrees with the reports of Jain et al., (35) and Shrestha et al., (1). Vancomycin and teicoplanin are anti-staphylococcal antibiotics to which resistance is rarely observed. These antibiotics should however be reserved for the treatment of staphylococcal infections resistant to all other antibiotics (32). The CoNS isolates were also largely sensitive to amikacin and tetracycline (>80%) but susceptibility to fluoroquinolones (ofloxacin, ciprofloxacin and laevofloxacin), clindamycin, pristinamycin, erythromycin and trimethoprim/sulfamethoxazole were reduced with resistance rate being over 20% for each of these isolates. Our findings are similar to what others have reported for CoNS clinical isolates (3, 28, 29, 36, 37, 38).

The ability of staphylococci to form biofilms helps the bacterium to resist host immune response and is considered responsible for chronicity as biofilm protects microorganisms from opsono-phagocytosis and antimicrobial agents. In view of the large number of infections caused by biofilm producing bacteria, a reliable method for their diagnosis is necessary. Nasr et al., (39) reported that 50% of CoNS isolated from intravascular blood cultures and catheters were biofilm producers using the CRA method, which is higher than 31.8% reported in our current study. The TCP method has however been reported to be a more sensitive, accurate and reproducible screen- ing method for detecting biofilm production in clinical staphylococci isolates with added advantage of being a quantitative tool for comparing the adherence of different strains (39). Oliveira et al., (26), Soumya et al., (8) and Shrestha et al., (1) reported that 73%, 87.5% and 85% CoNS respectively produce biofilm by the TCP method. This is similar to the high rate of 89.4% reported in our current study.

The ability of CoNS to be resistant to methicillin and produce biofilm is an important factor in infectivity which occurs through expression of *mecA* and *ica* genes respectively. Jain et al., (35) have reported that detection of *mecA* gene by PCR can be a beneficial complement to standard susceptibility test that can allow the identification of intrinsic resistance quickly and efficiently, as most strains carry the classical *mecA* gene. Six of the 9 (66.7%) selected CoNS isolates resistant to methicillin (by oxacillin E-test) in

our study amplified for *mecA* gene. However, other allotypes of *mecA* gene, have been described for subspecies of *S. sciuri* (*mecA1*) and for *S. vitulinus* (*mecA2*) among animal-derived isolates. Another *mecC* allotype, *mecC2*, was recently reported for methicillin resistant *S. saprophyticus* subsp. *saprophyticus* (40). The *icaAD* gene amplified in 5 of the 9 (55.5%) selected CoNS isolates in our study. This rate is higher than the 32% reported by Nasr et al., (39). It has been reported that the *icaAD* gene is not always associated with *in vitro* biofilm formation (39). The biofilm-forming ability of some isolates in the absence of *icaAD* gene highlights the need to investigate the genetic basis of *ica*-independent biofilm formation.

Conclusion:

In vitro resistance to methicillin (oxacillin) and biofilm formation were high among the CoNS isolates in Anti-Cancer Center, Batna, Algeria, but the association of these phenotypes with respective carriage of *mecA* and *icaAD* genes was low. Further studies are needed to investigate the genetic basis of biofilm formation independent of *icaAD* genes.

References:

1. Shrestha, L. B., Bhattarai, N. R., and Khanal, B. Antibiotic resistance and biofilm formation among coagulase-negative staphylococci isolated from clinical samples at a tertiary care hospital of eastern Nepal. Antimicrob Resist Infect Contr. 2017; 6 (89): 1-7.
2. Ahmed, R., Singh, S., Farooq, U., et al. Occurrence and Antimicrobial Susceptibility Pattern of Methicillin-resistant *Staphylococcus aureus* and Methicillin-resistant Coagulase-Negative Staphylococci Isolated from Different Clinical Specimens from the Patients Hospitalized in Teerthanker Mahaveer Medical College and Research Centre, Moradabad, India. Intern J. Sci Study. 2016; 3 (11): 41-47.
3. Cuevas, O., Cercenado, E., Vindel, A., et al. Evolution of the Antimicrobial Resistance of *Staphylococcus* spp. In Spain: Five Nationwide Prevalence Studies, 1986 to 2002. Antimicrob Agents Chemother. 2004; 48 (11): 4240-4245.
4. Cunha, Mde L., Sinzato, Y. K., and Silveira, L. V. A. Comparison of Methods for the Identification of Coagulase negative Staphylococci. Mem Inst Oswaldo Cruz. 2004; 99 (8): 855-860.
5. Xu, Z., Mkrtchyan, H. V., and Cutler, R. R. Antibiotic resistance and *mecA* characterization of coagulase-négtive stahylococci isolated from three hotels in London, UK. Front Microbiol.2015; 6: 1-6.
6. Koksal, F., Yasar, H., and Samasti, M. Antibiotic resistance patterns of coagulase negative *Staphylococcus* strains isolated from blood cultures of septicaemic patients in Turkey. Microbiol Res. 2009; 164: 404-410
7. Nagasrilatha, B., Manmohan, B., Sasidhar, M., et al. A Study on Methicillin Resistant Coagulase Negative Staphylococci Isolated from Medical Intensive Care Unit at a Tertiary Care Centre-India". Journal of Evolution of Medical and Dental Sciences. 2015; 4 (61): 10597-10601.
8. Soumya, K. R., Philip, S., Sugathan, S., et al. Virulence factors associated with Coagulase Negative Staphylococci isolated from human infections. Biotechnol. 2017; 7 (14): 1 - 10.
9. Hanssen, A. M., Kjeldsen, G., and Sollid, J. U. E. Local Variants of Staphylococcal Cassette Chromosome *mec* in Sporadic Methicillin-Resistant *Staphylococcus aureus* and Methicillin-Resistant Coagulase-Negative Staphylococci: Evidence of Horizontal Gene Transfer? Antimicrob Agents Chemother. 2004; 285: 296.
10. Zong, Z., Peng, C., and Lu, X. Diversity of *SCCmec* Elements in Methicillin-Resistant Coagulase-Negative Staphylococci Clinical Isolates. PLoS One. 2011; 6 (5): 1-6.
11. Bhatt, P., Tandel, K., Singh, A., et al. Prevalence and molecular characterization of methicillin resistance among Coagulase-negative Staphylococci at a tertiary care center. Med J Armed Forces India. 2016; 710: 1-5.
12. Arciola, C. R., Campoccia, D., Ravaioli, S., et al. Polysaccharide intercellular adhesin in biofilm: structural and regulatory aspects. Front Cell Infect Microbiol. 2015; 5: 1-10
13. Cho, S.H., Naber, K., Hacker, J., et al. Detection of the *icaADBC* gene cluster and biofilm formation in *Staphylococcus epidermidis* isolates from catheter-related urinary tract infections. Int J Antimicrob Agents. 2002; 19: 570-575
14. Christensen, G. D., Simpson, W. A., Yonger, J. J., et al. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol. 1985; 22: 996-1006.
15. Freeman, D. J., Falkiner, F. R., and Keane, C. T. New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol. 1989; 42: 872-874.
16. Heilmann, C., Schweitzer, O., Gerke, C., et al. Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. Mol Microbiol. 1996; 20: 1083-1091
17. Bauer, A. W., Kirby, W. M. M., Sherris, J. C., et al. Antibiotic Susceptibility Testing by a Standardized Single Disk Method. Am J Clin Pathol. 1966; 45: 493-496.
18. Clinical and Laboratory Standards Institute. Performance standards for Antimicrobial Susceptibility; Twenty-Fourth informational supplement, 2014 Philadelphia
19. Rodrigues, L. B., Santos, L. R. D., Tagliari,V. Z., et al. Quantification of biofilm production on polystyrene by *Listeria*, *Escherichia coli* and *Staphylococcus aureus* isolated from a poultry slaughterhouse. Braz J Microbiol. 2010; 41: 1082-1085.
20. Stepanovic, S., Vukovi, D., Hola, V., et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by Staphylococci. APMIS. 2007; 115 (89): 1-9.
21. Poulsen, A. B., Skov, R., and Pallesen, L. V. Detection of Methicillin Resistance in Coagulase-negative Staphylococci and in Staphylococci Directly from Simulated Blood Cultures Using the EVIGENE MRSA. J Antimicrob Chemother. 2003; 51: 419
22. Yazdani, R., Oshaghi, M., Havayi, A., et al. Detection of *icaAD* Gene and Biofilm Formation in *Staphylococcus aureus* isolates from wound infections. Iranian J Publ Health 2006; 35: 25-28.
23. Sanger, F., Nicklen, S., and Coulson, A. R. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA. 1977; 74: 5463-5467
24. Altschul, S. F., Gish, W., Miller, W., et al. Basic local alignment search tool. J Mol Biol. 1990; 215 (3): 403-410.
25. Shah, D. A., Wasim, S., and Abdullah, F. E.

Comparison of Oxacillin and Cefoxitin for the Detection of *mecA* Gene to Determine Methicillin Resistance in Coagulase Negative Staphylococci (CoNS). Journal of the College of Physicians and Surgeons Pakistan. 2017; 27 (8): 520-522.

26. Oliveira, A., and Cunha, M. L. R. S. Comparison of methods for the detection of biofilm production in coagulase-negative staphylococci. BMC Res Notes. 2010; 3: 260
27. Igbinosa, E. O., and Beshiru, A. Characterization of antibiotic resistance and species diversity of staphylococci isolated from apparently healthy farm animals. Afr J Clin Exper Microbiol. 2019; 20 (4): 289-298
28. Marsik, F. J., and Brake, S. Species Identification and Susceptibility to 17 Antibiotics of Coagulase-Negative Staphylococci Isolated from Clinical Specimens. J Clin Microbiol. 1982; 15: 640 - 645
29. Javadpour, S., Karimi, E., and Karmostaji, A. Frequency and antibiogram pattern of coagulase negative *Staphylococcus* in clinical specimens of Shahid Mohammadi Hospital in patients, Bandar-Abbas, Iran. Afr J Microbiol Res. 2010; 4 (14): 1581-1583.
30. Goudarzi, M., Seyedjavadi, S., Goudarzi, H., et al. Characterization of coagulase-negative staphylococci isolated from hospitalized patients in Tehran, Iran. Journal of Paramedical Sciences. 2014; 5 (2): 44-50.
31. Iorio, N. L. P., Azevedo, M. B., Frazão, V. H., et al. Methicillin resistant *Staphylococcus epidermidis* carrying biofilm formation genes: detection of clinical isolates by multiplex PCR. Int Microbiol. 2011; 14: 13-17.
32. Pinna, A., Zanetti, S., Sotgiu, M., et al. Identification and antibiotic susceptibility of coagulase negative staphylococci isolated in corneal/external infections. Br J Ophthalmol. 1999; 83: 771-773.
33. Pinheiro, L., Mello, P. L., Abraao, L. M., et al. Evaluation of reference values for phenotypic tests to detect oxacillin resistance in coagulase-negative staphylococci. Future Microbiol. 2018; 13 (5): 565-575.
34. Courvalin, P., Leclercq, R., et Bingen, E. Antibiogramme. Annexe. 2e édition ESKA; 2006.
35. Jain, A., Agarwal, J., and Bansal, S. Prevalence of methicillin-resistant, coagulase negative staphylococci in neonatal intensive care units: findings from a tertiary care hospital in India. J Med Microbiol. 2004; 53: 941-944.
36. Boukhatem, M. N., Ferhat, M. A., Mohamed, H., et al. Prevalence and antibiotic resistance of Staphylococci isolated from Kolea Hospital, Algeria. Journal of Fundamental and Applied Sciences. 2015; 7 (2): 260-270.
37. Farid, A., Naz, I., Ashraf, A., et al. Molecular detection of antimicrobial resistance in local isolates of *Staphylococcus epidermidis* from urinary tract infections in Faisalabad region of Pakistan. EXCLI Journal. 2015; 14: 697-705.
38. Gatermann, S. G., Koschinski, T., and Friedrich, S. Distribution and expression of macrolide resistance genes in coagulase-negative staphylococci. Clin Microbiol Infect. 2007; 13: 777-778.
39. Nasr, R. A., AbuShady, H. M., and Hussein, H. S. Biofilm formation and presence of *ica*AD gene in clinical isolates of staphylococci. The Egyptian Journal of Medical Human Genetics. 2012; 13: 269-274.
40. Becker, K., Heilmann, C. and Peters, G. Coagulase-Negative Staphylococci. CMR. 2014; 27: 870-926

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Original Article **Open Access**

Phenotypic and genotypic identification of *Staphylococcus aureus* resistant to clindamycin in Mansoura University Children Hospital, Egypt

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Abstract:

Background: Clindamycin has been a good alternative drug to penicillins in the treatment of infections caused by *Staphylococcus aureus* but resistance to this agent has led to therapeutic failure. Inducible clindamycin resistance in staphylococci carrying *erm* genes may not be detectable by routine disk diffusion test. The objective of this study is to phenotypically detect clindamycin resistance in clinical *S. aureus* isolates and determine the prevalence of *erm*A, *erm*B and *erm*C carriage among these isolates.
Methodology: A total of 230 non-duplicate *S. aureus* were isolated from children admitted to Mansoura University Children Hospital during the period January 2016 and June 2017 by conventional microbiology method. *Invitro* antibiotic susceptibility to selected antibiotics including erythromycin was performed by the disk diffusion technique. The „D-zone" test was used to phenotypically detect inducible clindamycin resistance. The presence of *erm*A, *erm*B and *erm*C genes was confirmed by multiplex polymerase chain reaction (m-PCR) assay.
Results: One hundred and seven (46.6%) isolates were phenotypically resistant to erythromycin while 109 (47.3%) were methicillin (cefoxitin) resistant *S. aureus* (MRSA). The macrolide- lincosamin-streptograminB (MLS_B) phenotypes among the erythromycin resistant isolates were 47 (44%) inducible MLS_B and 46 (43%) constitutive MLS_B, while 14 (13.0%) were MS phenotype. Although, the MLS_B phenotype was more predominant in MRSA (n=60, 56.1%) than MSSA (n=33, 30.7%) while the MS phenotype was more predominant in MSSA (n=9, 8.4%) than MRSA (n=5, 4.6%) isolates, the difference was not statistically significant (p=0.0777). The *erm*A (29.0%, n=31) and *erm*C (18.7%, n=20) were the most prevalent genes carried by the isolates while *erm*B was carried by a few (4.7%, n=5). Forty six (43%) isolates did not carry any detectable *erm* gene.
Conclusion: In this study, both inducible and constitutive clindamycin resistance phenotypes were common among *S. aureus* isolates. Although the genetic basis for this may be attributed to carriage of *erm*A, *erm*B and *erm*C genes, a number of the resistant isolates did not carry any of these genes.

Keywords: phenotypic, genotypic, macrolide-lincosamide-streptogramin B, *S. aureus*, children,

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Identification phénotypique et génotypique de *Staphylococcus aureus* résistant à la clindamycine à l'Hôpital Universitaire Mansoura, Egypte

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Abstrait:

Contexte: La clindamycine était un bon médicament alternatif aux pénicillines dans le traitement des infections causées par *Staphylococcus aureus*, mais la résistance à cet agent a entraîné un échec thérapeutique. La résistance inductible à la clindamycine chez les staphylocoques porteurs du gène erm peut ne pas être détectable par le test de diffusion systématique sur disque. L'objectif de cette étude est de détecter phénotypiquement la résistance à la clindamycine dans les isolats cliniques de S. aureus et de déterminer la prévalence du portage d'*erm*A, d'*erm*B et d'*erm*C parmi ces isolats.
sélection d"antibiotiques, notamment à l"érythromycine, a été réalisée par la technique de diffusion sur disque. Le test de la «zone D» a été utilisé pour détecter phénotypiquement la résistance inductible à la clindamycine. La présence des gènes *erm*A, *erm*B et *erm*C a été confirmée par un test de réaction en chaîne de la polymérase multiplexe (m-PCR).
Résultats: Cent sept isolats (46,6%) étaient phénotypiquement résistants à l'érythromycine, tandis que 109 (47,3%) étaient résistants à la méthicilline (céfoxitine) *S. aureus* (MRSA). Les phénotypes macrolide-lincosamin-streptogramine B (MLS_B) parmi les isolats résistants à l'érythromycine étaient 47 (44%) MLS_B inductibles et 46 (43%) MLS_B constitutifs, alors que 14 (13,0%) étaient des phénotypes MS. Bien que le phénotype MLS_B soit plus prédominant dans le SARM (n=60, 56,1%) que le MSSA (n=33, 30,7%), le phénotype MS était plus prédominant dans le MSSA (n=9, 8,4%) que le SARM (n=5, 4,6%), la différence n'était pas statistiquement significative (p=0,0777). *Erm*A (29,0%, n=31) et *ermC* (18,7%, n=20) étaient les gènes les plus prévalents portés par les isolats, tandis que ermB était porté par quelques-uns (4,7%, n=5). Quarante-six (43%) isolats ne portaient aucun *erm* gène détectable.
Conclusion: Dans cette étude, les phénotypes de résistance à la clindamycine tant inductibles que constitutifs étaient courants parmi les isolats de *S. aureus*. Bien que la base génétique de ceci puisse être attribuée au portage des gènes *erm*A, *erm*B et *erm*C, un certain nombre des isolats résistants ne portaient aucun de ces gènes.

Mots-clés: phénotypique, génotypique, macrolide-lincosamide-streptogramine B, *S. aureus*, enfants

Introduction:

Staphylococcus aureus is a common bacterial pathogen responsible for both community and hospital acquired infections. The infection caused by *S. aureus* ranges from mild skin and soft tissue to life threatening infections such as septicemia, meningitis and endocarditis (1). The major concern about this pathogen is its ability to acquire resistance to multiple antibiotics. Resistance to penicillin and methicillin has led to the use of alternative antibiotics especially macrolides and lincosamides such as clindamycin. Clindamycin can be given both orally and intravenously and it is effective against soft tissue infections as well as deep penetrating infections such as sepsis (2).

Resistance to clindamycin is usually associated with macrolides resistance especially erythromycin (3-7). The responsible mechanisms for macrolide and clindamycin resistance are attributed to two mechanisms (8). The first mechanism is due to the efflux of antibiotics outside the bacterial cell wall before its binding to ribosome. This mechanism is commonly reported in *S. aureus* and is controlled by *msr*A gene (9). The other mechanism is called MLS_B (macrolide, lincosamide, streptogramin B) which is due to changes on the ribosome affecting the binding site as a result of methylation of the 23S rRNA–binding site of macrolides. This results in resistance to macrolides and is controlled by the *erm*A, *erm*B and *erm*C genes (8, 9).

Clindamycin resistance can be detected phenotypically by two methods. The first method detects constitutive clindamycin resistance ($cMLS_B$) phenotype by the disk diffusion technique. The other method called the "D-zone" test detects inducible clindamycin resistance ($iMLS_B$) in susceptible isolates by adding erythromycin (which is a known strong methylase inducer in susceptible strains) to clindamycin disks, which results in appearance of a blunting of the clindamycin inhibition zone (appearing like a letter D) on the margin closest to the erythromycin disk (10).

Published data on the presence of *S. aureus* strains resistant to clindamycin in Egypt are lacking, however, information on this could have important implications for treatment protocols against staphylococcal infections in the country. The aim of this research therefore is to determine the carriage rate of *erm* genes responsible for inducible clindamycin resistance among clinical *S. aureus* isolates in Egypt.

Materials and method:

Study design and subjects

This cross sectional study was conducted on randomly selected children admitted to Mansoura University Children Hospital, Egypt, between January 2016 and June 2017, from whom 230 non-repetitive *S. aureus* isolates were obtained. The study was approved by Mansoura Faculty of Medicine Institutional Ethical Committee

Specimen collection

The samples for *S. aureus* isolation were from various clinical specimens such as

blood cultures (n=157), wound samples (n=41), and broncho alveolar lavage (n=32).

Isolation and identification of *S. aureus*

All specimens were inoculated on Sheep blood and MacConkey agar plates, and incubated at 37°C aerobically for 24 hours. Identification of *S. aureus* was first done by colony morphology on plates, followed by conventional identification methods of Gram stain, catalase activity, and slide and tube coagulase tests.

Antibiotic susceptibility test

Antibiotic susceptibility test (AST) was performed on all identified *S. aureus* isolates (n=230) in accordance with the Clinical and Laboratory Standards Institute (CLSI) agar disk diffusion method on Mueller-Hinton (MH) agar (11) with the following disks (Oxoid, England); erythromycin (15µg), ampicillin-sulbactam (20 µg / 20 µg), amoxicillin-clavulanic acid (20 µg / 10 µg), piperacillin (30µg), ciprofloxacin (5µg), ceftriaxone (5µg), cephradine (30µg), cefotaxime (30µg), ceftazidime (30µg), and cefoperazone (75µg).

Detection of methicillin resistance

Methicillin resistance was detected by the cefoxitin (30µg) disk diffusion test on MH plates which were inoculated with the standardized inoculum of the *S. aureus* isolates (n=230), and incubated at 37°C for 24 hours. *S. aureus* with cefoxitin inhibition zone ≤ 21mm were considered MRSA (10).

Detection of clindamycin resistance

All *S. aureus* isolates resistant to erythromycin in the AST (n=107) were evaluated for clindamycin resistance by the disk diffusion method as previously described (10). The isolated colonies were diluted in sterile normal saline to obtain 0.5 McFarland"s standard suspension. The inoculum was aseptically plated over MH agar plate, with both erythromycin (15µg) and clindamycin (2µg) disks placed ~15 mm apart edge to edge, and incubated aerobically at 37°C for 24 hours. Isolates resistant to erythromycin (zone of inhibition <13mm) with flattening of zone (D-zone) around the clindamycin disk(zone of inhibition≥21mm) were identified as inducible resistance phenotype (Fig 1). Isolates resistant to both erythromycin and clindamycin (zone of inhibition<21mm) were identified as constitutive resistance phenotype ($cMLS_B$) and isolates resistant to erythromycin but susceptible to clindamycin (zone of inhibition ≥21mm) with no D-zone were identified as MS phenotype (10).

Multiplex PCR for macrolide genes:

DNA extraction of purified colonies of all *S. aureus* resistant to erythromycin in the AST (n=107) was done by Qiagen extraction kit and kept frozen at -20°C until use. Multiplex PCR was performed for the isolates for detection of *erm*A, *erm*B and *erm*C genes using primers with sequences as shown in Table 1 (9). Amplification was performed in a 25µL PCR mixture (Qiagen) with the use of 50ng DNA template, and 25 pmol/L each, of forward and reverse primers. Thermal cycling was performed as follows; 5 min at 94°C, then 1min at 95°C, 30 sec at annealing temp of 55°C, and 1min at 72°C for 30 cycles, with a final extension at 72°C for 5min.

Ten µL of PCR product was resolved on 2% agarose gel containing 0.5mg/mL ethidium bromide at 90V for 1h and visualized in a gel documentation system. The reaction mix containing all materials except DNA was used as negative control.

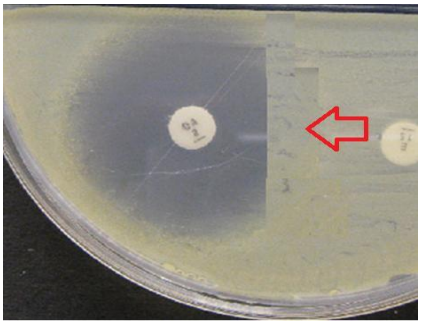


Fig 1: Mueller Hinton agar plate showing positive D-zone test in a *Staphylococcus aureus* isolate with inducible MLS_B phenotype

Table 1: Genes and the primers sequences used in the study

Target gene	Primer sequence	Product size, bp	Reference
ermA	F 5´-TAT CTT ATC GTT GAG AAG GGA TT-3	139	8
	R 5´-CTA CAC TTG GCT TAG GAT GAA A-3´´		
ermB	F 5´-CTA TCT GAT TGT TGA AGA AGG ATT-3´	142	8
	R 5´-GTT TAC TCT TGG TTT AGG ATG AAA-3´		
ermC	F 5´-CTT GTT GAT CAC GAT AAT TTC C-3´	190	8
	R 5´-ATC TTT TAG CAA ACC CGT ATT C-3´		

Results:

A total of 230 non-repetitive *S. aureus* were isolated from the subjects, mainly from blood cultures (n=157, 68.4%), wound samples (n=41, 17.9%) and broncho-alveolar lavage (n=32, 13.7%). All isolates were resistant to the tested antibiotics to varying degrees, with 109 resistant to cefoxitin, representing MRSA rate of 47.3%, and 107 (46.6%) resistant to erythromycin (Table 2).

Table 2: *Invitro* antibiotic resistance of *Staphylococcus aureus* in Mansoura University Children Hospital, Egypt

Antibiotic disk	Frequency of resistance (%)
Ciprofloxacin	64 (27.8)
Ceftazidime	44 (19.1)
Ceftriaxone	62 (26.9)
Ampicillin/Sulbactam	62 (26.9)
Cefoperazone	38| (16.3)
Cefotaxime	52 (22.6)
Cephradine	56 (24.3)
Piperacillin	32 (13.9)
Amoxicillin/Clavulanic acid	120 (52.1)
Erythromycin	107 (46.6)
Cefoxitin	109 (47.3)

The result of the D-zone test shows that 93 of the 107 (86.9%) erythromycin resistant *S. aureus* isolates were MLS_B phenotype (iMLS_B, 43.9% and cMLS_B, 43.0%) while 14 (13.1%) were MS phenotype (Table 3).

Although, the MLS_B phenotype was more predominant in MRSA (n=60, 56.1%) than MSSA (n=33, 30.8%) while the MS phenotype was more predominant in MSSA (n=9, 8.4%) than MRSA (n=5, 4.7%) isolates, the difference was not statistically significant (X^2 = 3.112, p = 0.0777).

Table 4 shows the carriage rates of *erm* genes among the 107 erythromycin resistant *S. aureus* isolates, with *erm*A (29.0%, n=31), *erm*C (18.7%, n=20) and *erm*B (4.6%, n=5). One isolate (1.0%) carried both *erm*A and *erm*C genes while 4 (3.7%) isolates each carried *erm*A, *erm*B and *erm*C genes. A total of 46 (43.0%) isolates did not contain any of the *erm* genes. Fig 2 shows gel electrophoresis of isolates with amplified *erm*A and *erm*C genes, and others with no amplified gene product.

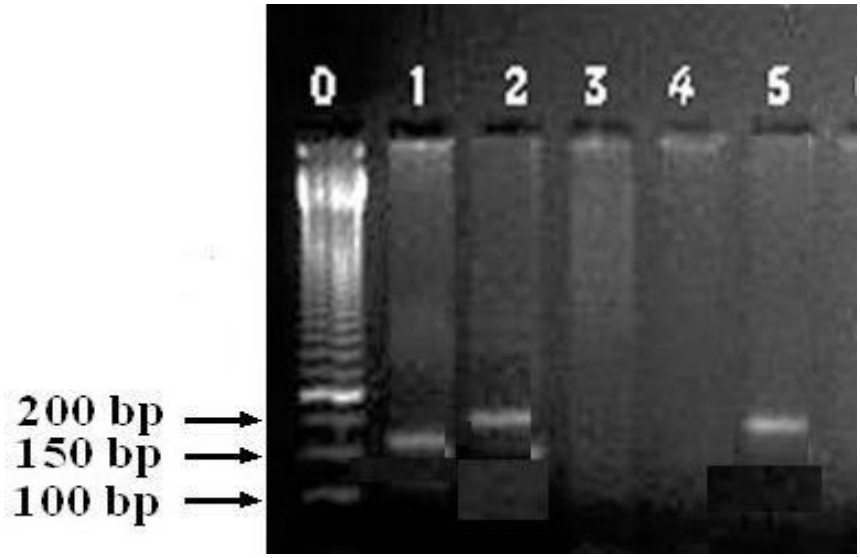
Table 4: Frequency of *erm* genes in phenotypic macrolide resistant *Staphylococcus aureus* isolates in Mansoura University Children Hospital, Egypt

Macrolide resistance genes	Frequency (%)
ermA	31(29.0)
ermB	5 (4.6)
ermC	20 (18.7)
ermA/ermC	1 (1.0)
ermA/ermB/ermC	4 (3.7)
No gene	46 (43.0)
Total	107

Table 3: Distribution of macrolide resistance phenotypes among *Staphylococcus aureus* isolates in Mansoura University Children Hospital, Egypt

Macrolide resistance phenotype	*Staphylococcus aureus* isolates		Total (%)	X^2	*p* value
	MRSA (%)	MSSA (%)			
Inducible MLS_B	27 (25.2)	20 (18.7)	47 (43.9)	3.112	0.0777
Constitutive MLS_B	33 (30.8)	13 (12.2)	46 (43.0)		
Sub-Total	60 (56.1)	33 (30.8)	93 (86.9)		
MS (ERY-R/CLI-S)	5 (4.7)	9 (8.4)	14 (13.1)		
Total	65 (60.8)	42 (39.2)	107 (100)		

Ery-R = erythromycin resistant; CLI-S = clindamycin sensitive; MRSA = methicillin resistant *Staphylococcus aureus*; MSSA= methicillin sensitive *Staphylococcus aureus*; MLSB = macrolide-lincosamide-streptograminB



0 = molecular weight marker; 1 = isolate with amplified *erm*A gene; 2 & 5 = isolates with amplified *erm*C gene; 3 & 4 = isolate with no gene amplified

Fig 2: Gel electrophoresis of amplified *erm* genes in some macrolide resistant *Staphylococcus aureus* isolates

Discussion:

The prevalence of MRSA has been increasing worldwide since last decades. This prevalence varies widely across different countries and also in hospitals of same country. In this study, isolated *S. aureus* strains showed high resistance to cefoxitin, representing MRSA rate of 47.3%, compared to a lower rate of 24% previously reported in a study from Minia, Egypt. However various studies have reported MRSA rate ranging from 50% to 68% (10,12,13).

There was also relatively high resistance to other beta lactam antibiotics such as ampicillin-sulbactam (26.9%), amoxicillin-clavulanic acid (52.1%), piperacillin (13.9%), ceftriaxone (26.9%), ceftazidime (19.1%), cefotaxime (22.6%), cephradine (24.3%), and cefoperazone (16.3%) in this study. This multidrug resistance among *S. aureus* can be explained by the common association of *mec*A (methicillin gene) with other resistant genes for beta lactamase production, leading to cross resistance to other beta lactam antibiotics. Previous studies have shown such association (14, 15).

Phenotypic resistance to erythromycin was 46.6% in this study. Similar rates of resistance to erythromycin were reported previously ranging from 46% to 51.7% (16-18) whereas lower rates ranging from 15.7% to 28.4% were reported by others (19,20). The MRSA isolates is our study had higher rate for both inducible MLS_B (25.2%) and constitive MLS_B (30.8%) phenotypes than the MSSA isolates (though not statistically significant), which agrees with the study of Prabhu et al., who reported 16.6% $cMLS_B$ phenotype in MRSA and 6.2% in MSSA (10). Also Gupta et al., reported $cMLS_B$ phenotype in 19% of the total isolates of which 46% were MRSA and 10% were MSSA (8). The phenotype of MLS_B (either constitutive or inducible) may show great variations based on the patient groups in different hospitals and geographical regions. This variation may be attributed to several factors such as usage rate of macrolide antibiotics in different hospitals, age of patients, and source of tested isolates (15).

Clindamycin resistance is usually attributed to the carriage of any of *erm*A, *erm*B or *erm*C genes. The association of MRSA with clindamycin resistance can be attributed to acquisition of one of these genes via transposition to become part of the SCC*mec* cassette chromosomes containing the methicillin resistant (*mec*A) genes (16). In our study, *erm*A and *erm*C genes were the two genes frequently carried by the *S. aureus* isolates, while a few isolate carried the *erm*B gene. This is similar to the report of a study in Denmark over a 30 year period (1959–1988) by Westh et al., which showed high carriage rates of *erm*A and *erm*C genes among the *S. aureus* isolates but no *erm*B gene detected (21). Similarly, a multicenter study of 24 European University Hospitals in the year 2000 reported high prevalence of *erm*A (64.0%) and low prevalence of *erm*C

(19.5%) but much lower prevalence of *erm*B (0.6%) genes among 851 *S. aureus* isolates (22). A more recent study on animal staphylococci showed a similar pattern with carriage rate of 55.6% *erm*A, 30.6% *erm*C and 11.1% *erm*B genes (23). These are similar to the findings in our study.

Conclusion:

From this study, we can conclude that clindamycin resistance is common among *S. aureus* isolates in Mansoura Children University Hospital, Egypt both inducible and constitutive types carrying mostly *erm*A and *erm*C genes that may be partly responsible for these phenotypes. Although, we did not find statistically significant association of clindamycin with methicillin resistance, nevertheless, we recommend routine mandatory testing of all *S. aureus* isolates for inducible clindamycin resistance in this hospital.

References

1. Projan, S. J., and Novick, R. P. The molecular basis of pathogenicity. In: Crossley K. B., and Archer, G. L. (eds). The staphylococci in human diseases, New York, Churchill, Livingstone: 1997: 55–81.
2. Bradley, J. R. Newer antistaphylococcal agents. Curr Opin Paediatr. 2005; 17: 71-77
3. Daurel, C., Huet, C., Dhalluin, A., et al. Differences in potential for selection of clindamycin-resistant mutants between inducible *erm*(A) and *erm*(C) *Staphylococcus aureus* genes. J Clin Microbiol. 2008; 46 (2): 546–550
4. Patil, N., Mali, U., Kulkarni, S., Ghorpade, M., and Mane, V. Detection of inducible clindamycin resistance among clinical isolates of *Staphylococcus aureus* in a tertiary care hospital. Int J Curr Microbiol App Sci. 2014; 127: 689-694
5. Lim, J. A., Kwon, A. R., Kim, S. K., Chong, Y., Lee, K., and Choi, E. C. Prevalence of resistance to macrolide, lincosamide and streptogramin antibiotics in Gram-positive cocci isolated in a Korean hospital. J Antimicrob Chemother. 2002; 49: 489–495
6. Gadepalli, R., Dhawan, B., Mohanty, S., Kapil, A., Das, B. K., and Chaudhry, R. Inducible clindamycin resistance in clinical isolates of *Staphylococcus aureus.* Indian J Med Res. 2006; 123: 571 - 573
7. Angel, M. R., Balaji, V., Prakash, J., Brahmadathan, K. N., and Mathews, M. S. Prevalence of Inducible clindamycin resistance in Gram positive organisms in a tertiary care center. Indian J Med Microbiol. 2008; 26: 262-264
8. Gupta, V., Datta, P., Rani, H., and Chander, J. Inducible clindamycin resistance in *Staphylococcus aureus*: a study from North India. J Postgrad Med. 2009; 55: 176-179
9. Martineau, F., Picard, F., Lansac, N., Menard, C., Roy, P. H., Ouellette, M., and Bergeron, M. G. Correlation between the Resistance Genotype Determined by Multiplex PCR Assays and the Antibiotic Susceptibility Patterns of *Staphylococcus aureus* and *Staphylococcus epidermidis*. Antimicrob Agents and Chemother. 2000; 44: 231-238
10. Prabhu, K., Rao, S., and Rao, V. Inducible clindamycin resistance in *Staphylococcus aureus* isolated from clinical samples. J Lab Phys. 2011; 3: 25-27
11. Clinical and Laboratory standards Institute (CLSI). Performance standards for Antimicrobial Susceptibility Testing; Twenty Second Informational Supplement, M100-S22, Wayne, 2012
12. Lewis, M. T., Gales, A. C., and Sader, H. S. Frequency of occurrence and antimicrobial susceptibility patterns for pathogens isolated from Latin-American patients with a diagnosis of pneumonia: results from the antimicrobial surveillance program (1998). Diagn Microbial Infect Dis. 2000; 37: 63–74.
13. Barisic, Z., Babic-Erceg, A., Borzic, E., et al. Urinary tract infections in South Croatia: aetiology and antimicrobial resistance. Int J Antimicrob Agents. 2003; 2: 61–64
14. Bashir, A., Mujahid, T. Y., and Jehan, N. Antibiotic resistance profile: isolation and characterization of clinical isolates of staphylococci from patients with community-acquired skin infections. Pak J Pharm Sci. 2007; 20: 299–304
15. Ojulong, J., Mwambu, T. P., Jalooba, M., Bwanga, F., and Kaddu-Mulindwa, D. H. Relative prevalence Methicillin Resistant *Staphylococcus aureus* and its susceptibility pattern in Mulago Hospital, Kampala, Uganda. Tanzania J Health Research. 2009; 11 (3): 149–153.
16. Leclercq, R. Staphylococcus resistant to antibiotic therapy. Annales Francaises d'anaestheise et de Reanimation. 2002; 21: 375–383
17. Lyall, K. S., Gupta, V., and Chhina, D. Inducible clindamycin resistance among clinical isolates of *Staphylococcus aureus*. J Mahatma Gandhi Inst Med Sci. 2013; 18: 112-115
18. Sande, S. V. Phenotypic detection and incidence of inducible clindamycin resistance among *Staphylococcus aureus* from tertiary care hospital. Int J Adv Med. 2015; 2 (3): 264-268.
19. Ajanta, G. S., Kulkarni, R. D., Shetty, J., Shukhada, C., and Jain, P. Phenotypic detection of Inducible clindamycin resistance among *S. aureus* isolates by using the lower limit of recommended inter disk distance. Indian J Pathol Microbiol. 2008; 51 (3): 376 - 378.
20. Goyal, R., Singh, N. P., Manchanda, V., and Mathur, M. Detection of clindamycin susceptibility in macrolide resistant phenotypes of *Staphylococcus aureus*. Indian J Med Microbiol. 2004; 22: 251-254
21. Westh, H., Hougaard, D. M., Vuust, J., and Rosdahl, V. T. Prevalence of *erm* gene classes in erythromycin resistant *Staphylococcus aureus* strains isolated between 1959 and 1988. Antimicrob Agents Chemother. 1995; 39 (2): 369–373. doi:10.1128/aac/39.2.369
22. Schmitz, F. J., Sadurski, R., Kray, A., Boos, M., Geisel, M., Köhrer, K., Verhoef, J., and Fluit, C. Prevalence of macrolide-resistance genes in *Staphylococcus aureus* and *Enterococcus faecium* isolates from 24 European university hospitals. J Antimicrob Chemother. 2000; 45: 891-894
23. Igbinosa, E. O., and Beshiru, A. Characterization of antibiotic resistance and species diversity of staphylococci isolated from apparently healthy farm animals. Afr J Clin Exper Microbiol. 2019; 21 (4): 289-298

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Original Article **Open Access**

Cytomegalovirus co-infection with HIV in children and adolescents on antiretroviral therapy in Abuja, Nigeria

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Abstract:

Background: Cytomegalovirus (CMV) co-infection with human immunodeficiency virus (HIV) is known to accelerate HIV disease progression. It has the potential of being a killer disease or a silent lifetime companion in HIV patients. There is dearth of information on CMV prevalence among HIV infected children and adolescents in our environment. We therefore conducted this study to determine its sero-prevalence, and risk factors for co-infection among HIV infected children and adolescents on highly active antiretroviral therapy (HAART) in our center.
Method: A descriptive cross sectional study of HIV-infected children and adolescents aged 2 months to 18 years on HAART was conducted over a 6 month period between October 2017 and March 2018 in our health facility. Blood samples of subjects were screened for CMV IgM using commercial test kits. Biodata of subjects, CD4 cell count, and viral load were collected into a designed proforma, and statistical analysis was done with SPSS version 22.0.
Result: A total of 161 HIV-infected children and adolescents were recruited, 103 (64.0%) were males, 83 (51.6%) were between the ages of 5 and <10 years, 113 (70.2%) were from lower socio-economic class, and 138 (85.7%) were on 1st line HAART. Of the 17 (10.6%) subjects positive for CMV IgM, 3 (17.6%) were less than 5 years old, 11 (64.7%) were between the ages of 5-10 years, and none was older than 15 years. Univariate analysis showed significant differences in the mean age, weight, length/height, and systolic blood pressure between CMV IgM positive and negative patients (p<0.05), but no significant difference in gender, socioeconomic class, types of antiretroviral drugs, CD4 cell count, and viral load (p>0.05). Multivariate analysis however did not show any significant difference in age, weight, length/height, and systolic blood pressure.
Conclusion: The prevalence of active CMV infections among HIV infected children and adolescents on HAART in our centre is high. Low CD4 cell count and high viral load were not associated with active CMV disease, and no risk factor for co-infection was also identified. Identifying those with primary/active infection will be necessary for possible treatment with anti-herpes drugs before development of reactivated CMV disease.

Keywords: CMV; HIV; co-infection; anti-retroviral; children; adolescents

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Co-infection par le cytomégalovirus et le VIH chez des enfants et des adolescents sous traitement antirétroviral à Abuja, au Nigéria

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Abstrait:

Contexte: On sait que la co-infection par le cytomégalovirus (CMV) et le virus de l'immunodéficience humaine (VIH) accélère la progression de la maladie. Il a le potentiel d'être une maladie mortelle ou un compagnon

silencieux à vie chez les patients VIH. Il existe peu d'informations sur la prévalence du CMV chez les enfants et les adolescents infectés par le VIH dans notre environnement. Nous avons donc mené cette étude pour déterminer sa séroprévalence et les facteurs de risque de co-infection chez les enfants et les adolescents infectés par le VIH sous traitement antirétroviral hautement actif (HAART) dans notre centre.
Méthode: Une étude transversale descriptive des enfants et adolescents infectés par le VIH et âgés de 2 mois à 18 ans sous multithérapie a été menée sur une période de 6 mois entre octobre 2017 et mars 2018 dans notre établissement de santé. Des échantillons de sang de sujets ont été testés pour l'IgM de CMV en utilisant des kits de test commerciaux. Les données biologiques des sujets, le nombre de cellules CD4 et la charge virale ont été recueillis dans un formulaire conçu à cet effet et une analyse statistique a été réalisée avec SPSS version 22.0.
Résultat: 161 enfants et adolescents infectés par le VIH ont été recrutés, dont 103 (64,0%) étaient des hommes, 83 (51,6%) étaient âgés de 5 à moins de 10 ans, 113 (70,2%) étaient issus de milieux socio-économiques inférieurs. et 138 (85,7%) suivaient la multithérapie de première ligne. Sur les 17 (10,6%) sujets positifs pour l'IgM du CMV, 3 (17,6%) avaient moins de 5 ans, 11 (64,7%) étaient âgés de 5 à 10 ans et aucun n'avait plus de 15 ans. Une analyse univariée a montré des différences significatives dans l'âge moyen, le poids, la taille / taille et la pression artérielle systolique entre les patients positifs et négatifs pour IgM anti-CMV (p<0,05), mais aucune différence significative entre le sexe, la classe socio-économique, les types de médicaments antirétroviraux et les cellules CD4 nombre et charge virale (p>0,05). L'analyse multivariée n'a cependant montré aucune différence significative d'âge, de poids, de taille / taille et de pression artérielle systolique.
Conclusion: La prévalence des infections à CMV actives chez les enfants et les adolescents infectés par le VIH sous HAART dans notre centre est élevée. Un faible nombre de cellules CD4 et une charge virale élevée n'étaient pas associés à la maladie à CMV active et aucun facteur de risque de co-infection n'a également été identifié. Identifier les personnes présentant une infection primaire / active sera nécessaire pour un traitement éventuel avec des médicaments anti-herpès avant le développement d'une maladie à CMV réactivée.

Mots-clés: CMV; HIV; co-infection; anti-rétroviral; Enfants; les adolescents

Introduction:

Cytomegalovirus also known as human herpes virus (HHV) 5 is one of the eight HHVs, belonging to beta herpesvirus subfamily. Infection is ubiquitous, and generally asymptomatic in healthy children and adults. Infants usually acquire the infection while in the uterus, during passage through the birth canal, or through breast milk. Young children are frequently infected by contaminated saliva when sucking and sharing toys. The virus can also be contacted through respiratory routes, pharyngeal secretions, tears, urine, faeces, seminal and vaginal fluids, during blood transfusion, organ transplant, or through sexual contact (1). Infection is largely contacted during childhood or during sexual activities in human population worldwide with some regional variations (1). Following primary infection, the virus becomes latent in a few cells in its episomal form (2). Reactivation with viral shedding occurs when immunity is compromised as seen in HIV co infection (2).

CMV is widely recognized as an opportunistic pathogen causing severe opportunistic infections in immuno-compromised individuals which generally manifests as retinitis with high tendency to cause rapid loss of vision, chronic crippling diarrhea, central nervous system diseases and sudden loss of neurologic function, and pneumonia (3, 4). CMV active infection might be a marker of extremely severe immunosuppression that can ultimately lead to a fatal outcome in immuno-compromised individual. Primary CMV infection occurs in a previously sero-negative individual and can be a potential killer in such individual, whereas secondary infection which is intermittent excretion of the virus in the presence of host immunity may be due to either reactivation of an endogenous virus or exposure to a new virus strain from an exogenous source. (5). Hence the presence of the IgM antibodies may be due to primary/active infection or re-infection (6).

Persistent immune activation is hallmark of human immunodeficiency virus infection, and in co-infected individual, CMV-induced T cell activation potentially contributes to increased HIV disease progression (7). The two viruses while infecting a number of similar cell types such as the endothelium of blood vessels, mononuclear cells, white blood cells, and epithelial cells, also interact through transactivation, secretion of cytokines that reactivate provirus, or increase in HIV tropism through CMV expression of receptor analogues or by formation of pseudoviruses (8). HIV infection induces immunosuppression through depletion of CD4 cell count with increasing CMV reactivation, the reactivation promote HIV replication through a complex interaction with the long terminal repeat region, transactivation of proviral HIV, release of inflammatory cytokines and chemokines, and up-regulation of CCR5 expression in central memory T cells (9-11). During the era of antiretroviral therapy (ART), accumulated data in adults suggests that CMV co-infection with HIV contributes

significantly to the accelerated HIV disease progression and development of non AIDS-defining comorb- idities (12-17). The complex interplay of these two chronic viral infections continues to be potentially significant especially in highly endemic area of sub-Saharan Africa, where both viruses are endemic (18).

In industrialized countries such as USA, Australia and Europe, CMV sero-prevalence among adult population is between 36 and 77%, in contrast to highly endemic areas of sub-Saharan Africa, where sero-prevalence approaches 100% (19). There is serological evidence of CMV infection in almost two thirds of infants from African origin by 3 months of age, and 85% are infected by a year (20). Among HIV-infected children in Africa, the majority are co-infected with CMV by their first birthday (20) and almost all by the time they reach their teens, in contrast to what is seen in industrialized nations (7, 21).

Antibody testing can be used to determine recent or past exposure to CMV infection. The first antibody to develop in response to CMV infection is IgM. This develops within a few days following primary infection. While CMV IgM remains detectable for six to nine months in the blood, medium to high levels of CMV IgM can be detected during the first three months of a primary infection. IgM is also detected during secondary infections either as re-activation or re-infection. By assessing the presence of IgM in a sample, active CMV infection can be ascertained. This study was therefore conducted to determine the prevalence of active CMV infection in HIV infected children and adolescents on ART in our health facility. The study also aimed to determine the risk factors for acquisition of the CMV, and the effects of co-infection on CD4 cell count and viral load of HIV patients.

Materials and method:

Study setting and design

A cross sectional hospital based study was conducted at the Paediatric Out-patient Special Treatment Clinic (POSTC) of the University of Abuja Teaching Hospital (UATH) over a 6 months period from October 2017 to March 2018. POSTC is an arm of out-patient service area of department of paediatrics where HIV infected children/adolescents and exposed babies are seen and followed up for treatment/monitoring. The clinic is opened for services from Monday-Friday, and from 7.30 am to 4 pm. UATH is a 350 bed capacity referral hospital, sub-serving the people of Federal Capital Territory (FCT) Abuja and neighbouring states of Nassarawa, Kogi, Kaduna, and Niger States. It is one of the first centers since 2005 to start offering free HIV/AIDS services in the country through the President Emergency Plan for AIDS Relief (PEPFAR) and the Federal Government of Nigeria (FGN).

Subjects

The subjects were paediatric patients' 2 months to 18 years old tested positive for HIV by either serological method or by polymerase chain reaction (PCR) test and started on anti retroviral therapy (ARV) therapy. Consecutive eligible children and adolescents 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 assent for the study. Inclusion criteria for the study were; HIV infected children and adolescents aged 2 months to 18 years on ARV therapy, parents/caregivers and older children accepting to be part of the study. Exclusion criteria included those unwilling to participate in the study, and exposed uninfected infants. Ethics clearance was obtained from the ethics committee of the hospital before the commencement of the study.

Clinical data and sample collection

Clinical and physical examination were carried out after enrollment by the attending physician. The demographic characteristics of the subjects were collected which included age, gender, religion, and socio economic statue (SES) of the parents. Using a vaccutainer needle, three milliliters (3 mls) of venous blood was collected from each subject and transported to the laboratory for analysis. In addition, the weight, blood pressure, CD4 cell count, and viral loads (VL) were retrieved from the patient's information record if done within 1 month of the study or if not, they were freshly performed.

Laboratory analysis

The serum was separated by centrifugation at 3500 rpm for 5 minutes and refrigerated (2-8°C) until analysis was done. At the time of analysis, the serum was carefully removed using a fine bore pipette to avoid extracting red cells and the test device removed from the sealed pouch. Screening for CMV IgM was done using commercial fortress diagnostics^{R} (CMV IgM) ELISA immunoassays

kits. The clinical sensitivity and specificity of the Cyto IgM ELISA kits was 99.6% on plasma and sera. The cut-off optical density (O.D) was obtained in accordance with the manufacturer's instructions; the CMV index of the samples was calculated by dividing the O.D value of each of the samples with the obtained Cut-off O.D values. OD interpretation was CMV index of <0.90 as negative and CMV index of 1.0 and above as positive.

CD4 cell count was measured using automated Partec Cyflow easy count kit (Partec code no. 05-8401 Western Germany), Viral load (VL) measurement was performed with Roche Smp/prep/cobs Taqman 96, USA, and Seca beam weighing scale was used to accurately measure weight to the nearest 0.01kg.

Data analysis

Data analysis was done using SPSS version 22.0, and percentage, means and standard deviations were calculated for the variables. The test for association was done using student t-test for continuous variables and Chi-square or Fisher's exact test for categorical variables where applicable. Multivariate logistic model was used to determine associated risk factors of CMV co-infection with HIV, unadjusted and adjusted odds ratio (OR) were reported, and *p* value of <0.05 was considered significant.

Results:

Table 1 shows the demographic and clinical characteristics of the study population. Of a total of 161 HIV infected children and adolescents screened for CMV IgM antibody, 103 (64.0%) were males, 83 (51.6%) were between the ages of 5-10 years, 110 (68.3%) were Christians, 113 (70.2%) were from low socio-economic class and 138 (85.7%) were on 1st line HAART. The mean body weight, CD4 cell count and VL were 30.0±12.4 kg, 979.5±457.7 cells/µl, and 9,136.1±306.0 copies/ml. There was no significant difference in the demographic characteristics between the male and female populations (p>0.05 for all the variables).

Table 2 shows the association of CMV IgM antibody and the co-variables. Seventeen (10.6%) of the 161 patients screened tested positive for the IgM antibody, while 144 (89.4%) were negative. Three (17.6%) of those tested positive were less than 5 years, 11 (64.7%) were between the ages of 5 and <10 years, and none was older than 15 years. On univariate analysis, significant difference was observed in mean age (p=0.025), body weight (p=0.004), length/height (p=0.039), and systolic blood pressure (p=0.001) between those who tested positive and negative for the CMV IgM. On multivariate logistic regression, none of the associated factors (age, body weight, length/height, and systolic blood pressure) showed significant association with CMV infection (p>0.05) (Table 3).

Although there were more male subjects (10, 58.8%), subjects in the age range of 5-<10 years (11, 64.7%), Christians (9, 52.9%), subjects from low socio-economic class (16, 94.1%), and subjects on 1st line ART (15, 88.2%) who tested positive for CMV IgM, the difference was not significantly different from CMV IgM negative subjects (p>0.05 for all the variables). Similarly, there were no statistically significant differences for other parameters; CD4 cell count, VL, BMI, ART duration, and diastolic blood pressure between those positive and negative for CMV IgM (p>0.05 for all variables).

Table 4 depicts the different values of CD4 cell count and VL in relation to CMV IgM positivity and negativity. Only 1 (5.9%) patient with CD4 cell count of <200 cells/µl and 2 (11.8%) with VL of >1000 copies/ml tested positive for CMV IgM. There was no significant difference in the values of CD4 cells and VL between CMV IgM positive and negative patients (p>0.05).

Discussion:

The prevalence of active or primary CMV infection in HIV infected children and adolescents on highly active anti-retroviral therapy (HAART) in this study, was 10.6%. This was comparable to 13% among HIV infected adolescents and adults from Kano by Musa et al., (22), 9.5% among AIDS patients from India by Basawaraju et al., (23) and 11.4% among infected prison inmates by Neusa et al., (24) in Brazil. The rate in our study is however much lower than 58.3% reported among HIV-infected infants initiated on ART in Cameroun by Kfutwah et al.,(26), 28% by Oladipo et al., (27) among blood donors in Nigeria, and 14% among HIV patients by Tsertsvadze et al., (28). The prevalence rates of 3.5% (29), 4.5% (30), 6.6% (31), 7.0% (32), 8.1% (33), and 8.4% (25) from various studies in Nigeria, Qatar, and Kenya were lower than the 10.6% recorded in this study. Although most of these referenced studies were from adult population, pregnant women and blood donors, with only two from HIV children and adolescents, the

Table 1: Demographic and clinical characteristics of HIV-infected children and adolescents in University of Abuja Teaching Hospital, Gwagwalada, Abuja, Nigeria

Characteristics of subjects	Male (%)	Female (%)	Total (%)	*p* value
No of subjects	103 (64.0)	58 (36.0)	161 (100)	0.64
Age group (years)				
<5	11(10.7)	2 (3.4)	13 (8.1)	
5-<10	52 (50.4)	31 (53.4)	83 (51.6)	
10-15	32 (31.1)	21 (36.2)	53 (32.9)	
>15	8 (7.8)	4 (6.9)	12 (7.4)	0.423
Religion				
Christianity	74 (71.8)	36 (62.1)	110 (68.3)	
Islam	29 (28.2)	22 (37.9)	51 (31.7)	0.201
Socio-economic class				
High	11 (10.7)	5 (8.6)	16(9.9)	
Middle	22 (21.4)	10 (17.2)	32(19.9)	
Low	70 (67.9)	43 (74.1)	113(70.2)	0.764
1st or 2nd line ARVT				
1st Line	89 (86.4)	49 (84.5)	138(85.7)	
2nd Line	14 (13.6)	9 (15.5)	23(14.3)	0.738
ARVT duration (years)*	6.7±3.3	7.0±3.5	6.9±3.4	0.695
Wt, CD4 and Viral Load*				
Weight (kg)	29.9±12.2	30.1±12.5	30.0±12.4	0.962
CD4 (cells/µl)	904.8±139.5	1054.1±75.9	958.6±37.8	0.058
Viral Load (copies/ml)	18507.9± 440.6	6206.6±343.4	9136.1±306.0	0.314

ARVT = Antiretroviral therapy; Wt = weight; *=mean values; CD = clusters of differentiation

Table 2: Clinical variables in HIV-infected children and adolescents with CMV co-infections in University of Abuja Teaching Hospital, Gwagwalada, Abuja, Nigeria

Clinical and associated risk factors/variables	**CMV positive (%)**	**CMV negative (%)**	**Total (%)**	**X^2**	***p* value**
Subjects	17 (10.6)	144 (89.4)	161 (100)		
Age Group (years)					
<5	3 (17.6)	10 (6.9)	13 (8.1)	5.558	0.135
5-<10	11 (64.7)	72 (50.0)	83 (51.6)		
10-15	3 (17.6)	50 (34.7)	55 (34.2)		
>15	0	12 (8.3)	16 (9.9)		
Gender					
Male	10 (58.8)	93 (64.6)	103 (63.9)	0.98	0.647
Female	7 (41.2)	51 (35.4)	58 (36.0)		
Religion					
Christianity	9 (52.9)	101 (70.1)	110 (68.3)	2.078	0.149
Islam	8 (47.1)	43 (29.9)	51 (31.7)		
Socio-economic class					
High	0	16 (11.1)	16 (9.9)	0.912	0.634
Middle	1 (5.9)	31 (21.5)	32 (19.9)		
Low	16 (94.1)	97 (67.4)	113 (70.2)		
Types of ARV Drugs					
1st line	15 (88.2)	123 (85.4)	138 (85.7)	0.099	0.753
2nd line	2 (11.7)	21 (14.5)	23 (14.3)		
Co-variables*	**CMV positive (%)**	**CMV negative (%)**	**Mean value**	**T- test**	**p value**
Age (years)	7.65±3.1	10.13±4.4	9.93±4.3	3.64	0.025**
ARV duration (years)	5.41±3.1	6.97±3.4	6.2±3.3	1.81	0.074
Weight (kg)	22.0±5.5	31.0±12.5	26.5±9.0	5.14	0.004**
Length/height (cm)	125.3±12.3	135.7±20.0	130.5±16.2	3.28	0.039**
BMI (kg/m^2)	13.9±1.9	16.9±7.9	15.4±4.9	0.711	0.164
Systolic BP (mmHg)	86.2±9.7	94.1±9.4	90.2±9.6	6.39	0.001**
Diastolic BP (mmHg)	53.5±7.8	57.2±9.4	55.4±8.6	0.64	0.119
CD4 (cells/µl))	919.6±34.1	1009.9±23.7	958.6±37.8	0.69	0.144
Viral Load (copies/ml)	10562.8±357	14491.±774.1	9136.1±306.0	0.87	0.837

ARV= antiretroviral therapy, *= mean values; ** = significant difference; X^2 = Chi square value

Table 3: Multivariate logistic regression of associated risk factors for CMV infection in HIV-infected children and adolescents in University of Abuja Teaching Hospital, Gwagwalada, Abuja, Nigeria

Variables	Unadjusted OR (95 % CI)	*p* value	Adjusted OR (95% CI)	*p* value
Age	0.7 - 0.975	0.017	0.85 - 1.17	0.954
Constant		0.545		0.924
Weight	0.85 - 0.974	0.007	0.69 - 0.99	0.055
Constant		0.685		0.924
Length/Height	0.95 - 0.99	0.049	0.98 - 1.146	0.094
Constant		0.501		0.924
Systolic Blood Pressure	0.86 - 0.968	0.002	0.88 - 1.015	0.118
Constant		0.020		0.924

OR = odd ratio; CI = confidence interval

Table 4: CD4 cell count and Viral Load of HIV infected children and adolescents with CMV IgM in University of Abuja Teaching Hospital, Gwagwalada, Abuja, Nigeria

Variables	CMV IgM positive (%)	CMV IgM negative (%)	Total (%)	X^2	*p* value
CD4 cell count (cells/µl)					
<200	1 (5.9)	2 (1.4)	3 (1.9)	2.631	0.268
200-500	1 (5.9)	22 (15.3)	23 (14.3)		
> 500	15 (88.2)	120 (83.3)	135 (83.8)		
Viral Load (copies/ml)					
< 20	10 (58.8)	65 (45.1)	75 (46.6)	1.389	0.499
20 - 1000	5 (29.4)	48 (33.3)	53 (32.9)		
> 1000	2 (11.8)	31 (21.5)	33 (20.5)		

CD = clusters of differentiation; CMV = cytomegalovirus; IgM = immunoglobulin M; X^2 = Chi square

differences however might not be unrelated to geographical locations of the studies, study populations, ethnic, social, cultural, and economic differences, and different sensitivity of the IgM screening tests used (34).

The high prevalence of active CMV infection in HIV children and adolescents in our study could signify either high level of primary infection or new strain of reactivated infections. This should be worrisome in this highly endemic area where vaccines for CMV have not yet been developed. However, the fact that all the patients in this study were on HAART with no clinical evidences of severe immune suppression such as retinitis, chronic crippling diarrhea, or other features of AIDS from CMV co-infection may suggest other associated factor(s) in the etiology of co- infection. Akinbami et al., (29) and Klatt and Shibata (36), however attributed such clinical manifestations of co-infection to reactivation of previous CMV infection rather than primary infection.

Our study showed non-statistically significant higher prevalence rate of CMV IgM in the male than female subjects, which is similar to the observations of Ojide et al., (31) and Fowotade et al., (35) among their HIV-infected adults cohort. Musa et al., (22) however had a contrary observation in Kano where they reported more female patients living with HIV having significantly higher CMV IgM than their male counterparts (8.7% vs 4.3%, $p<0.01$) although no reason for such finding was proffered. Udeze et al., (37) equally found significantly higher primary/ active CMV IgM among males than females HIV-infected children and adults on ART in their study cohort in Ilorin, and attributed such findings to more exposure of males to factors that lead to re-activation or re-infection of CMV in their locality.

There was also non-statistically significant highest prevalence of CMV IgM among subjects in 5-<10 year age group (64.7%) when compared to other paediatric age groups in this study ($p=0.135$). This is the period when parents usually send their children to day care services, nursery or primary schools. CMV transmission among children in

day care centers may be enhanced as a result of poor hand hygiene practices and overcrowding usually seen in such care centres (38). Exposure of children to such centers therefore increase the risk of CMV cross infection from school mates. Other studies however have shown significant association between prevalence of active CMV infection and other age groups (35, 37). In the study by Udeze et al., (37), higher IgM was significantly observed among children in age group ≤20 years (p=0.047) among their HIV cohorts aged 1 to 70 years on ART studied at Ilorin. The authors attributed such significant positive findings to the lower level of education and engagement in risky sexual behaviours in the age group but because the study did not separate the children into different paediatric age groups, it was difficult to appreciate which particular age group among the children had the highest prevalence of CMV IgM. CMV infection is ubiquitous with primary/active infection occurring commonly during childhood or adulthood in 4 out of every 5 person above 35 years old, but the infection is usually very mild and generally unrecognized (39).

Many previous studies identified low socio-economic class (SEC) as a risk factor for CMV infection (6,15,17). Our study however showed no significant association between low SEC and CMV IgM in the children and adolescents (p=0.634). Basha et al., (40) equally observed no significant association between CMV sero-prevalence and SEC (p=0.58) in their prospective cohort of non HIV Australian children aged 0-15 years. They however reported more congenital CMV in individuals with high SEC (55%) than the low SEC (9%) (p<0.001). Our study shows significant association between CMV IgM with respect to mean age, mean body weight, length/height and mean systolic blood pressure in the univariate analysis. These significant findings were not surprising considering that IgM were seen in younger age groups (none seen in >15years), and these variables were all age dependent. However on multivariate logistic analysis, all these variables were not statistically significant.

Low CD4 cell count in HIV infected person and high VL are indications of poor immunity and inadequate viral suppression. Although the prevalence of CMV IgM was higher among patients with high CD4+ cell counts and low VL in our study, this surprisingly, was not statistically significant. Similar studies in Benin (31) and Ilorin (37) in Nigeria, and in Iran (41) also found no statistically significant association between CD4+ cell count values and prevalence of CMV IgM in their study populations, and adduced such findings to the effects of ART and duration of treatment. Patients in the present study had mean CD4 cell count and ART treatment duration of 958.6±37.8 cells/µl and 6.9±3.4 years respectively, indicating adequate immune response and long duration on ART. Going by the suggestion of Ojide et al., (31) and Udeze et al., (37), the good immunological response of patients in our study and their long duration on ART could possibly explain the non-significant association between prevalence of CMV IgM and CD4 cell count and VL. However, many other studies have shown prevalence of CMV IgM to be significantly associated with CD4 cell count (22, 40, 41). Although significant association was observed between CD4 cell count and prevalence of CMV IgM among HIV infected patients in the study by Musa et al., (22), the authors noted that there was no positive correlation between CD4 cell count and the prevalence of CMV IgM.

Conclusion:

Our study reports high prevalence of CMV IgM, indicating active/primary CMV infection among HIV infected children and adolescents on HAART. This prevalence was not significantly associated with low CD4 cell count, high VL or any risk factor. Identifying those with primary/active infection is necessary for possible treatment with anti-herpes drugs before development of reactivated CMV disease.

References:

1. McGeoch, D. J., Cook, S., Dolan, A., Jamieson, F. E., and Telford, E. A. Molecular phylogeny and evolutionary timescale for the family of mammalian herpesviruses. J Mol Biol. 1995; 247: 443–458
2. Fishman, J. A. Overview: cytomegalovirus and the herpesviruses in transplantation. Am J Transplant. 2013; 13: 1–8
3. Drew, W. L. Cytomegalovirus infection in patients with AIDS. Clin Infect Dis. 1992; 14: 608– 615.
4. Gona, P., Van Dyke, R. B., Williams, P. L., et al. Incidence of opportunistic and other infections in HIV-infected children in the HAART era. JAMA. 2006; 296: 292– 300.
5. Vauloup-Fellous, C. M., Berth, M., Heskia, F., Dugua, J. M., and Grangeot-Keros, L. Reevaluation of the VIDAS Cytomegalovirus (CMV) IgG avidity assay: Determination of new cut-off values based on the study of kinetics of CMV-IgG maturation. J Clin Virol. 2013; 56: 118-123
6. Chakravarti, A, Kashyap, B, and Metlani, M. Cytomegalovirus Infections: An Indian Perspective. Indian J Med Microbiol. 2009; 27: 3 – 11.

7. Kovacs, A., Schluchter, M., Easley, K., Demmler. G., et al. Cytomegalovirus infection and HIV-1 disease progression in infants born to HIV-1-infected women. Pediatric pulmonary and cardiovascular complications of vertically transmitted HIV infection study group. N Engl J Med. 1999; 341: 77–84. 10.1056/NEJM199907083410203
8. Griffiths, P. D. CMV as a cofactor enhancing progression of AIDS. J Clin Virol. 2006; 35: 489–492
9. Crough, T., and Khanna, R. Immunobiology of human cytomegalovirus: from bench to bedside. Clin Microbiol Rev. 2009; 22: 76–98. 10.1128/CMR.00034-08.
10. Lichtner, M., Cicconi, P., Vita, S., et al. Cytomegalovirus co-infection is associated with an increased risk of severe non-AIDS-defining events in a large cohort of HIV-infected patients. J Infect Dis. 2015; 211: 178–186. 10.1093/infdis/jiu417.
11. Iwamoto, G. K., and Konicek, S. A. Cytomegalovirus immediate early genes upregulate interleukin-6 gene expression. J Investig Med. 1997; 45: 175– 182
12. Brunt, S. J., Cysique, L. A., Lee, S., Burrows, S., Brew, B. J., and Price, P. Short Communication: Do cytomegalovirus antibody levels associate with age-related syndromes in HIV patients stable on antiretroviral therapy? AIDS Res Hum Retroviruses. 2016; 32 (6): 567–572.
13. Gianella, S., Massanella, M., Wertheim, J. O, and Smith, D. M. The Sordid Affair between Human Herpesvirus and Human Immunodeficiency Virus. J Infect Dis. 2015: jiv148.
14. Freeman, M. L., Lederman, M. M., and Gianella, S. Partners in Crime: The Role of CMV in Immune Dysregulation and Clinical Outcome During HIV Infection. Curr HIV/AIDS Rep. 2016; 13 (1): 10–19.
15. Seale, H., MacIntyre, C. R., Gidding, H. F., Backhouse, J. L., Dwyer, D. E., and Gilbert, L. National sero-survey of cytomegalovirus in Australia. Clin Vaccine Immunol. 2006; 13: 1181-1184.
16. Smith, D. M., Nakazawa, M., Freeman, M. L., et al. Asymptomatic CMV Replication During Early Human Immunodeficiency Virus (HIV) Infection Is Associated With Lower CD4/CD8 Ratio During HIV Treatment. Clin Infect Dis. 2016; 63(11):1517-1524.
17. Gianella, S., and Letendre, S. Cytomegalovirus and HIV: A Dangerous Pas de Deux. J Infect Dis. 2016; 214 (2): S67–74.
18. Manicklal, S., Emery, V.C., Lazzarotto, T., Boppana, S.B., and Gupta, R.K. The -Silent‖ global burden of congenital cytomegalovirus. Clin Microbiol Rev. 2013; 26 (1): 86–102.
19. Cannon, M. J., Schmid, D. S., and Hyde, T. B. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. Rev Med Virol. 2010; 20: 202–213. 10.1002/rmv.
20. Gumbo, H., Chasekwa, B., Church, J. A., Ntozini R, Mutasa K, Humphrey JH. et al. Congenital and postnatal CMV and EBV acquisition in HIV-infected Zimbabwean infants. PLoS One. 2014; 9 (12): e114870.doi:10.1371/journal.pone.0114870.
21. Chakraborty, R., Rees, G., Bourboulia, D., et al. Viral co-infections among African children infected with human immunodeficiency virus type 1. Clin Infect Dis. 2003; 36: 922–924. 10.1086/368207.
22. Musa, A. M., Taura, D. W., Mukhtar, M. D., Koki, Y. A, and Adamu, S. Studies on Cytomegalovirus Infection among HIV Positive Patients Attending Infectious Diseases Hospital, Kano State, Nigeria. Greener J Epidemiol Publ Hlth. 2014; 2 (1): 32-36.
23. Basawaraju, A., Pratibha, M. M., and Vijayadurga, S. The Reactivation of Cytomegalovirus Infection in HIV Infected Patients. J Clin Diagnostic Res. 2011; 5: 749 – 751
24. Neusa, M., Antonio, F., and Incilia, C. Research of Antigen and Antibodies from Retrovirus, CMV, and HBV among Prisoners of Penitentiary Complex of the Region of Comparinces. S.P. Brazil. Rev Int Med Trop Sao Paulo. 1998; 40 (4) http://dx.doi.org/10.1590/S0036-46651998000400001.
25. Turbadkar, D., Mathur, M., and Rele, M. Seroprevalence of TORCH Infection in Bad Obstetric History. Indian J Med Microbiol. 2003; 21 (2): 108 – 110.
26. Kfutwah, A. K. W., Ngoupo P. A. T, Sofeu, C. L., et al. Cytomegalovirus infection in HIV-infected versus non-infected infants and HIV disease progression in Cytomegalovirus infected versus non infected infants early treated with cART in the ANRS 12140—Pediacam study in Cameroon. BMC Infect Dis. 2017; 17: 224.
27. Oladipo, E. K., Akinpelu, O. O., Oladipo, A. A., and Edowhorhu, G. Seroprevalence of Cytomegalovirus (CMV) among Blood Donors at Bowen University Teaching Hospital Ogbomoso. Am J Med Biol Res. 2014; 2 (3): 72-75
28. Tsertsvadze, T., Gochitashvili, N., Sharvadze, L., and Dvali, N. Cytomegalovirus Infection in HIV Patients. International Conference on AIDS, Infections Disease; AIDS and Clinical Immunology Research Center, Tbilisi, Georgia. 2002: 1 – 2
29. Akinbami, A. A., Akanmu, A. S., Adeyemo, T. A., Wright, K. O., Dada, M. O., and Dosunmu, A. O. Cytomegalovirus Antibodies among Immuno-compromised (HIV) Patients at Lagos University Teaching Hospital (LUTH) Idi Araba, Lagos. J Med. 2010; 11(2): 151-154. doi: 10.3329/jom.v11i2.5462
30. Marawan, A. A., Jerzy, M. B., and Haydee, A. D. *Toxoplasma gondii* Seropositivity and Co-Infection with TORCH Pathogens in High-Risk Patients from Qatar. Am J Trop Med Hyg. 2010; 82 (4): 626–633. doi:10.4269/ajtmh.2010.09-0530.
31. Ojide, C. K., Kalu, E. I., Nwadike, V. U., Ogbaini-Emovon, E., and Omoti, C. Seroprevalence of Cytomegalovirus among HIV-Infected Adult Patients on HAART. Int J Trop Dis Health. 2013; 3 (3): 233-241
32. Umeh, E. U., Onoja, T. O., Aguoru, C. U. and Umeh, J. C. Seroprevalence of Cytomegalovirus Antibodies in Pregnant Women, Benue State, Nigeria. Infect Dis Ther. 2015; 3: 242
33. Maingi, Z., and Nyamache, A. K. Seroprevalence of Cytomegalo Virus (CMV) among pregnant women in Thika, Kenya. BMC Res Notes. 2014, 7: 794.
34. Sufiawata, I., Widyaputra, S., and Djajakusamah, T. S. A study of cytomegalovirus serology among HIV infected patients in the highly active antiretroviral therapy era. Bandung Med J. 2013; 5 (2): 112-117.
35. Fowotade, A., Okonko, I. O., Agbede, O. O., and Suleiman, S. T. High seropositivity of IgG and IgM antibodies against cytomegalovirus (CMV) among HIV-1 seropositive patients in Ilorin, Nigeria. Afr Health Sci. 2015; 15 (1): 1-9.
36. Klatt, E. C., and Shibata, D. Cytomegalovirus infection in the acquired immunodeficiency syndrome: clinical and autopsy findings. Arch Pathol Lab Med. 1988; 112: 540–544
37. Udeze, A., Odebisi-Omokanye, M., and Ajileye, T. Cytomegalovirus infection among Human Immunodeficiency Virus (HIV) infected individuals

on highly active anti-retroviral therapy in North-Central Nigeria. Afr Hlth Sci. 2018; 18 (4): 1057 - 1065. https://dx.doi.org/10.4314/ahs.v18i4.27

38. Dowd, J. B., Aiello, A. E., and Alley, D. E. Socioeconomic disparities in the seroprevalence of cytomegalovirus infection in the US population: NHANES III. Epidemiol Infect 2009; 137: 58 – 65

39. Bernard, J. T. Human Cytomegalovirus Species. Classification of Viral Family (Herpesviridae). 2007; 2: 127–128

40. Basha, J. I., Iwasenko, J. M., Robertson, P., Craig, M. E., and Rawlinson, W. D. Congenital cytomegalovirus infection is associated with high maternal socio-economic status and corresponding low maternal cytomegalovirus seropositivity. J Paediatr Child Hlth. 2014; 50 (5): 368-72. doi:10.1111/jpc.12502.

41. Mehrkhani, F., Jam, S., Sabzvari, D., Fattahi, F., Kourorian, Z., and Seyed Alinaghi, S. Cytomegalovirus Co-Infection in Patients with Human Immunodeficiency Virus in Iran. Acta Medica Iranica. 2011; 49 (8): 551-555.

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Antagonistic effect and bacteriocinogenic activity of Lactic Acid Bacteria isolated from *Sorghum bicolor*-based 'ogi' on food borne bacterial pathogens from cabbage

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Abstract:

Background: Lactic acid bacteria (LAB) are important organisms recognized for fermentative ability as well as health and nutritional benefits. A large number of bacteriocins from LAB have been characterized and a number of studies have indicated the potential usefulness of bacteriocin in food preservative. The objective of this study was to evaluate the antagonistic effects and bacteriocinogenic activity of LAB isolated from *Sorghum bicolor*-based 'ogi' against selected food borne bacteria from cabbage samples.
Methodology: Five samples of *Sorghum bicolor*-based 'ogi' and 5 samples of suspected infected cabbage heads were randomly collected using sterile water proof material from Abakpa main market, Abakaliki, and processed at the Applied Microbiology Laboratory of Ebonyi State University, for isolation of LAB and food borne pathogen by conventional culture and biochemical identification tests. Antagonistic effects of LAB and its bacteriocinogenic activity were determined by agar well diffusion test.
Results: Three different *Lactobacillus* species designated A, B, and C, were isolated from the *Sorghum bicolor*-based 'ogi' and 5 bacterial species were isolated from cabbage heads; *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella*, and *Shigella* species. The *Lactobacillus* species had inhibitory effect against S. *aureus*, *E. coli*, and *Shigella* species with inhibition zone diameters (IZD) of 19 mm, 10 mm, and 10 mm respectively. The crude bacteriocin extracts from the *Lactobacillus* species showed higher inhibitory activity against tested bacterial isolates at 10^{-1} (0.1ml) than at 10^{-2} dilution (0.01ml), and the inhibitory activity was higher at pH 2 than pH 6 and 7, with no activity at pH 8.
Conclusion: This study showed that LAB and its extracted bacteriocin demonstrated *in vitro* inhibitory activity against food borne pathogens isolated from cabbage heads. There is the need to further characterize the active components of the bacteriocin for possible commercial use as preservatives and potential source of new antimicrobial agent.

Keywords: Lactic acid bacteria, bacteriocin, cabbage, fermented food, 'ogi'

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Effet antagonistes et activité bactériocinogène de bactéries de l'acide lactique isolées à partir d'un «ogi» à base de sorgho bicolore sur des agents pathogènes bactériens d'origine alimentaire issus du chou

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Abstrait:

Contexte: Les bactéries de l'acide lactique (LAB) sont des organismes importants reconnus pour leur aptitude à la fermentation ainsi que pour leurs bienfaits nutritionnels et de santé. Un grand nombre de bactériocines de LAB ont été caractérisées et un certain nombre d'études ont indiqué l'utilité potentielle de la bactériocine dans un conservateur alimentaire. L'objectif de cette étude était d'évaluer les effets antagonistes et l'activité bactériocinogène du LAB isolé de «ogi» à base de *Sorghum bicolor* sur certaines bactéries d'origine alimentaire prélevées dans des échantillons de chou.
Méthodologie: Cinq échantillons d'ogi à base de *Sorghum bicolor* et 5 échantillons de têtes de choux présumées infectées ont été prélevés au hasard à l'aide d'un matériau imperméable à l'eau stérile provenant du marché principal d'Abakpa, à Abakaliki, et traités au laboratoire de microbiologie appliquée d'Ebonyi State University pour l'isolement de LAB et agent pathogène d'origine alimentaire par culture conventionnelle et tests d'identification biochimiques. Les effets antagonistes de LAB et son activité bactériocinogène ont été déterminés par un test de diffusion sur gélose.
Résultats: Trois espèces différentes de Lactobacillus désignées par A, B et C ont été isolées à partir du «ogi» à base de *Sorghum bicolor* et 5 espèces bactériennes ont été isolées à partir de têtes de chou; *Staphylococcus aureus, Escherichia coli*, espèces de *Pseudomonas aeruginosa*, *Salmonella* et *Shigella*. Les espèces de Lactobacillus avaient un effet inhibiteur contre les espèces de *S. aureus, E. coli* et *Shigella* avec des diamètres de zone d'inhibition (IZD) de 19 mm, 10 mm et 10 mm respectivement. Les extraits de bactériocine bruts de l'espèce Lactobacillus ont montré une activité inhibitrice plus élevée contre les isolats bactériens testés à 10-1 (0,1 ml) qu'à une dilution de 10-2 (0,01 ml), et l'activité inhibitrice était supérieure à pH 2 à pH 6 et à 7, sans activité à pH 8.
Conclusion: cette étude a montré que le LAB et sa bactériocine extraite ont démontré une activité inhibitrice in vitro contre les agents pathogènes d'origine alimentaire isolés de la tête du chou. Il est nécessaire de mieux caractériser les composants actifs de la bactériocine pour une utilisation commerciale éventuelle en tant que conservateurs et source potentielle de nouvel agent antimicrobien.

Mots-clés: Bactéries lactiques, bactériocine, chou, aliment fermenté, 'ogi'

Introduction:

Traditional fermented foods prepared from millet (*Pennisetum typhoideum*), sorghum (*Sorghum bicolor*) and maize (*Zea mays*) are consumed in many West African countries (1). In southern Nigeria, maize and millet are processed by fermentation into 'ogi' also known as 'akamu' and consumed commonly. 'Ogi' has been known to exhibit health promoting properties such as in the control of gastroenteritis in animals and man (1). *In vitro* and *in vivo* data have shown the probiotic, hypolipidemic, hepatoprotective and antibacterial effects of some lactic acid bacteria isolated from 'ogi' (1,2). Lactic acid bacteria (LAB) are also known for their potentials to produce antimicrobial compounds and other valuable products that inhibit growth of pathogenic microorganisms, and degrade mycotoxins (3).

In fermented foods, LAB have been known to display antimicrobial activities through production of various metabolites, including lactic acid, hydrogen peroxide, and bacteriocins (3). Many bacteriocins are active against food-borne pathogens especially *Listeria monocytogenes* (3). The predominant LAB in 'ogi' fermentation is *Lactobacillus plantarum* which is responsible for production of the main acid (lactic acid) in 'ogi' with acidity usually below pH 4. At this pH, most pathogenic microorganisms in food cannot survive, hence lactic acid fermentation has been found to reduce the growth of pathogenic microorganisms in food (4).

There are increasing interests in bacteriocins as alternative to antibiotics and chemical food preservatives. This has prompted this study with the objectives of investigating the bacteriocinogenic effects of LAB recovered from a cereal-based food, *Sorghum bicolor*, on food borne pathogenic bacteria isolated from infected cabbage.

Materials and Method:

Study setting and collection of *Sorghum bicolor*-based 'ogi' samples

Five (5) samples of *Sorghum bicolor*-based 'ogi' were randomly purchased at Abakpa market, Abakaliki metropolis, Ebonyi State during the period August and November, 2018. The samples were collected aseptically with sterile water proof materials and transported within two hours to the Laboratory of Applied Microbiology Department, Ebonyi State University, for bacteriological analysis.

Isolation/identification of LAB from Sorghum-based 'ogi' samples

The pour plate technique was used for the isolation of LAB. One gram of each 'ogi' sample was dissolved in 10 ml of water and swirled to mix properly. A tenfold serial dilution was performed from the sample homogenate by adding 1 ml to 9 ml of sterile distilled water and 1 ml aliquot of 10^{-7} and 10^{-8} of the dilution

factors were inoculated on Mann Rogosa and Sharp (MRS) agar which has been incorporated with 50µg/ml of nystatin to suppress the growth of fungi (4). The inoculated plates were incubated at 37°C for 72 hours in anaerobic jar, and suspected LAB colonies were then sub-cultured on MRS agar to obtain pure culture (5, 6).

All isolates were identified using standard microbiological techniques such as Gram staining, and biochemical tests such as citrate utilization, oxidase, indole, methyl red, voges proskauer (VP), and sugar (lactose, glucose, sucrose, fructose, maltose and mannitol) fermentation (5,6,7).

Isolation of bacterial pathogens from cabbage

Cabbage samples collected from Abakpa main market, Abakaliki, Ebonyi State were processed by first removing their outer leaves and 20g of each of the samples were weighed, washed with distilled water and blended in an electric blender. The blended samples were put in a clean beaker containing 20 ml of sterile water and sieved. The resulting filtrate was serially diluted, and 1ml of 10^{-7} and 10^{-8} dilutions were then plated on Cysteine Lactose Electrolyte Deficient (CLED) agar, Mannitol salt agar (MSA) and *Salmonella-Shigella* (SS) agar. The plates were incubated at 37°C for 24 hours. The discrete colonies of each of the bacterial isolates were identified by standard morphological and biochemical tests (5,6,7) and then sub-cultured on nutrient agar to obtain pure cultures.

Antagonistic activity of LAB

The antagonistic activity of LAB isolates was determined by the agar well diffusion technique with the cell-free supernatant of each isolate. A standardized suspension of each isolated bacterium (*E. coli*, *S. aureus*, *Ps. aeruginosa*, *Shigella* and *Salmonella* species) from the cabbage samples was prepared and inoculated onto Mueller Hinton (MH) agar plates using sterile cotton swab. The plates were allowed to dry and a sterile cork borer with a diameter of 4mm was used to cut uniform well in the agar plates. Each of the wells was filled with 0.1 ml (100 µL) aliquot of the test *Lactobacillus* isolate. The plates were then incubated at 37°C for 72 hours. Isolates exhibiting highest zone of growth inhibition were selected and screened for bacteriocin production.

Bacteriocinogenic activity of LAB

Assay of crude bacteriocin production

Bacteriocin was extracted from LAB that had highest growth inhibition by growing them first in 1 litre MRS broth and incubating for 72hours at 30°C under anaerobic conditions (8). Extract was obtained by centrifuging the culture at 12,000 rpm for 15 minutes to pellet down the cells. The pH of cell-free cultured supernatant was adjusted to 6.5 with 1M NaOH. Then, catalase (1mg/ml) was added to remove hydrogen peroxide from the supernatant (9). The supernatant was filtered through a 0.45 µm pore size membrane and the protein was precipitated using 80 % (w/v) saturated ammonium sulphate. The mixture was stirred for 1hour, after which it was stored at 4°C.

After precipitation, the mixture was centrifuged at 16,000rpm at 4°C for 30 min, and pellet was stored at 4°C. The pellet was further separated from impurities by dissolving 1ml in distilled water in an Eppendorf tube and centrifuging at 10,000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet containing bacteriocin was washed with deionized water, dispensed into another Eppendorf tubes, and centrifuged once again at 10,000 rpm for 15 minutes at 4°C to pellet down the proteins. After discarding the supernatant, the pellet was dissolved in 500 µl of 0.1M sodium phosphate buffer (pH 7.0) and the total volume was made up to 2 ml.

The sample was then loaded in a pre-treated dialysis tubing cellulose membrane (18-20 length) and dialyzed in a 3 litre 0.1M sodium phosphate buffer (pH 7.0) for 2 hours, following which the buffer was changed and the sample further dialyzed overnight at 4°C. After 24 hours dialysis, the sample was reloaded in Eppendorf tubes and centrifuged at 10,000 rpm at 4°C for 30 minutes and then stored at -20 °C.

Partial precipitation of proteins from crude bacteriocin

The crude bacteriocin samples were dispensed into Eppendorf tubes and centrifuged at 12,000 rpm for 15 mins at 4°C to pellet down the cells. The supernatant was poured into a 50 ml capacity beaker in an ice pack at a temperature of 4°C and 15g of ammonium sulphate [$(NH_4)_2SO_4$] in dry form was measured and dissolved in the supernatant. The mixture was stirred for 1 hour and stored at 4°C for 24 hours. The stirring was carefully done to prevent foaming that may lead to protein denaturation (10).

Determination of bacteriocin activity

The agar well diffusion assay was used to determine the bacteriocin activity of the LAB isolates (11). Ten ml of partially purified bacteriocin was serially diluted up to 10^{-2} using saline diluent. An overnight culture of bacteria

isolated from cabbage grown in Tryptic soy broth (TSB) at 37°C was diluted in saline to a 0.5 McFarland standards. The suspension was inoculated on MH agar plates using a sterile cotton swab. The plates were allowed to dry and a sterile cork borer with a diameter of 4mm was used to cut uniform circular wells in the agar plates. Each of the wells was filled with 0.1ml (100µL) aliquot of the partially purified bacteriocin from the LAB. The plates were kept at 4°C for 2hours to ensure diffusion of the supernatant fluid into the agar, and then incubated at 37°C for 24 hours. With the aid of meter rule, the antimicrobial activity was determined by measuring the diameter of zones of inhibition around the wells.

Effect of pH on crude bacteriocin activity

A 5ml aliquot of crude bacteriocin from the LAB was distributed into different test tubes. To each of the respective test tubes, 1 ml of sodium hydroxide (NaOH) and hydrogen chloride (HCl) was added to obtain pH values of 2, 3, 4, 5, 6, 7, and 8 which was confirmed with the aid of calibrated Jenway pH meter. The solutions were allowed to stand at room temperature for 2 hours. Aliquots of 50 µl from each test tube were placed in wells (4mm diameter) of MH agar plates that have been inoculated with overnight broth cultures of the isolated bacteria (*S. aureus*, *E. coli*, *Ps. aeruginosa, Salmonella*, and *Shigella* species) from cabbage. These were incubated at 30°C for 24hours, following which the zones of inhibition around the wells were measured in mm with a meter rule.

Results:

Morphological and biochemical characteristics of LAB

Table 1 shows the morphological and biochemical characteristics of LAB isolated from samples of *Sorghum bicolor*-based 'ogi' collected from Abakpa main market, Abakaliki, Ebonyi State. Three different LAB groups; *Lactobacillus* species A*, Lactobacillus* species B, and *Lactobacillus* species C were identified. Table 2 shows that *S. aureus*, *E. coli*, *Ps. aeruginosa*, *Salmonella* and *Shigella* species were isolated from cabbage samples collected from the same market.

Table 1: Morphological, microscopic and biochemical characteristics of the Lactic Acid Bacteria isolated from Sorghum-based 'ogi'

Morphological Characteristics		Microscopic Characterization	Biochemical Tests Sugar Fermentation											
Shape	Colour	Gram reaction	Motility test	Catalase Test	Mannose	Arabinose	Lactose	Ribose	Glucose	Mannitol	Sucrose	Fructose	Maltose	Suspected LAB
Rod	White	+	-	-	+	-	+	+	-	+	+	-	+	*Lactobacillus* species A
Rod	Cream	+	-	-	-	-	+	+	+	-	+	+	+	*Lactobacillus* species B
Rod	Dirty white	+	-	-	-	+	+	+	+	+	+	-	-	*Lactobacillus* species C

+ = positive - = negative

Tables 2: Morphological, microscopic and biochemical characteristics of bacteria isolated from cabbage sample

Morphological Characteristics		Biochemical Tests						Sugar Fermentation Test							
Shape	Colour	Gram reaction	Motility test	Citrate Test	Oxidase Test	Coagulase Test	Indole Test	Lactose	Glucose	Sucrose	Catalase Test	Urease Test	Voges Proskauer	Methyl Red	Probable Organism
Cocci	Yellow on mannitol salt agar	+	-	-	-	+	-	+	+	-	+	+	+	+	*Staphylococcus aureus*
Rods	Opaque yellow on CLED	-	+	-	-	-	+	+	+	+	+	-	-	+	*Escherichia coli*
Rods	Greenish on CLED	-	+	-	+	-	-	+	+	+	+	-	-	+	*Pseudomonas aeruginosa*
Rod	Pink on SS agar	-	-	-	-	-	-	-	+	-	+	-	-	+	*Shigella* species
Rods	Black on SS agar	-	+	-	-	+	-	-	+	-	+	-	-	+	*Salmonella* species

+ = positive; - = negative

Antagonistic effects of isolated LAB

Lactobacillus species group A produced inhibitory zone diameters of 19, 10, 10, 12 and 14mm against *S. aureus, E. coli, Ps. aeruginosa, Salmonella,* and *Shigella* species respectively (Fig1). *Lactobacillus* species group B produced inhibitory zone diameters of 16, 10, 12, 14 and 10mm against *S. aureus, E. coli, Ps. aeruginosa, Salmonella,* and *Shigella* species respectively. *Lactobacillus* species group C produced inhibitory zone diameters of 14, 14, 12, 12 and 10mm against *S. aureus, E. coli, Ps. aeruginosa, Salmonella*, and *Shigella* species respectively.

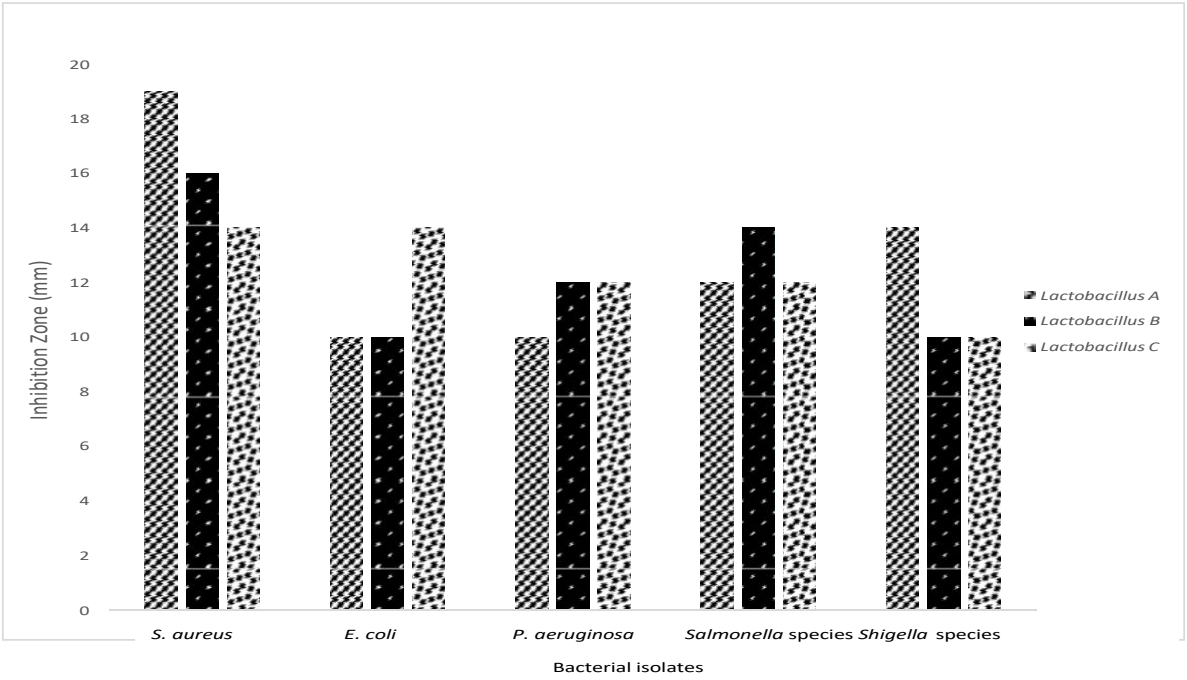

Fig 1: The Inhibition Zone Diameter (mm) of LAB isolated from *Sorghum bicolor*-based 'ogi' against food borne bacterial isolates from cabbage

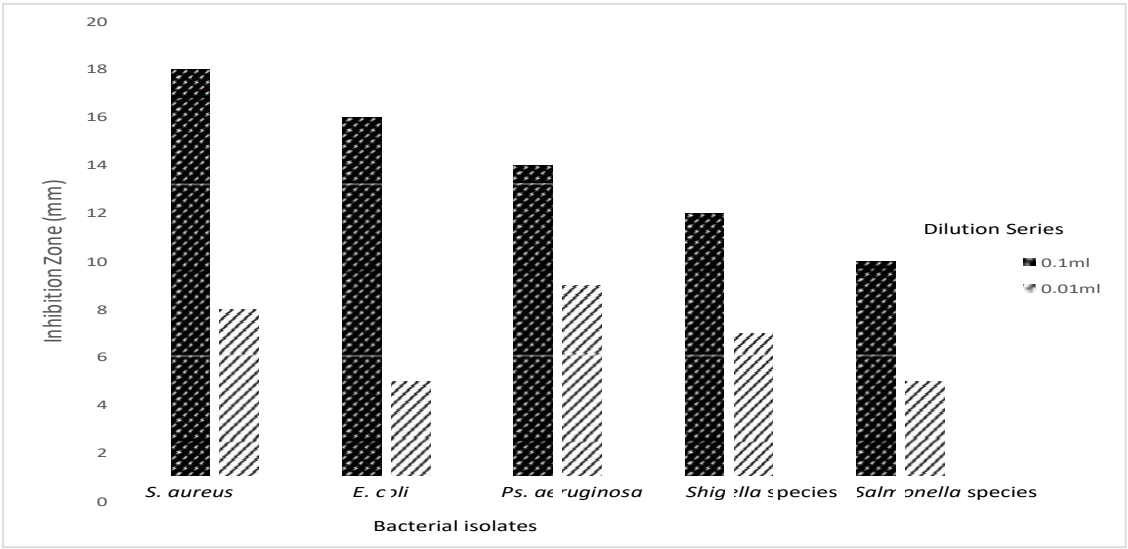

Fig 2: Antimicrobial activity of diluted crude bacteriocin from *Lactobacillus* species A isolated from *Sorghum bicolor*-based 'ogi' against bacterial isolates from cabbage samples

Bacteriocinogenic activity of LAB

The antimicrobial activity of diluted crude bacteriocin from *Lactobacillus* species A as depicted in Fig 2 showed that 10^{-1} (0.1ml) dilution produced inhibition zone diameters of 18, 16, 14, 12, and 10mm against *S. aureus, E. coli, Ps. aeruginosa, Shigella* species, and *Salmonella* species respectively, while 10^{-2} (0.01ml) dilution produced inhibition zone diameters of 8, 5, 9, 7, and 5mm against *S. aureus, E. coli, Ps. aeruginosa, Shigella* and *Salmonella* species respectively.

The antimicrobial activity of the crude bacteriocin from *Lactobacillus* species group B showed that 10^{-1} dilution produced inhibition zone diameters of 16, 11, 13, 10, and 15mm against *S. aureus, E. coli, Ps. aeruginosa, Shigella*, and *Salmonella* species respectively, while 10^{-2} dilution produced inhibition zone diameters of 7, 4, 5, 8, and 11mm against *S. aureus, E. coli, Ps. aeruginosa, Shigella,* and *Salmonella* species respectively (Fig 3).

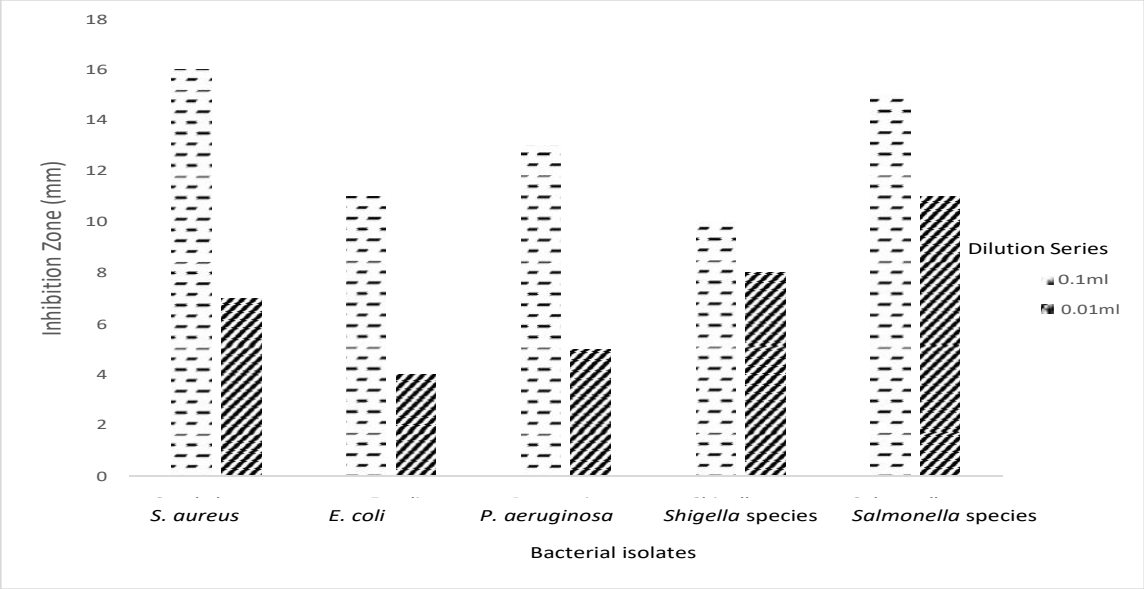

Fig 3: Antimicrobial activity of diluted crude bacteriocin from *Lactobacillus* species B from *Sorghum bicolor*-based 'ogi' against bacterial isolates from cabbage samples

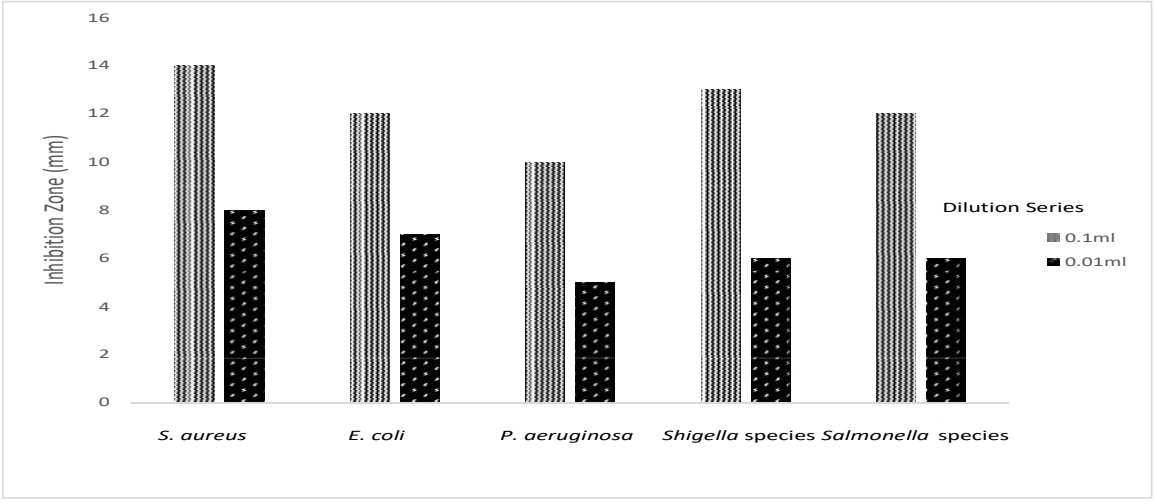

Fig 4: Antimicrobial activity of diluted crude bacteriocin from *Lactobacillus* species C isolated from *Sorghum bicolor*-based 'ogi' against bacterial isolates from cabbage samples

Table 3: Effect of pH on antimicrobial activity of bacteriocin against bacteria isolated from *Sorghum bicolor*-based 'ogi'

pH	Isolates zone of inhibition (mm)				
	S. aureus	*E. coli*	*Ps. aeruginosa*	*Salmonella* species	*Shigella* species
2	21	17	14	19	18
3	14	13	11	12	11
4	13.5	10	9	10	11
5	11	11	10	9	10
6	9	7	4	5	4
7	5	3	5	2	3
8	3	1	4	NI	1

NI = no inhibition

The antimicrobial activity of the crude bacteriocin from *Lactobacillus* species group C depicted in Fig 4 showed that 10^{-1} dilution produced inhibition zone diameters of 14, 12, 10, 13, and 12mm against *S. aureus, E. coli, Ps. aeruginosa, Shigella*, and *Salmonella* species respectively, while 10^{-2} dilution produced inhibition zones of 8, 7, 5, 6, and 6mm for *S. aureus, E. coli, Ps. aeruginosa, Shigella*, and *Salmonella* species respectively (Fig 4).

Effect of pH on stability of bacteriocin

Table 3 showed that at pH of 2, 3, 4, 5, 6, 7 and 8, inhibition zone diameters (IZDs) of 21, 14, 13.5, 11, 9, 5 and 3mm respectively were recorded against *S. aureus;* IZDs of 17, 13, 10, 11, 7, 3 and 1mm against *E. coli;* IZDs of 14, 11, 9, 10, 4, 2, and 0mm against *Ps. aeruginosa*; IZDs of 19, 12, 10, 9, 5, 2, and 0mm against *Salmonella* species and IZDs of 18, 11, 11, 10, 4, 3, and 1mm against *Shigella* species.

Discussion:

Lactic acid bacteria satisfy the increasing demand from consumers for food that contain lower concentration of chemical preservatives, as bacteriocin are natural antimicrobials, produced by bacteria normally present in fermented food. The present study sought to determine the effect of partially purified bacteriocin on some indicator organisms that are known to cause food spoilage. In this study, *Lactobacillus* species were isolated from five samples of *Sorghum bicolor*-based 'ogi' which Ohenhen *et al*., had previously isolated from fermented 'ogi' samples in their study (12). *Lactobacillus* species have also been reported to be predominant in fermented foods (13).

Staphylococcus aureus, *E. coli*, *Ps. aeruginosa*, *Shigella*, and *Salmonella* species were also isolated from infected cabbage collected from Abakpa main market, which is similar to the study of Sujeet and Vipin (14), who reported the presence of the same bacterial species in cabbage and other salad vegetables. These bacteria have been implicated as common food borne infectious pathogens (15). It is well established that food is a valuable source of nutrients for certain microbes, and their growth on the food may result in unpleasant smell, bad taste, and poor appearance of food (16).

The isolated LAB from *Sorghum bicolor* showed antagonistic activity against the food borne bacteria isolated from cabbage. It was observed that all the *Lactobacillus* species and their purified bacteriocins (at the two dilutions) produced highest IZDs against *S. aureus* and lowest against *E. coli*. This implies that they possess higher activity against Gram positive than Gram negative bacteria. Our finding is in agreement with that of Rammelsberg *et al*. (17), who observed that antimicrobial activity of purified bacteriocin extracted from *L. parecasei* subsp *tolerans* was more active against *S. aureus* and *Listeria monocytogenes* than *E. coli*. The cell wall of Gram positive bacteria is made of large amount of peptidoglycan which constitutes about 90% of the dry cell wall mass (18), and which may be the site of action of the bacteriocin. In contrast, the more complex cell wall composition of Gram negative bacteria made them resistant to many antimicrobial compounds especially those with high molecular size that may be unable to penetrate the cell wall to reach their possible sites of inhibitory action on the bacteria (15). Our observation however contrast that of Ohenhen *et al*., (12), who observed highest and lowest zone of inhibitions by *Lactobacillus plantarum* for *E. coli* and *S. aureus* respectively.

The actions of the bacteriocin on *S. aureus* showed dose-dependent inhibitory effects with larger IZDs at 10^{-1} dilution (higher concentration) compared to 10^{-2} dilution (lower concentration), which agrees with the report of Ohenhen *et al*., (12), who observed similarly that 10^{-1} dilution produced higher inhibitory activity against indicator bacteria than 10^{-2}

dilution. Because bacteriocins do not act equally against target species, many researchers have examined the affinity of bacteriocin to specific species and strains (19). The crude bacteriocin extract in our study demonstrated higher antimicrobial activity at pH 2 compared to pH 6 and 7, and no activity was demonstrated at pH 8. This could be due to the fact that the producer organism (*Lactobacillus* species) has a high tolerance for low pH. This observation is similar to that of Ogunbanwo *et al*., (8), who reported that purified bacteriocin extract recovered from *L. plantarum* was more active at pH 2 and 6, than at pH 10 and 12.

Conclusion:

Our study showed that LAB isolated from *Sorghum bicolor*-based 'ogi' and partially purified bacteriocins from them demonstrated inhibitory activity maximal at pH 2 against Gram positive *(S. aureus*) and Gram-negative bacteria (*E. coli, Ps. aeruginosa, Salmonella*, and *Shigella* species). The LAB have potential for use as safer bio-preservatives in acidic food products in preference to chemical preservatives. This study indicates that partially purified bacteriocins have the potential to replace chemical preservatives in food products. Additionally, the bacteriocin produced by the LAB isolates in this study were noted to have maximum activity at acidic pH 2-6, which supports their use as bio-preservatives in acidic food products such as fruits juices, in preference to chemical preservatives that may have adverse effects on human body system.

Conflict of interest:

Authors declare no conflicts of interest

References:

1. Oyetayo, V. O., and Osho, B. Assessment of Probiotic Properties of a Strain of *Lactobacillus plantarum* Isolated from Fermenting Corn Slurry. J Food Agric Environ. 2004; 2 (1): 132–134.
2. Aderiye, B. I., Laleye, S. A., and Odeyemi, A. Thypolipidemic Effect of *Lactobacillus* and *Streptococcus* Species from some Nigerian Fermented Foods. Res J Microbiol. 2007; 2 (6): 538-544.
3. Mokoena, M. P. Lactic Acid Bacteria and Their Bacteriocins: Classification, Biosynthesis and Applications against Uropathogens. Molecules. 2017; 2: 45-47.
4. Sahlin, P. Fermentation as a Method of Food Processing Production of Organic Acids, pH-Development and Microbial Growth in Fermenting Cereals. Lund Institute of Technology, Lund University Sweden. 1999; 2: 23-25.
5. Harley, J. P., and Prescott, L. M. Laboratory Exercises in Microbiology. 5th Edition Mc Graw Hill, New York. 2002: 65-64.
6. Sharma, K. Manual of Microbiology. Ane Books. Pvt. Ltd. New Delhi. 2009: 23.
7. Holt, J. G. Krig, N. R., Statey, J. T., and Williams, S. T. Bergey's Manual of Determinative Bacteriology 9th Edition, Preston Street, Baltimore, Maryland 21202, USA. 1994: 528-540.
8. Ogunbanwo, S. T, Sanni, A. I., and Onilude, A. A. Characterization of Bacteriocin Produced by *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1. Afr J Biotechnol. 2003; 5: 275–286.
9. Daba, H., Pandean, S., Gosselin, J. F., Simard, R. E, Huang, J., and Lacroix, C. Detection and Activity of Bacteriocin Produced by *Leuconostoc mesenteriodes*. J Appl Environ Microbiol. 1991; 57:3450-3455.
10. Hensyl, W. R., Williams and Wilkins. Bergey's Manual of Systematic Bacteriology, 9th Edition. 1994: 45-47.
11. Khalid, K. An Overview of Lactic Acid Bacteria. Int J Biosci. 2011; 3: 1-13.
12. Ohenhen, R. E., Isibor, J. O., Emonfonmwan, G., and Enabulele, S. A. Effect of pH and Storage Temperature on Antimicrobial Activity of Bacteriocin Produced by Lactic Acid Bacteria Isolated from Ogi. Br Microbiol Res J. 2015; 9 (3): 1-9.
13. Ogunshe, A. A., Omotosho, M. A., and Adeyeye, J. A. *In vitro* Antimicrobial Characteristic of Bacteriocin Producing *Lactobacillus* strains from Nigeria Indigenous Fermented Food. Afr J Biotechnol. 2007; 6 (4): 445-453.
14. Sujeet, K. M., and Vipin, K. A Study on Prevalence of Microbial Contamination on the Surface of Raw Salad Vegetable. Int J Food Microbiol. 2017; 6: 411-418.
15. Cleveland, J., Montville, T. J., Nes, I. F., and Chikindas, M. L. Bacteriocins: Safe, Natural Antimicrobials for Food Preservation. Int J Food Microbiol. 2001; 71: 1-20.
16. Pal, M. Food spoilage. Ph.D., Lecture Notes. Addis Abba University College of veterinary Medicine, Debre Zeit, Ethiopia: 1-9.
17. Rammelsberg, M., and Radler, F. Antibacterial Polypeptides of *Lactobacillus* species. J Appl Bacteriol. 1990; 69: 177-184.
18. Castro, M. P., Palarecino, N. Z., Herman, C., Garro, A.O., and Campos, C. A. Lactic Acid Bacteria Isolated from Artisanal Dry Sausage: Characterization of Antimicrobial Compounds and Study of the Factor Affecting Bacteriocin Production. Meat Sciences. 2011; 87: 321-329.
19. Omar, N. B., Abriouel, H., Lucas, R., Martinez, Canamero, M., Guyot, J., and Galvez, A. Isolation of bacteriocinogenic *Lactobacillus plantarum* Strains from Ben Salga, a Traditional Fermented Gruel from Burkina Faso. Int J Food Microbiol. 2006; 112: 44-50

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Underutilization of the Clinical Microbiology Laboratory by Physicians in Nigeria

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Abstract:

Background: Clinical laboratories are critical to correct diagnosis of medical conditions to ensure appropriate management. Point prevalence survey (PPS) of antimicrobial use and resistance performed in Nigeria in 2015 and 2017 showed high rates of antibiotic use, but poor laboratory utilization for definitive diagnosis of the infections for which the antimicrobials were prescribed. This study investigated the reasons for clinicians" poor utilization of the clinical laboratory for definitive diagnosis and treatment of infections.
Methods: A cross sectional survey of clinicians attending the 2018 annual scientific conference and general meeting of the National Postgraduate Medical College of Nigeria (NPMCN) in Owerri, Southeastern Nigeria, was conducted using self-administered structured questionnaire to obtain information on the sub-optimal utilization of the clinical microbiology laboratory.
Results: Of 283 respondents, 14.8% were general practitioners and 85.2% were specialists who have been in practice for a median period of 20 years (range 3 – 48 years). The specialists included surgeons (26%), family physicians (19.8%), internists (14.3%), pathologists (13.9%), paediatricians (8.8%), obstetricians and gynecologists (8.1%), community medicine physicians (6.2%), and dental surgeons (2.6%). Majority of the respondents (90.8%) work in public, 88.3% work in tertiary and 9.9% in secondary care hospitals. For diagnosis of infections, 16% and 49.8% reported using laboratory "always" and "very often" respectively. Among these, the most commonly utilized investigations were microscopy, culture and sensitivity (62.4%), DNA detection (18.3%), GeneXpert for tuberculosis (17.2%), and antigen detection (16.7%). Among clinicians that "hardly make use" of the laboratory, their reasons for non-use were; clinical diagnosis being sufficient (39.7%), delayed results (17.2%), having knowledge of „potent" antibiotics (15.5%), lack of access to microbiology laboratory (13.8%), absence of pathologists to assure quality of tests (12.1%), and no need of the laboratory to manage patients with infections (8.6%).
Conclusion: These findings indicate that poor use of the microbiology laboratory seems mainly associated with perception and attitude of the physicians to the relevance of the laboratory, and perceived inadequacy of microbiology practice in some others. There is need to raise physicians" awareness on the relevance and what constitutes optimal use of the clinical microbiology laboratory for accurate diagnosis of infections and appropriate antimicrobial use.

Key words: utilization, microbiology laboratory, diagnosis, antimicrobials, infectious diseases

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Sous-utilisation du laboratoire de microbiologie clinique par des médecins au Nigéria

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Abstrait:

Contexte: Les laboratoires cliniques sont essentiels pour corriger le diagnostic des conditions médicales et assurer une prise en charge appropriée. Une enquête de prévalence ponctuelle (PPS) sur l'utilisation et la résistance aux antimicrobiens réalisée au Nigéria en 2015 et 2017 a montré des taux élevés d'utilisation d'antibiotiques, mais une faible utilisation en laboratoire pour le diagnostic définitif des infections pour lesquelles les antimicrobiens ont été prescrits. Cette étude a examiné les raisons de la faible utilisation du laboratoire par les cliniciens pour le diagnostic définitif et le traitement des infections.
Méthodes: Une enquête transversale sur les cliniciens participant à la conférence scientifique annuelle et à l'assemblée générale de 2018 du Collège national des médecins diplômés du Nigéria (NPMCN) à Owerri, dans le sud-est du Nigéria, a été réalisée à l'aide d'un questionnaire structuré auto-administré visant à obtenir des informations sur le sous-optimal. utilisation du laboratoire de microbiologie clinique.
Résultats: Sur 283 répondants, 14,8% étaient des omnipraticiens et 85,2% des spécialistes exerçant depuis 20 ans en moyenne (de 3 à 48 ans). Les spécialistes comprenaient des chirurgiens (26%), des médecins de famille (19,8%), des internistes (14,3%), des pathologistes (13,9%), des pédiatres (8,8%), des obstétriciens et des gynécologues (8,1%), des médecins de santé communautaires (6,2%), et chirurgiens dentistes (2,6%). La majorité des répondants (90,8%) travaillent en public, 88,3% dans le tertiaire et 9,9% dans les hôpitaux de soins secondaires. Pour le diagnostic des infections, 16% et 49,8% ont déclaré utiliser le laboratoire «toujours» et «très souvent» respectivement. Parmi ceux-ci, les examens les plus couramment utilisés étaient la microscopie, la culture et la sensibilité (62,4%), la détection de l'ADN (18,3%), GeneXpert pour la tuberculose (17,2%) et la détection de l'antigène (16,7%). Parmi les cliniciens qui «utilisent à peine» le laboratoire, les raisons de leur non-utilisation étaient: diagnostic clinique suffisant (39,7%), résultats tardifs (17,2%), connaissance d'antibiotiques «puissants» (15,5%), manque d'accès au laboratoire de microbiologie (13,8%), absence de pathologistes pour garantir la qualité des tests (12,1%), et aucun laboratoire n"a besoin de prendre en charge des patients infectés (8,6%).
Conclusion: Ces résultats indiquent que la mauvaise utilisation du laboratoire de microbiologie semble principalement associée à la perception et à l'attitude des médecins à l'égard de la pertinence du laboratoire, et à l'insuffisance perçue de la pratique de la microbiologie chez certains autres. Il est nécessaire de sensibiliser les médecins à la pertinence et à l'utilisation optimale du laboratoire de microbiologie clinique pour un diagnostic précis des infections et une utilisation appropriée des antimicrobiens.

Mots-clés: utilisation, laboratoire de microbiologie, diagnostic, antimicrobiens, maladies infectieuses

Introduction:

Antimicrobial resistance is increasing globally and locally (1,2,3). Multidrug resistant organisms have been isolated from clinical and environmental sources in different parts of Nigeria and their rates are on the increase (3,4,5). Rational antibiotic use is needed to reduce selection pressure on bacteria and curb development of antibiotic resistance. This involves use of antibiotic policy and guidelines for antibiotic prescriptions. To produce a meaningful antibiotics guideline, locally generated data on the antibiogram of the prevailing pathogenic bacteria in an area are recommended (6). Targeted antimicrobial therapy, as recommended by antimicrobial stewardship, is one of the ways to achieve rational antibiotic use (7). This involves the identification and determination of antimicrobial susceptibility pattern of the offending pathogens.

Antibiotic guideline and targeted antimicrobial therapy require the utilization of the clinical microbiology laboratory. To optimize accurate diagnosis of infectious diseases, clinicians need to ensure that diagnostic

specimens are properly collected, transported and promptly submitted to the clinical microbiology laboratory, preferably before the initiation of antimicrobial therapy (8). Even in life-threatening conditions such as sepsis and septic shock where prompt initial antibiotic therapy is associated with better outcome, it is still recommended that blood culture samples be obtained before initiation of antibiotic treatment, though without delaying the latter.

Many infectious conditions hardly require instant antimicrobial administration, therefore, the time spent for proper diagnosis and prudent selection of antibiotics will be of great benefit to the patient and the health care system in the long run. Biomarkers such as procalcitonin and C-reactive protein levels are usually employed to guide empiric antibiotic therapy, which is usually broad spectrum or involves combination therapy. Treatment may need to be de-escalated and the antibiotic spectrum narrowed as soon as culture and susceptibility pattern are ascertained. This reduces the incidence of adverse events and cost, as well as delay the emergence of antimicrobial resistance.

Point prevalence survey (PPS) of anti-microbial prescription and resistance conducted in Nigeria in 2015 and 2017 showed very high rates of antibiotic use and poor laboratory utilization for definitive diagnosis of infections for which the antimicrobials were prescribed (9,10). These findings raised the question as to why clinicians are not optimally utilizing the clinical microbiology laboratory. The objective of this study was therefore to determine the reasons for clinicians" suboptimal utilization of the clinical microbiology laboratory in Nigeria for diagnosis of infections.

Materials and method:

Study setting

The study setting is Nigeria which operates a three-tiered hierarchical health structure; primary, secondary and tertiary health facilities (11). Each tier is manned by certain categories of health manpower that render specified range of services to the public. Irrespective of the tier, the health facility could be publicly or privately funded. The secondary and tertiary health facilities are more likely to have full laboratory support compared to the primary health facilities. Health facilities without laboratory support often depend on the privately-owned laboratory facilities. Most of the specialists practice in the secondary and tertiary health facilities.

Study design, population and sample size

We conducted a descriptive cross-sectional survey of specialists attending the annual scientific conference and general meeting of the National Postgraduate Medical College of Nigeria (NPMCN) in Owerri, South-eastern Nigeria, in August 2018. The NPMCN has trained over 7,000 specialists, with about 4,000 currently practicing in Nigeria. The conference was attended by 800 delegates (personal communication).

The sample size was calculated using the formula for estimating single independent proportion (12). We assumed 50% utilization of laboratory services and 5% level of significance. We also adjusted for a population less than 10,000 and 75% response rate. This gave us the sample size of 468, which was approximated to 470.

Sampling technique and data collection

We used a semi-structured pre-tested questionnaire to collect information on socio-demographic characteristics, use of clinical microbiology laboratory for diagnosis of infections, and reasons for non-use of the laboratory, from each participant. The participants from different specialties were selected by ballot without replacement method of simple random sampling technique, and the questionnaires were hand-delivered to and self-administered by each participant.

The degree of utilization of the clinical microbiology laboratory services was ranked as „always", „very often", „occasionally", „not often", „rarely" and „never". Responses of „always" and „very often" were categorized as „good use" while others were categorized as „poor use" of the laboratory.

Data analysis

Data were analyzed using EPI INFO version 7.2 (US CDC). The socio-demographic characteristics and the degree of use of clinical microbiology laboratory were presented as frequency and proportions. The relationship between use of clinical microbiology laboratory and socio-demographic characteristics were examined using Chi square test at 5% level of significance. The odd ratio and 95% confidence interval of the odds were estimated.

Results:

Only 283 participants returned completed questionnaires giving a response rate of 60.2%. There were 207 (73.1%) males, with male: female ratio of 2.7:1. The age group 45-

49years constituted the largest group (25.2%). Forty-two (14.8%) respondents were general practitioners while 241 (85.2%) were specialists who have been in practice for a median period of 20 years (3–48years). The specialists included general surgeons (26%), family physicians (19.8%), internists (14.3%), pathologists (13.9%), paediatricians (8.8%), obstetricians and gynecologists (8.1%), community medicine physicians (6.2%), and dental surgeons (2.6%) (Table 1). Majority of the respondents (90.8%, n=257) work in public hospitals, 250 (88.3%) work in tertiary and 28 (9.9%) in secondary care hospitals

One hundred and eighty six (65.7%) of the respondents made good use of the laboratory; 45 (15.9%) always and 141 (49.8%) very often, while 97 (34.3%) made use of the laboratory poorly; 35 (12.4%) use the facility occasionally, 39 (13.8%) not often, 18 (6.3%) rarely, and 5 (1.8%) never (Table 2). The reasons for utilization were microscopy, culture and sensitivity (62.4%), nucleic acid detection (18.3%), GeneXpert (17.2%) and antigen detection (16.7%) (Table 2). Nucleic acid detection was used mainly for follow up for hepatitis B management.

Reliance on clinical diagnosis (39.7%) and perceived patient"s inability to afford the cost of the laboratory test were the most common reasons given by the respondents for non-use of the laboratory. Others were the prolonged turnaround time, lack of access to medical microbiology laboratory, and having knowledge of potent antibiotics (Table 2). None of the factors examined was significantly associated with good use of the clinical microbiology laboratory for diagnostic purposes (Table 3).

Table 1: Socio-demographic characteristics of respondents

Variable	Frequency	Percentage (%)
Gender		
Male	207	73.1
Female	76	26.9
Age group (years)*		
35-39	17	6.0
40-44	51	18.1
45-49	71	25.2
50-54	54	19.2
55-59	42	14.9
60-64	28	9.9
≥65	19	6.7
Mean age ± SD	50.7±8.4	
Branch of Medicine		
Physician	177	62.5
Surgeon	106	37.5
General Practitioner		
Yes	42	14.9
No	241	85.1
Specialty*		
General Surgeon	72	26.4
Family Physician	54	19.8
Internist	39	14.3
Pathologist	38	13.9
Paediatrician	24	8.8
Obstetrics & Gynaecologist	22	8.1
Community Physician	17	6.2
Dental Surgeon	7	2.6
Place of Practice		
Public	257	90.8
Private	15	5.3
Faith based	11	3.9
Level of practice		
Tertiary	250	88.3
Secondary	28	9.9
Primary	5	1.8
Duration of practice (years)		
1-10	29	10.3
11-20	122	43.1
21-30	64	22.6
>30	68	24.0

*missing values

Table 2: Patterns of utilization of clinical microbiology laboratory by clinicians for patient management

Variable	Frequency	Percentage (%)
Use of microbiology laboratory for diagnosis of infections (n = 283)		
Always	45	15.9
Very often	141	49.8
Occasionally	35	12.4
Not often	39	13.8
Rarely	18	6.3
Never	5	1.8
Laboratory investigations often requested for (n = 186) *		
Bacteria culture and susceptibility tests	116	62.4
Nucleic acid detection	34	18.3
GeneXpert	32	17.2
Antigen detection	31	16.7
Microscopy	4	2.2
Others	59	31.7
Nucleic acid detection (n = 34)		
Hepatitis B	7	20.6
Paternity dispute	2	5.9
TB MDR	2	5.9
Sex determination	1	2.9
Hepatitis C	1	2.9
Reasons for non-use of laboratory for diagnosis of infections (n = 58) *		
Clinical diagnosis is sufficient	23	39.7
Patients cannot afford the cost	12	20.7
Results are always delayed so irrelevant to patient management	10	17.2
Already know potent antibiotics and no need for test	9	15.5
No access to medical microbiology laboratory	8	13.8
No pathologist to ensure quality of test	7	12.1
I don"t need lab to manage patients with infections	5	8.6
Others	19	32.8

*Multiple responses allowed

Table 3: Factors associated with laboratory utilization by clinicians

Variable	Laboratory utilization		Crude Odds ratio
	Good (%)	Poor (%)	(95% CI)
Gender			
Male	137 (66.2)	70 (33.8)	1.1 (0.62-1.87)
Female	49 (64.5)	27 (35.5)	1
General Practitioner			
Yes	28 (66.7)	14 (33.3)	1.1 (0.53-2.11)
No	157 (65.4)	83 (34.6)	1
Branch of Medicine			
Physicians	117 (66.1)	60 (33.9)	1.0 (0.63-1.93)
Surgeons	69 (65.1)	37 (34.9)	1
Level of Practice			
Tertiary	164 (65.6)	86 (34.4)	1.0 (0.44-2.05)
Others	22 (66.7)	11 (33.3)	1
Duration of Practice (years)			
1 – 10	16 (55.2)	13 (44.8)	0.6 (0.26-1.56)
11 – 20	77 (63.1)	45 (36.9)	0.9 (0.49-1.69)
21 – 30	48 (75.0)	16 (25.0)	1.6 (0.73-3.35)
>30	45 (66.2)	23 (33.8)	1

Discussion:

This study revealed suboptimal use of the clinical microbiology laboratory, contrary to the practice in western countries where studies have shown that there is over utilization of the clinical laboratories and efforts are actually being made to reduce this (13,14,15). The clinical microbiology laboratory plays a vital role in the management of infectious diseases. Non utilization of the laboratory has dire consequences as diagnosis of infectious diseases based on symptoms and signs lead to both over and under-diagnosis or outright

misdiagnosis of some infections. These have clinical implications including over and inappropriate prescription of antibiotics, wastages, infection complications, economic consequences, and potential fatal outcome (10,16,17,18).

One of the reasons for non-use of the clinical laboratory by physicians in this survey is the presumed sufficiency of clinical diagnosis, thereby making microbiology invest- igations unnecessary. Similar findings have been reported in other African countries where doctors express doubt about accuracy of laboratory results or are confused by them and therefore prefer to follow up patients rather than use the laboratory (19,20,21,22). In these countries, poor laboratory infrastructure or personnel have been blamed for this, but in our study, respondents who work in reputable tertiary hospitals with good clinical micro-biology laboratories and qualified pathologists did not make effective use of the laboratories (19,20,21,22).

Some of the respondents claim to already have knowledge of potent antibiotics and therefore do not require the laboratory. This view may be the result of lack of knowledge of the critical role clinical microbiology laboratory plays in the diagnosis and treatment of infectious diseases, as well as low awareness and/or absence of antimicrobial stewardship programmes in most Nigerian hospitals at the time of the survey. This may be responsible for high use of antibiotics noted in some surveys (9,10,23,24). The obvious lack of association between poor clinical microbiology utilization and any of the sociodemographic variables analysed highly suggests that poor clinical microbiology laboratory utilization is a habit or behavioural anomaly that cuts across the spectrum of doctors.

The low utilization of nucleic acid detection tests suggests possibility of poor diagnosis of viral infections. This is a cause for concern as a significant proportion of viral infections are often mistreated with antibiotics thus paving way for subsequent development of antimicrobial resistance. Previous studies have reported over prescribing of antibiotics for viral diseases (25).

One limitation of the study was the use of subjective adjectives in describing the degree of laboratory utilization. The study participants were specialists whom we believed have good understanding of the words and were honest in their responses. However, in view of the very low rate targeted antibiotic therapy obtained in previously performed surveys, the rate of non-use of the micro-biology laboratory confessed may well be an underestimate and need to be corroborated with other studies (9,10). Another limitation may be the high level of non response rate that is typical of such settings.

Conclusion:

The results of this study confirm that physicians" use of the clinical microbiology laboratory in Nigeria for diagnosis of infection is suboptimal and needs to be improved on. The reasons for these are mainly physicians" perception that clinical diagnosis is sufficient, delayed laboratory results, poor access to microbiology laboratory, and lack of qualified pathologists. There is urgent need for education of physicians to optimally utilize the microbiology laboratory to improve their antibiotics prescribing practice, and also the need to raise awareness among them on what constitutes optimal use of the laboratory for diagnosis and appropriate management of infections.

References:

1. Prestinaci, F., Pezzotti, P., and Pantosti, A. Anti-microbial resistance: a global multifaceted phenomenon. Pathog Glob Health. 2015; 109 (7): 309–318.
2. Bernabé, K. J., Langendorf, C.. Ford, N., Jean-Baptiste., R. J., and Murphy, A. R. Antimicrobial resistance in West Africa: a systematic review and meta-analysis. Int J Antimicrob Agents. 2017; 50 (5): 629-639
3. Oduyebo, O. O., Onwuezobe, I. A., Olayemi, S. O., and Akintunde, A. S. Correlating Antibiotic Consumption With Antimicrobial Resistance of Uropathogens In a University Teaching Hospital In Lagos, Nigeria. Nig J Clin Pract. 2008; 11(4): 305-308
4. Shittu, A. O., Okon, K., Adesida, S., Oyedara, O., Witte, W., Strommenger, B., Layer, F., and Nübel, U. Antibiotic resistance and molecular epidemiology of *Staphylococcus aureus* in Nigeria. BMC Microbiol. 2011; 11: 92
5. Nmema, E. E. Peculiar pattern of antibiotic resistance in bacteria isolated from various sources in South-East Nigeria and the implications in health and economy. J Appl Sci Environ Manag. 2013; 17 (4): 529-534
6. World Health Organization SEARO: Step-by-step approach for development and implementation of hospital antibiotic policy and standard treatment guidelines, 2011
7. CDC. Core Elements of Hospital Antibiotic Stewardship Programs, Available from https://www.cdc.gov/antibiotic-use/core-elements/hospital.html
8. Leekha, S., Terrell, C. L., and Edson, R. S. General Principles of Antimicrobial Therapy. In Mayo Clin Proc. 2011; 86 (2): 156-167.
9. Oduyebo, O, O., Iregbu, K. C., Chukwuma, U., et al. The Global Point Prevalence Survey of Antimicrobial Consumption and Resistance (Global-PPS): Results of Antimicrobial Prescribing

in Nigerian Hospitals. In proceedings of 28th ECCMID conference; 2018 April 21-24, Madrid, Spain. ESCMID abstract publications; 2018. E0034
10. Oduyebo, O. O., Olayinka, A. T., Iregbu, K.C., et al. A point prevalence survey of antimicrobial prescribing in four Nigerian Tertiary Hospitals. Ann Trop Pathol. 2017; 8: 42-46
11. National Health Act 2014. Federal Republic of Nigeria, Official Gazette No. 145 (101) Notice No. 208
12. Cochran, W. G. Sampling Techniques, 3rd Ed., New York: John Wiley and Sons, Inc., 1977.
13. Minerowicz, C., Abel N., Hunter K., Behling, K. C., Cerceo, E., and Bierl, C. Impact of weekly feedback on test ordering patterns. Am J Manag Care. 2015; 21: 763–768
14. Kobewka, D. M., Ronksley, P. E., McKay, J. A., Forster, A. J., and Van Walraven, C. Influence of educational, audit and feedback, system based, and incentive and penalty interventions to reduce laboratory test utilization: A systematic review. Clin Chem Lab Med. 2015; 53: 157–183.
15. Zhi M., Ding, E. L., Theisen-Toupal J., Whelan, J., and Arnaout, R. The landscape of inappropriate laboratory testing: a 15-year meta-analysis. PLoS One. 2013; 8: e78962.
16. Bindraban, R. S., ten Berg, M. J., Naaktgeboren, C. A., Kramer, M. H., van Solinge, W. W., and Nanayakkara, P. W. Reducing Test Utilization in Hospital Settings: A Narrative Review. Ann Lab Med. 2018; 38 (5): 402–412.
17. Ngwu, B. A., and Agbo, J. A. Typhoid fever: clinical diagnosis versus laboratory confirmation. Niger J Med. 2003; 12: 187-192
18. Petti, C. A., Polage C. R., Quinn, T. C., Ronald, A. R., and Sande, M. A. Laboratory Medicine in Africa: A Barrier to Effective Health Care. Clin Infectious Dis. 2006; 42 (3): 377-382. https://doi.org/10.1086/499363
19. Oladosu, O. O. and Oyibo, W. A. Over diagnosis and Overtreatment of Malaria in Children That Presented with Fever in Lagos, Nigeria. ISRN Infect Dis. 2013; 2013: 6. http://dx.doi.org/10.5402/2013/914675
20. Vanker, N., and Faull, N. H. Laboratory test result interpretation for primary care doctors in South Africa. Afr J Lab Med. 2017; 6 (1): 1-6 https://doi.org/10.4102/ajlm.v6i1.453
21. Moyo, K., Porter, C., Chilima, B., et al. Use of laboratory test results in patient management by clinicians in Malawi. Afr J Lab Med. 2015; 4(1):1-8
22. Benatar, S. R. Health care reform and the crisis of HIV and AIDS in South Africa. N Engl J Med. 2004; 351: 81-92.
23. Mepham, S. O., Squire, S. B., Chisuwo, L., Kandulu, J., and Bates, I. Utilisation of laboratory services by health workers in a district hospital in Malawi. J Clin Pathol. 2009; 62 (10): 935–938.
24. Fadare, O. J., Ogunleye, O., Iliyasu, G., Adeoti, A., Schellack, N., Engler, D., Massele, A., and Godman, B. Status of antimicrobial stewardship programmes in Nigerian tertiary healthcare facilities: Findings and implications. J Glob Antimicrob Resist. 2019; 17: 132-136
25. Imanpour, S., Nwaiwu, O., McMaughan, D. K., DeSalvo, B., and Bashir, A. Factors associated with antibiotic prescriptions for the viral origin diseases in office-based practices, 2006-2012. JRSM Open. 2017; 8: 1-8

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Short Communication **Open Access**

Otitis externa in a tertiary care hospital in Zagazig, Egypt: isolated pathogens and their antibiotic sensitivity patterns

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Abstract:

Introduction: Recurrent otitis externa is a worldwide problem. This study aims to identify the different aetiological organisms isolated from otitis externa and their sensitivity to different antibiotics.
Methods: A total of 27 patients with clinical presentation of otitis externa for a period of three weeks or more were enrolled for the study. Two swab samples collected from each infected ear were cultured for bacterial and fungi, and growth identified using standard microbiological methods including analytical profile index (API) system. Antibiotic susceptibility of isolated bacteria was performed by the disk diffusion technique.
Results: Thirty one organisms were isolated from the 27 patients; 12 (38.7%) fungi and 19 (61.3%) bacteria species. *Aspergillus* spp was the most frequently isolated organism (35.4%) while *Pseudomonas aeruginosa* was the most frequently isolated bacteria (19.3%), and was most sensitive to amikacin. Four of 11 patients with *Aspergillus* infection showed clinical resistance to econazole local treatment but had complete clinical response to itraconazole oral treatment.
Conclusion: Otitis externa in Egypt is caused by antibiotic resistant bacteria or fungi, and the most causative organisms are *Aspergillus* spp and *Ps. aeruginosa*.

Keywords: Otitis externa, antibiotic resistance, *Pseudomonas aeruginosa,* Egypt

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Otite externe à l'hôpital de soins tertiaires de Zagazig, en Égypte: agents pathogènes isolés et leur profil de sensibilité aux antibiotiques

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Abstrait:

Introduction: L'otite externe récurrente est un problème mondial. Cette étude vise à identifier les différents organismes étiologiques isolés d'une otite externe et leur sensibilité à différents antibiotiques.
Méthodes: Un total de 27 patients présentant une présentation clinique de l'otite externe sur une période de trois semaines ou plus ont été inclus dans l'étude. Deux échantillons de prélèvement prélevés sur chaque oreille infectée ont été mis en culture pour détecter la présence de bactéries et de champignons, et leur croissance a été identifiée à l'aide de méthodes microbiologiques standard, notamment d'un système d'indice de profil analytique (API). La

sensibilité aux antibiotiques de bactéries isolées a été réalisée par la technique de diffusion sur disque. **Résultats:** Trente et un organismes ont été isolés parmi les 27 patients; 12 espèces de champignons (38,7%) et 19 espèces de bactéries (61,3%). *Aspergillus* spp était l'organisme le plus fréquemment isolé (35,4%), tandis que *Pseudomonas aeruginosa* était la bactérie la plus fréquemment isolée (19,3%) et était la plus sensible à l'amikacine. Quatre des 11 patients infectés par *Aspergillus* ont présenté une résistance clinique au traitement local à l'éconazole, mais ont présenté une réponse clinique complète au traitement oral à l'itraconazole.
Conclusion: L'otite externe en Egypte est causée par une bactérie ou un champignon résistant aux antibiotiques. Les organismes les plus responsables sont *Aspergillus* spp et *Ps. aeruginosa*.

Mots-clés: otite externe, résistance aux antibiotiques, *Pseudomonas aeruginosa*, Égypte

Introduction:

Otitis externa, also known as external otitis or swimmer's ear, is an inflammation of the pinna and external ear canal (1)**.** Swimming in polluted water is a common means by which swimmer's ear is contracted but it is also possible to contract the disease from water trapped in the ear canal after a shower, especially in a humid climate. In addition to the presence of microorganisms, impairment of the integrity of the skin of the ear canal is required for external otitis to develop (2). Acute otitis externa is usually caused by bacteria while fungal involvement is commoner with chronic otitis externa.

Otitis externa is one of the most commonly encountered diseases by otorhino-laryngologists, and is common in certain parts of world with hot humid climate. Due to humid climate of Egypt, there is a high occurrence of otitis externa, especially in the summer. A number of patients are seen without evidence of eczematoid-type chronic otitis externa or other clear-cut causes of their recurrent bouts of otitis externa (3).

Although, the course of the disease is usually self-limited and responds quickly to basic treatment, some cases of otitis externa become resistant or are associated with multiple recurrences. This study aims to identify the microbial pathogens of otitis externa and determine their susceptibility to different antibiotics.

Materials and method:

Study setting and design

This cross sectional study was conducted at the Otorhinolaryngology Department of Zagazig University Hospital and Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, Egypt. The subjects included 27 patients with clinical diagnosis of otitis externa, who were enrolled among those attending the outpatient clinic on complaints of otitis externa for a period of three weeks or more, irrespective of previous therapy. The Institutional Review Board of Faculty of Medicine, Zagazig University approved the study. Informed consent of each patient was obtained prior to enrollment.

Clinical features of subjects

Each patient had full medical history including duration of illness, symptoms and history of previous treatment, and a general examination. The patient ears were then examined after thoroughly dry-mopping with cotton pads, and signs such as tenderness, erythema, scales, exfoliation and ear discharge were recorded.

Specimen collection and culture isolation

Two swab samples were collected from each infected ear, one swab for bacterial culture and antibiotic susceptibility, and the other for fungal culture. The swabs were rapidly transferred to laboratory within one hour. For bacterial isolation, swab specimens were cultured on Blood, Chocolate and MacConkey agar plates. The Chocolate agar plate was incubated in candle extinction jar while the Blood and MacConkey agar plates were incubated in air. All media were incubated at 37°C for 48 hours. For fungi isolation, swab specimens were inoculated on two plates of Sabouraud Dextrose agar (SDA), one plate was incubated at 25°C (room) temperature and the other at 37°C for 14 days.

Microbial identification from culture

Isolated bacteria were identified according to standard bacteriology techniques of Gram stain, colony morphology and biochemical reactions including the API system (4). Antibiotic susceptibility test was performed by the disc diffusion method and interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (5). Filamentous fungi isolated were identified using modified slide culture technique (6) while *Candida albicans* was identified by the germ tube test.

Modified slide culture technique:

A 1 by 1 cm block of SDA was cut with sterile scalpel and transferred to a plate of SDA. The isolated fungus was inoculated using

sterile wire needle into the four sides of the agar block. Sterile cover slip was put over the block with slight pressure to ensure adherence and the plate cover replaced afterwards. The plates were incubated at 25ºC (room temperature). The growth of the fungus was examined periodically. The closed Petri dish was placed on the microscope stage and the slide culture examined with the low power. When reproduced structures had developed, a forcep was used to remove the cover slip and put on a second slide for examination. The agar block was removed using flamed wire needle and put into a container of antifungal disinfectant. A drop of Lactophenol cotton blue stain was put on the remaining slide and was then covered with a new cover slip (6).

Therapy and follow up of patients:

Patients were treated with appropriate antibacterial and antifungi agents, and follow-up visit was at the seventh day after the baseline visit. Treatment was continued for patients whose symptoms persist with follow-up visits at 2 and 3 weeks. At each follow up, clinical information such as pain, itching, otorrhoea, stuffy feeling, burning, irritation and compliance with treatment were obtained from patients and recorded.

Results:

The study was conducted on 27 patients with clinical otitis externa, 18 males and 9 females (M: F ratio of 2:1). The age range of the subjects was 15-64 years with mean of 42.37±14.01. Table 1 summarizes the demographic characteristics and clinical manifestations of the patients.

Table 2 shows that 31 organisms were isolated from the patients; 12 (38.7%) fungi and 19 (61.3) bacteria species. *Aspergillus* spp was the most frequently isolated organism (35.4%). Bacteria isolated were 9 (29%) Gram positive and 10 (32.3%) Gram negatives. *Pseudomonas aeruginosa* was the most frequently isolated bacteria (19.3%), followed by *Staphylococcus epidermidis* (16.1%), *Klebsiella* spp (9.7%) and *Staphylococcus aureus* (9.6%). As shown in Table 3, there was no significant association between the types of microorganism isolated (bacteria or fungi) and symptoms of otalgia (p=0.8137), itching (p=0.7347) or ear discharge (p=0.2522).

The antibiotic susceptibility of the Gram positive bacteria as depicted in Table 4 shows that they were mostly sensitive to ciprofloxacin, levofloxacin and linezolid, and resistant to clindamycin, tetracycline and ceftriaxone. The Gram negative bacteria were mostly sensitive to imipenem, levofloxacin, ciprofloxacin and colistin, and largely resistant to chloramphenicol, gentamicin, tobramycin and ceftriaxone.

Patients with *Aspergillus* infection (n=11) were treated with econazole but only 7 had complete clinical response to econazole local treatment while the remaining 4 had complete clinical response to itraconazole oral treatment. The patients with *Candida* infection had clinical response to terbinafine cream.

Table 1: Socio-demographic characteristics and clinical history of patients with clinical otitis externa in Zagazig University Hospital, Egypt

Characteristics	Frequency	Percentage
Gender		
Male	9	33.3
Female	18	66.7
Marital status		
Married	25	92.5
Single	1	3.75
Widow	1	3.75
Occupation		
Employed	13	48.2
Unemployed	14	51.8
Smoking history		
None	21	77.8
Smokes	6	22.2
Past medical history		
No previous surgery	14	51.8
Adenotonsillectomy	1	3.75
Tonsillectomy	2	7.5
Allergy from drugs	1	3.75
Not relevant	9	33.3
Clinical manifestations		
Otalgia	26	96.3
Discomfort	22	88.5
Itching	26	96.3
Edema of ear canal	27	100

Table 2: Microorganisms isolated from patients with otitis externa in Zagazig University Hospital, Egypt

Microorganism	Frequency	Percentage
Fungi	12	38.7
Aspergillus spp	11	35.4
Candida spp	1	3.2
Gram positive bacteria	9	29
Diphtheroids	1	3.2
Staphylococcus aureus	3	9.6
Staphylococcus epidermidis	5	16.1
Gram negative bacteria	10	32.3
Pseudomonas aeruginosa	6	19.3
Klebsiella spp	3	9.7
Proteus spp	1	3.2
Total	31	100

Table 3: Relationship between symptoms of otitis externa and microorganisms isolated among patients in Zagazig University Hospital, Egypt

Symptoms	Microorganism			X^2	95% CI	*p* value
	Bacteria (n=19)	Fungi (n=12)	Total (n=31)			
Otalgia						
Positive	19	11	30	0.05552	0.4385-0.8005	0.8137*
Negative	0	1	1			
Ear discharge						
Positive	10	3	13	1.311	0.8878-2.666	0.2522*
Negative	9	9	18			
Ear itching						
Positive	18	11	29	0.1149	0.3015-5.111	0.7347*
Negative	1	1	2			

* = not significant

Table 4: Antibiotic susceptibility profile of bacteria isolates of otitis externa in Zagazig University Hospital, Egypt

Antibiotics	Gram positive bacteria (n=9)		Gram negative bacteria (n=10)	
	Sensitive (%)	Resistant (%)	Sensitive (%)	Resistant (%)
Oxacillin	1 (11.1)	8 (88.9)	NA	NA
Tetracycline	1 (11.1)	8 (88.9)	NA	NA
Clindamycin	1 (11.1)	8 (88.9)	NA	NA
Linezolid	9 (100)	0	NA	NA
Chloramphenicol	NA	NA	0	10 (100)
Tobramycin	NA	NA	4 (40)	6 (60)
Amikacin	NA	NA	7 (70)	3 (30)
Colistin	NA	NA	9 (90)	1 (10)
Aztreonam	NA	NA	6 (60)	4 (40)
Amoxicillin-Clavulanic acid	3 (33.3)	6 (66.7)	1 (10)	9 (90)
Ceftriaxone	1 (11.1)	8 (88.9)	4 (40)	6 (60)
Gentamicin	5 (55.6)	4 (44.4)	2 (20)	8 (80)
Ciprofloxacin	9 (100)	0	9 (90)	1 (10)
Laevofloxacin	9 (100)	0	9 (90)	1 (10)
Imipenem	8 (88.9)	1 (11.1)	9 (90)	1 (10)

NA = Not Applicable

Discussion:

Otitis externa is a common disease and its severity varies from mild, self-limited condition to severe malignant otitis externa with involvement of cartilage and bone of the external auditory canal, and a tendency to break through to the brain (7). In the current study, *Ps. aeruginosa* was the most frequently isolated bacteria pathogen and constituted 19.5% of the isolates, which agrees with previous studies that established this pathogen as the most frequent in acute otitis externa (8, 9)**.** The rate for *Ps. aeruginosa* in this study is however lower than 41.7% reported from a study conducted on ear discharge of malignant otitis externa in Jordan (10) and 52.9% in India (11), but the rate is higher than 12% reported from a study in Iran (12).

Among the fungi, *Aspergillus* spp are the predominant organisms implicated in oto-mycosis (7). In the current study, *Aspergillus* spp was the most frequently isolated organism with 35.4%, which agrees with studies from

Turkey (13) and Iran (14), where *Aspergillus* spp constituted 78.6% of otomycosis agents, followed by *Candida* species (6.8%) and other saprophytic fungi (4.7%). The high prevalence of fungal otitis externa may be secondary to over use of broad spectrum antibiotics and increased topical use of fluoroquinolones (13), which encourages fungi overgrowth.

Fungal otitis externa is often asymptomatic, with discomfort being the most frequent complaint, but there may be pruritus and a feeling of fullness in the ear. *Aspergillus* species, particularly *Aspergillus niger* may grow in the cerumen and desquamated keratinaceous debris in the external auditory canal, sometimes forming a visible greenish or blackish fluffy colony (6). In this study, there was no significant difference between the symptoms of otalgia, ear itching or discharge between bacteria and fungi otitis externa ($p>0.05$).

In the current study, the Gram negative bacteria were mostly sensitive (90%) to imipenem, laevofloxacin, ciprofloxacin and colistin, but largely resistant to chloramphenicol, gentamicin, tobramycin and ceftriaxone. The Gram positive bacteria on the other hand were totally sensitive (100%) to laevofloxacin, ciprofloxacin and linezolid, and 89% sensitive to imipenem, but were largely resistant to clindamycin, tetracycline and ceftriaxone. The most frequently isolated Gram negative bacteria, *Pseudomonas* spp was mostly sensitive to amikacin, imipenem, laevofloxacin, ciprofloxacin and colistin, but largely resistant to amoxicillin-clavulanate, gentamicin, tobramycin and chloramphenicol. All *S. aureus* and *S. epidermidis* isolates were resistant to oxacillin, while the only diphtheroid was sensitive to the antibiotic. The issue of bacterial resistance to antibiotics is a global challenge (15). In developed countries such as the United States, contemporary ear culture isolates at quaternary care center show higher rates of methicillin resistant *S. aureus* (MRSA) compared to historical reports in the literature (16). However, the challenge of antibiotic resistance is more in developing countries such as Egypt (17), as a result of low socioeconomic state and behavioral pattern of the populace regarding antibiotic use (18). In Egypt, antibiotics can be purchased from pharmacies without medical authorization or prescription.

In this study, fungi were isolated in 38.7% of cases with half of them having bacteria pathogen concurrently involved, which explains the chronicity of the lesion. These cases were previously treated as pure bacterial infection while the fungal aetiology was hidden by the discharge and erythema usually seen in bacterial infection. On the other hand, fungal infection is usually associated with itching as seen in 11 out of 12 (91.7%) cases in this study.

Treatment of acute otitis externa should include an active antimicrobial agent and not just an acidifying agent such as acetic acid (19). Moderate to severe cases will require ototopical agents in addition to antiseptic solution (20). Ciprofloxacin ear drop was the commonest agent used in the initial treatment of our patients followed by gentamicin with or without corticosteroid, and then econazole antifungal agent. Our study observed that many of the locally used drugs such as gentamicin, chloramphenicol and tetracycline have poor activity against most pathogens. This may be due to the injudicious use of these agents which has resulted in the emergence of more resistant strains, especially among the troublesome pseudomonads.

The resistant otitis externa cases could also be associated with contact dermatitis (7), therefore antiinflammatory corticosteroid combination in the ear drops may be required. In the case series by Smith et al., (21) in 1990, 23.5% of their patients with otitis externa had contact dermatitis, and this was even less than what Holmes and Johnson (22) had reported in 1982. Infestation by *Demodex* species can also cause persistent itching in resistant cases especially in immuno-compromised patients (23) or with prolonged use of local steroid which may increase the frequency of the parasite in the external ear canal of affected patients (24). In this study, local treatment of candida otitis externa was totally responsive to terbinafine cream but only 7 out of 11 patients with *Aspergillus* infection had complete clinical response to econazole local treatment. The four patients resistant to econazole had complete clinical response to itraconazole oral treatment. This observation agrees with a study in India (25), which reported that 5 days course of itraconazole treatment was very effective for recurrent otomycosis in diabetic patients.

Conclusion:

Our study reports that otitis externa in Egypt is caused by antibiotic resistant bacterial and fungi, the most common pathogens being *Aspergillus* spp and *Ps. aeruginosa*. Oral itraconazole was effective for recurrent fungal otitis externa. We recommend that in cases of otitis externa resistant to antibacterial agents,

fungi aetiology should be investigated by culture on Saboraud Dextrose agar.

Conflict of interest:

Authors declared no conflict of interest

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Authors contributions:

AAA, AEET and KAEM were responsible for concept and design of the study. NAME undertook acquisition and interpretation of data and drafting of the manuscript. All authors contributed to data collection. AAA undertook critical review of the manuscript. NAME, EAM and MAEE undertook statistical analysis and review of the manuscript.

References:

1. Moharram, A. M., Ahmed, H. E., and Nasr, S. A. M. Otomycosis in Assiut, Egypt. Journal of Basic and Applied Mycology (Egypt); 2013; 4: 1-11.
2. Roland, P. S., and Stroman, D. W. Microbiology of acute otitis externa. Laryngoscope. 2009; 112: 1166-1177. https://doi.org/10.1097/00005537-200207000-00005
3. Shapiro, D. P. Recurrent Otitis Externa. Arch Otolaryngol Head Neck Surg. 1989; 115 (3): 394. https://doi.org/10.1001/archotol.1989.01860270136034
4. Mackie, T. J., Collee, J. G., and McCartney, J. E. Mackie and McCartney Practical Medical Microbiology. New York: Churchill Livingstone, 1996.
5. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial disk susceptibility tests; 9th approved standard. Document M2-A9. Wayne, PA: CLSI; 2006.
6. Rosana, Y., Matsuzawa, T., Gonoi, T., and Karuniawati, A. Modified Slide Culture Method for Faster and Easier Identification of Dermatophytes. Microbiol Indonesia. 2014; 8: 135 - 139. https://doi.org/10.5454/mi.8.3.
7. Mustafa, M., Patawari, P., Sien, M. M., Muniandy, R. K., and Zinatara, P. Acute Otitis Externa: pathophysiology, clinical presentation, and treatment. IOSR Journal of Dental and Medical Sciences. 2015; 14: 73 – 78 https://doi.org/10.9790/0853-14717378
8. Hajioff, D., and Mackeith, S. Otitis externa. Clinical Evidence. BMJ. 2015; 6 (51): 1 - 23.
9. Treviño González, J. L., and Moreno, K. D. Otitis Externa: an update. Ann Otolaryngol Rhinol. 2017; 4 (8): 1195.
10. Battikhi, M. N., and Ammar, S. I. Otitis externa infection in Jordan. Clinical and microbiological features. Saudi Med J. 2004; 25 (9): 199 - 203.
11. Shamanna, K., and Ganga, B. V. Changing trend in the management of malignant otitis externa: our experience. Research in Otolaryngology. 2018; 7 (1): 9-14. https://doi.org/10.5923/j.otolaryn.20180701.03
12. Kiakojuri, K., Mahdavi, O. S., Jalili, B., et al. Bacterial Otitis Externa in Patients Attending an ENT Clinic in Babol, North of Iran. Jundishapur J Microbiol. 2016; 9 (2): e23093. https://doi.org/10.5812/jjm.23093.
13. Enoz, M., Sevinc, R., and Florencio Lapeña, J. Bacterial and fungal organisms in otitis externa patients without fungal infection risk factors in Erzurum, Turkey. Braz J Otorhinolaryngol. 2009; 75: 721 - 725. http://dx.doi.org/10.1590/S1808-8694-20090005 00018
14. Gharaghani, M., Seifi, Z., and Mahmoudabadi, A. Z. Otomycosis in Iran: A Review. Mycopathologia. 2015; 179: 415–424. https://doi.org/10.1007/s11046-015-9864-7
15. Roca, I., Akova, M., Baquero, F., et al. The global threat of antimicrobial resistance: science for intervention. New Microbes and New Infections. 2015; 6: 22-29. https://doi.org/10.1016/j.nmni.2015.02.007
16. Duarte, M. J., Kozin, E. D., Bispo, P. J. M., el al. Methicillin resistant *Staphylococcus aureus* in acute otitis externa. World J Otorhinolaryngol Head Neck Surg. 2018; 4: 246-252. https://doi.org/10.1016/j.wjorl.2017.09.003
17. Ayukekbong, J. A., Ntemgwa, M., and Atabe, A. N. The threat of antimicrobial resistance in developing countries: causes and control strategies. Antimicrob Resist Infect Contr. 2017; 6: 47. https://doi.org/10.1186/s13756-017-0208-x
18. Okeke, I. N., Lamikanra, A., and Edelman, R. Socioeconomic and behavioural factors leading to acquired bacterial resistance to antibiotics in developing countries. Emerg Infect Dis. 1999; 5 (1):18-27. https://doi.org/10.3201/eid0501.990103
19. Dohar, J. E. Evolution of management approaches for otitis externa. Pediatr Infect Dis J. 2003; 22: 299-305. https://doi.org/10.1097/01.inf.0000059444.02851.1e.
20. Osguthorpe, J. D., and Nielsen, D. R. Otitis externa. Review and clinical update. Am Fam Phys. 2006; 74 (9): 1510-1516.
21. Smith, I. M., Keay, D. G., and Buxton, P. K. Contact hypersensitivity in patients with chronic otitis externa. Clin Otolaryngol. 1999; 15: 155-156. https://doi.org/10.1111/j.1365-2273.1990.tb00449.x
22. Holmes, R. C., Johns, A. N., Wilkinson, J. D., et al. Medicament contact dermatitis in patient with chronic inflammatory ear disease. J R Soc Med. 1982; 75 (1): 27-30.
23. Bilal, N., Kirişci, Ö., and Özkaya, E. *Demodex* species infestation in patients with ear itching and its relationship to itch severity. Turkiye Parazitol Derg. 2017; 41: 87-91. https://doi.org/10.5152/tpd.2017.5070
24. Cevik, C., Kaya, O., Akbay, E., et al. Investigation of *Demodex* species frequency in patients with a persistent itchy ear canal treated with a local steroid. J Laryngol Otol. 2014; 128 (8): 698-701. https://doi.org/10.1017/S0022215114001510.
25. Venkataramanan, R., and Kumar, R. S. Efficiency of 5 Day Course Oral Itraconazole in Management of Recurrent Otomycosis in Diabetic Patients-a Randomized Control Clinical Trial. Indian J Res. 2016; 5 (1): 96-98.

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Short Communication **Open Access**

Laboratory survey of extended spectrum beta-lactamase producing enterobacteriaceae in clinical infections among hospitalised patients at LAUTECH Teaching Hospital, Ogbomoso, Nigeria

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Abstract:

Background: The extended-spectrum beta-lactamase (ESBL) producing enterobacteriaceae are a major public health threat globally, causing both community and healthcare associated infections (HAIs). Due to multi-resistant nature of these strains, infections caused by them are associated with treatment failure, high mortality, and increased healthcare costs. This laboratory survey determined the prevalence of infections caused by ESBL-producing enterobacteriaceae in LAUTECH Teaching Hospital, Ogbomoso
Methodology: Over three years (January 2016 to December 2018), non-duplicate clinical samples (sputum, blood, urine, and wound swabs) collected from hospitalised patients with suspected clinical infections were routinely processed at microbiology laboratory of our hospital for aerobic culture and isolation of enterobacteriaceae. Antibiotic susceptibility of each isolate to routinely used antibiotics was determined by the disk diffusion method and 'double disk' synergy test was used to routinely confirm ESBL production. Demographic and clinical data were extracted from the requisition form.
Results: Of the total 4,198 hospitalised patients over the three year period, 1,222 (29.1%) had clinical infections, out of which 689 (16.4%) were laboratory confirmed. Enterobacteriaceae were isolated from 343 patients (prevalence rate, 8.2%) while ESBL producers were isolated from 46 (prevalence rate, 1.1%). The most frequent enterobacteriaceae were *Klebsiella* spp (54.5%) and *Escherichia coli* (35.9%) recovered mainly from urinary tract infection (UTI, 45.2%), skin and soft tissue infection (SSTI, 27.9%) and lower respiratory tract infection (LRTI, 17.5%) but ESBL producers were frequently associated with osteomyelitis (50%), LRTI (18.3%) and SSTI (14.6%). The ESBL producers were all resistant to ampicillin, cefotaxime, ceftazidime, cefepime, gentamicin, and ciprofloxacin but susceptible to imipenem. The non-ESBL producers were comparatively less resistant with 43.8%, 34.3%, 29%, 35%, 43%, 37%, and 4% resistant to ampicillin, cefotaxime, ceftazidime, cefepime, gentamicin, ciprofloxacin and imipenem respectively.
Conclusion: The prevalence of clinical infections among hospitalised patients in our facility is high but the rate of ESBL-producing enterobacteriaceae is relatively low. In spite of this, there is need for continuous surveillance of ESBL and other antibiotic resistant pathogens as part of the infection prevention and control (IPC) programme, with implementation of measures that will reduce the incidence of these infections in our hospital.

Keywords: Laboratory survey; hospitalised patients; ESBL; multidrug resistance

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Enquête de laboratoire sur les entérobactéries productrices de bêta-lactamases à spectre étendu lors d'infections cliniques chez des patients hospitalisés à l'hôpital universitaire LAUTECH, à Ogbomoso, au Nigéria

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Abstrait:

Contexte: Les entérobactéries productrices de bêta-lactamase à spectre étendu (BLSE) constituent une menace majeure pour la santé publique dans le monde, provoquant à la fois des infections dans la communauté et des infections associées aux soins de santé. En raison de la nature multirésistante de ces souches, les infections qu'elles provoquent sont associées à un échec du traitement, à une mortalité élevée et à une augmentation des coûts de soins de santé. Cette enquête en laboratoire a permis de déterminer la prévalence d'infections causées par des entérobactéries productrices de BLSE à l'hôpital universitaire LAUTECH, à Ogbomoso.
Méthodologie: Sur trois ans (janvier 2016 à décembre 2018), des échantillons cliniques non dupliqués (expectorations, sang, urine, et plaies oreilles) prélevés chez des patients hospitalisés présentant des suspicions d'infections cliniques ont été systématiquement traités dans le laboratoire de microbiologie de notre hôpital pour culture aérobie et isolement d'entérobactériacées. La sensibilité aux antibiotiques de chaque isolat aux antibiotiques utilisés en routine a été déterminée par la méthode de diffusion sur disque et un test de synergie «double disque» a été utilisé pour confirmer en routine la production de BLSE. Les données démographiques et cliniques ont été extraites du formulaire de demande.
Résultats: Sur un total de 4198 patients hospitalisés au cours de la période de trois ans, 1222 (29,1%) ont présenté une infection clinique, dont 689 (16,4%) ont été confirmés en laboratoire. Des entérobactéries ont été isolées chez 343 patients (taux de prévalence de 8,2%), tandis que les producteurs de BLSE ont été isolés chez 46 patients (taux de prévalence de 1,1%). Les entérobactériacées les plus fréquentes étaient *Klebsiella* spp (54,5%) et *Escherichia coli* (35,9%) principalement dues à une infection des voies urinaires (UTI, 45,2%), une infection de la peau et des tissus mous (SSTI, 27,9%) et des voies respiratoires inférieures (LRTI, 17,5%), mais les producteurs de BLSE étaient fréquemment associés à l'ostéomyélite (50%), au LRTI (18,3%) et au SSTI (14,6%). Les producteurs de BLSE étaient tous résistants à l'ampicilline, au céfotaxime, à la ceftazidime, au céfépime, à la gentamicine et à la ciprofloxacine, mais sensibles à l'imipénem. Les producteurs non BLSE étaient comparativement moins résistants, avec respectivement 43,8%, 34,3%, 29%, 35%, 43%, 37% et 4% de résistance à l'ampicilline, au céfotaxime, au ceftazidime, au céfépime, à la gentamicine, à la ciprofloxacine et à l'imipénème.
Conclusion: La prévalence d'infections cliniques chez les patients hospitalisés dans notre établissement est élevée mais le taux d'entérobactéries productrices de BLSE est relativement faible. Malgré cela, il est nécessaire de surveiller en permanence les BLSE et les autres agents pathogènes résistants aux antibiotiques dans le cadre du programme de prévention et de contrôle des infections (IPC), avec la mise en œuvre de mesures permettant de réduire l'incidence de ces infections dans notre hôpital.

Mots clés: Enquête de laboratoire; patients hospitalisés; BLSE; résistance multiple aux médicaments

Introduction:

Infections caused by the extended spectrum beta lactamase (ESBL) producing enterobacteriaceae are a major public health threat globally (1, 2). ESBLs confer resistance to all beta lactam antibiotics except carbapenems and cephamycins (2). They may also confer resistance to additional antibiotic classes such as aminoglycosides, sulfonamides and fluoroquinolones from carriage of plasmids containing resistant genes (3). Infections by these strains are therefore associated with treatment failure, high mortality, and increased healthcare costs (4, 5).

It is important to periodically survey these resistant pathogens in health care institutions for the purpose of infection prevention and control. The objectives of this survey therefore is to identify ESBL producing enterobacteriaceae and determine the prevalence in clinical infections among hospitalized patients in Ladoke Akintola University of Technology (LAUTECH) Teaching Hospital, Ogbomoso, Nigeria.

Methodology:

Study design and setting

This is a descriptive cross sectional survey of all hospitalised patients with suspected clinical infections at LAUTECH Teaching Hospital, Ogbomoso, during the period January 2016 and December 2018.

Subjects

All patients on admission with fever and other clinical features suggestive of sepsis routinely investigated for microbial aetiology of infections during the study period were eligible.

Specimen and data collection

From each hospitalised patient, non-duplicate clinical specimens such as swabs, sputum, blood or urine as appropriate were collected following recommended guidelines and methods (6). Demographic and clinico-laboratory data were obtained from the patients requisition and laboratory records.

Isolation and susceptibility test

Cell-free culture media appropriate for each sample were inoculated and incubated in aerobic atmosphere at 37^{o}C for 24 hours (6). Suspected colonies of enterobacteriaceae on culture media were identified to species level using conventional biochemical test scheme of indole, motility, methyl red, vogues proskauer and citrate utilization (IMMVC) (7).

Invitro antibiotic susceptibility test (AST) of each isolate was determined by the disk diffusion method on Mueller Hinton (MH) agar (8) to routinely used antibiotics (ampicillin 10µg, cefotaxime 30µg, ceftazidime 30µg, cefepime 30µg, gentamicin 10µg, ciprofloxacin 5µg, and imipenem 10µg) in our hospital, and AST results interpreted according to CLSI guideline (9). *Escherichia coli* ATCC 25922 was used as control strain.

ESBL detection

All isolates resistant to cefotaxime or ceftazidime in the AST were tested for ESBL production on MH agar plate using the classical 'double disk' synergy test (10) with amoxicillin (20µg)-clavulanic acid (10µg) disk and cefotaxime (30µg) or ceftazidime (30µg) disk. ESBL production was confirmed when there was greater than 5mm increase in the inhibition zone size produced by the cefotaxime and amoxicillin-clavulanic or ceftazidime and amoxicillin-clavulanic disk over the inhibition zone size produced by the cephalosporin disk alone. *Escherichia coli* ATCC 25922 was used as negative control while *Klebsiella pneumoniae* ATCC 700603 was used as positive control strain.

Statistical analysis

Data were analysed using GraphPad InStat software (San Diego, CA 92105) and presented as frequency distribution tables. Association between categorical variables was measured by Chi square test and $p < 0.05$ was taken as significant value.

Results:

Table 1 shows the prevalence of clinical infections among hospitalised patients in LAUTECH Teaching Hospital, Ogbomoso, during the three year period of survey. Of the total 4,198 patients hospitalised, 1,222 had clinical infections, giving an overall infection prevalence of 29.1% with 24.6% in 2016, 36.4% in 2017 and 29.1% in 2018 ($p<0.0001$).

Bacteria were isolated from samples of 689 (16.4%) hospitalised patients, entero-bacteriaceae from 343 (8.2%) and ESBL producing enterobacteriaceae from 46 (1.1%). The most frequently isolated members of the enterobacteriaceae were *Klebsiella* spp (54.5%), followed by *Escherichia coli* (35.9%), *Proteus* spp (9.3%) and *Enterobacter* spp (0.3%) but ESBL producers were mainly *E. coli* (n=30) and *Klebsiella* spp (n=16) (Table 2).

The clinical infections caused by the enterobacteriaceae included urinary tract infection (UTI, 45.2%), skin and soft tissue infection (SSTI, 27.9%), lower respiratory tract infection (LRTI, 17.5%), blood stream infection (BSI, 2.0%), osteomyelitis (1.7%), and others (5.5%). However, ESBL producing entero-bacteriaceae were frequently associated with osteomyelitis (50%), LRTI (18.3%), SSTI (14.6%), BSI (14.3%) and UTI (10.9%) (Table 3).

The antibiotic susceptibility profile shows that ESBL producing enterobacteriaceae were totally resistant to all tested antibiotics (ampicillin, cefotaxime, ceftazidime, cefepime, gentamicin and ciprofloxacin) but susceptible to imipenem (Table 4). In contrast, the non-ESBL producing enterobacteriaceae showed lower resistance to ampicillin (51.3%), cefotaxime (43.1%), ceftazidime (38.5%), cefepime (34.4%), gentamicin (51.3%) and ciprofloxacin (45.5%) ($p<0.0001$), and only 4% were resistant to imipenem ($p=0.3388$).

Discussion:

The prevalence of clinical infections among hospitalised patients in our hospital is high (prevalence rate of 29.1% over a 3 year period) and this increased significantly in 2017 and 2018 compared to 2016 (p <0.0001). This increasing rate may be a pointer to some problems with our infection control practices. The infection prevention and control (IPC) programme of our hospital has not fully taken off and the IPC committee was only officially inaugurated in late 2018 (11). This committee is currently developing IPC plan and policies to address challenges of hospital and community associated infections in our facility.

The prevalence rate of 1.1% among hospitalised patients for clinical infectio n cause

Table 1: Prevalence of clinical infections caused by ESBL producing enterobacteriaceae in LAUTECH Teaching Hospital, Ogbomoso (2016-2018)

Year	No of hospitalized patients	No of clinical infection (*prevalence)	No of clinical infections caused by enterobacteriaceae (*prevalence)	No of clinical infections caused by ESBL producing enterobacteriaceae (*prevalence)		
				Klebs spp	*E. coli*	Total
2016	1747	**430 (24.6%)	177 (10.1%)	14	4	18 (1.0%)
2017	1076	**392 (36.4%)	74 (6.9%)	4	3	7 (0.7%)
2018	1375	**400 (29.1%)	92 (6.7%)	12	9	21 (1.5%)
Total	4198	1222 (29.1%)	343 (8.2%)	30 (0.7%)	16 (0.4%)	46 (1.1%)

*Prevalence of clinical infection was calculated by dividing the number of infection (numerator) by the number of hospitalised patients (denominator);
**Significant difference in infection prevalence between years 2016, 2017 & 2018 ($X^2 = 45.005$, $p < 0.0001$)

Table 2: Frequency of enterobacteriaceae isolated from patients with clinical infections in LAUTECH Teaching Hospital, Ogbomoso (2016-2018)

Bacteria isolates	No of ESBL producing enterobacteriaceae	No of non-ESBL producing enterobacteriaceae	Total number of enterobacteriaceae (%)
Klebsiella spp	30	157	187 (54.5)
Escherichia coli	16	107	123 (35.9)
Proteus spp	0	32	32 (9.3)
Enterobacter spp	0	1	1 (0.3)
Total	46 (13.4)	297 (86.6)	343 (100)

ESBL = Extended Spectrum Beta Lactamase

Table 3: Clinical infection types and ESBL producing enterobacteriaceae in LAUTECH Teaching Hospital, Ogbomoso (2016-2018)

Clinical infection	No of patients with enterobacteriaceae infection (%) (n=343)	No of patients with ESBL producing enterobacteriaceae infection (%)		
		Escherichia coli (n=16)	*Klebsiella* spp (n=30)	Total (%) (n=46)
Urinary tract infection	155 (45.2)	10	7	17 (10.9)
Skin and soft tissue infection	96 (27.9)	5	9	14 (14.6)
Lower respiratory tract infection	60 (17.5)	0	11	11 (18.3)
Blood stream infection	7 (2.0)	0	1	1 (14.3)
Osteomyelitis	6 (1.7)	1	2	3 (50.0)
*Others	19 (5.5)	0	0	0

ESBL = Extended Spectrum Beta Lactamase; *includes gastroenteritis, ophthalmitis, meningitis

Table 4: *Invitro* antibiotic resistance profiles of enterobacteriaceae isolated from patients with clinical infections in LAUTECH Teaching Hospital, Ogbomoso (2016-2018)

Antibiotics	No of ESBL producers resistant to antibiotics (%) (n=46)	No of non-ESBL producers resistant to antibiotics (%) (n=297)	Total of number of isolates resistant to antibiotics (%) (n=343)	X^2	95% CI	*p* value
Ampicillin	46 (100)	130 (43.8)	176 (51.3)	48.182	0.1985-0.3327	< 0.0001*
Cefotaxime	46 (100)	102 (34.3)	148 (43.1)	67.344	0.2375-0.3925	< 0.0001*
Ceftazidime	46 (100)	86 (29.0)	132 (38.5)	81.944	0.2673-0.4366	< 0.0001*
Cefepime	46 (100)	72 (35.0)	118 (34.4)	97.968	0.3019-0.4834	< 0.0001*
Gentamicin	46 (100)	130 (43.8)	176 (51.3)	48.182	0.1985-0.3327	< 0.0001*
Ciprofloxacin	46 (100)	110 (37.0)	156 (45.5)	61.167	0.2242-0.3734	< 0.0001*
Imipenem	0 (0)	12 (4.0)	12 (3.5)	0.9151	0.02107-0.06947	0.3388**

ESBL = Extended Spectrum Beta Lactamase; X^2 = Chi square; CI = Confidence Interval; * = Statistically significant; ** = Not statistically significant

by ESBL producing enterobacteriaceae in this survey appears relatively low because the rate was calculated in relation to all hospitalised patients (with and without infections) during the survey period, which gives a true reflection of the prevalence in our facility. However, most studies on ESBL producing enterobacteriaceae usually report prevalence among only infected or colonised hospitalised patients, which tends to over-estimate the true prevalence (12-14).

Considering only patients with entero-bacteriaceae infection, Iroha et al., in Nigeria reported 20.3% ESBL rate (12) while Ibrahim et al., in Saudi Arabia reported 27% (14). In the current study, we report a rate of 13.4%, which is similar to 12% rate reported by Obebe et al., (13) in the same geographical region as ours. ESBL colonization or infection of patients in healthcare facility and community have been reported to depend on many factors including antibiotic use/misuse, previous hospitalisation and residence in a long term care facility, use of medical devices, old age, co-morbidity, and adequacy of infection control programmes (15-18).

The ESBL producers in this survey were multiply and highly resistant to the antibiotic disks routinely used in our laboratory (which mirrors commonly prescribed antibiotics in the hospital) compared to the non-ESBL producers with the exception of imipenem to which both groups were highly susceptible. This high resistance rate may have occurred in part from poor antibiotic prescribing practice as antimicrobial stewardship programme is not yet in place in our facility. The misuse/overuse of antibiotics especially the third and fourth generation cephalosporins, fluoroquinolones, and aminoglycosides can select ESBL strains carrying multidrug resistant (MDR) plasmids or integrons (3,19,20), which may be circulating in our facility.

There are a number of limitations to this survey; (i) laboratory surveys have low sensitivity for infection surveillance because positive laboratory result may not indicate infection and negative result does not rule out infection; (ii) the findings of this cross sectional survey have limited application because data were collected only once from infected patients, therefore assessment of infection risk factors could not be done; and (iii) the study design did not differentiate healthcare from community associated infections. Inspite of these shortcomings, the survey provides background information on infection rate in our facility, particularly from ESBL producing enterobacteriaceae, which may be useful for the hospital IPC intervention programme.

Conclusion:

In conclusion, the prevalence of clinical infections among hospitalised patients in our facility is high but the rate due to ESBL-producing enterobacteriaceae is low. In spite of this, there is need for continuous surveillance of antimicrobial resistance in enterobacteria-ceae and other nosocomial pathogens as part

of the IPC programme, with implementation of control measures to reduce the incidence of infections from ESBL and other multidrug resistant pathogens in our hospital.

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References:

1. Chaudhary, U., and Aggarwal, R. Extended Spectrum Lactamases (ESBL): An Emerging Threat to Clinical Therapeutics. Indian J Med Microbiol. 2004; 22: 75-80
2. Pitout, J. D, and Laupland, K. B. Extended-spectrum beta lactamase producing Enterobacteriaceae: an emerging public-health concern. Lancet Infect Dis. 2008; 8: 159–166.
3. Ben-Ami, R., Schwaber, M. J., Navon-Venezia, S., et al. Influx of extended spectrum beta-lactamase-producing enterobacteriaceae into the hospital. Clin Infect Dis. 2006; 42: 925–934.
4. Schwaber, M. J., and Carmeli, Y. Mortality and delay in effective therapy associated with extended-spectrum beta-lactamase production in Enterobacteriaceae bacteraemia: a systematic review and meta-analysis. J Antimicrob Chemother. 2007; 60: 913–920
5. Trecarichi, E. M., Cauda, R., and Tumbarello, M. Detecting risk and predicting patient mortality in patients with extended-spectrum β-lactamase-producing Enterobacteriaceae bloodstream infections. Future Microbiology, 2012; 7 (10): 1173–1189
6. Cheesbrough, M. District Laboratory Practice in Tropical Countries. 2nd edn. Cambridge, UK; Cambridge University Press; 2000.
7. Barrow, G. I., and Feltham, R. K. A. Cowan and Steel Manual for the Identification of Medical Bacteria. Third edition. Cambridge University Press, London, 1993.
8. Bauer, A. W., Kirby, W. M. M., Sherris, J. C., and Turck, M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966; 45 (4): 493–496.
9. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; 26th informational supplement. CLSI document M100-S26. Clinical and Laboratory Standards Institute, Wayne, PA, 2016
10. Iqbal, R., Ikram, N., Shoaib, M., Javaid asad, M., Mehmood, R. T., Niazi, A., Asghar, A., Ishfaq, B., and Naseer, F. Phenotypic confirmatory disc diffusion test (PCDDT), double disc synergy test (DDST), E-test OS diagnostic tool for detection of extended spectrum beta lactamase (ESBL) producing Uropathogens. J Appl Biotechnol Bioeng. 2017; 3 (3): 344–349
11. Taiwo, S. S. Infection Prevention and Control Programme in LAUTECH Teaching Hospital (LTH), Ogbomoso. A lecture delivered to LTH Management during the official inauguration of LTH Infection Prevention and Control Committee (IPCC), 2nd Aug 2018
12. Iroha, I. R., Amadi, S. E., Adikwu, M. U., and Esimone, C. O. Detection of Plasmid Borne Extended Spectrum Beta-Lactamase enzymes in Clinical Isolates of *Escherichia coli* from a Community General Hospital. Int J Mol Med Adv Sci. 2008a; 4 (2): 46-49.
13. Obebe, A. O., Deji-Agboola, A. M., and Olawuyi, O. J. prevalence of extended spectrum beta lactamase producing gram negative bacteria in a university Hospital in Ilisan Remo, Ogun State, Nigeria. World J Med Sci. 2014; 11(4): 497-503.
14. Ibrahim, M. E., Abbas, M., Al-Shahrai, A. M., and Elamin, B. K. Phenotypic Characterization and antibiotic Resistance Patterns of Extended spectrum Beta lactamase and Amp-C Beta Lactamase Producing Gram Negative Bacteria in a Referral Hospital, Saudi Arabia. Canad J Infect Dis Med Microbiol. 2019, Article ID 6054694. https://doi.org/10.1155/2019/6054694
15. Tham, J., Odenholt, I., Walder, M., Andersson, L., and Melander, E. Risk factors for infections with extended-spectrum beta lactamase producing *Escherichia coli* in a county of Southern Sweden. Infect Drug Resist. 2013; 6: 93–97
16. Ben-Ami, R., Rodriquez-Bano, J., Arslan, H., et al. Multinational Survey of Risk Factors for Infection with Extended-Spectrum *β*-Lactamase-Producing Enterobacteriaceae in Non-hospitalized Patients. Clin Infect Dis. 2009; 49 (5): 682-690
17. Ikeda, Y., Mamiya, T., Nishiyama, H., Koseki, T., Mouri, A., and Nabeshima, T. Risk factors for extended spectrum β lactamase producing *Escherichia coli* infection in hospitalized patients. Nagoya J Med Sci. 2012; 74 (1-2): 105–114
18. Harris, A. D., McGregor, J. C., Johnson, J. A., et al. Risk Factors for Colonization with Extended-Spectrum β-Lactamase–producing Bacteria and Intensive Care Unit Admission. Emerg Infect Dis. 2007;13(8):1144. https://dx.doi.org/10.3201/eid1308.070071
19. Moghaddam, M. J. M., Mirbagheri, A. A., Salehi, Z., and Habibzade, S. M. Prevalence of Class 1 Integrons and Extended Spectrum Beta Lactamases among Multi-Drug Resistant *Escherichia coli* Isolates from North of Iran. Iran Biomed J. 2015 19 (4): 233 – 239. doi:10.7508/ibj.2015.04.007
20. Pérez-Etayo, L., Berzosa, M., González, D., and Vitas, A. I. Prevalence of Integrons and Insertion Sequences in ESBL-Producing *E. coli* Isolated from Different Sources in Navarra, Spain. Int J Environ Res Public Health. 2018; 15 (10): 2308.

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Short Communication **Open Access**

Ectoparasitic infestations of cats and dogs in Izzi Local Government Area of Ebonyi State, Nigeria: brief communication for 'One Health' approach to control of potential zoonoses

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Abstract:

Background: Cats and dogs are important companion animals that paradoxically pose risks of zoonotic infections to their owners. This study determined the ectoparasitic infestations of cats and dogs in a semi-rural setting of Ebonyi State, so as to establish the prevalence of the ectoparasites among the companion animals for creation of public health awareness relevant to prevention of zoonoses in the area.
Methods: One hundred dogs and 21 cats from Izzi Local Government Area of Ebonyi State, were examined for ectoparasitic infestations, using standard parasitological techniques. Systematic random sampling technique was employed in the study. Parasites were identified with standard identification guides. Data were analysed using aspects of Bush infection statistics and Chi-square. Statistical significance was established at $p<0.05$.
Results: Out of the 100 dogs examined, 80 (80%), 8 (8%), 6 (6%), 2 (2%) and 4 (4%) were infested with *Rhipicephalus sanguineus, Haemaphysalis longicornis, Ctenocephalides canis, Ctenocephalides felis* and *Sarcoptes scabiei* respectively. A significant association was observed between *R. sanguineus* and the dogs ($X^2=100.00$; $p=0.000$). Six (28.6%) of the 21 cats examined were infested with *C. felis*, with significant statistical association ($X^2=21.000$; $p=0.000$) and 2 (9.5%) were infested with *Otodectes cynotis* but no significant association ($X^2=5.526$; $p=0.063$).
Conclusion: Based on the observed prevalence of ectoparasites among the animals, collaborative efforts of the medical and veterinary personnel are solicited in the spirit of 'one health' in order to protect the health of the pets and those of their owners.

Keywords: Ectoparasitism, Cats, Dogs, Ebonyi State, Zoonoses

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Infestations ectoparasites de chats et de chiens dans la zone de gouvernement local d'Izzi, dans l'État d'Ebonyi, au Nigéria: communication succincte concernant l'approche «One Health» de la lutte contre les zoonoses potentielles

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Abstrait:

Contexte: Les chats et les chiens sont des animaux de compagnie importants qui, paradoxalement, présentent des risques d'infections zoonotiques pour leurs propriétaires. Cette étude a déterminé les infestations ectoparasites de chats et de chiens dans un cadre semi-rural de l'État d'Ebonyi, de manière à établir la prévalence des ectoparasites parmi les animaux de compagnie afin de sensibiliser la santé publique à la prévention des zoonoses dans la région.

Méthodes: Des infestations d'ectoparasites ont été examinées chez 100 chiens et 21 chats de la région du gouvernement local d'Izzi, dans l'État d'Ebonyi, à l'aide de techniques parasitologiques classiques. La technique d'échantillonnage aléatoire systématique a été utilisée dans l'étude. Les parasites ont été identifiés avec des guides d'identification standard. Les données ont été analysées à l'aide d'aspects des statistiques d'infection de Bush et du chi carré. La signification statistique a été établie à $p<0,05$.
Résultats: Sur les 100 chiens examinés, 80 (80%), 8 (8%), 6 (6%), 2 (2%) et 4 (4%) étaient infestés par *Rhipicephalus sanguineus, Haemaphysalis longicornis, Ctenocephalides canis, Ctenocephalides felis* et *Sarcoptes scabiei* respectivement. Une association significative a été observée entre *R. sanguineus* et les chiens ($X^2=100,00$; $p=0,000$). Six (28,6%) des 21 chats examinés étaient infestés par *C. felis*, avec une association statistique significative ($X^2=21\ 000$; $p=0,000$) et 2 (9,5%) étaient infestés par *Otodectes cynotis* mais aucune association significative ($X^2=5,526$; $p=0,063$).
Conclusion: sur la base de la prévalence observée d'ectoparasites chez les animaux, des efforts de collaboration du personnel médical et vétérinaire sont sollicités dans l'esprit de «one health» afin de protéger la santé des animaux et de leurs propriétaires.

Mots clés: Ectoparasitisme, Chats, Chiens, Etat d'Ebonyi, Zoonoses

Introduction:

Cats and dogs are globally recog- nized as beneficial companion animals. Pet animals play tremendous social, emotionaland psychological roles for their owners (1). In addition to being used as pets, they have been employed for hunting, security, sport and for the purpose of breeding (2,3). Cats are also involved in warding off rodents in households.

Zoonosis is a disease of animals that is transmissible to humans from its primary animal hosts (4). They can be transmitted directly through contact with infected animals, indirectly through cont- aminated animal environments, or by vertebrate-borne routes (arthropods such as the acarines, fleas, and other insects) (5). Despite the important roles played by companion animals in households, it is paradoxical that they are incriminated in the transmission of many zoonotic infections that are of significant public health importance (5).

Certain groups of people including pregnant women, children, aged and immunocom- promised individuals are at great risks of zoonotic infections.

Research findings have implicated wild life as an important zoonotic pool of novel pathogens from which the companion animals derive, elaborate and intensify the disease causing organism at homes (6). Globalizations, urbanization, climate change, population explosion among other factors, are reported to be responsible for the sustenance of emerging and re-emerging zoonotic infections and diseases (7). Desertification, deforestation and landscape alterations observed in Libya (8) and in some parts of Africa and the rest of the world have led to the liberation and migration of wild animals from their natural habitats to both human and domestic animal dwellings thereby making many known small wild animals to become synanthropic species.

Increase in pet ownership and expansion of pet definition by including new and exotic animals have been reported as factors making pet zoonoses emerging public health issues (7).

Ectoparasites are a wide range of parasitic arthropods that consist of ticks, mites, fleas, chewing and sucking lice (9). Zoonotic ectoparasitic infestations are common with varying signs and symptoms that depend on the causative agents and hosts involved (10). The role of ecto- parasites in disease transmission is of great public health importance and cannot be over-emphasized (11). The fleas and ticks can infest humans causing dermatitis and tick-borne paralysis respectively (12, 13). Physical discomfort, irritation, itching, inflammation, self trauma, release of neurotoxins (may result in tick paralysis), systemic illness, and hyper- sensitivity reactions are some of the consequences of tick bite (14).

Tick and other parasitic infestations have been reported to be more prevalent among stray dogs than the pet ones (15). However, direct or indirect exposure by mingling of the pet dogs with the stray ones, aid frequent transmission of different parasitic infest- ations among the two classes of dogs (15). Other ectoparasitic infestations common among companion animals include canine demodiciosis caused by *Demodex canis, Sarcoptic* mange caused by infestation with *Sarcoptes scabiei* var *canis,* and otoacariosis cause by *Otodectes cynotis* (16). Mosallanejad *et al.,* (16) also reported other studies as having documented *Ctenocephalides felis, Ctenocephalides canis* and *Pulex irritans* as the three most common flea species that infest dogs.

The expanded definition of pet animals, the global snowball increase in acquisition of companion animals and their public health zoonotic impacts have informed this brief communication on zoonotic ectoparasitic

infestations on cats and dogs in Izzi LGA, a semi-rural setting of Ebonyi State, Nigeria.

Materials and method:

Study area

This study was conducted in Izzi Local Government Area of Ebonyi State, Nigeria. Ebonyi State lies within longitude $7^{0}30^{1}$ and $8^{0}30^{1}$E and latitude $5^{0}40^{1}$ and $6^{0}45^{1}$N in southeastern region of Nigeria (19,20). Farming and hunting form part of the economic livelihood of the inhabitants of the study area**.**

Sampling technique

Forty one (10%) out of the total 412 houses in Izzi LGA of Ebonyi State, were selected by systematic random sampling technique. The houses were first serially numbered with tags (1 to 412), and the interval of sampling of the houses for survey was based on the formula; sample interval (n^{th}) = total population divided by the sample size (412/41=10). The first 10 tags were removed from the houses and shuffled in a bowel, and one number (tag 8) was selected by simple random sampling. House to house survey therefore started with house number 8, and then every other 10^{th} house until the 41^{st} house (house number 408) was surveyed. In the 41 households surveyed, 34 households had dogs (19 had 3 each, 11 had 2 each, 3 had 5 each and 1 had 6 dogs) and 16 households had cats (10 had 1 each, 5 had 2 cats each and 1 household had 1 cat). In all, a total of 21 cats and 100 dogs were selected.

Examination and specimen collection: House to house screening and examination of cats and dogs was carried out among the selected households. A total of 21 cats and 100 dogs were examined for ectoparasites. Parasite specimen collection and preservation followed the methods described by ESCCAP (9) and Bhati et al., (21). The entire body surface of each animal was thoroughly examined for ectoparasites. The body surfaces of the animals were carefully combed with fine combs and brushes. Any present parasite was collected on a clean white cloth that was spread on the ground. A sterilized forcep was used to carefully remove the entire tick, including the mouthparts from the body of the animals. The recovered parasites were preserved in 70% alcohol.

Skin scrapings were made with the use of sterilized blunt knives. The scrapings were collected in Petri dishes with the edges smeared in vaseline gel. All the specimens were transported to the Teaching and Research Laboratory of the Department of Medical Laboratory Science, Ebonyi State University, Abakaliki, Nigeria, for onward analysis.

Laboratory analysis

In the laboratory, hand lenses were used to examine the dorsal and ventral anatomy of the animals. Morpho- logical features of the ectoparasites were used for proper identification. Further examination and clarification was carried out using the Olympus CX23 microscope (India) at magnification of 400x (10x primary and 40x secondary).

The scrapings from the skin were put in 10% KOH to dissolve debris and hair particles. Afterwards, the preparation was centrifuged 4 times and dehydrated in ascending grades (70%, 90% and 100%) of alcohol followed by clearing in xylene. DPX was applied to a portion of the sediment and examined under the microscope. Recovered parasites from the animals were identified using pictorials and guidelines (21, 22, 23, 24).

Statistical analysis

Data collected were analysed using mean intensity and mean abundance of infection statistics according to Bush *et al*., (25) which states that mean intensity = number of parasites species in all infested hosts divided by the number of hosts infested by parasites, and mean abundance = number of a parasite species in all infested hosts divided by the number of hosts examined (infested and non-infested). The Chi square test was used to determine associations, and statistical significant values were established at $p<0.05$.

Results:

Of the 100 dogs examined in this study, 80 (80%), 8 (8%), 6 (6%), 2 (2%) and 4 (4%) were infested by *R. sanguineus, H. longicornis, C. canis, C. felis* and *S. scabiei* respectively. A significant association was observed between *R. sanguineus* and the number of dogs infested (X^{2}_{4} =100.00; p=0.000). However, no significant association was observed between other ectoparasitic infestations and the number of infested dogs ($p>0.05$) (Table 1).

Table 2 depicts species-specific distribution of ectoparasites among the cats in the study area. Out of the 21 cats examined, 6 (28.6%) were infested with *C. felis* indicating a significant association between the infestation

Table 1: Species –specific distribution of ectoparasites among dogs in Izzi LGA of Ebonyi State

Parasites Species	Number Examined	Number Infested (%)	Number of Parasites	Mean Intensity	Mean Abundance	X^2 value	*p* value
Rhipiaphalus sarguineus	100	80 (80)	148	1.85	1.48	100.000	.000
Haemaphysalis longicornis	100	8 (8)	16	2.00	0.16	2.174	0.704
Ctenocephalides canis	100	6 (6)	10	1.67	0.10	1.596	0.810
Ctenocephalides felis	100	2 (2)	2	1.00	0.02	0.550	0.973
Sarcoptes scabiei	100	4 (4)	5	1.25	0.05	1.042	0.903

Table 2: Species-specific distribution of ectoparasites among cats in Izzi LGA of Ebonyi State

Parasites Species	Number Examined	Number Infested (%)	Number of Parasites	Mean Intensity	Mean Abundance	X^2 value	*p* value
Ctenocephalides felis	21	6 (28.6)	14	2.33	0.67	21.000	0.000
Otodectes cynotis	21	2 (9.5)	3	1.50	0.14	5.526	0.63

and the cat hosts (X^2_2=21.00; *p*=0.000). On the other hand 2 (9.5%) of the cats were infested with *O. cyanotis* with no significant association between ectoparasitic infestations and the animals (X^2_2=5.526; *p*=0.063). No evide- nce of ectoparasitism was observed in 13 of the 21 (61.9%) cats

Discussion:

The study investigated the ecto-parasitic infestations in cats and dogs in Izzi LGA of Ebonyi State, Nigeria in order to add more information on such studies for one health approach in controlling potential zoonoses that could arise from such infestations. The high prevalence of ectoparasites (especially due to *R. sanguineus)* observed in the dogs is a reflection of the level of the local environmental contamination and poor status of veterinary awareness and practices that prevail in the study area. The ectoparasites were more intense, abundant and diverse among the dogs in comparison with the cats. However, this observation might be attributed to the number of owned cats examined in the study area. The use of dogs for hunting that exposes them more to the wildlife might have accounted for this. However, this may be attributed to the small sample size (n = 21) of the examined cats. In the study area, people utilize more of dogs than cats as companion animals because of their multipurpose usage.

C. felis was the least prevalent (2%) and least abundant (0.02) ectoparasite among the dogs. This report is in agreement with the findings of Elom *et al.,* (26) in their study in Ikwo and Ezza localities of Ebonyi State, Nigeria. However, the finding disagrees with Durden *et al*., (27) and Tavassoli *et al.,* (28), who reported *C. felis* as the most abundant ectoparasites in their studies in the USA and Iran respectively. The disparity in abundance of *C. felis* could be attributed to environmental factors prevalent in the geographical areas as some of the studies were conducted in tropical areas where as others were carried out in temperate zones. In addition, survival of ectoparasites is dependent on availability of hosts. That *C. felis* was most prevalent in studies carried out in the USA and Iran could be attributed to more dense population of dogs as opposed to sparse population of dogs in the study area (Nigeria). This could be because more dogs are being utilized as companion animal in the USA and Iran than in Nigeria.

The nature of ectoparasitism observed

in this study is similar to that of Omonijo and Sowemimo (11) in their study in Ekiti State, Nigeria. However, the report of the present study differs from Omonijo and Sowemimo (11) by observing *Ctenocephalides* species infestation to be statistically high in cats only and not in both cats and dogs as reported in the previous study. The 28.6% infestation of cats with *C. felis* is similar with the documented report of 28.3% dog infestation with fleas in Jos, Plateau area of Nigeria (29).

Eighty percent of the dogs in this study were infested with *R. sanguineus,* which was the most prevalent ectoparasite; while the remaining 20% were infested with different species of ticks, fleas, and mites. This observation is similar to that of Abdulkareem *et al*., (30), who reported greater than 80% overall ectoparasitic infestations of dogs in their study in Kwara State, Nigeria. However, the present study is not in conformity with Adbulkareem *et al*., (30) by not observing multiple infestations on the animals. The rate for *R. sanguineus* infestation in this study is also similar to the previous report of 88.6% reported in Ethiopia (31) but lower than 98.5% reported in Southwestern Nigeria (32). The scabies mite has been reported to be highly communicable among dogs and may infest humans, but cats are known to be relatively resistant (5). The recognized resistance by cats might have been responsible for the absence of this infestation among the cats in the present study.

Although no co-infestation was observed in this study, it has been reported that companion animals and humans can be sequentially or simultaneously infected with more than one tick species and that a single tick has the potential to transmit more than one pathogen, leading to co-infestations in the infested hosts (5). The parasites reported in this study have been previously documented globally, with inter-regional differences in prevalence and density of infestation (33). It has also been reported that such differences could be strongly determined by changes in climate, host availability and vegetation which influence the microclimate (34).

Conclusion:

The high prevalence, intensity and abundance of some ectoparasites with zoonotic potentials were observed among cats and dogs from the study area. It is therefore pertinent that collaborative efforts of medical and veterinary practitioners, and public health officers, are established for 'one health' approach in prevention and control of potential zoonoses.

Authors' contribution:

MOE was involved in conception, writing and reviewing of manuscript; NNO was involved in collection and analysis of data; AN was involved in editing and reviewing of manuscript; and VU was involved in literature search and writing of manuscript. All authors agreed to the final manuscript draft submitted.

Conflict of interest:

No conflict of interest is declared

References:

1. Robertson, I. D., Irwin, P. J., Lymberg, A. J., and Thompson, R. C. The role of companion animals in emergence of parasitic zoonoses. Int J Parasitol.. 2000; 30 (2000): 1369 - 1377.
2. Abere, T., Bogale, B., and Melaku, A. Gastrointestinal helminth parasites of pet and stray dogs as potential risks for human health in Bahir Da Town, North Western Ethiopia. Veterinary World. 2013; 6: 388-392.
3. Pirzada, N. H., Sahito, H. A., Gopang, M. A., Memon, M. R., Pirzada, M., Sanjrani, M. I., Memon, M. A., and Khuhro, A. P. Prevalence of intestinal parasites and risk perception of zoonotic infection for humans. Dyn Microbiol Infect Dis. 2014; 1 (1): 1-7.
4. Mosby's Medical Nursing and Allied Health Dictionary. Anderson K. N. (eds). Rand McNally, USA, 1994
5. Day, M. J. Pet-related infections. American Family Physician. 2016; 94: 794-802.
6. Diamond, J., and Ford, L. E. Guns, germs and steel: the fate of human societies. Bio Med. 2000; 43: 609.
7. Smith, A., and Whitfield, Y. Household Pets and Zoonoses. National Collaborating Centre for Environmental Health. Ontario Veterinary College, University of Guelph, 2012
8. Hosni, M. M., and Maghrbi, A. A. Ectoparasitic infestation of free-ranging hedgehog (*Eteterix algirus*) in north Western Libya. Open Veterinar J. 2014; 4 (1): 12-15.
9. European Scientific Counsel Companion Animal Parasites. Control of Ectoparasites in Dogs and Cats. ESCCAP Guideline 03 Sixth Edition, 2018
10. Ogbaje, C. I., Ofukwu, R. A., and Ajogi, I. A. Zoonotic gastrointestinal parasite burden of local dogs in Zaria, Northern Nigeria: Implications for human health. International J One Health. 2015; 1: 32-36.
11. Omonijo, A. O., and Sowemimo, O. A. Prevalence of ectoparasites of cats and dogs in Ijero and Moba LGAs, Ekiti State, Nigeria. Nig J Parasitol. 2017; 38 (22): 278-283.
12. Scott, D. W., Miller, W. H., and Griffin, C. E. Miller and Kirks Small Animal Dermatology, 6th Edition. W. B. Sanders, Philadelphia, U.S.A. 2001
13. Xhaxhin, D., Kusi, I., Rapt, D., Visser, M., Knaus, M., and Linder, T. H. Ectoparasites of dogs and cats in Albania. Parasitol Res. 2009; 105: 1577-1587.

14. Taylor, M. A., Coop, R. L. and Wall, R. L. Veterinary Parasitology. Third Edition. Blackwell Publishing Limited, Oxford, United Kingdom, London. 2007
15. Sahu, A., Mohanty, B., Panda, M. R., Sander, K. K., and Dehuri, M. Prevalence of tick infestation in dogs in and around Bhubaneswar. Veterinar World, 2013; 6: 981-985.
16. Mosallanejad, B., Alborzi, A. R., and Katvandi, N. A survey on ectoparasite infestations in companion dogs of Alvaz District, South West of Iran. Journal of Arthropod-Borne Diseases, 2012; 6 (1): 70-78.
17. Rabinowtz, P. M., Gordon, Z., and Odofin, L. Pet-related infections. Am Fam Phys. 2007; 76 (9): 1314 - 1322.
18. Karesh, W. B., Cook, R. A., Bennett, E. L., and Newcomb, J. Wild trade and global disease emergence. Emerg Infect Dis. 2005; 11 (7): 1000-1002.
19. Uzuakpunwa, A. B. Abakaliki Pyroclastics, Eastern Nigeria: new age and tectonic implications. Geological Magazine. 1974; 3: 65-70.
20. Uneke, C. J., and Ibeh, I. M. Impact of deforestation on malaria in south eastern Nigeria: the epidemiological, socio-economic and ecological implications. Intern J Third World Med. 2008; 8 (1): 3-9.
21. Bhatia, B. B., Pathak, K. M. L., and Juyal, P. D. Textbook of Veterinary Parasitology, Third Edition, Kalyani Publishers, New Delhi, India. 2012
22. Wall, R. D., and Shearer, D. Veterinary Ectoparasites: Biology Pathology and Control. Second Edition. Blackwell Sciences Limited, Oxford, London, 2001.
23. Walker, A. R., Bouattour, A., Camicas, J. L., Estrada–Peria, A., Horak, I. G., Latif, A. A., Pegram, R. G., and Preston, P. M. Ticks of Domestic Animals in Africa: A Guide to Identification of Species, 2014
24. Mathison, B. A., and Pritt, B. S. Laboratory identification of arthropod ectoparasites. Clin Microbiol Rev. 2014; 27 (1): 48-67.
25. Bush, A. O., Laggerty, K. D., Lotz, J. M., and Shostak, A. W. Parasitology meets ecology on its own term. J Parasitol. 1997; 83 (4): 575-583.
26. Elom, M. O., Alo, M. N., Nworie, A., Usanga, V. U., Ugah, U. I., and Alegu, L. U. Ecto- and intestinal parasitic fauna of domestic dogs in two rural areas of Ebonyi State, Nigeria: public health zoonotic jeopardy. Journal of Entomology and Zoology Studies. 2015; 3 (4): 444-448.
27. Durden, L. A., Judy, J. N., Martin, J. E., and Spedding, L. S. Fleas parasitising domestic dogs in Georgia, U.S.A: species composition and seasonal abundance. Veterinar Parasitol. 2005; 130 (1-2): 157-162.
28. Tavassoli, M., Alimadi, A., Imam, A., Alimadiara, E, Javadi, S. H., and Hadian, M. Survey of flea infestation in dogs in different geographical regions of Iran. Korean J Parasitol. 2010; 48 (2): 145-149.
29. James-Rugu, N. N., and Iwuala, M. O. E. Ticks of Nigerian Livestock with different for conditions and colour shades. Afr J Natur Sci. 2002; 3: 102-106.
30. Abdulkareem, B. O., Christy, A. L., and Samuel, U. U. Prevalence of ectoparasite infestations in owned dogs in Kwara State, Nigeria. Parasite Epidemiology and Control. 2018; 3: e 00079.
31. Testaye, A., and Chanie, M. Ectoparasites are major skin diseases of dogs in Gondar, Amhara National Regional State, Ethiopia. Intestinal Journal of Animal and Veterinary Advances. 2011; 3 (5): 392-396.
32. Agbolade, O., Soetan, E., Awesu, A., Ojo, J., Somoye, O., and Raufu, S. Ectoparasites of domestic dogs in some Ijebu communities, southeast, Nigeria. World Appl Sci J. 2008; 3 (6): 916-920.
33. Ugbomoiko, U. S., Ariza, I., and Heukelbach, J. Parasites of importance for human health in Nigerian dogs: high prevalence and limited knowledge of pet owners. BMC Veterinar Res. 2008; 4: 49-52
34. Noor-Un-Nisa., Khan, A., and Ghazi, R. R. Prevalence of ticks associated with Samoyed dogs in different localities of Karachi, Pakistan. Pakistan J Parasitol. 2018; 66: 39-43.