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Review Article **Open Access**

Pathologic changes in patients infected with SARS-CoV-2: a review

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Abstract:

Severe acute respiratory syndrome–coronavirus-2 (SARS-CoV-2) enters cells using the angiotensin converting enzyme 2 (ACE2), which are expressed by the respiratory tract endothelium, epithelial cells of the stomach, duodenum, ileum, rectum, cholangiocytes, and hepatocytes. Pathological examinations of these organs are not feasible method of diagnosis but can explain pathological changes, pathogenesis of the disease, and the cause of death in COVID-19 cases. In this review, we performed a literature search for COVID-19-related pathological changes seen during post-mortem examinations in different organs of the body including the lungs, gastrointestinal tract, liver, kidney, skin, heart and blood. Our findings showed that SARS-CoV-2 has damaging effects on many organs, probably due to the host immune responses to the presence of the virus. It is recommended that both antiviral and immunomodulatory agents should be considered in the management of COVID-19 patients for better prognosis, and clinical outcome.

Keywords: COVID-19, SARS-CoV-2, ACE-2, pathology, autopsy findings

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Changements pathologiques chez les patients infectés par le SRAS-CoV-2: une revue

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Abstrait:

Le syndrome respiratoire aigu sévère-coronavirus-2 (SARS-CoV-2) pénètre dans les cellules à l'aide de l'enzyme de conversion de l'angiotensine 2 (ACE2), qui est exprimée par l'endothélium des voies respiratoires, les cellules épithéliales de l'estomac, du duodénum, de l'iléon, du rectum, des cholangiocytes, et les hépatocytes. Les examens pathologiques de ces organes ne sont pas une méthode de diagnostic réalisable, mais peuvent expliquer les changements pathologiques, la pathogenèse de la maladie et la cause du décès dans les cas de COVID-19. Dans cette revue, nous avons effectué une recherche bibliographique sur les changements pathologiques liés au COVID-19

observés lors d'examens post-mortem dans différents organes du corps, notamment les poumons, le tractus gastro-intestinal, le foie, les reins, la peau, le cœur et le sang. Nos résultats ont montré que le SRAS-CoV-2 a des effets néfastes sur de nombreux organes, probablement en raison des réponses immunitaires de l'hôte à la présence du virus. Il est recommandé que les agents antiviraux et immunomodulateurs soient pris en compte dans la prise en charge des patients COVID-19 pour un meilleur pronostic et des résultats cliniques.

Mots-clés: COVID-19, SARS-CoV-2, ACE-2, pathologie, résultats d'autopsie

Introduction:

In December 2019 an outbreak of a novel coronavirus disease was reported and subsequently declared a global pandemic (1). The World Health Organization (WHO) officially named the disease caused by the severe acute respiratory syndrome coronavirus - 2 (SARS-CoV-2) as coronavirus disease 2019 (COVID-19) (2). SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA virus belonging to the beta coronavirus family and is the seventh coronavirus that cause human infections (3). Among the other six coronaviruses that can cause diseases in humans are SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), which is associated with high mortality (4,5). SARS-CoV-2 is highly homologous to SARS-CoV and enters the cell via the angiotensin converting enzyme 2 (ACE-2).

COVID-19 notably present with symptoms of fever, dry cough, fatigue, muscle aches, shortness of breath, headache, diarrhea, and indigestion (6). It has been observed that from the onset of symptoms to death from the disease is between 6 and 41 days, and the average is 14 days (7). The aim of this review was to examine changes in various organs including the skin following infection by SARS-CoV-2. It is hoped that adequate knowledge of the pathology of the disease will aid early diagnosis and management of COVID-19, which can lead to reduction in mortality and length of hospital stay for the patients.

Methodology:

Online databases including the Web of Science, PubMed, Scopus, and Google Scholar were searched for relevant publications on the clinical features, pathogenesis, organ changes and complications in COVID-19 following the PRISMA guideline (8). We searched for pathological changes in terms of gross and microscopic morphology on autopsy, immuno-histochemistry, electron microscopy, fluorescence in-situ hybridization (FISH) and RT-PCR used to confirm SARS-COV-2 in these organs or tissues.

Search terms and phrases used include; "COVID-19 pathology", "COVID-19 autopsy findings", "COVID-19 diagnostic methods", "angiotensin-converting enzyme 2 (ACE2)", "SARS-CoV-2", "COVID-19", "2019-nCoV" and "organ changes in COVID-19". There was no restriction on the date, place, type of study, and inclusion/ exclusion criteria but publications not written in English were excluded. With greatest sensitivity search, we found 382 articles on external databases collected using Endnote Software. All the articles from the cited databases were then unified to avoid duplicates. Following review of the titles and abstracts, non-relevant articles were excluded, leaving a total of 60 eligible articles for the review (Fig 1).

Results and Discussion:

COVID-19 and the lungs

The lungs are the most affected organ in COVID-19 (9). However, severity of its involvement ranges from lack of symptoms or mild pneumonia to severe hypoxia, shock, respiratory failure, and multiorgan failure or death is associated with critical form of the disease (10). Grossly the lungs are heavy, often 3-5 times the normal size with evidence of congestion and haemorrhagic necrosis (11). In over 80% of cases, microscopic examination revealed various stages of diffuse alveolar damage, including the exudative phase with hyaline membrane formation, the proliferative phase with type 2 pneumocyte hyperplasia, and the early repair phase with interstitial spindle cell hyperplasia and/or intra-alveolar organization (12).

Other methods have been used to detect SARS-CoV-2 in the lung tissues including electron microscopy, immunohistochemistry to detect viral antigens, immunofluorescence viral nucleic acid detection by in-situ hybridization (FISH), and reverse transcriptase polymerase chain reaction (RT-PCR) (13). Effort has been made to detect SARS-CoV-2 on electron microscopy which reported "virus-like particles" (14).

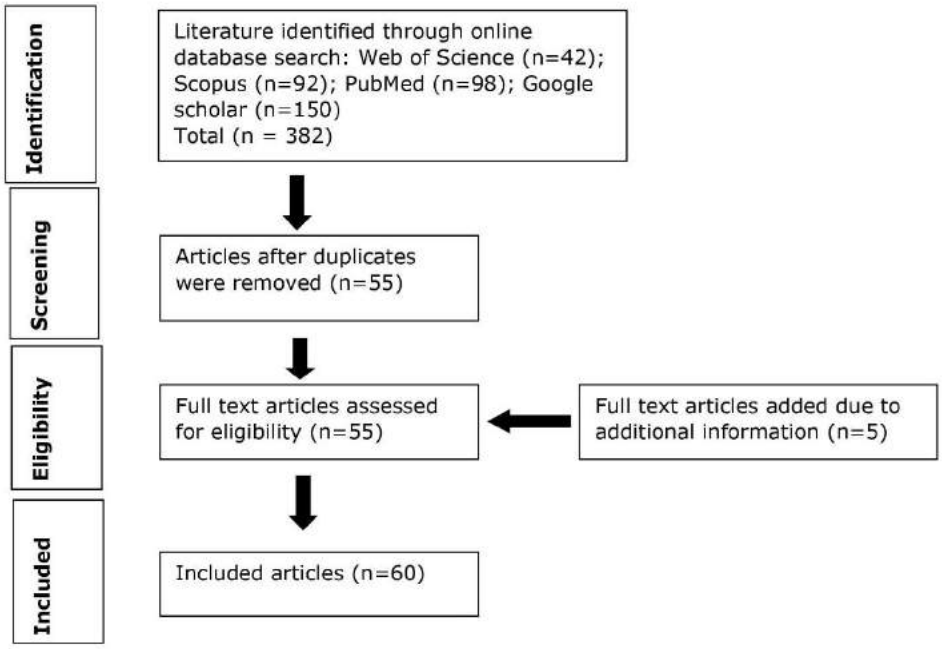

Fig. 1: Process of selection of publications (PRISMA guide) used for the review

In another study, it was reported that detection of SARS-CoV-2 by immuno-histochemistry was successful only in the lung, while no virus could be detected by this method in the heart, liver, kidney, small intestine, skin, adipose tissue, and bone marrow (15). Compared to immunohistochemistry, immunofluorescence has been used less frequently for the detection of SARS-CoV-2 proteins/antigens (16), and this could partly be due to stronger autofluorescence as a cofounding factor and potential infectivity of frozen tissues.

COVID-19 and the heart

Clinical features in COVID-19 patients suggest that SARS-CoV-2 has a major impact on the heart. Patients have features which suggest acute myocardial injury, including consistently elevated serum troponin level and ECG changes (17). The pathophysiology and the degree of myocardial injury, along with the short and long-term cardiovascular outcomes in COVID-19 survivors remain unclear. However, autopsy studies of the heart in these patients suggest that pre-existing heart diseases were dominant, particularly hypertensive heart disease (18). In other post-mortem studies, the findings showed endothelitis, as well as myocardial infiltration with lymphocytes, while direct injury of cardiomyocytes by the virus was not confirmed (19-21)

One autopsy case series study revealed severe right ventricular dilatation in patients, along with drop out myocyte necrosis and apoptosis. However there was no evidence of lymphocytic myocarditis suggesting that elevated troponin levels in these patients was due to extreme stress from acute pulmonary disease (22). Viruses with particular tropism for heart directly gain entrance to cardiomyocytes to cause their degeneration or to infect endothelial cells. These often lead to significant endothelial dysfunction, ischemia, cytokine release, and infiltration of myocardial tissue with immune cells (23). Consequently, innate and adaptive immune systems are activated, both being responsible for cytokine storm syndrome, as well as viral clearance.

Left ventricular systolic function impairment and cardiomyocytes dysfunction often lead to death (23). Of particular interest is the report of severe myocarditis and decreased systolic function after SARS-COV-2 infection, leaving us to wonder if the virus induces new cardiac pathologies or merely exacerbates underlying pathologies. It is obvious that as more studies are conducted and the disease evolves, more will be known about the long term effect of this virus on the heart (24).

COVID 19 and the kidney

Although, the first organs to be affected in SARS-COV-2 infection are the lungs, as initial clinical sign for the detection of COVID-19 is

pneumonia (25), many organ damages have been reported (6) and some cases of COVID-19 pneumonia present with kidney injury (26). Autopsy findings from patients who died of COVID-19 also revealed renal damage (27). Many studies have reported that SARS-CoV and SARS-CoV-2 use the ACE-2 to enter into target cells (28), and ACE-2 is well expressed on the surface of kidney tubular cells, which explains SARS-CoV-2 tropism for the kidney.

Many studies recognized the relevance of the inflammatory/immune-mediated reaction with the release of high levels of circulating harmful mediators such as IL-1, IL-6, TNF-α and chemokines capable of interacting with kidney-resident cells to cause endothelial dysfunction, microcirculatory derangement, and tubular injury (29). Acute kidney injury (AKI) developed on the average 9 days after admission together with secondary infections and acute cardiac damage (30). Many factors such as age, severity of illness, and the presence of diabetes mellitus contribute to AKI in patients with acute respiratory diseases (ARDS) (31).

The immune system responses to SARS-CoV-2 and direct cytotropic effect of the virus may be responsible for AKI in COVID-19 (32). Studies have shown that severe COVID-19 patients have reduced levels of $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T lymphocytes, NK cells, and high levels of inflammatory cytokines. As soon as SARS-CoV-2 infiltrates into the renal cells, innate immune system and inflammatory responses might be triggered causing a cytokine storm syndrome, which is responsible for hypoxia, shock, rhabdomyolysis and acute kidney injury (33,34).

COVID 19 and gastrointestinal system

Similar to abundant expression of ACE-2 in the lungs, it has also been demonstrated on the epithelial cells of the stomach, duodenum, ileum, rectum, cholangiocytes, hepatocytes of the liver and esophageal mucosa (35). From physiological point of view, the kidney and the gut share a strong association or synergy during the maintenance of internal milieu known as the gut-kidney axis, which is further divided into metabolism-dependent and immune pathways (34). Gastrointestinal symptoms such as vomiting, diarrhea, loss of appetite and abdominal pain have been reported in SARS-CoV and COVID-19 patients (36-38).

Post-mortem changes in the gastrointestinal linings include vasculitis and increased inflammatory infiltrates. The spleen demonstrates increased neutrophil numbers, while the mesenteric lymph nodes show increased plamoblasts and congestion (39).

COVID-19 and the liver

Angiotensin converting enzyme-2 (ACE-2) is abundantly expressed in both the gastrointestinal epithelial cells and the liver, which explains a potential direct damage by SARS-CoV-2 on the liver (40). The liver enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, are elevated in COVID-19 patients, which indicate liver injury in these patients. AST elevation is more common than ALT, reflecting the contribution of AST from sources outside the liver. Hypoalbuminemia and slight increase in total bilirubin have been reported from several studies (41). Abnormal liver biochemistries are uncommon in children (42). Many drugs used in the treatment of COVID-19, such as antipyretic analgesics, antivirals, antibiotics and glucocorticoids, might have potential to cause drug-induced liver injury (43).

Autopsy findings from the first case of COVID-19 patient revealed that the liver tissue showed mild active inflammatory lesions in the hepatic lobular portal area, which suggest liver injury (44). *In vitro* studies have shown that SARS-CoV can cause direct liver injury (45). There is expression of moderate microvascular steatosis and mild lobular and portal activity, which indicate that liver injury could be caused by either SARS-CoV-2 infection or drug-induced liver injury (44).

COVID-19 and the skin

Viral illnesses are mostly associated with cutaneous manifestations, and may have diagnostic or prognostic value. With COVID-19, cutaneous manifestations range from rashes to eruptions.

Morbilliform rash

This is commonly seen with viral exanthemas. Studies from Italy reported that 78% of patients with COVID-19 had an erythematous/ morbilliform eruption (46).

Urticaria

A study from France reported that a patient developed an urticarial eruption along with odynophagia and diffuse arthralgia 48 hrs before onset of fever, chills and COVID-19 diagnosis (47). This unusual presentation of urticaria before the more well-known symptoms, signals the possibility that cutaneous eruptions can be a presenting symptom of COVID-19.

Vesicular eruptions

These resembles herpes vesicular eruptions and have been reported in COVID-19 patients (48). It was also observed that the median latency time from COVID-19 systemic symptoms to the rash was 3 days, and the median duration of skin manifestations was 8 days (49).

COVID toes

Research on cutaneous manifestations of coronavirus disease 2019 (COVID-19) are still going on. Acral cutaneous lesions also known as Covid toes have been reported in patients with COVID-19. the pattern of acral lesions is described as erythematous to purple, purpuric macules, papules and/or vesicles (50).

Livedoid eruptions

Many cases of Livedo reticularis-like eruptions have been reported in US patients with COVID-19 (51). It has been hypothesized that SARS-CoV-2 induces immune complexes formation with inflammation and vasculitis. This was further demonstrated on skin biopsies which showed small vessel thrombosis with no viral skin identification (52), demonstrating the possibility that tissue abnormalities are due to systemic toxicity triggered by a disproportionate immune response, rather than to direct viral spread. Another hypothesis is that the virus itself causes vascular damage, binding to ACE-2, which is widely expressed in endothelial cells (35). Early recognition of these cutaneous lesions may help to rapidly start treatment, since their worsening may be related to a severe systemic involvement**.**

COVID 19 and the neurologic system

Despite the fact that SARS-CoV-2 has been noted to principally affect the respiratory system, many studies have reported the involvement of neurological system. Neurological involvement in COVID-19 has been discussed in three sections; first is neurological features of viral infection, second is post-infective neurological complications, and third is infection in patients with neurological co-morbidity (53). Haematogenous spread and retrograde axonal transport have been described as routes for neuro-invasion by a coronavirus (54). Like other viruses, SARS-CoV-2, has been observed to directly invade the brain leading to clinical encephalitis (55). Other heterogeneous mechanisms such as cytokine storm and secondary hypercoagulability caused by the virus are thought to be involved.

Neurological manifestations in SARS-CoV-2 infection can be grouped into central nervous system (CNS) and peripheral nervous system (PNS). CNS features include headache, dizziness, ataxia, altered sensorium, encephalitis, stroke and seizures, while PNS features are seen as skeletal muscle injury and peripheral nerve involvement in the form of hyposmia and hypogeusia.

Post infective neurological complications including demyelinating conditions were previously reported (56). Guillain-Barré syndrome (GBS) is an inflammatory polyradiculoneuropathy associated with numerous viral infections. Recently, there have been many case reports describing the association between COVID-19 and GBS. However, despite numerous case reports of GBS associated with COVID-19, the prevalence remains unclear (57). In most of the cases reported, the patients were over 50 years of age, with male predominance (58). Coronavirus infection of the CNS has long provided a model for studying demyelinating diseases such as multiple sclerosis, vaccine design, and novel immunotherapeutic to limit virus spread (59). Even though the neurological manifestations in the majority of infected patients are mild, management of these patients should be a multidisciplinary approach.

The post-mortem nuclear magnetic resonance (NMR) findings of COVID-19 patients were parenchymal brain abnormalities such as subcortical macrobleeds and microbleeds, and edematous changes, which were suggestive of posterior reversible encephalopathy syndrome (PRES), and non-specific changes in the white matter (60).

Conclusion:

SARS-CoV-2 directly damage the lungs, heart, kidneys, liver, skin and brain. However, most of the injuries to these organs is caused by abnormal host immune responses to the virus. In the management of COVID-19 patients, it is recommended that both antiviral and immunomodulatory agents be applied for better prognosis and disease outcome.

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Review Article **Open Access**

A review of the possible prognostic values of biochemical changes in patients with SARS-CoV-2 infections

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Abstract:

Because of high mortality and long-term hospital stay among patients with SARS-CoV-2 infections, it is important to search for biochemical changes in different organs and systems that could be useful in diagnosis and prognosis of COVID-19. We conducted a literature search of online databases including PubMed, Web of Science, Scopus and Google scholar for relevant materials on biochemical changes in SARS-COV-2 infections published between December 2019 and March 2021. The review shows that SARS-COV-2 uses the angiotensin converting enzyme 2 (ACE2) for attachment and entry into host cells. These ACE2 are abundantly expressed by the epithelial cells of the respiratory tract and moderately expressed by the epithelial cells of the esophagus, stomach, duodenum, ileum, rectum, cholangiocytes, liver hepatocytes, pancreatic beta cells, and kidney tubular cells. This explains the systemic nature of SARS-COV-2 infection, and the high morbidity and mortality associated with COVID-19. Although, tests to assess biochemical changes are not specific enough for the diagnosis of SARS-CoV-2 infection, they may be useful for predicting outcome of COVID-19. This review highlights biochemical parameters that are significantly elevated or reduced in SARS-COV-2 infections, and which can be used as predictive factors of the severity and prognosis in COVID-19 patients.

Keywords: SARS-CoV-2; COVID-19; ACE2; Biomarkers; Diagnosis; Prognosis

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Un examen des valeurs pronostiques possibles des changements biochimiques chez les patients infectés par le SRAS-CoV-2

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Abstrait:

En raison de la mortalité élevée et du séjour à l'hôpital à long terme chez les patients infectés par le SRAS-CoV-2, il est important de rechercher des changements biochimiques dans différents organes et systèmes qui pourraient être utiles pour le diagnostic et le pronostic de COVID-19. Nous avons effectué une recherche documentaire dans des bases de données en ligne, notamment PubMed, Web of Science, Scopus et Google Scholar, pour rechercher des documents pertinents sur les changements biochimiques dans les infections par le SRAS-COV-2 publiés entre décembre 2019 et mars 2021. La revue montre que le SRAS-COV-2 utilise l'enzyme de conversion de l'angiotensine 2 (ACE2) pour la fixation et l'entrée dans les cellules hôtes. Ces ACE2 sont abondamment exprimés par les cellules épithéliales des voies respiratoires et modérément exprimés par les cellules épithéliales de l'œsophage, de

l'estomac, du duodénum, de l'iléon, du rectum, des cholangiocytes, des hépatocytes du foie, des cellules bêta pancréatiques et des cellules tubulaires rénales. Cela explique la nature systémique de l'infection par le SRAS-COV-2, ainsi que la morbidité et la mortalité élevées associées au COVID-19. Bien que les tests pour évaluer les changements biochimiques ne soient pas assez spécifiques pour le diagnostic de l'infection par le SRAS-CoV-2, ils peuvent être utiles pour prédire l'issue du COVID-19. Cette revue met en évidence les paramètres biochimiques qui sont significativement élevés ou réduits dans les infections par le SRAS-COV-2, et qui peuvent être utilisés comme facteurs prédictifs de la gravité et du pronostic chez les patients COVID-19.

Mots-clés: SARS-CoV-2; COVID-19; ACE2; Biomarqueurs; Diagnostic; Pronostic

Introduction:

Severe acute respiratory syndrome–coronavirus-2 (SARS-CoV-2) is responsible for the current coronavirus disease-2019 (COVID-19) pandemic. Like other coronaviruses, it is a highly pathogenic and transmissible virus (1). Millions of people globally have been affected since the World Health Organization (WHO) declared it a pandemic on March 11, 2020 (2, 3). The earliest coronaviruses (called infectious bronchitis virus) were grown from infected chickens (4). Subsequent isolates grown from the anterior nares of man were called coronavirus 229E and OC43 (4). A few other members of this family have also been branded, including SARS-CoV, CoV-NL63, HKU1, MERS-CoV, and 2019-nCoV, now known as SARS-COV-2 (4). Almost all of these have been linked to severe respiratory tract infections. The SARS-COV-2 is considered a relative of the deadly SARS and the Middle East respiratory syndrome (MERS) coronaviruses, both of which are characterized by flu-like symptoms, including fever, cough, and nasal congestion, and have the potential of transmission from animals to humans (5). Other studies suggested that bats and snakes could be the potential natural reservoirs of SARS-COV-2 (6,7,8).

The guidelines for diagnosis and treatment of this novel coronavirus infection have clear criteria for severe COVID-19, including respiratory rate, hemoglobin oxygen saturation (SaO2), and oxygenation index (PaO2/FiO2) (9). However, these criteria are highly subjective and lack objectivity, which may lead to an extended time for diagnosis and the possibility of misdiagnosis in severe COVID-19. Therefore, it is pertinent to search for biochemical parameters associated with this virus infection that could effectively aid in the early diagnosis and prompt management of COVID-19.

Methodology:

Online databases including PubMed, Web of Science, Scopus and Google scholar were comprehensively searched for published articles from December 2019 to March 2021 using the keywords and Boolean search terms; ["laboratory" OR "chemistry" OR "clinical"] AND ["coronavirus 2019" OR "COVID-19" OR "2019-nCoV" OR "SARS CoV-2"], and the PRISMA guidelines for selection of relevant materials. We searched for biochemical features seen in patients with COVID-19 in the published articles including C-reactive protein, interleukin-6, triglycerides, and other biomarkers responsible for hyper-inflammatory or 'cytokine storm syndromes' associated with COVID-19.

Initially, with greatest sensitivity search, we found 310 articles on external databases collected using Endnote software. Then unifying the articles from all the cited databases and bringing out duplicate articles, we separately reviewed all the articles and excluded the articles that were not related to the topic. After further review of the titles and abstracts, several other articles were excluded, leaving 70 eligible articles. The reference lists of all the eligible articles were also scrutinized, from which 6 additional articles were identified, given a total of 76 eligible published articles for the review (Fig 1).

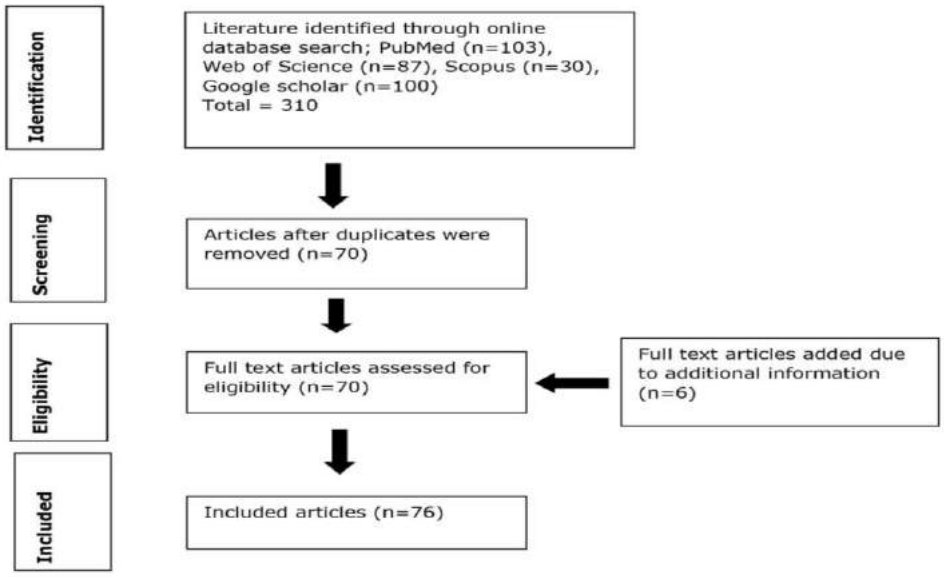

Fig. 1: Process of selection of publications (PRISMA guide) used for the review

Results and Discussion:

Biology of SARS-COV-2

Coronaviruses are enveloped, positive-sense single-stranded RNA viruses with helical nucleocapsid symmetry (10). They are members of the order *Nidovirales*, family *Coronaviridae*, and sub-family *Coronavirinae*, which contains four genera; *alpha, beta, gamma* and *deltacoronaviruses* (4). This classification is on the basis of their phylogenetic relationships and genomic structures. *Alpha* and *beta-coronaviruses* are mammalian pathogens while the *gamma* and *delta-coronaviruses* infect birds, but some of them can also infect mammals (11).

Alpha and *beta-coronaviruses* are respiratory tract pathogens of man and enteric pathogens in animals. SARS-CoV (beta-coronavirus), 229E (alpha-coronavirus), HKU1 (beta-coronavirus), NL63 (alpha-coronavirus), OC43 (betacoronavirus), and MERS-CoV (betacoronavirus) can all cause infections in humans (4).

Inflammatory markers in COVID-19

It has been demonstrated that there is direct cytopathic effect of SARS-CoV-2 on lymphocytes, along with a number of morphological changes seen on the peripheral blood smear of infected patients with 'cytokine storm syndrome' (12). Secondary haemophagocytic lymphohistiocytosis (sHLH) is an under recognized hyper inflammatory syndrome characterized by a fulminant and fatal hypercytokinaemia with multi-organ failure, and is most commonly triggered by viral infections (12). A cytokine profile resembling sHLH is associated with COVID-19 severity, characterized by increased interleukin (IL)-2, IL-7, granulocyte colony stimulating factor (GCSF), interferon-γ inducible protein 10 (IGIP-10), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1-α (MIP-1α), and tumor necrosis factor-α (TNF-α) (13). It is therefore plausible that measurement of some of these inflammatory biomarkers will be crucial for early and accurate identification of COVID-19 patients who may be at high risk of unfavorable progression.

C-reactive protein (CRP) is a commonly measured nonspecific biomarker of inflammation. Increased CRP concentration has consistently been shown to be associated with poor outcome in SARS-CoV-2 infection (14). Ferritin is a positive acute phase protein, which is easily measured and may be a marker of adverse outcomes in individuals infected with SARS-CoV-2 (15). Procalcitonin (PCT) is also a main inflammatory marker routinely measured in clinical practice. However, it has been reported that the overall number of COVID-19 patients with increased PCT values seem limited (16).

Biomarkers of cardiovascular functions in COVID-19

COVID-19 patients who are co-morbid with hypertension, heart failure and coronary artery disease are at greater risk of serious

illness and ICU admission (17). Cardiac troponin (cTnI) is the 'gold standard' necrotic biomarker for risk assessment of myocardial injury worldwide (18). It is released exclusively in the presence of myocardial injury irrespective of the mechanism of insult. SARS-CoV-2 has been shown to induce expression of multiple cytokines and chemokines, resulting in vascular inflammation, plaque instability and myocardial inflammation (13). Several studies have shown that the blood levels of cardiac troponins are higher in patients with more severe illness, compared to those with milder disease (19). An increased cardiac troponin in COVID-19 patients is probably reflecting an acute myocardial injury caused by either the virus or host immune response, rather than myocardial infarction due to rupture of an atherosclerotic plaque (20). Other cardiac biomarkers, including creatine kinase-MB, myoglobin and atrial natriuretic peptides have been shown to have similar relationships (21).

Physiological parameters of respiratory functions in COVID-19

Severe COVID-19 is associated with hypoxemia and metabolic acidosis (22), which may lead to acute respiratory distress syndrome (ARDS). Hence the need for the measurement of arterial blood gas parameters especially pH, pO_2, pCO_2, HCO_3 and lactate (23). COVID-19 is adjudged to be severe if respiratory rate is ≥ 30 breaths/min, SpO_2 < 93% while breathing room air or PaO_2/FIO_2 ≤ 300 mmHg or 40 kPa (24).

Biomarkers of muscle injury in COVID-19

Oxidative stress, which is preceded by excessive production of proinflammatory cytokines in hypercatabolic conditions, is associated with production of corrosive molecules that cause severe myocyte damage (25). Myokines and adipokines produced by sarcopenic muscle and adipose tissue stimulate signaling pathway of inflammation and oxidative stress resulting in hyper-catabolism, especially in people with advanced age and those with metabolic disorders (26). Remarkable elevation in biomarkers of muscle loss, such as creatine kinase (CK), in up to 27% of hospitalized COVID-19 patients, was a condition described as hyperCKemia (27, 28). In a meta-analytical study, report shows elevated levels of CK, LDH, and myoglobin in severe than mild COVID-19 cases (29).

Fluid and electrolytes disturbances in COVID-19

The kidneys and gastrointestinal (GI) involvement in COVID-19 result in fluid and electrolyte disturbances. The notable disturbances are hyponatremia, hypernatremia, hypokalemia, hypocalcemia, hypochloremia, hypervolemia, and hypovolemia, which if left unattended to, can lead to high mortality. Fluid and electrolyte disturbances are more common among hospitalized patients and those in intensive care units. Deranged renal function leads to fluid and electrolyte disturbances (30), and many studies on COVID-19 have confirmed electrolyte disturbances such as sodium, potassium, chloride, and calcium imbalances (13, 31). However, the most common electrolyte disorder is hyponatremia, which is associated with increased risk of mortality among hospitalized COVID-19 patients (32).

Some drugs used in the treatment for patients with COVID-19 such as chloroquine and hydroxychloroquine, have also been implicated to cause electrolyte imbalance (33). Hypokalemia, another complication in COVID-19, can exacerbate acute respiratory distress syndrome (ARDS) and increase the risk of heart injuries (34). Hypocalcemia has also been observed as one of the electrolytes disorders in patients with COVID 19, which if not controlled, can increase mortality rate (35). Syndrome of inappropriate antidiuretic hormone (SIADH) has been reported in some COVID-19 patients and implicated in causing fluids and electrolyte disturbances (36).

Effects of SARS-CoV-2 on the liver and biomarkers of hepatic injury in COVID-19

The liver performs protective functions in host defense against microbes. Therefore, SARS-CoV-2 infections can be associated with liver injury during disease progression and treatment in COVID-19 patients with or without preexisting liver disease. Elevated serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and bilirubin, and low albumin and prealbumin concentrations have all been associated with poor outcome of COVID-19 (37). It was reported in a study that some drugs used in treatment of COVID-19 were associated with elevated biomarkers of liver injury (38).

SARS COV-2 RNA has been detected in stool highlighting the possible means of transmission of the virus from the gut through the portal circulation to the liver. SARS-COV-2 may directly bind to ACE2 positive cholangiocytes to exert a cytopathic effect. The disruption of cholangiocyte function is associated with hepatobiliary damage (39). Multiple factors are responsible for liver dysfunction or damage in patients with COVID-19. This includes a direct cytopathic effect of SARS-COV-2 from contin-

uous replication of the virus inside the hepatocytes, indirect effects following drug-induced liver injury (DILI) from the current armoury of therapeutic agents deployed against SARS-COV-2, and liver injury related to accentuated immune response such as 'cytokine storm syndrome' and immune mediated hepatitis. Also, hypoxia and shock induced by COVID-19-related complications such as systemic inflammatory response syndrome and multiple organ failure, may cause hepatic ischaemia and hypoxia-reperfusion dysfunction (40).

Between 37.2 and 76.3% of COVID-19 patients have abnormal liver function (41), and the commonly elevated parameters are aspartate transaminases (AST), alanine transaminases (ALT), alkaline phosphatase (ALP), total bilirubin, and the gamma glutaryl transferase (GGT) while albumin is usually reduced (42). Liver test abnormalities defined using the Yale New Haven Health System (YNHHS) laboratory reference range standards include; AST >33 U/L, ALT > 34 U/L, ALP > 122 U/L, total bilirubin > 1.2 mg/dL, and albumin < 3.5 mg/d L (43). Abnormal liver function in COVID-19 is more common in adult males than females, and less common in children (44). Liver dysfunction in COVID-19 is usually mild in majority of the patients with many parameters returning to normal without treatment. However, severe liver injury (AST 1445 U/L and ALT 7590 U/L) have been reported, with 61.5% of intensive care unit (ICU) patients having higher number of liver injury than non-ICU patients (25%). Among non-survivors, the incidence of liver injury might reach as high as 58%-78% (45, 46,47). Therefore, liver function could be used as an indicator of disease progression, and special attention should be given to any liver dysfunction while managing COVID-19 patients (48).

In a study of 417 patients with COVID-19 in Shenzhen, China by Cai et al., (41), 76.3% of them were found to have abnormal liver tests results and 21.5% had liver injury during hospitalization. The patterns of the liver abnormalities were hepatocellular, cholestatic or mixed. Hepatocellular abnormalities resulted in elevations of ALT and/or AST, cholestatic type resulted in elevation of ALP and/or GGT, while mixed type was associated with elevation of combination of the liver enzymes. On the other hand, a decrease in serum albumin level and/ or prolonged prothrombin time was considered synthetic type of liver abnormality (41). Similarly, a study done by Fan et al., (44) on 148 COVID-19 patients reported incidence of abnormal liver function tests as lactate dehydrogenase (35.1%), AST (21.6%), ALT (18.2%), GGT (17.6%), total bilirubin (6.1%) and ALP (4.1%).

A review of more than 20 publications showed abnormal levels of transaminases in COVID-19 patients (48,49), with a correlation between the level of liver dysfunction and severity of the disease (48). The incidence of raised ALT and AST ranged from 2.5-50.0% to 2.5%-61.1% respectively (44,47,49,50). These studies reported elevation of the hepatocellular markers of liver abnormalities (AST and ALT) in 14%-53% of patients (45,46,51). In a large cohort study of 1099 patients from 552 hospitals, Guan et al., (52) observed elevated levels of only AST in 18.2% of COVID-19 patients with mild to moderate disease, and 56% with severe disease. Moreover, in the same study, 28.1% of patients with severe disease were observed to have abnormal ALT compared to 19.8% of mild cases. Lei et al., (53) reported AST to be the first elevated marker on hospital admission for COVID-19, and this was also associated with the highest mortality. A retrospective study done Xu et al., (7) reported a patient with severe hepatitis due to COVID-19 having ALT of 7590 U/L and AST of 1445 U/L. AST elevation is the commoner of the transaminases, reflecting the extrahepatic contribution of this enzyme (54).

Other studies have reported abnormal levels of parameters of liver dysfunction such as LDH and albumin in COVID-19, with Kukla et al., (55) reporting 76% and 98% of their patients with abnormal LDH and albumin levels respectively. SARS-COV-2 caused decrease in albumin secretion in severe COVID-19 cases in their study to values of about 26.3-30.9 g/L. In a systematic review and meta-analysis of 128 studies (17), the most frequent liver function abnormalities were hypoalbuminemia (61.3%), and elevated GGT (27.9%), AST (23.4%) and ALT (23.3%). Another large systematic review of 11 studies (56) which evaluated liver parameters of 2541 patients with SARS-COV-2 infection, reported elevated AST and/or ALT (25%), LDH (20%), bilirubin (3%), but normal ALP in almost all the COVID-19 patients (56). Patients with chronic liver disease who contract SARS-COV-2 are at increased risk of severe COVID and a sizable number of them tend to develop decompensated liver as a systemic inflammatory response induced by the virus (57).

Effects of SARS-COV-2 on the kidneys and biomarkers of renal dysfunctions in COVID-19

The involvement of kidneys in COVID-19 has been said to be multifactorial. Cytopathic effects of kidney-resident cells and the 'cytokine storm syndrome' damage are known

modes of tubular damage (58). Elevations of both serum creatinine and urea (blood urea nitrogen, BUN) have been associated with unfavorable clinical outcome (14). SARS-COV-2 uses the angiotensin converting enzyme 2 (ACE2), which is highly expressed in the kidneys, as receptors for cellular entry (59). The viral spike protein (S) binds to renal ACE2, enabling membrane fusion and endocytosis processes through the cellular transmembrane serine protease 2 (TMPRSS2), and within the cell, viral polyproteins are synthesized, assembled and released (60). The mechanisms by which SARS-COV-2 causes renal injury include the release of nephrotoxic substances from 'cytokine storm syndrome', prothrombotic coagulopathy, and an imbalance of the renin-angiotensin-aldosterone system (RAAS) with negative haemodynamic effects (61). Naicker (62) outlined other possible aetio-pathological mechanisms for renal injury during the course of COVID-19 as renal hypoperfusion-related acute tubular necrosis or cellular damage, dysregulated inflammatory response, micro-circulatory dysfunction, metabolic reprogramming, 'cytokine storm syndrome' precipitated by sepsis, and direct viral injury.

SARS-CoV-2 causes kidney injury that typically involves the glomerulus and tubules. Impaired glomerular filtration manifests as increased blood urea nitrogen and creatinine while tubular damage manifests as abnormalities in urinalysis (63,64). Proteinuria in patients with COVID-19 is often mild. In a study by Li et al., (65), 27% of COVID-19 patients had elevated urea nitrogen while 19% of same group of patients had elevated creatinine. Another study on 99 patients showed 6% of them had elevated serum urea nitrogen, 3% had elevated serum creatinine and incidence of acute kidney injury (AKI) was 3% (66). The incidence of abnormal renal function, defined as an increase in serum creatinine reported by Zhu et al., (67) in a meta-analysis of 3062 COVID-19 was found to be 25.5%.

The prevalence of kidney injury among hospitalized COVID-19 patients vary from one study to another with a reported highest prevalence of 69% (68). Kidney injury is identified with any of increased serum creatinine levels, proteinuria, and haematuria, with some of the patients requiring renal replacement therapy (69). A large proportion of COVID-19 patients manifest kidneys dysfunction manifesting as proteinuria, haematuria, elevated serum creatinine and blood urea nitrogen (BUN) (22). In a review of 24 studies with 10,180 COVID-19 patients by Yang et al., (70), the pooled prevalence of acute kidney injury (AKI) was 16.2%, increased serum creatinine 8.3%, increased BUN 6.2%, increased D-dimer 49.8%, proteinuria 50.1%, and haematuria 30.3%. They also observed that these analytes were higher in ICU/severe cases in comparison with non-ICU/non-severe patients, with serum creatinine 6.4-folds, BUN 1.8-folds, D-dimer 0.67-folds and AKI 30-folds (70). Wald et al., (69) also observed that majority of COVID-19 patients admitted into the ICU had AKI with 20% of them receiving renal replacement therapy. Survivors of AKI due to COVID-19 often have incomplete recovery of kidney function at the time of hospital discharge (71).

On admission and/or during hospitalization, studies (56,62) have shown that COVID -19 patients present with massive albuminuria (34%), elevated BUN (27%) along with haematuria (44%), and increased serum creatinine (15.5%). Importantly, signs of kidney dysfunction were found in a large percentage of patients on hospital admission for COVID-19 including 44 - 65% with proteinuria, 27 - 44% with haematuria, and 10-14% with increased serum creatinine (58,70,72). Different studies have observed proteinuria in 7% - 63% of COVID-19 patients (61,65) who present either as low abundance proteinuria attributed to tubular injury or as abundant proteinuria which is suggestive of glomerular impairment. Activation of RAAS, injury to podocytes and nephron endocytosis with consequent increase in glomerular permeability may be responsible for proteinuria (73). Kaliuresis with hypokalaemia due to activation of RAAS in a cohort of 175 COVID-19 patients was associated with the most severe forms of SARS-COV-2 infection (74). Mabillard and Sayer, reported 26.7% of haematuria in COVID-19 patients (36).

AKI is reported in 6.0-36.6% of COVID-19 cases and has been reported to be an independent predictor of in-hospital mortality in COVID-19 patients (45,52,75). Several factors contribute the onset and progression of AKI in COVID-19, some of which include ventricular dysfunction due to COVID-19 pneumonia, renal endothelial damage, rhabdomyolysis, activation of RAAS and 'cytokine storm' following SARS-COV-2 replication (76). A study of 710 COVID-19 patients in Huazhong, China reported 44% with proteinuria and haematuria, 26.9% of whom had simple haematuria, 15.5% had elevated serum creatinine, and 14.1% had elevated BUN. Elevations in serum creatinine, BUN levels and occurrence of AKI increase hospital mortality rate by 3.61, 2.51 and 2.21 respectively (45,48).

Conclusion:

SARS-CoV-2 infection affects multiple organs and systems of the body, from which several biochemical substances are released as a result of damage to these organs. The knowledge of the biochemical parameters associated with prognosis in COVID-19 is very important to prevent lengthy stay in ICU and mortality associated with dreaded disease. This review has highlighted various biochemical markers (Table 1) that are significantly elevated or reduced as evidence of serious damage to the organs involved, and which can be explored for diagnostic and prognostic purposes, as well as monitoring of COVID-19 patients on therapy.

It is however important to concentrate more on those parameters that have been found to be strongly associated with worse outcomes. These include elevated levels of C-reactive protein (CRP), ferritin, cardiac troponins (cTnI), serum creatinine, creatinine kinase, LDH and BUN. On the other hand, values of $PaO_2/FIO_2 \leq 300$ mmHg or 40 kPa, and $SpO_2 < 93\%$ while breathing room air are associated with poor pulmonary function.

Table 1: Biochemical parameters of organ dysfunctions in COVID-19

Organ/system of the body	Elevated parameters	Reduced parameters
Inflammatory and immune system	Interleukin (IL)-2 Interleukin (IL)-7 Granulocyte colony stimulating factor Interferon-γ inducible protein 10 Monocyte chemoattractant protein 1 Macrophage inflammatory protein 1-α Tumor necrosis factor-α *C-reactive protein (CRP) *Ferritin	
Cardiovascular system	*Cardiac troponins (cTnI) Creatine kinase-MB Myoglobin Natriuretic peptides	
Liver	Aspartate aminotransferase (AST) Alanine aminotransferase (ALT) Bilirubin	Albumin Prealbumin
Kidneys	*Serum creatinine, *Blood urea nitrogen (BUN) *Lactate dehydrogenase (LDH)	
Respiratory system	Respiratory rate ≥30 breaths/min	*$PaO_2/FIO_2 \leq 300$ mmHg/40 kPa, *$SpO_2 < 93\%$ while breathing room air
Fluid and electrolytes		*Hyponatraemia Hypocalcaemia Hypochloraemia *Hypocalcemia
Muscle	*Creatinine kinase LDH Myoglobin	

* = parameters associated with higher probability of the need for intensive unit (ICU) care and high mortality rate

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Review Article **Open Access**

Hosts and transmission of *Mycobacterium ulcerans*: a systematic review

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Abstract:

The control of Buruli ulcer (BU), a debilitating neglected tropical disease, is hampered by the inadequate understanding of the mode of transmission of its causative agent, *Mycobacterium ulcerans* (*M. ulcerans*). The DNA of *M. ulcerans* has been detected in some living organisms and non-living environmental samples of both aquatic and terrestrial sources. However, it is unclear whether the identified organisms support *in vivo* multiplication of the bacterium or play any role in its transmission. This paper identifies hosts of *M. ulcerans*, reviews progress made in unravelling the exact mode of transmission of *M. ulcerans* and identifies research gaps in this aspect of BU epidemiology. Using the search terms, 'niche, *Mycobacterium ulcerans'* and 'mode of transmission, *Mycobacterium ulcerans'* as well as defined inclusion criteria, information was obtained from the PubMed database and reviewed to assess their importance to the research question. Aquatic bugs of the genera *Appasus* and *Diplonychus* as well as *Naucoris cimicoides* and possums were identified to support *in vivo* multiplication of the bacterium. Bite of *M. ulcerans* contaminated *Aedes notoscriptus*, bite of aquatic bugs harboring or contaminated with *M. ulcerans,* and *M. ulcerans* contaminated skin-puncturing materials present in nature create opportunity for its transmission and infection. Appropriate protective measures may be useful to reduce the risk of exposure to *M. ulcerans* in BU endemic areas, and incorporation of trophic interactions of aquatic organisms known to support *in vivo* multiplication of *M. ulcerans* is needed in future research for better understanding of the spread of *M. ulcerans* in nature.

Keywords: *Mycobacterium ulcerans*; niche; transmission

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Hôtes et transmission de *Mycobacterium ulcerans*: une revue systématique

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Abstrait:

Le contrôle de l'ulcère de Buruli (UB), une maladie tropicale négligée débilitante, est entravé par la compréhension insuffisante du mode de transmission de son agent causal, *Mycobacterium ulcerans* (*M. ulcerans*). L'ADN de *M. ulcerans* a été détecté dans certains organismes vivants et des échantillons environnementaux non vivants de sources aquatiques et terrestres. Cependant, il n'est pas clair si les organismes identifiés favorisent la multiplication *in vivo* de la bactérie ou jouent un rôle dans sa transmission. Cet article identifie les hôtes de *M. ulcerans*, passe en

revue les progrès réalisés pour démêler le mode exact de transmission de *M. ulcerans* et identifie les lacunes de la recherche dans cet aspect de l'épidémiologie de l'UB. À l'aide des termes de recherche « niche, *Mycobacterium ulcerans* » et « mode de transmission, *Mycobacterium ulcerans* » ainsi que des critères d'inclusion définis, des informations ont été obtenues à partir de la base de données PubMed et examinées pour évaluer leur importance pour la question de recherche. Des punaises aquatiques des genres *Appasus* et *Diplonychus* ainsi que *Naucoris cimicoides* et possums ont été identifiées pour soutenir la multiplication *in vivo* de la bactérie. La piqûre *d'Aedes notoscriptus* contaminé par *M. ulcerans*, la piqûre d'insectes aquatiques hébergeant ou contaminés par *M. ulcerans* et les matériaux de perforation de la peau contaminés par *M. ulcerans* présents dans la nature créent une opportunité de transmission et d'infection. Des mesures de protection appropriées peuvent être utiles pour réduire le risque d'exposition à *M. ulcerans* dans les zones d'endémie UB, et l'incorporation d'interactions trophiques d'organismes aquatiques connus pour favoriser la multiplication *in vivo* de *M. ulcerans* est nécessaire dans les recherches futures pour une meilleure compréhension de la propagation de *M. ulcerans* dans la nature.

Mots clés: *Mycobacterium ulcerans*; niche; transmission

Introduction:

Buruli ulcer (BU) is a chronic, necrotizing and indolent disease of the skin, subcutaneous tissue and occasionally bones (1), caused by *Mycobacterium ulcerans*. It usually occurs in the vicinity of rural tropical wetlands, and its discovery dated back to 1897. The disease has been reported in 33 countries globally (2), mostly in tropical (3,4) and subtropical regions (3). However, the largest number of cases has been reported from riverine areas in distinct regions of Benin, Côte d'Ivoire and Ghana as well as Cameroon and the Democratic Republic of Congo (3) in West and Central Africa respectively. The profound morbidity in BU victims and the devastating nature of its complications (5) have enormous adverse socioeconomic implications. This calls for more research in the grey areas of the disease, such as the mode of transmission of the bacterium, to help improve our understanding and control of the disease.

The mode of transmission of *M. ulcerans* has been a subject of investigation since 1948 (6) when the bacterium was identified as the causative agent of BU. However, the reservoir and mode(s) of transmission of the bacterium are not definitively known, posing great challenge to BU epidemiology. *M. ulcerans* is reported to adopt a biofilm-like structure *in vitro* and *in vivo*, and displays abundant extracellular matrix (ECM), which enhances colonization of insect vectors and mammalian hosts and confers to it increased resistance to antimicrobial agents (7). Additionally, *M. ulcerans* and, to a greater extent, its deoxyribonucleic acid (DNA), have been identified in various environmental samples such as inanimate materials (8-10), plants (11,12), invertebrates (13 -16) and vertebrates (9,17-21) of both aquatic and terrestrial habitats.

Two major research gaps are whether these samples support active multiplication of the bacterium and/or play any active role in its transmission. This paper identifies hosts of *M. ulcerans*, reviews progress made in the quest to unravel the exact mode(s) of transmission and identifies specific gaps that may generate interest in research in this aspect of BU epidemiology.

Methodology:

This systematic review was undertaken using the PRISMA guidelines (22) developed by the Centre for Review Dissemination (CRD). The following search terms were used to obtain information for all years from PubMed database; 'niche, *Mycobacterium ulcerans'* (NMU) and 'mode of transmission, *Mycobacterium ulcerans'* (MTMU). The searches were carried out on 2nd September 2020 and the filters applied; abstract, free full text, full text and journal article limited the years from 2002 to 2020 and 2001 to 2020 for NMU and MTMU respectively.

The diagnosis of BU by the polymerase chain reaction (PCR) is based on the amplification of the insertion sequence IS*2404* in the genome of *M. ulcerans* (23-25) using appropriate primers, and IS*2404* PCR is considered the most sensitive method for laboratory confirmation of the disease (26). However, the detection of *M. ulcerans* from environmental samples requires confirmatory PCR targeting additional insertion sequence, IS*2606*, and the ketoreductase B domain (KR) of *M. ulcerans* mycolactone polyketide synthase genes, to differentiate *M. ulcerans* from other environmental mycobacteria that may carry IS*2404*, and other non-mycolactone-producing mycobacteria (8). Therefore, studies with environmental samples having culture-confirmed *M. ulcerans* and/or PCR-positive *M. ulcerans* DNA (IS*2404*, KR and IS*2606*) (8) as well as IS*2404* and KR with cycle thresholds (Ct) of less than 34 and 36 for IS*2404* and KR-B respectively (8,27) were included in the review. The

inclusion of IS*2606* and KR of the *M. ulcerans* mycolactone polyketide synthase genes is based on the observation that their detection by PCR augment the specificity of IS*2404* PCR for the analysis of various environmental samples (8). Other studies, including laboratory based, reporting on successful and/or proposed *M. ulcerans* transmission, were also included in the review. Studies that did not meet these criteria, including review articles, were excluded from the review.

Results:

Number and selection of literature

The total number of articles obtained from both searches, in PubMed database, using NMU and MTMU search terms were 58 and 24 respectively with no duplicates. Three additional relevant articles were downloaded from Google Scholar. Out of the total of 85 identified articles, 57 were excluded, as they were deemed to be irrelevant to the research question after reading the titles and abstracts. After assessing full-text articles of the remaining 28 for eligibility, 15 of these were included in the review. The literature selection process is shown in Fig 1.

Sources and reservoirs of *M. ulcerans* in nature

M. ulcerans and its DNA were identified from various sources including aquatic, living and inanimate sources. Table 1 describes the type and source of sample from which *M. ulcerans* and its DNA were detected as well as the detection method and key findings.

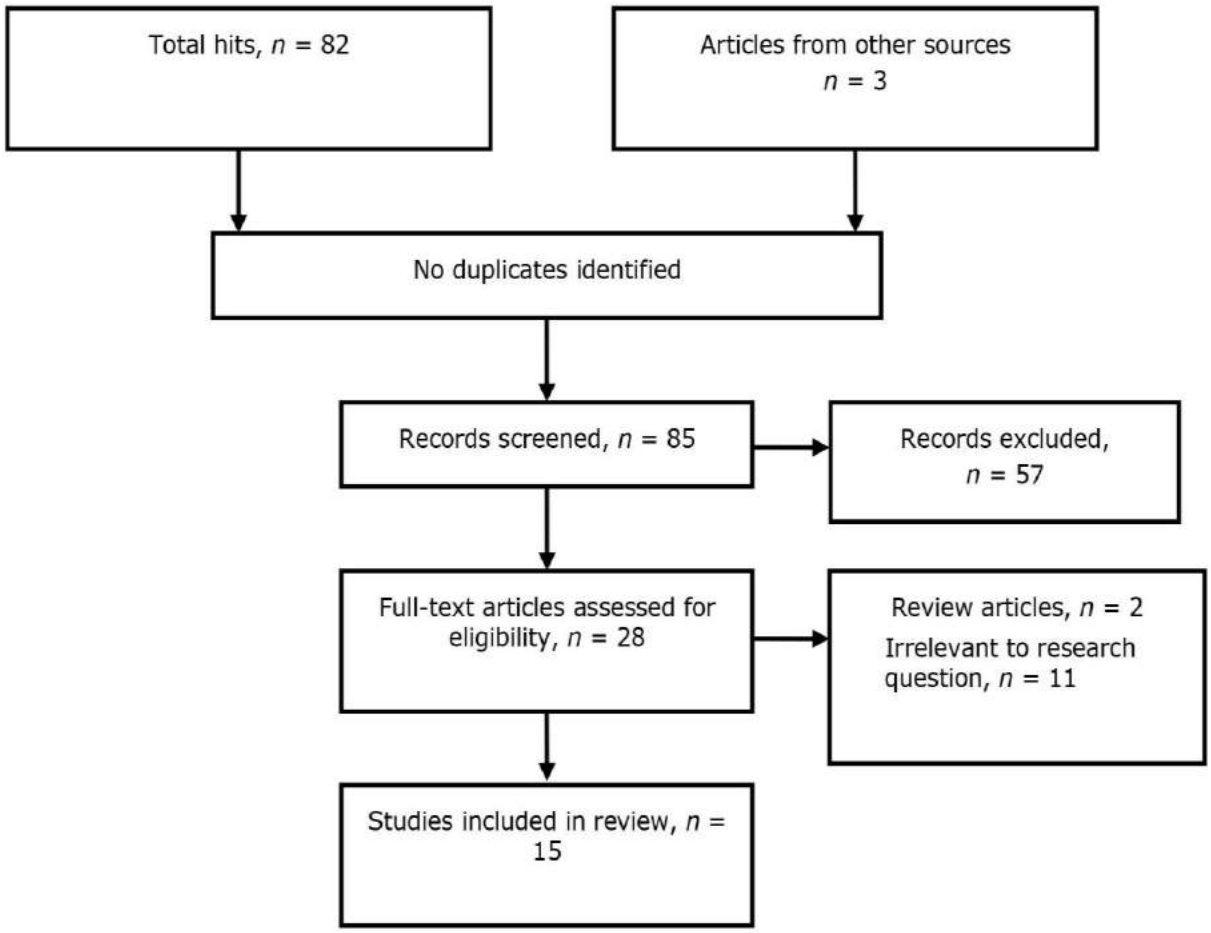

Fig 1: Flow chart of study selection process (PRISMA guide)

Table 1: Identified sources of *Mycobacterium ulcerans* and its DNA (IS*2404*, IS*2606* and KR)

Sample type (reference)	Sample size and source of study	Detection method and result
Freshwater green algae (11)	Two green algae, *Rhizoclonium* sp. and *Hydrodictyon reticulatum*, on mud or rock surface in freshwater in BU endemic and non-endemic areas, Ivory Coast.	*M. ulcerans* biofilm formed on *Rhizoclonium* sp. One positive BACTEC culture supplemented with algal extract, IS*2404* PCR positive on culture and IS*2404* PCR positive for two aquatic plant samples in BU endemic area.
Amoeba (28)	Amoeba cultures from water (*n* = 13), herbaceous plant biofilms (*n* = 90) and aquatic detritus samples (*n* = 45) in and around water bodies in BU endemic and non-endemic communities, Ghana.	Real-time PCR (RT-PCR) positive for IS*2404*, IS*2606* and KR in 1 of 148 environmental samples. IS*2404* positive for 7 out of 166 amoeba cultures from 124 samples from BU endemic and non-endemic communities.
Amoeba (29)	Free-living amoeba (FLA) cultures from plant and tree trunk biofilms (*n* = 428), water (*n* = 53), detritus (*n* = 45) and aerosols in BU endemic and non-endemic communities, Ghana.	Quantitative PCR (qPCR) positive for IS*2404* in 25 (4.64%) out of 370 FLA cultures from 539 specimens, but negative for IS*2606* and KR. Green fluorescence protein (GFP) expressed *M. ulcerans* in laboratory-infected *Acanthamoeba castellanii* by flow cytometry.
Creeping water bugs (14)	Salivary glands of adult Naucoridae (*n* = 80) from a river in BU endemic area, Ivory Coast. .	Two Culture positive for *M. ulcerans* and 5 of 80 IS*2404* nested-PCR positive detection in naturally-infected Naucoridae. Culture positive strains inoculated into mice tail produced inflammatory lesions with edema that were PCR positive for *M. ulcerans*.
Aquatic Heteroptera (30)	Single-taxon batches (*n* = 283) of Belostomatidae, Naucoridae, Corixidae, Ranatridae Nepidae, and Saliva of *Diplonychus* sp. (*n* = 69) from ponds near villages in BU endemic area, Ivory Coast.	Real-time PCR positive for IS*2404* and KR in 26 of 283 single-taxon batches of insect families and 6 of 69 random saliva samples of *Diplonychus* sp.
Mosquitoes (16)	Mosquitoes (*n* = 11,504), mainly *Aedes camptorhynchus*, from BU endemic area, Australia.	Of the 11,504 mosquitoes tested, 13 pools were positive for IS*2404*, KR and IS*2606*. VNTR locus 9 (2 positive pools) sequence identical to local *M. ulcerans* human strain.
Mosquitoes (31)	Adult mosquitoes (*n* = 41,797), mainly *Aedes camptorhynchus*, from BU endemic areas, Australia.	Real-time PCR for IS*2404* (± IS*2606* and KR) detection rate ranged from 1.02 to 10.80 per 1,000 mosquitoes. Highest proportions of *M. ulcerans*-positive mosquitoes detected in areas with highest BU incidences.
Mosquitoes/flying insects, aquatic plants, invertebrate and vertebrate (32)	Flying insects (*n* = 7,230), including mosquito spp. (*n* = 4,322), Macro-invertebrate and vertebrate (*n* = 3,377), plants ((*n* = 95) from water sources near villages in highly BU endemic areas, Benin.	qPCR positive for IS*2404* and KR in 8.7% (28/322 pools) of aquatic insects including water bugs, but not in mosquitoes or other flying insects. Positive-PCR for 2.1% (2/95) plants in the Poaceae family.
Mosquitoes and march flies (33)	Mosquitoes (*n* = 16,900) allocated to 845 pools and march flies (*n* = 296) from BU endemic areas, Australia.	Real-time PCR positive for IS*2404*, IS*2606* and KR in one pool of mosquito (*Verrallina* sp.) out of 845 pools screened, but negative in march flies.
Ringtail and brushtail possums (9)	Faecal samples from ringtail (*n* = 589) and brushtail (*n* = 250) possums from BU highly, low and non-endemic areas, Australia.	PCR positive for *M. ulcerans* DNA (IS*2404*, IS*2606* and KR) highest in highly BU endemic areas. Culture negative, but VNTR positive for *M. ulcerans* human strain in faecal samples.

	Ringtail (*n* = 42) and brushtail (*n* = 21) possums from the BU highly endemic area examined for lesions.	Laboratory-confirmed (PCR ± culture) *M. ulcerans* lesions and/or PCR-positive faeces in 16 (38%) ringtail and 5 (24%) brushtail possums.
Inanimate materials, terrestrial and aquatic plants (9)	Suspended solids (*n* = 33), detritus (*n* = 47), sediment (*n* = 28), soil (*n* = 49), aquatic plant biofilm (*n* = 19), aquatic plants (*n* = 21) and terrestrial vegetation [leaves, bark, flowers, seeds] (*n* = 79) from water bodies and terrestrial habitat in high and low BU endemic areas, Australia.	Low levels of *M. ulcerans* DNA (weak positive real-time PCR signals for IS*2404*, IS*2606* and KR), but relatively higher number of positive samples in the high BU endemic area.
Ringtail and brushtail possums (34)	Possum faecal samples (*n* = 57), possum blood (*n* = 63), buccal swab (*n* = 67), urine (*n* = 16), pouch swab (*n* = 15) and cloacal (*n* = 20) samples, and clinically affected possum (*n* = 27) from BU endemic areas, Australia.	Culture positive for skin lesions (19), liver, spleen, mandibular lymph node (1) and skin lesions, liver, lung and small intestinal contents (1) cases. IS*2404* PCR positive for faecal (14), buccal swab (7), pouch swab (3) and cloacal (1) samples, but negative for blood and urine samples.
Bandicoot, white-tailed rats and possum (35)	Scat samples of bandicoot (*n* = 140), white-tailed rat (*n* = 4), possums (*n* = 2) and bandicoot ulcer swab sample (*n* = 1) from BU endemic areas, Australia.	Real-time PCR positive for IS*2404*, IS*2606* and KR in 2 out of 140 bandicoot scat samples, but negative in other scat and swab samples.
Aquatic bugs (Heteroptera) (36)	Water bug tissues from BU endemic (*n* = 3647 [616 pools]) and non-endemic (*n* = 422 [80 pools]) areas and saliva (*n* = 293) samples of *Appasus* sp. from endemic area, Cameroon.	qPCR positive for IS*2404* and KR in 68 pools out of 616 (11%) in BU endemic area, but all 80 pools negative in non-endemic area. qPCR positive for IS*2404* and KR in 17.4% saliva (51/293) and tissue samples of *Appasus* sp. in endemic area. *M. ulcerans* DNA was detected in five out of seven analyzed insect families.
Domestic animals (21)	Swabs of skin lesions (*n* = 25) out of 361 domestic animals surveyed in BU endemic areas, Benin. Swabs of skin lesions (*n* = 44) out of 397 domestic animals surveyed in BU endemic areas in Cameroon.	qPCR positive for IS*2404*, IS*2606* and KR in 2 (8%) external lesions of a goat and a dog out of 36 animals with lesions in Benin, but none in communities in Cameroon.
Domestic animals (37)	Faecal samples (*n* = 180) of chickens, goats, sheep, dogs and lizards from BU endemic and non-endemic villages in Ghana.	qPCR negative for *M. ulcerans* DNA targets IS*2404* and KR-B.

Discussion:

Ecology of *M. ulcerans*

Mycobacterium ulcerans or its DNA (IS *2404*, IS*2606* and KR) is found associated with various aquatic and terrestrial organisms as well as inanimate materials of aquatic and terrestrial sources (Table 1). In BU endemic areas in particular, *M. ulcerans* or its DNA is found associated with freshwater green algae (11), amoeba (28,29), aquatic bugs of the Order Hemiptera, including Naucoridae [creeping water bugs] (14,30), Belostomatidae [giant water bugs] (30,36), Corixidae [water boatmen], Ranatridae, and Nepidae [water scorpions] (30). Similar observations were made in mosquitoes (16,31,33), bandicoot scat samples (35), ringtail and brushtail possums (9,34), goats and dogs (21), as well as inanimate materials such as suspended solids, detritus, sediment and soil samples (9) in BU-endemic areas. Interestingly, higher levels of *M. ulcerans* DNA tend to be detected in BU-endemic areas compared with non-endemic ones.

The type of association of *M. ulcerans* with these hosts will provide insight into the maintenance and distribution of *M. ulcerans* in the environment. *M. ulcerans* is known to survive best under low oxygen tension, such as

exist in mud in the bottom of swamps (38) where the roots of the aquatic plants, *Cyperus*, *Panicum* and *Eichhornia* shelter aquatic bugs (39). Crude organic extracts from two freshwater green algae, *Rhizoclonium* sp and *Hydrodictyon reticulatum*, from BU-endemic areas in tropical and temperate regions respectively, are able to stimulate the growth of *M. ulcerans* in a culture medium (11). This discovery is supported by the fact that aquatic plants are able to secrete many organic compounds, including amino acids and polysaccharides, which are used by bacteria as substrates for growth (40-42). Interestingly, genotypic analysis carried out previously (11) showed that plant-associated *M. ulcerans* had the same profile as *M. ulcerans* isolates recovered in the same region from both aquatic insects and clinical specimens. By virtue of their habitat and predatory habit, it is probable that aquatic bugs get contaminated with *M. ulcerans* or acquire it through their food chain, calling for better understanding of the ecology of these bugs.

The identification of other reservoirs and hosts that support active multiplication and shedding of *M. ulcerans* cells into the environment will be an important step in our understanding of the spread of the bacterium. *M. ulcerans* remains viable in experimentally-infected *Acanthamoeba polyphaga* (28) and its IS*2404* is detectable in amoeba cultures isolated from the environment (28,29), suggesting that amoebae are potential natural hosts for *M. ulcerans*. The exclusive localization and survival of *M. ulcerans* within the salivary glands of *Naucoris cimicoides* (14), detection of *M. ulcerans* DNA in saliva of *Diplonychus* sp. (30), saliva and tissue samples of *Appasus* sp. (36), as well as the successful cultivation of *M. ulcerans* from water striders [*Gerris* sp.] (15), are evidence that aquatic bugs support active multiplication of *M. ulcerans* and may shed them into the environment. The common ringtail (*Pseudocheirus peregrinus*) and brushtail (*Trichosurus vulpecula*) possums may also shed viable *M. ulcerans* into the environment, as they are implicated as reservoirs for *M. ulcerans* (9), having had *M. ulcerans* PCR positive faeces and developed laboratory-confirmed *M. ulcerans* skin lesions.

The discovery of a biofilm sample, from water body, with similar variable number of tandem repeat (VNTR) profile to a patient sample in BU endemic community (43) suggests that the victim might have been infected following exposure to the water body. Several other environmental samples have been reported to be sources of *M. ulcerans* infection in humans. However, the possibility of BU victims shedding viable *M. ulcerans* into the environment requires investigation. The routes of spread of *M. ulcerans* and how they contribute to infection and development of BU should be a research priority.

Transmission of *M. ulcerans*

The search for the exact mode(s) of transmission of *M. ulcerans* has been challenging since its discovery as the causative agent for BU in 1948 (6). Laboratory investigations suggest that contact of mammalian skin with *M. ulcerans* does not result in infection, as mouse tails coated in *M. ulcerans* (44) and introduction of *M. ulcerans* onto skin abrasions in guinea pig models (45) are not enough to cause BU. However, the introduction of *M. ulcerans* into skin greatly facilitates infection.

Multiple proposed modes of transmission of *M. ulcerans*, including insect bite and contamination of traumatic skin sites, are documented in literature. The idea that mosquitoes may be involved in the transmission of *M. ulcerans* is premised on the association of *M. ulcerans* or its DNA with several species of mosquitoes in nature (16,31,33) as well as the positive correlation between the proportion of *M. ulcerans*-positive mosquitoes and the number of BU cases (16,46-48). The larvae of several species of mosquito remain infected with *M. ulcerans* throughout larval development, although the infections are not carried over into the pupae or adult mosquitoes (46). This observation suggests that mosquitoes may not serve as biological vectors for *M. ulcerans*. However, mechanical transmission of *M. ulcerans* involving blood feeding *Aedes notoscriptus* has been proposed (44). It appears the size of the mosquito's penetrating appendage or structure contaminated by *M. ulcerans* is important, as relatively larger *A. notoscriptus* established BU in mice model unlike *A*. *aegypti* (44). Further study involving multiple mosquito bites at *M. ulcerans*-contaminated skin surfaces, and *M. ulcerans* infection doses in proposed vectors, is recommended.

Mechanical transmission of *M. ulcerans* by predatory aquatic bugs, through biting (17), has strongly been proposed. The experimental infection of aquatic bugs *(Naucoris cimicoides)* following feeding on grubs experimentally-infected with *M. ulcerans* and its transmission to mice through the bite of these insects (14) is documented as the first strong evidence implicating insects in the transmission of the bacte-

rium. Belostomatidae insects of the genera *Appasus* (36) and *Diplonychus* (30) as well as *Naucoris cimicoides* (14) support multiplication of viable *M. ulcerans* in their salivary glands. These aquatic bugs inflict painful bites on humans, creating opportunity for the introduction of *M. ulcerans* into the skin and facilitating infection.

Trauma may be essential for the introduction of *M. ulcerans* into the skin (49), since lesions often develop at sites of skin trauma. Interestingly, mechanical transmission of *M. ulcerans* involving anthropogenic or natural skin-puncturing microtrauma has been suggested (44). This proposed method of transmission complicates the search for definitive mode of transmission of *M. ulcerans*, as any *M. ulcerans*-contaminated material capable of causing minor injuries has the potential of transmitting it. It highlights the importance of avoiding exposure to skin-puncturing microtrauma and insect bites in BU endemic areas.

Understanding *M. ulcerans* spread: the way forward

One of the key challenges in the control of BU is the inadequate understanding of the spread of *M. ulcerans* in nature. The contamination of various environmental samples with *M. ulcerans* DNA complicates the spread and mode of transmission of the bacterium. Fundamental to understanding the mode of spread of *M. ulcerans* in nature is the identification of factors that drive the spread.

Organisms that support *in vivo* multiplication of *M. ulcerans* such as aquatic hemipterans of the genera *Appasus* (36), *Diplonychus* (30), *Naucoris* (14) and *Gerris* (15) may play important role in its spread. Therefore, epidemiological studies of *M. ulcerans* that incorporate trophic interactions of such organisms may be important future research direction.

Conclusion:

This review reveals that transmission of *M. ulcerans* requires the introduction of viable organism into the skin of its host. Aquatic bugs of the genera *Appasus* and *Diplonychus* as well as *Naucoris cimicoides* support multiplication of viable *M. ulcerans* in their salivary glands. The bite of insects, such as aquatic bugs, harboring or contaminated with viable *M. ulcerans* creates opportunity for infection following the introduction of the bacterium into host skin. Similarly, skin-puncturing materials found in nature that are contaminated by *M. ulcerans* can also cause infection when these materials cause traumatic injuries to the hosts.

Although the mode of transmission of *M. ulcerans* is less-definitive, appropriate protective measures may reduce the risk of exposure to the bacterium in BU-endemic areas. Future research on the epidemiology of *M. ulcerans* should incorporate trophic interactions of aquatic organisms known to support *in vivo* multiplication of the bacterium to improve our understanding of its spread in nature.

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Evaluation of the antimicrobial susceptibility testing process in clinical microbiology laboratories at Niamey, Niger

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Abstract:

Background: Risk assessment is the means of identifying and evaluating potential errors or problems that may occur in testing process. The aim of this study was to perform risk assessment of antimicrobial susceptibility testing (AST) process in clinical microbiology laboratories of Niamey, Niger Republic.
Methodology: We conducted a descriptive cross-sectional study from October 1 to December 31, 2019, to evaluate AST performance in seven clinical microbiology laboratories at Niamey, the capital city of Niger republic. The evaluation focused on the determination of the criticality index (CI) of each critical point (frequency of occurrence of anomalies, severity of the process anomaly, and detectability of the anomaly during the process) in the AST process and the performance of the AST through an observation sheet using two reference strains; *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213.
Results: The criticality index (CI) was greater than 6 for most of the critical points related to material, medium, equipment, method and labour for the AST process in all the laboratories. A range of 18-100% errors on the inhibition zone diameters of the reference strains were observed. Major and/or minor categorization (Sensitive S, Intermediate I and Resistance R) discrepancies were found at all the laboratories for either one or both reference strains. The antibiotics most affected by the S/I/R discrepancies were trimethoprim (100%), vancomycin (100%), amoxicillin (80%) and amoxicillin + clavulanic acid (70%).
Conclusion: This study showed a deficiency in the control of critical control points that impacts the performance of the AST reported by the laboratories in Niger. Corrective actions are needed to improve the performance of AST in clinical microbiology laboratories in Niger.

Keywords: Quality control; antibiogram; criticality index; clinical microbiology laboratories; Niger

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Evaluation du processus de réalisation de l'antibiogramme dans les laboratoires d'analyses de biologie médicale de la ville de Niamey, Niger

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Résumé:

Contexte: L'évaluation des risques est le moyen d'identifier et d'évaluer les erreurs ou les problèmes potentiels qui peuvent survenir dans le processus de test. L'objectif de cette étude était de réaliser une évaluation des risques du processus d'antibiogramme (ABG) dans les laboratoires de microbiologie clinique de Niamey, en République du Niger.
Méthodologie: Nous avons mené une étude transversale descriptive du 1er octobre au 31 décembre 2019 pour évaluer la performance des ABG dans sept laboratoires de microbiologie clinique à Niamey, capitale de la république du Niger. L'évaluation a porté sur la détermination de l'indice de criticité (IC) de chaque point critique (fréquence d'apparition des anomalies, gravité de l'anomalie du processus et détectabilité de l'anomalie au cours du processus) dans le processus et la performance des AGB à travers une fiche d'observation en utilisant deux souches de référence; *Escherichia coli* ATCC 25922 et *Staphylococcus aureus* ATCC 29213.
Résultats: L'indice de criticité était supérieur à 6 pour la plupart des points critiques liés au matériel, au milieu, à l'équipement, à la méthode et à la main-d'œuvre pour le processus AST dans tous les laboratoires. Une fourchette d'erreurs de 18 à 100% sur les diamètres des zones d'inhibition des souches de référence a été observée. Des écarts de catégorisation majeurs et/ou mineurs (Sensible: S, Intermédiaire: I et Résistance: R) ont été constatés dans tous les laboratoires pour l'une ou les deux souches de référence. Les antibiotiques les plus touchés par les écarts S/I/R étaient la triméthoprime (100%), la vancomycine (100%), l'amoxicilline (80%) et l'amoxicilline + acide clavulanique (70%).
Conclusion: Cette étude a montré une déficience dans le contrôle des points de contrôle critiques qui a un impact sur la performance de l'antibiogramme rapportée par les laboratoires au Niger. Des actions correctives sont nécessaires pour améliorer la performance des ABG dans les laboratoires de microbiologie clinique au Niger.

Mots clés: Contrôle de qualité, antibiogramme; indice de criticité; laboratoires de microbiologie clinique; Niger

Introduction:

Antibiotic susceptibility test (AST) is a common test in clinical microbiology laboratory. AST is a critical component of a quality control plan to ensure reliable patient results and management of bacterial infections. AST results guide clinicians to prescribe appropriate antibiotics for treatment by testing the effectiveness of antimicrobial drugs against specific organisms. Numerous methods are used to perform AST in clinical laboratory, but the Kirby-Bauer disk diffusion test has long been widely used for evaluating the susceptibility of specific organisms to antimicrobial drugs (1). Different actors are involved in the process of performing an AST using the Kirby Bauer method and each of them carries error risk that can directly affect the results. Risk assessment is the means of identifying and evaluating potential errors or problems that may occur in testing process. The fishbone diagram, also known as the Ishikawa diagram, identifies five components that must be evaluated in risk assessment that includes the specimen, the test system, the reagents, environment, and testing personnel (2).

Risk assessment can be used in AST as a brainstorming session that immediately sorts ideas into the potential source of error and risk factor. It can provide to clinical laboratory the basis for taking action to manage the negative consequences of unreliable AST. It is for this reason, that internal quality control (IQC) should be performed regularly with reference strains (3). In Niger, few clinical bacteriology laboratories control the quality of their AST process. The objective of this study was therefore to perform risk assessment of the AST process in clinical microbiology laboratories of Niamey, Niger.

Materials and method:

Study design

We conducted a descriptive cross-sectional study from October 1 to December 31, 2019 to evaluate the quality of the AST process in public and private clinical microbiology laboratories where bacteriological analysis and AST were performed at Niamey, the capital city of Niger republic. This design allowed the evaluation of the critical points and the process of carrying out AST in each selected laboratory.

Selection of laboratories

In this study, all laboratories in the city of Niamey that perform bacteriology analyses were eligible but the inclusion criterion was availability of bacteriology examinations particularly AST. A total of 7 eligible laboratories were selected; 3 from the public and 4 from the private sector.

Evaluation of the process and procedure of AST and determination of criticality index

The critical points and the measures taken to control the risks were analyzed according to the 5-M Model (Man, Medium, Machine, Management and Mission) method (4), and then checked onsite using a FMECA (Failure Mode, Effects and Criticality Analysis)

type observation sheet to determine the sources of error (3,5). The criticality index (CI) of each point was evaluated on a scale of three items; Frequency (F) of occurrence of anomalies, Severity (G) of the process anomaly, and Detectability (D) of the anomaly during the process. Each item was rated from 1 to 3 according to the 2017 adapted first edition of QUAMIC (2), which allowed the establishment of a criticality index (CI) from multiplication of the 3 items i. e. criticality index (CI) = F x G x D, and the threshold cut off was CI ≥ 6.

The evaluation of the performance of the AST process was an experimental verification onsite, according to the procedure for carrying out AST established by each laboratory using supplied reference strains. These AST processes were, among others, samples and reagents, temperature of conservation and that of the laboratory rooms, working materials, methods used, technical integrity of reagents and competence of the staff. The relevant parameter verified was the accuracy of the results. For this purpose, two reference strains (S1: *Escherichia coli* ATCC 25922 and S2: *Staphylococcus aureus* ATCC 29213) were made available to the laboratories on a blinded basis. Each laboratory (LBM1 – LBM7) performed AST on each of the reference strains after identification according to the procedure in use in the laboratory and under the same technical conditions as the clinical samples, but no clinical strains were involved in the study.

Results:

A total of 8 laboratories in the city of Niamey were selected but only 7 (3 public and 4 private) met the inclusion criteria while 1 was excluded due to suspension of AST in the laboratory during the study period.

Evaluation of critical control points in the AST process

Risks related to materials (samples and reagents)

The criticality index (CI) was ≥ 6 for the inoculum suspension at 6 of the 7 laboratories (LBM1, LBM2, LBM3, LBM4, LBM5 and LBM7). The CI was also ≥ 6 for the antibiotic disks at all the laboratories (LBM1 to LBM7). The CI score was 6 for the culture media at 6 of the 7 laboratories (LBM1, LBM2, LBM3, LB M5, LBM6 and LBM 7). However, the CI was < 6 for bacterial identification in all 7 laboratories (Fig 1)

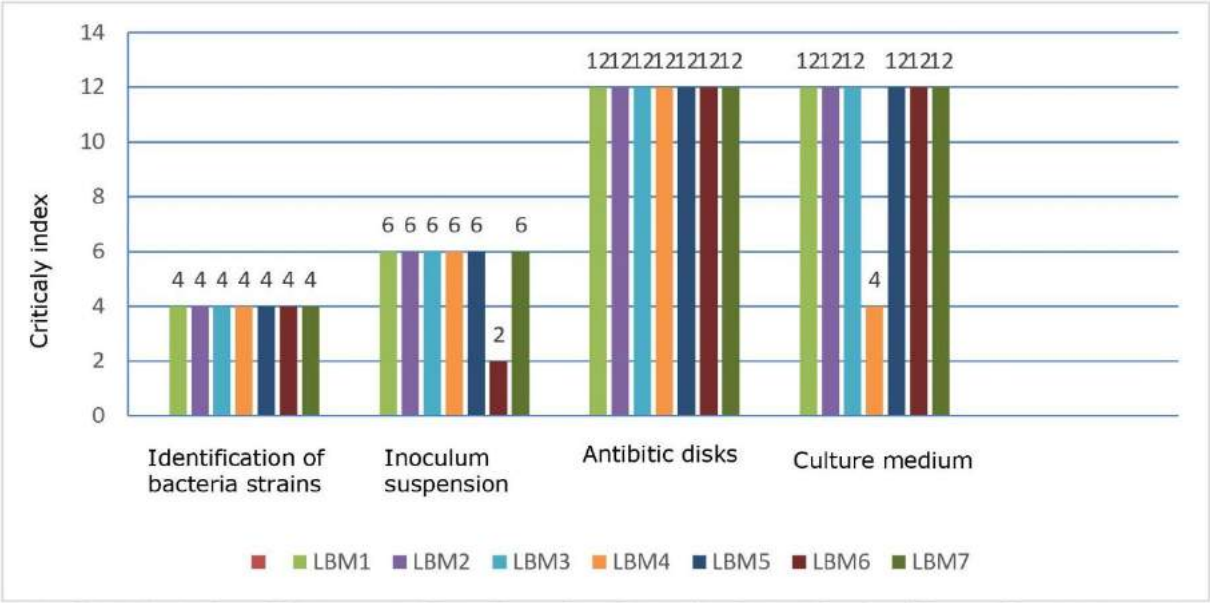


Fig 1: Criticality index of the material (sample and antibiotic discs) used by the different laboratories in the antibiotic susceptibility testing process

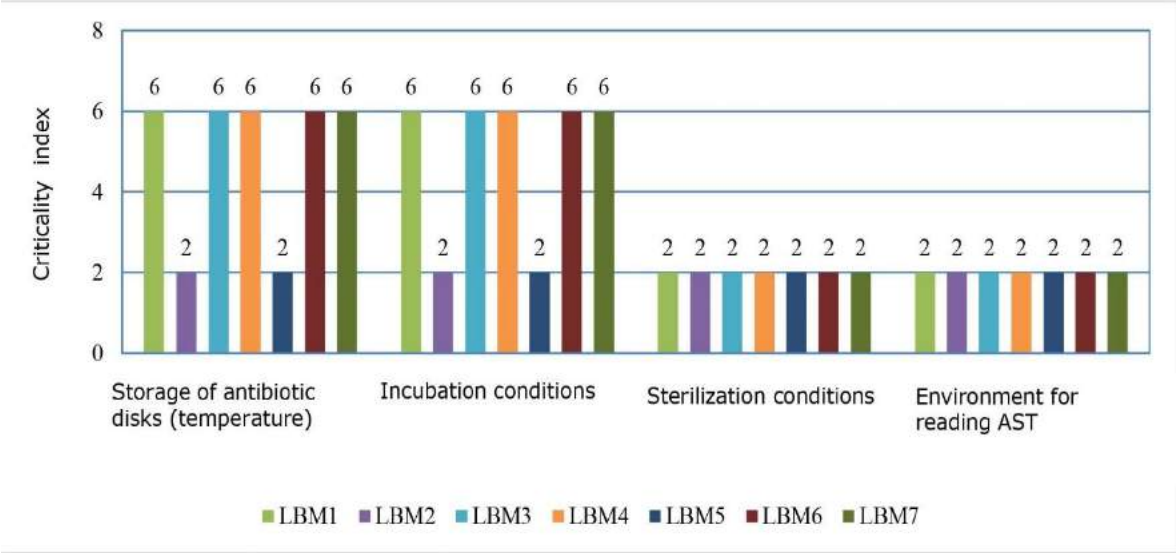


Fig 2: Criticality index of the environment (storage and room temperatures) used by the different laboratories in the process of performing the antibiogram

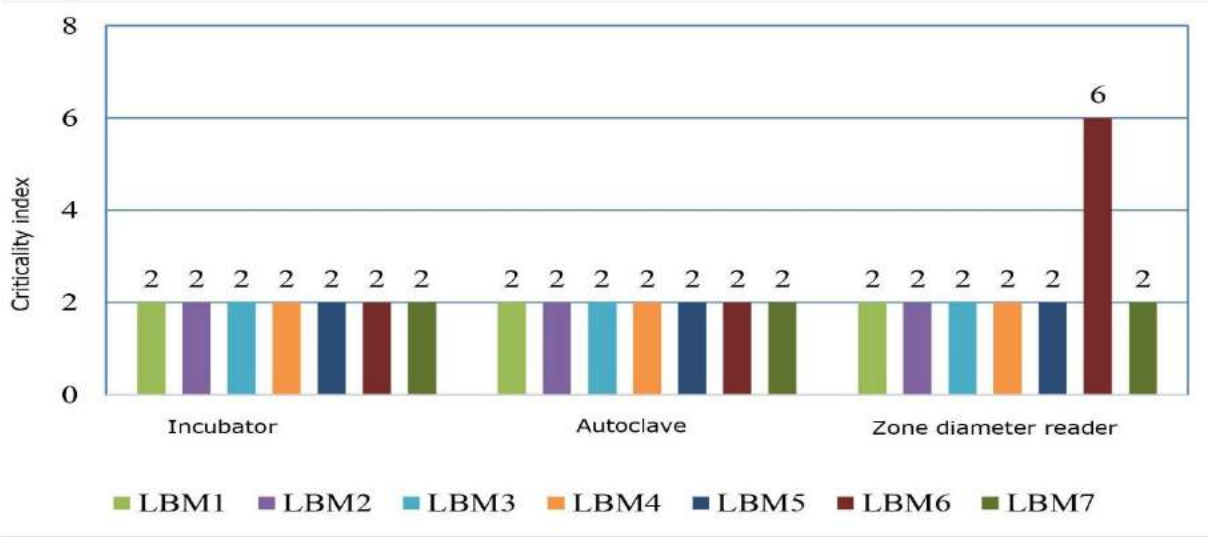


Fig 3: Criticality index of the equipment used by the different laboratories in the AST process

Risks related to media

The CI score was 6 for storage and incubation conditions at 5 of the 7 laboratories (LBM1, LBM3, LBM4, LBM6 and LBM7), while the CI score for sterilization and environmental condition for reading of AST was < 6 at all the 7 laboratories (Fig 2).

Risks related to AST materials

The CI score of 6 for AST reader was reported for only one laboratory (LBM6) while the CI score for incubator and autoclave was < 6 for all the laboratories (Fig 3)

Risks related to AST method and procedure

The CI score for AST procedure, quality control and reading of the inhibition diameters was 6 at all the laboratories (LBM1 to LBM7) while the CI score of AST result reporting was 6 for 6 of the 7 laboratories (LBM1, LBM3, LBM4, LBM5, LBM6 and LBM7) (Fig 4).

Risks related to the workforce

The CI score for the choice of antibiotic disks used by the laboratory staff was 9 (≥ 6) for 4 of the 7 laboratories (LBM1, LB M3, LBM5 and LBM6) while the CI score for staff qualification was <6 for all the laboratories (Fig 5).

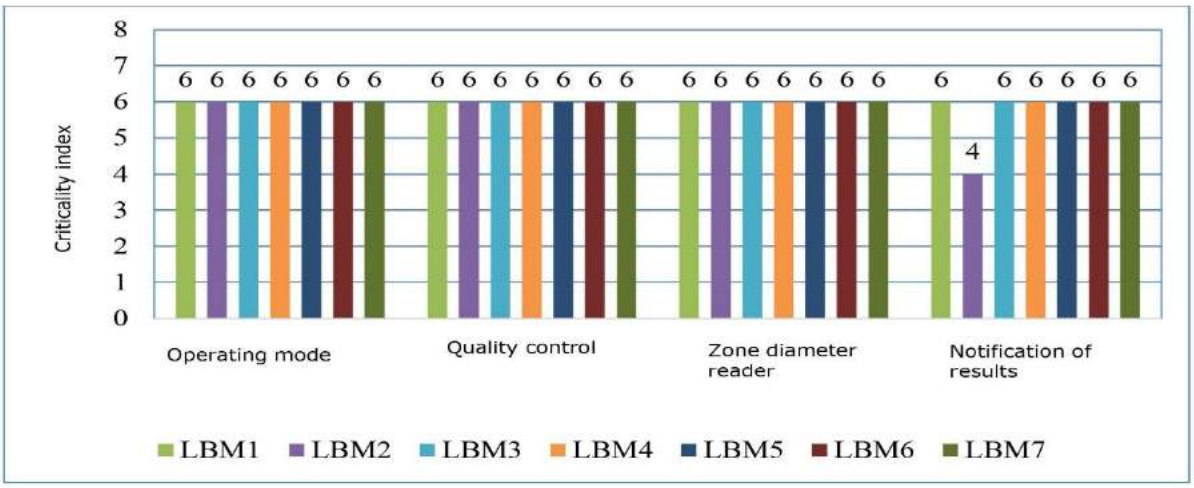


Fig 4: Criticality index of the method used by the different laboratories in the AST process

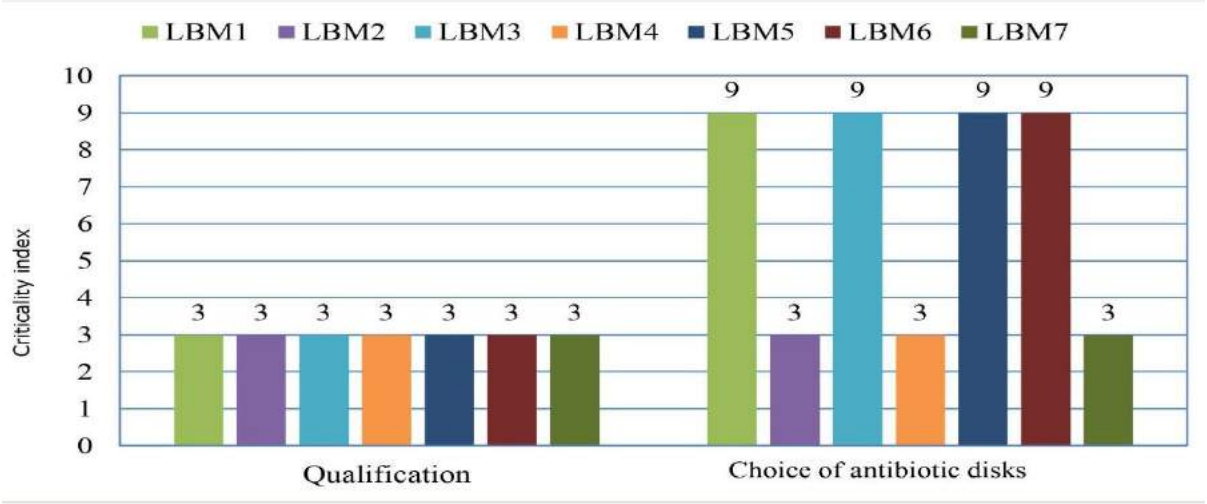


Fig 5: Criticality index of the manpower used by the different laboratories in the antibiogram process.

Evaluation of AST performance

Essential matches (EC) and measurement errors of diameters of inhibition by the laboratories for reference strains S1 and S2

The essential measurement agreement of the diameters of the inhibition zones for *E. coli* (S1) and *S. aureus* (S2) strains was 100% for LBM3. The frequencies of inhibition zone diameter measurement errors in excess or by default on the reference strain of *E. coli* (S1) were 52%, 50%, 42%, 40%, 32% and 28% for LBM2, LBM4, LBM5, LBM6, LBM4, LBM7, and LBM1, respectively. For the reference *S. aureus* (S2) strain, the frequencies of over- or under-inhibition zone diameter measurement errors were 100%, 40%, 32%, 29%, 29%, and 20% for LBM4, LBM6, LBM1, LBM2, LBM5, and LBM7, respectively (Fig 6).

Types of errors in inhibition zone diameters reported by laboratories for reference strains S1 and S2

On strain S1, all (100%) of the inhibition zone diameter errors encountered at LBM1, LBM4, LBM5 and LBM7 were default errors. Similarly, errors made by the laboratories LBM2 and LBM7 on the diameters of the inhibition zones of the S2 strain were errors by excess. On strain S2, all (100%) of the inhibition zone diameter errors encountered at LBM2 and LBM7 were excess errors (Fig 7).

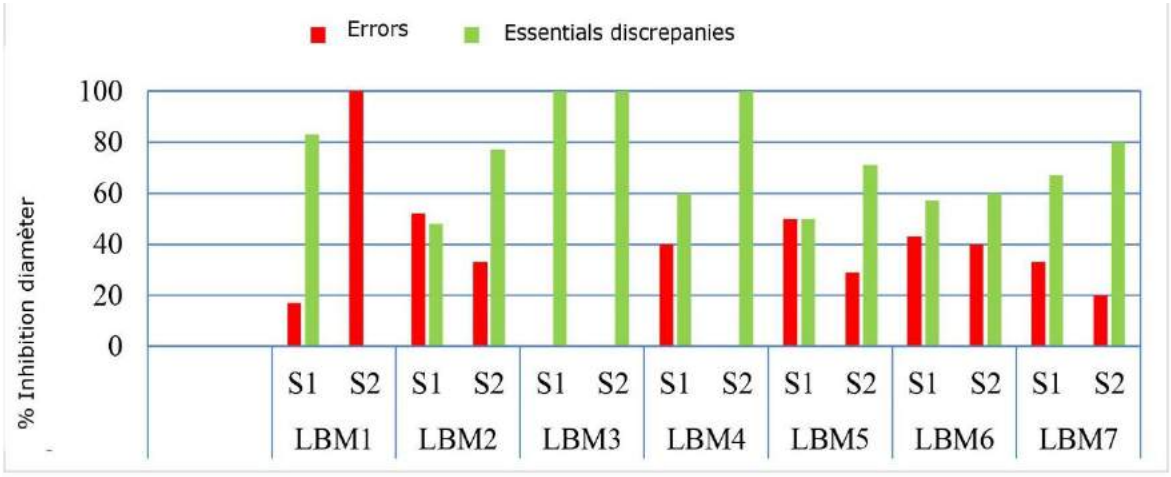


Fig 6: Distribution of essential concordance (EC) and inhibition zone diameter errors by laboratory and reference strains

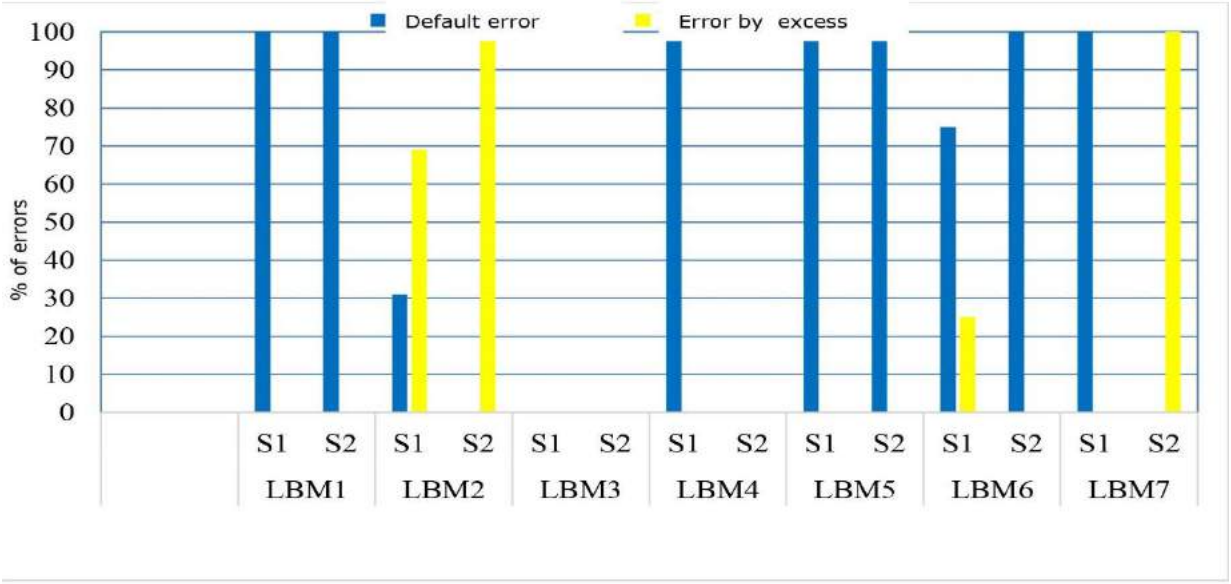


Fig 7: Distribution of the types of errors made on the diameters of the inhibition zones according to the laboratories and the reference strains

S/I/R discrepancies and categorical S/I/R matches reported by the laboratories

The S/I/R categorical discrepancies observed for the reference strain of *E. coli* (S1), were less than 20% except for LBM5, LBM6, and LBM7 where they were 50%, 29%, and 33%, respectively. On the reference *S. aureus* strain (S2), the highest S/I/R category discrepancies were found at LBM1 (37%) and LBM6 (50%) (Fig 8).

S/I/R categorization discrepancies reported by the laboratories

All (100%) the categorization discrepancies reported by LBM2, LBM3 and LBM7 were major discrepancies while those reported by LBM1 and LBM4 were minor discrepancies (Fig 9).

Antibiotic discs with S/I/R categorization discrepancy reported by laboratories for reference strains S1 and S2

The antibiotics most affected by the S/I/R misclassification were amoxicillin (80 %), amoxicillin + clavulanic acid (70%), trimethoprim (100%) and vancomycin (100%) (Fig 10).

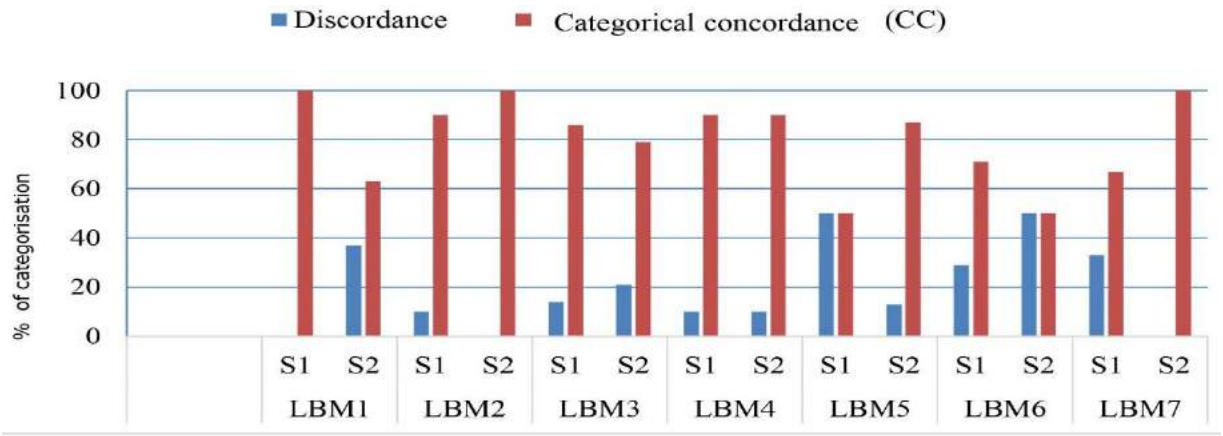


Fig 8: Distribution of S/I/R categorical mismatches and matches by laboratory and reference strain

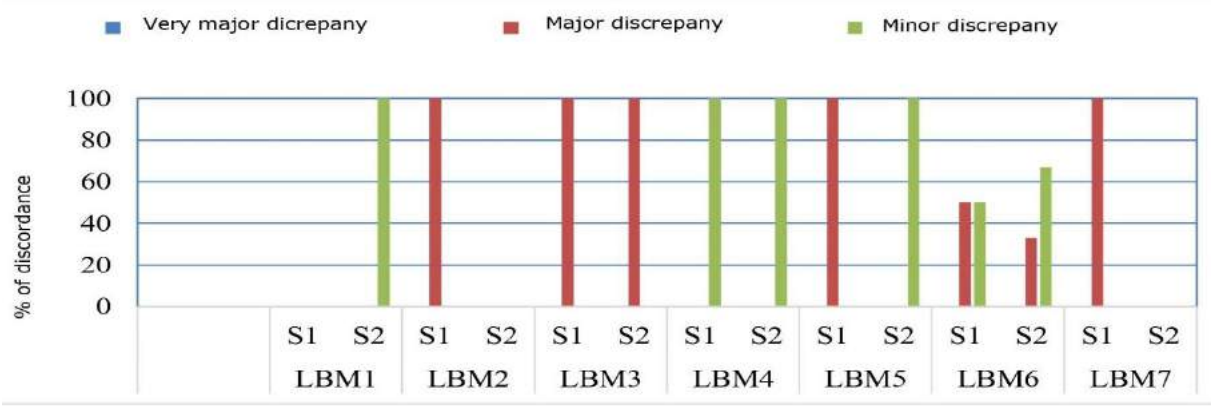
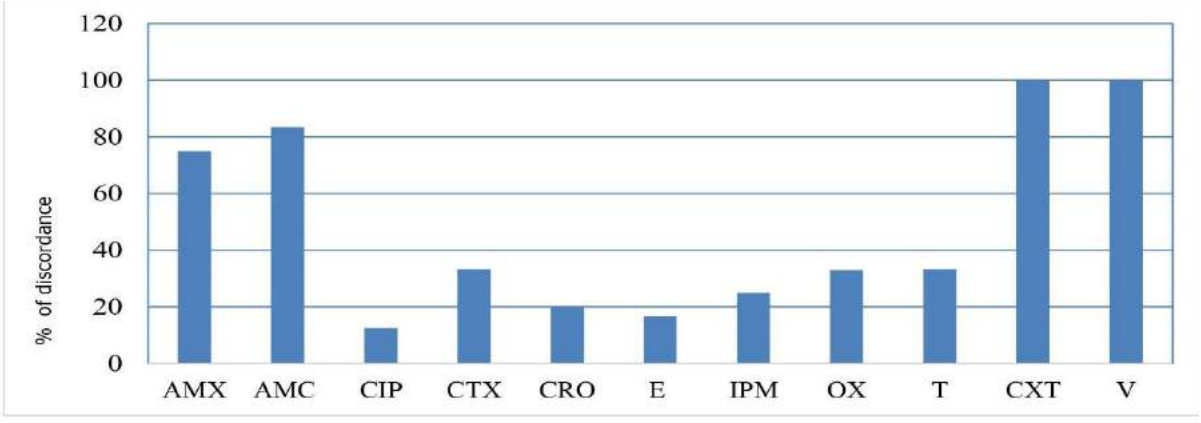


Fig 9: Distribution of S/I/R categorization mismatches by laboratory and reference strains



AMX = Amoxicillin; CIP = Ciprofloxacin; CRO = Ceftriaxone; IPM = Imipenem; AMC = Amoxicillin + clavulanic acid; CTX = Cefotaxime; E = Erythromycin; OX = Oxacillin; T = Tobramycin; CXT = Trimethoprim + sulfamethoxazole; V = Vancomycin

Fig 10: Distribution of S/I/R categorization mismatches by antibiotic disk

Discussion:

At the material level (sample and reagents) evaluation, inoculum was not standardized in most laboratories because they did not have a standardized method of inoculum preparation. This was similarly reported by Salou et al., (6) in 2016 in Lomé laboratories in Togo. According to Weber (7), "*poorly controlled inoculum is a common error especially if a densitometer is not used for calibration*". Non-standardization of the inoculum could have a very important impact on the final result of the antibiotic susceptibility test, as heavy inoculum leads to smaller zone diameters and vice versa (8). Thabaut et al., (9) demonstrated that poor standardization of the inoculum resulted in a large variability in the diameters of the inhibition zones for the same bacterial strain.

Moreover, according to the work of Weber et al., (7) in France excessively heavy inoculum gave results that affected all antibiotics, particularly beta-lactams and glycopeptides, giving falsely intermediate or resistant results. In this study, only one of the seven laboratories controlled the agar thickness according to the EUCAST recommendations (4 mm ±0.5 mm) (8). The lack of agar thickness control could lead to inhibition zone diameters outside the performance limits (10).

At the environmental level (storage and room temperatures) in this study, 5 of 7 laboratories did not comply with the conditions for storage of disks and culture media and incubation of culture media for AST. These risks were mainly due to the fact that these laboratories did not have mapping or metrological records of the cold chambers or oven. Discharge of the disks leads to reduced inhibition zones and is a common source of errors (8)**.** On hardware evaluation, the risk of error factors related to the materials used in the process of performing the AST i. e. procedures for using ovens, autoclaves and inhibition diameter readers, were relatively well controlled by the laboratories. Nevertheless, we found that one of the laboratories (LBM6) had a very high CI compared to its inhibition zone diameter reader. A survey carried out in this laboratory showed that it did not use any instrument to read the inhibition zone diameters, but only read this with the naked eye. This result confirmed the one reported by Védy et al., (11) in France, which showed that visual reading of AST was subject to variation depending on the eye of the operator.

With regard to evaluation of the performance of AST, all the laboratories had high criticality indexes. This could be due to the lack of standard operating procedures (SOPs) for performing AST, lack of quality control, and absence of double reading by technicians/biologists. In this respect, our results corroborated those of Védy et al., (11) in France, which reported that AST procedure was a risk factor for error and AST results were likely to fluctuate between different technicians on workstation if uniform AST procedures were not applied by all staff.

At the level of the workforce, we discovered that all the laboratories used skilled workforce, but the risk of error was mainly due to the choice of antibiotic disks used by the technicians, which did not correspond to the disks recommended specifically for the performance of AST. Our results justify the use of a single reference system across the country in order to harmonize practices.

Considering accuracy of the results of AST on the reference strains, most of the laboratories reported inhibition zone diameters beyond acceptable reference limits for one or both strains. This could be attributed to the fact that no risk factor for error was fully controlled by the laboratories. The LBM3 laboratory that did not report zone diameters out of bounds for either of the two strains also did not have full control of the risk factors. The results from this laboratory were due to the wrong choice of antibiotics tested on the different strains. The errors made by LBM1, LBM4, LBM5 and LBM7 on the diameters of the inhibition zones for the reference (wild) *E. coli* ATCC 25922, which is a susceptible strain, were all default errors. This type of error could be due to too thick agar, too heavy inoculum, or insufficient loading of the antibiotic disks. Insufficient disk loading could be due to manufacturing error and storage conditions such as temperature or exposure to light or humidity (11). Furthermore, when the inoculated culture boxes are left at laboratory temperature too long before the antibiotic disks are deposited, the bacteria could start to grow, leading to a false decrease in the size of the inhibition zones (7).

The errors made by LBM2 and LBM7 on the inhibition zone diameters of the low beta-lactamase producing *S. aureus* reference strain ATCC 29213 were all oversized errors. An excess error would be much more problematic than a default error, as it predisposes to a higher risk of treatment failure (11). According to the 2018 EUCAST guideline/standard (8), culture boxes should ideally be incubated within 15 minutes after disk deposition, but no longer than 30 minutes. If they are left at room temperature after disk deposition, the pre-diffusion of antibiotics would result in falsely enlarged inhibition zones. The errors on the inhibition zone diameters of the reference strains made evident the misclassification inconsistencies that we found in the results from all the laboratories. All categorization discrepancies reported by LBM2, LBM3 and LBM7 were major discrepancies, i. e. reported as resistant (R) when the strain was susceptible (S). This type of discrepancy in result can lead to selection of inappropriate antimicrobials for effective treat ment from which the patient could have benefited, but this will not expose the patient to the risk of therapeutic failure. These discrepancies were due to default errors, the sources of which could be too thick agar, excess inoculum, or insufficient loading of the antibiotic disks.

The antibiotic disks most affected by the discordances were; amoxicillin, amoxicillin + clavulanic acid, trimethoprim, and vancomycin, as well as cefotaxime, ceftriaxone, imipenem and oxacillin. This result showed that the affected antibiotic disks were predominantly beta-lactam antibiotics. Accor-

ding to Adam et al., (12), amoxicillin, cefalotin, amoxicillin + clavulanic acid, and ticarcillin + clavulanic acid were "fragile" antibiotic molecules and had to be stored permanently at 4°C in a desiccant container. Similarly, Weber (7) showed that some disks impregnated for AST were particularly sensitive to humidity. In an open cartridge stored at +4°C, even in a dispenser containing a desiccant, the antibiotic load loss of a disk was very rapid. For this reason, they recommended that an open cartridge should not be stored for more than 5 days in the refrigerator and that cartridges of particularly sensitive antibiotics, such as beta-lactams should be stored at -20°C.

In this study, we noted default errors in ciprofloxacin, which were similar to those reported by King and Brown (10) in their study on quality assurance of disk diffusion sensitivity tests. Fluoroquinolones are known to be inactivated by prolonged exposure to light. From our study, we showed that the discrepancies presented by antibiotic disks were mostly major discrepancies and ranged from 75 to 100% for amoxicillin, amoxicillin + clavulanic acid, trimethoprim, vancomycin, cefotaxime, imipenem and oxacillin. These findings are similar to those reported by Dougnon et al., (3) in Benin in 2016.

This study has some limitations. The reference strains used by the laboratories in our study received special treatments with respect to AST performance, different from those routinely performed in the standard practice. There was also the possibility of bias in the comparison of results from different laboratories, because the nature and number of antibiotic disks tested per strain differed between the laboratories. However, the findings met the set objectives of our study.

Conclusion:

The findings of our study showed that the criticality index was high (≥ 6) for most of the critical points related to material, medium, equipment, method, and workmanship at the level of the laboratories assessed. Major and/or minor categorization discrepancies occurred at the level of all laboratories for both reference strains used. Corrective actions are required by these laboratories to improve their performance.

Evaluation of the quality of AST practices should be formalized, carried out periodically and extend to all laboratories in Niger. This could be done within the framework of surveillance of antibacterial resistance, with standardization of the performance of AST at the national level.

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Original Article **Open Access**

Knowledge, attitude and practice of infection prevention and control among healthcare workers: one year after an outbreak of nosocomial Lassa fever in a tertiary hospital in southeast Nigeria

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Abstract:

Background: With the rise in cases of Lassa fever in recent times in West Africa, the healthcare setting continues to pose significant risk especially among healthcare workers (HCWs) for diseases like Lassa fever that are transmitted via contact with blood and other body fluids. We therefore assessed the knowledge, attitude and practice (KAP) of infection prevention and control (IPC) one year after an outbreak of nosocomial transmission of Lassa fever in the study hospital.
Methodology: A cross-sectional study of HCWs was conducted in Alex Ekwueme Federal University Teaching Hospital, Abakaliki, Ebonyi State, a tertiary hospital designated for Lassa fever treatment in southeast Nigeria. A total of 631 HCWs selected by systematic random sampling were surveyed using self-administered questionnaire to determine the KAP of IPC. Data analysis was done with EPI INFO version 7.2 and Microsoft Excel 2016, and Chi square statistic was used to examine relationship between variables at 5% level of significance.
Results: Only 287 (51.1%) of the 562 respondent HCWs had good knowledge of IPC, 442 (78.6%) had good attitude towards IPC, and 268 (47.7%) had good practice of IPC. Socio-demographic predictors of IPC knowledge included being a medical laboratory scientist (AOR=0.5; 95% CI=0.29-0.83; p=0.009), tertiary education level (AOR=7.0; 95% CI=1.11-44.60; p=0.038), and work experience of ≥ 7 years (AOR=2.3; 95% CI=1.47-3.57; p<0.001). Male gender (AOR=1.9; 95% CI=1.06-3.42; p=0.031), nurse professional (AOR=6.5; 95% CI=2.67-15.81; p<0.001) and work experience of ≥ 7 years (AOR=2.5; 95% CI=1.37-4.54; p=0.003) were predictors of good attitude towards IPC. Also, nurse professional (AOR=3.1; 95% CI=1.79-5.20; p<0.001) and married status (AOR=1.6; 95% CI=1.05-2.55; p=0.028) were predictors of good practice of IPC among the respondents.
Conclusions: The study demonstrated that knowledge and practice of IPC was low in the study location despite the interventions that had been instituted to improve the IPC framework. Therefore, there is need to adapt approaches that will influence behavior change towards IPC in the course of the in-service trainings being conducted in the hospital.

Keywords: Infection prevention, Lassa fever, Nosocomial, KAP, Healthcare workers

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Connaissances, attitude et pratique de la prévention et du contrôle des infections chez les agents de santé: un an après une épidémie de fièvre de Lassa nosocomiale dans un hôpital tertiaire du sud-est du Nigeria

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Abstrait:

Contexte: Avec l'augmentation récente des cas de fièvre de Lassa en Afrique de l'Ouest, le milieu de la santé continue de présenter un risque important, en particulier chez les travailleurs de la santé (TS) pour des maladies comme la fièvre de Lassa qui se transmettent par contact avec le sang et d'autres fluides corporels. Nous avons donc évalué les connaissances, l'attitude et la pratique (CAP) de la prévention et du contrôle des infections (PCI) un an après une épidémie de transmission nosocomiale de la fièvre de Lassa dans l'hôpital de l'étude.
Méthodologie: Une étude transversale des travailleurs de la santé a été menée à l'hôpital universitaire fédéral Alex Ekwueme, à Abakaliki, dans l'État d'Ebonyi, un hôpital tertiaire désigné pour le traitement de la fièvre de Lassa dans le sud-est du Nigéria. Un total de 631 TS sélectionnés par échantillonnage aléatoire systématique ont été interrogés à l'aide d'un questionnaire auto-administré pour déterminer le CAP de la CIP. L'analyse des données a été effectuée avec EPI INFO version 7.2 et Microsoft Excel 2016, et la statistique du Chi carré a été utilisée pour examiner la relation entre les variables à un niveau de signification de 5 %.
Résultats: Seuls 287 (51,1%) des 562 TS interrogés avaient une bonne connaissance de la CIP, 442 (78,6%) avaient une bonne attitude envers la CIP et 268 (47,7%) avaient une bonne pratique de la CIP. Les prédicteurs sociodémographiques des connaissances en CIP comprenaient le fait d'être un scientifique de laboratoire médical (AOR=0,5; IC à 95%=0,29-0,83; p=0,009), le niveau d'études supérieures (AOR=7,0; IC à 95% =1,11-44,60; p=0,038) et une expérience de travail ≥ 7 ans (AOR=2,3; IC à 95%=1,47-3,57; p<0,001). Sexe masculin (AOR=1,9; IC à 95%=1,06-3,42; p=0,031), infirmier professionnel (AOR=6,5; IC à 95%=2,67-15,81; p<0,001) et expérience professionnelle de ≥ 7 ans (AOR=2,5; IC à 95%=1,37-4,54; p=0,003) étaient des prédicteurs d'une bonne attitude envers la CIP. De plus, l'infirmière professionnelle (AOR=3,1; à 95% IC=1,79-5,20; p<0,001) et le statut de personne mariée (AOR=1,6; à 95% IC=1,05-2,55; p=0,028) étaient des prédicteurs de bonne pratique de la CIP parmi les répondants.
Conclusions: L'étude a démontré que les connaissances et la pratique de la CIP étaient faibles dans le lieu de l'étude malgré les interventions qui avaient été instituées pour améliorer le cadre de la CIP. Par conséquent, il est nécessaire d'adapter les approches qui influenceront le changement de comportement envers la CIP au cours des formations en cours d'emploi menées à l'hôpital.

Mots clés: Prévention des infections, Fièvre de Lassa, Nosocomiale, CAP, Personnel de santé

Introduction:

In West Africa, Lassa fever is one of the infectious diseases with a rise in cases in recent times (1). Within the West African region, an estimated 300000-500000 cases of the disease occur with associated annual deaths of about 5000 (2). Nigeria contributed the highest burden of the disease with upsurge in the number of cases in the past few years (3). The healthcare setting poses significant risk for diseases such as Lassa fever that are transmitted via contact with blood and other body fluids. Healthcare workers (HCWs) are at high risk of transmission of infectious diseases in the course of their work (4). This may be worse in resource-constrained settings due to deficiencies in human resources for health as well as poor infection prevention and control (IPC) (5).

Ebonyi is one of the high-burdened States for Lassa fever in Nigeria. The State has been recording outbreak of Lassa fever since 2005 (6), with several events of nosocomial spread of the infection among HCWs in the State (6–8). Following the outbreak of Lassa fever in 2018, an assessment was done in the study location by the National Rapid Response Team deployed for the outbreak in the State, and the assessment revealed that there were gaps in the practice and infrastructure for IPC in the hospital (9). Recommendations were also made by the Team which included raising awareness of IPC among the HCWs and provision of necessary materials for IPC (9). Some interventions had been instituted to improve the IPC framework in the State and the study location.

IPC is a scientific approach and practical solution designed to prevent harm caused by infection to patients and HCWs especially in hospital setting (10). It is vital to HCWs and patients at every single healthcare encounter. The aim of this study therefore is to assess the situation of infection control one year after an outbreak of nosocomial Lassa fever in the study hospital.

Materials and method:

Study design and setting

We conducted a cross-sectional study among HCWs in Alex Ekwueme Federal University Teaching Hospital designated for Lassa fever treatment in southeast Nigeria. It is a 720-bed hospital located in Abakaliki, Ebonyi State (11). The hospital has over 4,000 staff comprising core and non-core HCWs. Doctors, nurses, medical laboratory scientists and the cleaning staff were recruited for the study due to their frontline roles requiring strict infection control.

Sample size and sampling technique

We calculated the sample size of 631 using the Cochran formula for cross-sectional studies based on a prevalence of 70% of HCWs with good knowledge of IPC from a similar study in Nigeria (12), 4% precision and 20% non-response rate. Using a systematic

sampling technique, we selected the 631 respondents who met our selection criteria from the different groups of HCWs in the hospital, comprising 855 doctors, 1150 nurses, 362 medical laboratory scientists and 300 cleaning staff. The sample size was allocated to each group of HCWs proportionately. The list of workers in each group was drawn, and the sampling interval was estimated for each group and used to systematically sample the required number.

Study instrument and data collection

A self-administered questionnaire was used to collect information from each respondent. The questionnaire was adapted from previous studies (9,12), and consisted of 4 sections. Section A obtained data on the socio-demographic characteristics of the respondents. Section B obtained data on knowledge of IPC among respondents and questions were on knowledge of most effective method of controlling hospital-acquired infections (HAIs), modes of transmission of HAIs, and the WHO 5 moments of hand hygiene. Section C obtained data on the attitude of respondents towards IPC and the questions were on attitude towards handling of body fluids, hand washing, adherence to IPC policies, IPC training, and recapping of needles. Section D obtained data on practice of IPC among respondents and the questions were on the practice of hand washing and the use of hand gloves.

Data management

Data were cleaned by checking for consistency and completeness. Analysis was done with EPI INFO version 7.2 and Microsoft Excel 2016. Knowledge of IPC was tested using 12 questions, while 8 and 4 questions were used to assess attitude towards IPC and practice of IPC respectively. Each correct question attracted a score of one and a wrong answer was scored zero. Respondents were classified as having good knowledge if they scored ≥75% of the total scores, fair if scores were 50-74% and poor if <50% (9).

We examined the relationship between good knowledge, attitude and practice of IPC and socio-demographic characteristics using Chi square statistics. The scores were dichotomized (fair and poor were merged together as poor) before the bivariate analysis. All variables with *p*-value less than 0.2 in the bivariate analysis were selected and modeled into the multivariate logistic regression to determine the predictors of good knowledge, attitude and practice of IPC at 5% level of significance. The crude odds ratio (COR) and adjusted odds ratio (AOR) were reported.

Ethical consideration

Ethical approval was obtained from the Research and Ethics Committee of Alex Ekwueme Federal University Teaching Hospital. Respondents were informed of their voluntariness to participate in the study, and confidentiality and anonymity of data collected were maintained by avoiding inclusion of possible identifiers, such as names and contact details.

Results:

Of the 631 HCWs selected for this study, 562 HCWs aged between 22 years and 70 years responded, representing a response rate of 89.1%. The mean age of the respondents was 37.4±8.1 years with higher proportion of the respondents (361, 64.2%) being < 40 years of age. There were more females (350, 62.3%), 327 (58.2%) had < 7 years work experience, and 447 (79.5%) had previous training on IPC. Doctors and nurses constituted 67.2% of the respondents (Table 1).

Among the respondents, 287 (51.1%) had good knowledge of IPC, 442 (78.6%) had good attitude towards IPC, and 268 (47.7%) had good practice of IPC. Sociodemographic predictors of IPC knowledge were being a medical laboratory scientist (AOR=0.5; 95% CI=0.29-0.83; p=0.009), tertiary education level (AOR=7.0; 95% CI = 1.11-44.60; p= 0.038), and work experience of ≥ 7 years (AOR=2.3; 95% CI = 1.47-3.57; p<0.001), while gender, age group, nurse professional, orderly/cleaner, marital status, and previous training in IPC were not significantly associated with knowledge of IPC (Table 2).

In relation to respondents' attitude to IPC, male gender (AOR=1.9; 95% CI=1.06-3.42; p=0.031), nurse professional (AOR= 6.5; 95% CI=2.67-15.81; p<0.001) and work experience of ≥ 7 years (AOR=2.5; 95% CI=1.37-4.54; p=0.003) were predictors of good attitude towards IPC, while age group, medical laboratory scientist, orderly/cleaner, marital status, educational level, and previous training in IPC were not significantly associated with attitude towards IPC (Table 3).

Assessment of respondents' practice of IPC identified nurse professional (AOR=3.1; 95% CI=1.79-5.20; p<0.001) and married status (AOR=1.6; 95% CI=1.05-2.55; p= 0.028) as predictors of good practice of IPC, while gender, age group, medical laboratory scientist, orderly/cleaner, educational level, work experience, and previous training in IPC were not significantly associated with practice of IPC (Table 4).

Table 1: Socio-demographic characteristics of selected healthcare workers in Alex Ekwueme Federal University Teaching Hospital, Abakaliki, Nigeria, 2019

Socio-demographic characteristics	Frequency (%)
Gender	
Female	350 (62.3)
Male	212 (37.7)
Age group	
<40 years	361 (64.2)
≥40 years	201 (35.8)
Profession	
Doctor	189 (33.6)
Nurse	189 (33.6)
Medical Laboratory Scientist	92 (16.4)
Orderly/Cleaner	92 (16.4)
Religion	
Christianity	556 (98.9)
Islam	4 (0.7)
Traditional Religion	1 (0.2)
Others	1 (0.2)
Marital Status	
Single	126 (22.4)
Married	423 (75.3)
Separated/Divorced	13 (2.3)
Level of Education	
No Education	1 (0.2)
Primary Education	6 (1.1)
Secondary Education	80 (14.2)
Tertiary education	475 (84.5)
Work Experience	
<7 years	327 (58.2)
≥7 years	235 (41.8)
Previous Training on IPC	
Yes	447 (79.5)
No	115 (20.5

Table 2: Knowledge of infection prevention and control among selected healthcare workers in Alex Ekwueme Federal University Teaching Hospital, Abakaliki, Nigeria, 2019

Variable	Knowledge		COR (95% CI)	*p*-value	AOR (95% CI)	*p*-value
	Good	Poor				
Gender						
Female	185 (52.9)	165 (47.1)	1.2 (0.86-1.70)	0.276		
Male	102 (48.1)	110 (51.9)	1			
Age						
≥40 years	127 (63.2)	74 (36.8)	2.2 (1.51-3.07)	<0.001	1.3 (0.80-1.98)	0.329
<40 years	160 (44.3)	201 (55.7)	1		1	
Profession						
Nurses	136 (72.0)	53 (28.0)	2.0 (1.31-3.08)	0.001	1.4 (0.89-2.25)	0.144
Medical Laboratory scientist	40 (43.5)	52 (56.5)	0.6 (0.36-0.99)	0.048	0.5 (0.29-0.83)	0.009*
Orderly/Cleaner	5 (5.4)	87 (94.6)	0.1 (0.02-0.12)	<0.001	0.2 (0.04-1.10)	0.064
Doctors	106 (56.1)	83 (43.9)	1		1	
Marital Status						
Married	234 (55.3)	189 (44.7)	2.0 (1.36-2.97)	<0.001	1.1 (0.66-1.71)	0.803
Others	53 (38.1)	86 (61.9)	1		1	
Level of Education						
Tertiary Education	283 (59.6)	192 (40.4)	30.6 (11.03-84.81)	<0.001	7.0 (1.11-44.60)	0.038*
Others	4 (4.6)	83 (95.4)	1		1	
Work Experience						
≥7 years	153 (65.1)	82 (34.9)	2.7 (1.90-3.80)	<0.001	2.3 (1.47-3.57)	<0.001*
<7 years	134 (41.0)	193 (59.0)	1		1	
Previous Training on IPC						
Yes	226 (50.6)	221 (49.4)	0.9 (0.60-1.37)	0.635		
No	61 (53.0)	54 (47.0)	1			

*Statistically significant; COR=Crude Odds Ratio; AOR = Adjusted Odds Ratio; CI = Confidence Interval

Table 3: Attitude towards infection prevention and control among selected healthcare workers in Alex Ekwueme Federal University Teaching Hospital, Abakaliki, Nigeria, 2019

Variable	Attitude		COR (95% CI)	*p*-value	AOR (95% CI)	*p*-value
	Good	Poor				
Gender						
Male	175 (82.6)	37 (17.4)	1.5 (0.96-2.26)	0.079	1.9 (1.06-3.42)	0.031*
Female	267 (76.3)	83 (23.7)	1		1	
Age						
≥40 years	174 (86.6)	27 (13.4)	2.2 (1.40-3.58)	0.001	1.0 (0.52-1.77)	0.891
<40 years	268 (74.2)	93 (25.8)	1		1	
Profession						
Nurses	181 (95.8)	8 (4.2)	5.9 (2.67-12.97)	<0.001	6.5 (2.67-15.81)	<0.001*
Medical Laboratory Scientist	71 (77.2)	21 (22.8)	0.9 (0.48-1.60)	0.674	0.9 (0.47-1.72)	0.741
Orderly/Cleaner	40 (43.5)	52 (56.5)	0.2 (0.12-0.34)	<0.001	1.1 (0.24-5.06)	0.911
Doctors	150 (79.4)	39 (20.6)	1		1	
Marital Status						
Married	347 (82.0)	76 (18.0)	2.1 (1.37-3.27)	0.001	1.2 (0.69-1.97)	0.561
Others	95 (68.4)	44 (31.6)	1		1	
Level of Education						
Tertiary education	406 (85.5)	69 (14.5)	8.3 (5.07-13.70)	<0.001	4.4 (0.97-19.57)	0.055
Others	36 (41.4)	51 (58.6)	1		1	
Work Experience						
≥7 years	208 (88.5)	27 (11.5)	3.1 (1.92-4.89)	<0.001	2.5 (1.37-4.54)	0.003*
<7 years	234 (71.6)	93 (28.4)	1		1	
Previous Training on IPC						
Yes	348 (77.9)	99 (22.1)	0.8 (0.47-1.33)	0.364		
No	94 (81.7)	21 (18.3)	1			

*Statistically significant; COR=Crude Odds Ratio; AOR = Adjusted Odds Ratio; Confidence Interval

Discussion:

This study was an assessment of the state of IPC, one year after an outbreak of nosocomial Lassa fever among HCWs in the study location. Routine assessment of health systems is key to improving healthcare services delivery through identifying strengths and weaknesses and working towards fixing identified weaknesses. In our study location, there was an initial assessment of IPC which identified gaps during the nosocomial outbreak in 2018 (8,9). Several interventions had been carried out in the hospital to improve IPC after the initial assessment, and the hospital management in conjunction with her partners had organized series of training on IPC among its health workforce. The partners had supported the hospital in improving its IPC infrastructures.

Despite the series of training that had been conducted in the preceding year prior to this study, only about half (51.1%) of the respondents had good knowledge of IPC. Findings from the initial assessment in 2018 (9) had recorded a higher proportion (71.9%) of hospital staff with good knowledge of IPC (9) however, the sample size used in this initial assessment was small (n=135) and the professional distribution of the study participants might have been different. Some other findings from Nigeria and Ethiopia reported higher proportions with good IPC knowledge (12–14). But these studies used a smaller sample size and a lower cut-off to adjudge good knowledge of IPC. A study in Ethiopia with similar sample size (n=649) had a similar proportion (53.7%) of HCWs with good knowledge of IPC; hence small sample size might be the major factor for the difference in good knowledge of IPC observed in other studies. The finding showed that there are still gaps in the knowledge of IPC among HCWs. This has implication on practice and risk of healthcare associated infections. It also calls for the need to institutionalize the IPC interventions instead of one-off training interventions. Reinforcing the training and practice of IPC will likely have synergistic effect on knowledge and practice of IPC in healthcare settings.

There were several predictors of good knowledge of IPC. Although doctors were twice more likely to have good knowledge of IPC compared to medical laboratory scientists,

Table 4: Practice of infection prevention and control among selected healthcare workers in Alex Ekwueme Federal University Teaching Hospital, Abakaliki, Nigeria, 2019

Variable	Practice		COR (95% CI)	p-value	AOR (95% CI)	*p*-value
	Good	Poor				
Gender						
Female	180 (51.4)	170 (48.6)	1.5 (1.06-2.11)	0.023	0.9 (0.58-1.45)	0.718
Male	88 (41.5)	124 (58.5)	1		1	
Age group						
≥40 years	110 (54.7)	91 (45.3)	1.6 (1.10-2.20)	0.013	1.0 (0.62-1.45)	0.808
<40 years	158 (43.8)	203 (56.2)	1		1	
Profession						
Nurses	126 (66.7)	63 (33.3)	3.3 (2.18-5.07)	<0.001	3.1 (1.79-5.20)	<0.001*
Medical Laboratory Scientist	39 (42.4)	53 (57.6)	1.2 (0.74-2.03)	0.437	1.3 (0.73-2.14)	0.424
Orderly/Cleaner	32 (34.8)	60 (65.2)	0.9 (0.53-1.49)	0.650	1.0 (0.25-4.36)	0.963
Doctors	71 (37.6)	118 (62.4)	1		1	
Marital Status						
Married	222 (52.5)	201 (47.5))	2.2 (1.50-3.34)	<0.001	1.6 (1.05-2.55)	0.028*
Others	46 (33.1)	93 (66.9)	1		1	
Level of Education						
Tertiary Education	238 (50.1)	237 (49.9)	1.9 (1.18-3.08)	0.007	1.1 (0.26-4.56)	0.903
Others	30 (34.5)	57 (65.5)	1		1	
Work Experience						
≥7 years	130 (55.3)	105 (44.7)	1.7 (1.21-2.38)	0.002	1.2 (0.78-1.78)	0.438
<7 years	138 (42.2)	189 (57.8)	1		1	
Previous Training on IPC						
Yes	215 (48.1)	232 (51.9)	1.1 (0.72-1.64)	0.700		
No	53 (46.1)	62 (53.9)	1			

*Statistically significant; COR=Crude Odds Ratio; AOR = Adjusted Odds Ratio; Confidence Interval

this was still far below the proportion of nurses with good knowledge of IPC, while the proportion of orderlies/cleaners with good knowledge of IPC was abysmally too low. Those with tertiary education were seven times more likely to have good knowledge compared with others, and those with 7 or more years of work experience were twice more likely to have good knowledge compared to those with less than 7 years of work experience. Some other factors that have been associated with knowledge of IPC included age, gender, work experience, educational status (14), profession (15), and types of facility (16,17), were also reported in this study.

About three-quarter (78.6%) of the respondents had good attitude towards IPC. This may be due to the nosocomial Lassa fever infection that had affected their colleagues severally and sometimes resulting in deaths. However, a similar study reported a higher percentage (93.4%) of HCWs with good attitude towards IPC (18) while another study reported a lower percentage (55.6%) of HCWs who had positive attitude about infection prevention (17). The difference noticed in these studies may be due to variation in study setting and the composition of study respondents. A good attitude towards IPC is desired because this is expected to result in good practice which is protective for the HCWs. We also found that males were about twice more likely to have good attitude towards IPC compared to the females while nurses were about 7 times more likely to have good attitude compared to doctors. However, the finding of males being more likely to have good attitude of IPC may have been influenced by confounders. Females have been reported to have better IPC attitude as some IPC practices may be associated with household-type chores, which may be seen as women's work and therefore feminine (19). This may also be the reason for nurses being more likely to have good attitude compared to doctors as nursing is dominated by females why medical practice in Nigeria is dominated by males. Also, those with 7 or more years of work experience were about three times more likely to have good attitude compared to those with lesser years of work experience. It is possible

that with increasing years of work experience, HCWs may tend to have a better attitude towards IPC due to increasing understanding of the risks associated with healthcare.

In this study, less than half (47.7%) of the respondents had good practice of IPC. A recent study in Nigeria reported 68% of HCWs with good practice of IPC (16). As observed in our study, it was noticed that good practice of IPC was lower than knowledge and attitude about IPC as reported in some other studies (12,16–18). This might suggest that knowledge of IPC does not translate to good practice. Some researchers have reported weak correlation between good knowledge and good practice of IPC (12,20). This may be connected to the fact that other factors such as availability of the IPC infrastructure, personal protective equipment (PPE) and presence or otherwise of organizational cultures and policy determine how individuals practice IPC in different settings. Our hospital has an IPC committee, and has developed IPC policy but this has not been distributed in the hospital while supply of PPE are still not optimum. These could also be contributory and need to be addressed.

Other factors have also been reported to be associated with good practice of IPC. In our study, nurses were three times more likely to have good practice of IPC compared to doctors. Also, the married HCWs were 1.6 times more likely to practice IPC compared to the unmarried. In some studies, age, educational status, and years of work experience (14,15,17), profession (15), level of healthcare facility (17), continuous supply of PPE (17), presence of IPC guidelines (15), working in department with continuous water supply (21), gender and type of department (18), have been reported to be associated with good practice of IPC.

Conclusion:

The study shows that knowledge and practice of IPC are still low in the study location despite the interventions that had been instituted to improve the IPC framework. Therefore, there is need to adapt approaches that will influence behavior change towards IPC in the course of the series of in-service training being conducted in the hospital. Also, there is need to set up an IPC surveillance structure in the study location so as to support HCWs in the course of their routine work in the hospital.

Conflict of interest:

Authors declare no conflict of interest

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Original Article **Open Access**

Phenotypic and genotypic characterization of plasmid-mediated AmpC beta-lactamases in enteric Gram-negative bacteria from patients with lower respiratory tract infections in a tertiary hospital, southwest Nigeria

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Abstract:

Background: AmpC or class C or group 1 beta lactamases are class C cephalosporinases that hydrolyse a wide variety of beta-lactam antibiotics including alpha methoxy beta-lactams (cefoxitin), narrow and broad spectrum cephalosporins. This study was conducted to characterize plasmid-mediated AmpC producing enteric Gram-negative bacteria from patients with lower respiratory tract infections in Obafemi Awolowo University Teaching Hospital Complex (OAUTHC) Ile Ife, Osun State, Nigeria
Methodology: A total of 149 patients with clinical features of lower respiratory tract infections (LRTI) were selected by simple random sampling for the study. All Gram-negative isolates recovered from standard microbiological cultures of respiratory specimens of these patients were tested against cefoxitin, third generation cephalosporins (3GCs), and other antibiotics using the disc diffusion AST method, and also screened for production of AmpC beta-lactamases phenotypically by the CLSI method. Plasmid DNA extraction was carried out on twenty-nine cefoxitin-resistant selected isolates using the Kado and Lin method, while genotypic detection of plasmid-mediated *AmpC* gene was carried out by the polymerase chain reaction (PCR) assay.
Results: The results showed that 204 (43.3%) of 471 isolates recovered from the 149 selected patients were resistant to 3GC in the AST assay, among which 121 (59.3%) were resistant to cefoxitin, and 189 of the 471 isolates (40.1%) were AmpC producers. The AmpC producers concurrently showed multiple resistance pattern to other antibiotics tested in this study. Ninety six percent of the 29 selected isolates for plasmid analysis contained plasmids, 45% of which amplified positive on PCR for *CMY*, 38% for *FOX*, and 31% for *ACC* types of *AmpC* genes.
Conclusion: This study showed a high degree of antibiotic resistance among enteric Gram-negative bacteria recovered from patients with LRTIs, as well as high degree of plasmid-encoded *AmpC* genes responsible for this high antibiotic resistance among the isolates. Proper antibiotic policy and regulation are required to limit the spread of plasmid mediated AmpC β-lactamase producing organisms because they can lead to therapeutic failure in infected patients in the nearest future.

Keywords: Enteric Gram-negatives, AmpC beta-lactamase, plasmid, lower respiratory tract infection, PCR

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Caractérisation phénotypique et génotypique des bêta-lactamases AmpC à médiation plasmidique dans les bactéries entériques Gram-négatives de patients atteints d'infections des voies respiratoires inférieures dans un hôpital tertiaire, sud-ouest du Nigéria

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Abstrait:

Contexte: Les bêta-lactamases AmpC ou de classe C ou de groupe 1 sont des céphalosporinases de classe C qui hydrolysent une grande variété d'antibiotiques bêta-lactamines, y compris les alpha-méthoxy bêta-lactamines (céfoxitine), les céphalosporines à spectre étroit et large. Cette étude a été menée pour caractériser les bactéries à Gram négatif entériques produisant de l'AmpC à médiation plasmidique chez des patients atteints d'infections des voies respiratoires inférieures du complexe hospitalier universitaire d'Obafemi Awolowo (OAUTHC) Ile Ife, État d'Osun, Nigéria
Méthodologie: Un total de 149 patients présentant des caractéristiques cliniques d'infections des voies respiratoires inférieures (LRTI) ont été sélectionnés par échantillonnage aléatoire simple pour l'étude. Tous les isolats à Gram négatif récupérés à partir de cultures microbiologique standard d'échantillons respiratoires de ces patients ont été testés contre la céfoxitine, les céphalosporines de troisième génération (3GC) et d'autres antibiotiques en utilisant la méthode AST de diffusion sur disque, et également criblés pour la production de bêta-lactamases AmpC phénotypiquement par le Méthode CLSI. L'extraction de l'ADN plasmidique a été réalisée sur 29 isolats sélectionnés résistants à la céfoxitine en utilisant la méthode Kado et Lin, tandis que la détection génotypique du gène AmpC à médiation plasmidique a été réalisée par le test de réaction en chaîne par polymérase (PCR).
Résultats: Les résultats ont montré que 204 (43,3%) des 471 isolats récupérés des 149 patients sélectionnés étaient résistants à la 3GC dans le test AST, parmi lesquels 121 (59,3%) étaient résistants à la céfoxitine et 189 des 471 isolats (40,1%) étaient des producteurs d'AmpC. Les producteurs d'AmpC ont montré simultanément plusieurs profils de résistance à d'autres antibiotiques testés dans cette étude. Quatre-vingt-seize pour cent des 29 isolats sélectionnés pour l'analyse des plasmides contenaient des plasmides, dont 45% amplifiés positifs par PCR pour *CMY*, 38% pour *FOX* et 31% pour les types *ACC* des gènes *AmpC*.
Conclusion: Cette étude a montré un degré élevé de résistance aux antibiotiques parmi les bactéries entériques Gram-négatives récupérées chez des patients atteints de LRTI, ainsi qu'un degré élevé de gènes AmpC codés par plasmide responsable de cette résistance élevée aux antibiotiques parmi les isolats. Une politique et une réglementation appropriées en matière d'antibiotiques sont nécessaires pour limiter la propagation des organismes producteurs β-lactamase d'AmpC à médiation plasmidique car ils peuvent conduire à un échec thérapeutique chez les patients infectés dans un avenir proche.

Mots clés: Gram-négatifs entériques, AmpC, bêta-lactamase, plasmide, infection des voies respiratoires inférieures, PCR

Introduction:

The resistance rate of multidrug-resistant (MDR) Gram-negative bacteria has increased continuously in the past few years, and bacterial strains that produce AmpC-type or class C beta-lactamases and the extended spectrum beta-lactamases (ESBLs) are now of concern (1). However, the increased presence of plasmid-mediated AmpC beta-lactamases in the world is becoming of greater concern. Gram-negative organisms resistant to carbapenems or third generation cephalosporins due to ESBL production were defined as highly resistant isolates. However, strains resistant to two or more agents of the antimicrobial classes (e. g. quinolone and aminoglycoside) were also defined as highly resistant strains (2).

AmpC-type beta-lactamases are cephalosporinases that hydrolyze cephamycins as well as other extended-spectrum cephalosporins and are poorly inhibited by clavulanic acid. They are clinically significant because they confer resistance to groups of antibiotics such as cephalosporin, penicillin, cephamycin and monobactam. The *AmpC* gene located on plasmids or chromosomes can be moved from one microorganism to another through transformation and conjugation. The presence of such genes on plasmids facilitates their spread between the family of Enterobacteriaceae (3). The AmpC enzyme-producing strains show broader and higher resistance, making them more challenging for physicians to treat associated infections by such strains.

There are two types of AmpC β-lactamases; plasmid-mediated (usually constitutive and rarely inducible) and chromosomal mediated (inducible). The chromosomal AmpC enzymes are communicated constitutively at a low level and are constantly found in bacteria such as *Citrobacter freundii*, *Enterobacter cloacae*, *Morganella morganii*, *Hafnia alvei* and *Serratia marcescens.* They are inducible normally by β-lactam antimicrobial agents such as cefoxitin and imipenem which are interceded by the *AmpR* regulator. However, they are inadequately induced by the third or fourth generation cephalosporins (4,5). Infections caused by AmpC producing bacteria are of particular clinical and epidemiological importance, and are often associated with high morbidity and mortality in infected patients (6,7).

Plasmid-mediated AmpC beta-lactamases (PMACBL) have been found all over the world but are much less common than ESBLs (5,8). Most strains of PMACBL have been isolated from patients after several days of hospitalization, but culture of the isolates from long-term care facilities, rehabilitation centers, and outpatient clinics have been reported (9,10). Risk factors for bloodstream infection (BSI) caused by AmpC producing strains of *Klebsiella pneumoniae* and other enteric pathogens include prolonged hospital stay, care in an emergency unit, central venous

catheterization, indwelling urinary catheter, and administration of broad spectrum cephalosporins and beta-lactamase inhibitors, which are similar risk factors for infections caused by ESBL producing *K*. *pneumoniae* strains (5,11, 12).

There is paucity of data on the prevalence of AmpC beta-lactamases among Gram negative isolates in Ile-Ife, Nigeria. Hence this study aimed to determine the prevalence and characterize AmpC producing Gram-negative enteric bacteria isolates from patients with lower respiratory tract infections (LRTI) in Obafemi Awolowo University Teaching Hospital Complex (OAUTHC) Ile-Ife, Nigeria.

Materials and method:

Study setting, design and sampling

Patients who reported at the Obafemi Awolowo University Teaching Hospital Complex (OAUTHC) chest clinic, adult emergency and chronic obstructive pulmonary disease (COPD) clinic with symptoms of lower respiratory tract infections were selected by simple random sampling and recruited for this study. Sputum and nasal swabs samples were collected aseptically from subjects between the period of January, 2015 and February, 2016. Sample size was determined using Cochran's formula (22).

Ethical consideration

Ethical approval was obtained from the Hospital Ethical and Research Committee of the institution. Informed consent of each subject was also obtained.

Specimen collection, processing and bacterial isolation

Sputum and nasal swabs of selected subjects with lower respiratory infections were collected into sterile universal bottles and sterile swab sticks respectively. The samples were cultured on cetrimide agar medium and MacConkey agar (Oxoid Ltd., Basingstoke, UK) and incubated aerobically at 37°C for 18–24 h. Bacteria growth colonies on the culture plates were identified microbiologically using conventional methods (colonial morphology, Gram staining, and biochemical tests) according to Cheesebrough (13) and confirmed by the API 20E commercial identification kit.

Antibiotic susceptibility test (AST)

Antibiotic susceptibility testing (AST) was performed by the Kirby Bauer disc diffusion method. Mueller-Hinton (MH) agar plates were inoculated with 18-24 h culture inoculum of each bacterial isolate standardized with 0.5 McFarland standards. Single antibiotics discs (Oxoid, Basingstoke, Hampshire, England) of; cefotaxime (30µg), piperacillin (10µg), augmentin (2µg), cefoxitin (30µg), ceftazidime (30µg), cefuroxime (30µg), ceftriaxone (30 µg), ofloxacin (5µg), cefixime (5µg), imipenem (10µg), gentamicin (10µg), ciprofloxacin (5µg) and nitrofurantoin (300µg) were firmly placed on the surface of the inoculated MH agar plate using a sterile forcep. The plates were incubated aerobically in an inverted position at 37^{o}C for 18–24 h. The diameters of zone of inhibition of each bacterial isolate to each antibiotic disk on the culture plate was measured and compared with the Clinical and Laboratory Standards Institute (CLSI) guidelines (14) to determine resistivity or resistance of each isolate.

Screening of AmpC production

The primary screening of the AmpC enzyme-producing strains was conducted by the Kirby-Bauer disk diffusion method described by Kazemian (15). AmpC-producing bacteria were detected using FOX (30µg) drug-sensitive disc according to the CLSI standards. A clear zone of ≤18 mm indicated strains producing AmpC enzyme (16).

Extraction of plasmid DNA

Plasmid DNA extraction was carried out according to the method of El-deeb (17), and Ganesh et al, (18). The plasmid DNA of 29 selected cefoxitin resistant *Klebsiella* isolates was extracted by suspending the bacterial cell in an Eppendorf tubes containing 1ml of sterile distilled water. The tube was then vortex-mixed and centrifuged at 13,000 rpm for 5 min using BIO-RAD model 16K micro-centrifuge, and the supernatant was decanted. A 200µl of lysing solution (Tris buffer and 0.1% sodium dodecyl sulfate) was added to the pellet and the cell pellet was re-suspended completely to achieve complete lysis (17,18). The cell pellet was incubated at 65^{o}C for 30 minutes.

Phenol-chloroform (1:1) solution (200 µl) was added to the pellet and mixed by inversion until complete emulsion is formed so as to precipitate the protein and to remove the cell debris (17). The tube was then centrifuged at high speed for 10 minutes. One hundred and fifty microliters (150µl) of the supernatant were transferred into a fresh tube and mixed well with 3µl of 5M NaCl and 150µl propanol was added to the tube to precipitate the DNA and mixed by inverting the tubes 3-5 times until the mixture became sticky. Samples were kept on ice for 20 minutes (to prevent them from the degradation of chromosomal DNA which may be co-precipitated with plasmid) and spinned at 12,000 rpm for 20 minutes and the supernatant was removed immediately. A 100µl of cold 80% ethanol was added to wash the DNA and spinned at 12000 rpm for 3 min, the supernatant was immediately removed. The pellet was then dried completely and re-suspended in 30µl of TE (Tris Ethylene diamine tetraacetate) buffer or distilled water and

RNase (to degrade RNA) for further use.

Purified plasmids were electrophoresed in 0.8% (w/v) agarose for size estimation and pattern comparison. Plasmid DNA bands were visualized by UV-transilluminator, photographed with a Polaroid camera and documented using a gel electrophoresis documentation system. The molecular sizes of the unknown plasmid DNA were then estimated by comparing the distance travelled with that of the molecular weight of the standards (17,18).

Molecular detection of *AmpC* beta-lactamase genes by PCR assay

The extracted plasmid DNA served as the PCR template. The genes encoding the PMACBL in cefoxitin-resistant *Klebsiella* isolates were detected by PCR technique (19). The PCR was performed using 25 µl of AmpliTaq Gold PCR Master Mix (Applied Biosystems), 0.5 µM of the forward and reversed primers for *ACC*-M-F-AACAGCCTCAGCAGCCGGTTA, *ACC*-M-R-TTCGCCGCAATCATCCCTAGC (346bp), and *CMY*-F-TGGCCAGAACTGACAGGCAAA, *CMY*-R-TTTCTCCTGAACGTGGCTGGC (462bp), 0.4 µM of the primers for *FOX*-M-F-AACATGGGG TAT CAGGGAGATG, and *FOX*-M-R-CAAAGCGCGTA ACCGGATTGG (190bp) (20), and 2µl of plasmid DNA template. The final reaction volume of 50µl was made up with sterile distilled water.

The amplification conditions were 10 min at 95^{o}C; 25 cycles of 94^{o}C for 30 s, 64^{o}C for 30, 72^{o}C for 60 s; and final extension of 72^{o}C for 7 minutes. The amplicons were electrophoresed on 0.8% (w/v) agarose, and bands were visualized with ultraviolet transilluminator and photographed using gene gel bioimaging system.

Statistical analysis

The data obtained were analysed using GraphPad Prism Version 6.01. The Chi-square test was used to test the significance of association of the variables with $p < 0.05$ considered as significance value.

Results:

The baseline characteristics of the 149 patients are depicted in Table 1. From the 471 isolates recovered, 40% (189/471) were AmpC producers, with the most frequent isolates being *Enterobacter* sp (66.6%, 42/63), followed by *Yersinia* sp (60%, 6/10) and the least is *Escherichia coli* (12.1%, 4/33) (Table 2). The isolates were most resistant to penicillins (100%), cephems (51.9%), beta-lactam inhibitors (76.6%), and least resistant to fluoroquinolones (4.4%) (Table 3). The multiple antibiotic resistance (MAR) phenotypes of the bacterial isolates showed that diversities of MAR patterns occurred among the isolates of which 0.75% and 0.25% developed resistance to six and seven classes of antibiotics tested respectively, 5.5% developed resistance to five classes of antibiotics, 21.6% to four and 65.3% to three classes of antibiotics. All isolates exhibited different resistance profiles with "AMC^{R}/AUG^{R}/PRL^{R}" appearing the most frequent.

Ninety-six percent (96%) of the selected cefoxitin resistant and AmpC producing *Klebsiella* (n=29) isolates harboured single plasmid with molecular weight of 1300 bp. The PCR result showed that 13 (45%) of the 29 isolates were positive for *CMY* gene, 11 (38%) amplified for *FOX* gene while only 9 (31%) amplified for *ACC* gene (Figs 1-3).

Discussion:

The results of the plasmid DNA analysis showed that the representatives multiple antibiotic resistant isolates harboured single plasmid. Ganesh et al., (18) reported similar single plasmid in *E. coli*. Plasmids are environmental and genetic factors that are highly mobile and can confer resistance genes on several classes of antimicrobials. The prevalence of plasmid-mediated *AmpC* type resistance is imperative to give helpful data and information expected to focused antimicrobial treatment and better disease control (21). The isolates were highly resistant to third generation cephalosporins including cefoxitin as previously reported by Thonda et al., (22). Reasons may be that the third generation cephalosporins antibiotics have been misused for a long period by individuals, therefore, over time the pathogens have developed resistance to these antibiotics (22).

In this study, 40% of the isolates were producers of PMACBL. AmpC production in these isolates could be due to a mechanism similar to that of ESBL producing organism that appear susceptible to ceftazidime by the disc diffusion method. This finding is similar to the study of Manchanda and Singh (23) who reported 39% cefoxitin susceptibility in AmpC producers. Our study showed that not all cefoxitin resistant isolates possess plasmid *AmpC* β-lactamase gene by PCR assay. This finding agreed with the study of Fam et al., (24) and Yilmaz et al., (25) which reported that not all cefoxitin resistant isolates are AmpC β-lactamase producers. Firstly, the reason may be because cefoxitin resistance is not due to production of AmpC β-lactamase but also may be as a result of other enzymatic mechanism such as extended spectrum beta lactamases (ESBLs) and metallo beta-lactamase (MBL) or because of the non-enzymatic mechanism such as porin channel mutation (26). Another reason may be cefoxitin resistant phenotype in *E. coli* can result from overexpression of the chromosomal *AmpC* gene

Table 1: Socio-demographic and clinical characteristics of patients with lower respiratory tract infections in Obafemi Awolowo University Teaching Hospital Complex (OAUTHC), Ile-Ife, Nigeria

Characteristics	No of patients (%)
Gender:	
Female	61 (40.9)
Male	88 (59.0)
Age group (years)	
≤10	17 (11.4)
11-20	22 (14.8)
21-30	8 (5.4)
31-40	14 (9.4)
41-50	30 (20.1)
51-60	29 (19.5)
>60	29 (19.5)
Marital status:	
Single	21 (14.0)
Married	128 (85.9)
Patient status:	
Outpatients	57 (38.2)
Inpatients	92 (61.7)
Religion:	
Christianity	117 (78.5)
Islamic	32 (21.4)
Occupation:	
Farming	24 (16.1)
Trading	41 (27.5)
Civil servant	18 (12.0)
Teaching	11 (7.3)
Motor vehicle mechanics	4 (2.6)
Others	50 (33.5)
Occupational exposure:	
Yes	33 (22)
No	116 (78)
Cough duration:	
< 3 months	102 (68)
3 months and above	47 (32)
Smoking status:	
Current	4 (2.6)
Former	27 (18.1)
Never	118(79.1)
Hospitals	
OAUTHC	131 (87.9)
SDAH	16 (10.7)
Wesley, Ilesa	2 (1.3)

OAUTHC = Obafemi Awolowo University Teaching Hospital Complex; SDA = Seventh Day Adventist Hospital

Table 2: Prevalence and distribution of AmpC producing enteric Gram-negative bacteria isolates in Obafemi Awolowo University Teaching Hospital Complex (OAUTHC), Ile-Ife, Nigeria

Isolated Organism	No of isolates resistant to 3GC	No of AmpC positive Isolates (%)
Enterobacter sp (n=63)	21	42 (66.6)
Klebsiella sp (n=166)	116	50 (30.1)
P. aeruginosa (n=47)	20	27 (57.5)
Escherichia coli (n=33)	29	4 (12.1)
Serratia sp (n=20)	13	7 (35)
Aeromonas sp (n=29)	16	13 (44.8)
Citrobacter sp (n=37)	25	12 (32.4)
Salmonella sp (n=32)	17	15 (46.9)
Providencia sp (n=4)	3	1 (25)
Yersinia sp (n=10)	4	6 (60)
Shigella sp (n=28)	16	12 (42.9)
Total (n=471)	**204 (43.3)**	**189 (40.1)**

3GC = 3rd generation cephalosporins

Table 3: Antibiotic resistance (%) of bacterial isolates from patients with lower respiratory tract infections

Classes of antibiotics tested	Penicillins (PRL)	Cephems (CTX)	Beta-Lactamase Inhibitor (AMC)	Carbapenems (IPM)	Nitrofurans (NIT)	Aminoglycosides (GEN)	Fluoroquinolones (OFL)
Percentage resistance (%)	100	51.9	76.6	4.4	15.9	12.3	3.92

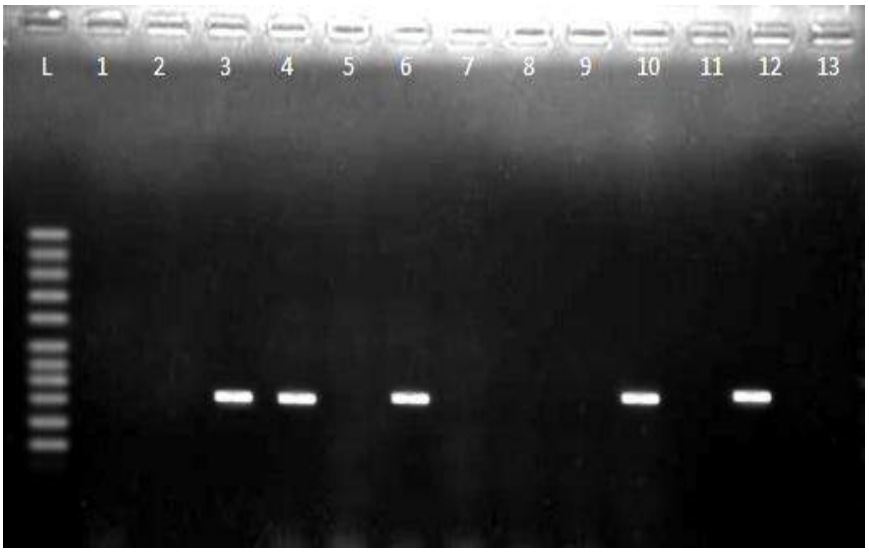
Piperacillin-PRL, Cefotaxime-CTX, AMC-Amoxicillin-clavulanic acid, Imipenem-IPM, Nitrofurantoin-NIT, Ofloxacin-OFL, Gentamicin-GEN

Lane N=DNA Ladder (100bp), Lane A-M = Positive for *CMY* gene

Fig 1: Agarose gel electrophoresis of the amplification product coding *CMY* gene (462bp) in selected multiple antibiotic resistant *Klebsiella* Isolates

Lane L=DNA Ladder (100bp), Lane 1-13 = Positive for *FOX* gene

Fig 2: Agarose gel electrophoresis of the amplification product coding *FOX* gene (190bp) in selected multiple antibiotic resistant *Klebsiella* Isolates



Lane L=DNA Ladder (100bp), Lane 1-13 = Positive for *ACC* gene

Fig 3: Agarose gel electrophoresis of the amplification product coding *ACC* gene (346bp) in selected multiple antibiotic resistant *Klebsiella* isolates

because of mutations that could occur in the promoter and attenuator regions (27). Cefoxitin has been demonstrated as a substrate to active efflux pump in clinical isolates (28). Genes for AmpC beta-lactamases are commonly found among members of the family Enterobacteriaceae including *Enterobacter* sp*, Shigella, Providencia, Citrobacter freundii, Morganella morganii, Serratia marcescens* and *E. coli*.

In this study, PCR revealed *CMY* genes to be the most prevalent among the *AmpC* genes. The prevalence rate was similar to the study of Soha and Lamina (5), which reported *Klebsiella* sp to be 44% AmpC producers, but much lower than the study of Wassef et al., (39), which reported that 26.9% harboured the plasmid-mediated *AmpC* gene and that of Yilmaz et al., (25) who reported that 3.6% *K. pneumoniae* were AmpC-producing strains. The molecular detection of plasmid- mediated *AmpC* using PCR techniques showed that the most prevalent *AmpC* gene belongs to family *CMY* which was detected in 40% of all selected isolates tested while *FOX* and *ACC* genes were 33% and 27% respectively. Cefoxitin resistance in non-AmpC producers could be due to some other resistance mechanism(s). Hernandez-Alles et al., (30) have demonstrated the interruption of a porin gene by insertion sequences which could be a common type of mutation which causes loss of porin expression and high cefoxitin resistance in *K. pneumoniae*.

The results of the present study indicate that screening should include all the clinical isolates showing resistance to any of the cephalosporins irrespective of their cefoxitin susceptibility status. Plasmid-mediated AmpC beta-lactamases may have arisen through the transfer of chromosomal genes for the inducible AmpC beta-lactamases onto plasmids. This transfer has resulted in plasmid-mediated *A*mpC beta-lactamases in isolates of *E. coli, K. pneumoniae, Salmonella* sp, *Citrobacter freundii, Enterobacter aerogenes* and *Proteus mirabilis*. AmpC beta-lactamase producing bacterial pathogens may cause a major therapeutic failure if not detected and reported in time.

Conclusion:

In our study, the prevalence of AmpC beta-lactamase resistance is high among patients with respiratory infection and who had prior exposure to antibiotics. Detection and identification of types of PMACBL producing organisms is also important to ensure effective therapeutic intervention and optimal clinical outcomes so as to aid in hospital infection control and to help the clinicians to prescribe the most appropriate therapeutic agents, thereby decreasing the selective pressure, which generates antibiotic resistance.

Conflict of interest:

Authors declare no conflict of interest

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Original Article **Open Access**

Phenotypic identification of soil bacterial and fungal communities inhabiting an archaeological monument at Augustine University, Ilara Epe, southwest Nigeria

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Abstract:

Background: The Sungbo Eredo Monument is an ancient public work with a system of defensive walls and ditches located in Eredo Local Council Development Area of Epe, Lagos State, southwest Nigeria. A huge section of the monument cuts through the Augustine University campus, forming two-sided vertical walls with a deep ridge in-between. The objective of this investigative study is to determine the microbial profile of soil samples from the monument in the University campus.
Methodology: Soil samples were collected from the topsoil at a depth of 7.5cm from four randomly selected points along the edge of the monument. The samples were transported to the microbiology laboratory of the Department of Biological Sciences of Augustine University for analysis. Samples were cultured on Nutrient agar (NA) and incubated aerobically for 24-48 hours for bacteria isolation and on Sabouraud's Dextrose agar (SDA) for 72 hours for fungi isolation. Bacterial colonies on NA were preliminarily identified to genus level by Gram reaction and conventional biochemical test scheme for Gram-positive (catalase, coagulase, starch hydrolysis) and Gram-negative isolates (oxidase, urease test, indole, methyl red, Voges Proskauer and sugar fermentation tests). Fungi colonies on SDA were identified using conventional macroscopic and microscopic characteristics. Antibiotic susceptibility test of the bacterial isolates to selected antibiotics was done using the Kirby Bauer disc diffusion method.
Results: A total of twenty-three bacterial isolates in four genera; *Bacillus, Staphylococcus, Cellobiococcus* and *Micrococcus* and nine fungal isolates in three genera; *Saccharomyces*, *Aspergillus* and *Botrytis* were identified from the cultures. The bacterial isolates were sensitive (>50% sensitivity) to only gentamicin and ofloxacin, with 65.2% and 78.3% sensitivity rates respectively, while they were largely resistant to all other antibiotics such as ceftriaxone, erythromycin, cefuroxime, cloxacillin, ceftazidime and augmentin, with resistance rates of 65.2%, 65.2%, 73.9%, 82.6%, 86.9%, 91.3% respectively.
Conclusion: The results of this investigative study revealed the presence of antibiotic-resistant bacteria (mainly Gram-positive) and fungi on the archaeological monument of Augustine University, adding to the existing data on microbial spectrum of archaeological monuments that could be useful for unraveling human cultural habits and microbe-related human diseases. However, further studies on molecular identification of these microbial spectrum will be required to ascertain their genetic relatedness and ancestral phylogeny, which will be useful for archaeologists in their study of the Sungbo-Eredo ancestral monument.

Keywords: Archaeology; microbial profile; soil; antibiotic resistance; southwest Nigeria

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Identification phénotypique des communautés bactériennes et fongiques du sol habitant un monument archéologique à l'Université Augustine, Ilara Epe, sud-ouest du Nigeria

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Abstrait:

Contexte: Le monument Sungbo Eredo est un ancien ouvrage public doté d'un système de murs défensifs et de fossés situé dans la zone de développement du conseil local d'Eredo à Epe, dans l'État de Lagos, au sud-ouest du Nigéria. Une énorme section du monument traverse le campus de l'Université Augustine, formant des murs verticaux à deux côtés avec une crête profonde entre les deux. L'objectif de cette étude d'investigation est de déterminer le profil microbien d'échantillons de sol provenant du monument du campus universitaire.
Méthodologie: Des échantillons de sol ont été prélevés dans la couche arable à une profondeur de 7,5 cm à partir de quatre points choisis au hasard le long du bord du monument. Les échantillons ont été transportés au laboratoire de microbiologie du Département des sciences biologiques de l'Université Augustine pour analyse. Les échantillons ont été cultivés sur gélose nutritive (NA) et incubés en aérobie pendant 24 à 48 heures pour l'isolement des bactéries et sur gélose au dextrose de Sabouraud's (SDA) pendant 72 heures pour l'isolement des champignons. Les colonies bactériennes sur NA ont été préalablement identifiées au niveau du genre par réaction de Gram et schéma de test biochimique conventionnel pour les isolats Gram-positif (catalase, coagulase, hydrolyse de l'amidon) et Gram-négatif (oxydase, test à l'uréase, indole, rouge de méthyle, Voges Proskauer et sucre essais de fermentation). Les colonies de champignons sur SDA ont été identifiées en utilisant des caractéristiques macroscopiques et microscopiques conventionnelles. Le test de sensibilité aux antibiotiques des isolats bactériens à des antibiotiques sélectionnés a été effectué en utilisant la méthode de diffusion sur disque de Kirby Bauer.
Résultats: Un total de vingt-trois isolats bactériens dans quatre genres; *Bacillus*, *Staphylococcus*, *Cellobiococcus* et *Micrococcus* et neuf isolats fongiques de trois genres; *Saccharomyces, Aspergillus* et *Botrytis* ont été identifiés à partir des cultures. Les isolats bactériens étaient sensibles (sensibilité >50%) uniquement à la gentamicine et à l'ofloxacine, avec des taux de sensibilité de 65,2 % et 78,3 % respectivement, alors qu'ils étaient largement résistants à tous les autres antibiotiques comme la ceftriaxone, l'érythromycine, la céfuroxime, la cloxacilline, la ceftazidime et l'augmentine avec des taux de résistance de 65,2%, 65,2%, 73,9%, 82,6%, 86,9%, 91,3% respectivement.
Conclusion: Les résultats de cette étude d'investigation ont révélé la présence de bactéries résistantes aux antibiotiques (principalement à Gram positif) et de champignons sur le monument archéologique de l'Université Augustine, ajoutant aux données existantes sur le spectre microbien des monuments archéologiques qui pourraient être utiles pour démêler l'homme. les habitudes culturelles et les maladies humaines liées aux microbes. Cependant, d'autres études sur l'identification moléculaire de ces spectres microbiens seront nécessaires pour déterminer leur parenté génétique et leur phylogénie ancestrale, ce qui sera utile aux archéologues dans leur étude du monument ancestral Sungbo-Eredo.

Mots-clés: Archéologie; profil microbien; sol; résistance aux antibiotiques; sud-ouest du Nigéria

Introduction:

The soil harbors many forms of micro-organisms, majorly bacteria and fungi (1). The soil microbial community shows multiple levels of biological organization which encourages genetic variability as well as evenness among communities (2). The Sungbo Eredo Monument is a system of defensive walls and ditches built in honour of the Ijebu noblewoman, Oloye Bilikisu Sungbo in 800-1000 AD. The Sungbo Eredo is the outer enclosure of Ijebuland in southwestern Nigeria, approximately 180 km in length, forming a large feature that shows how involved the socio-political system was long before the opening of the Atlantic trade (3). It is a system of ditch and bank that surrounds the whole of Ijebu kingdom (4), and indeed the most significant earthwork of the ancient ramparts, boundary embankments and ditches that stretched across Nigeria (5).

Analyses of samples at various archaeological sites have witnessed new direction of analysis that has led to drastic changes in the formulation of theories. There are adequate records of such observations obtained from the analysis of samples from excavations and other archaeological sites from different parts of the world (4,6). Garg and Shukla (7) reported that efforts should be concentrated on performing microbiological analysis of rare archaeological samples and rationalizing the need for such integration towards better scientific and comprehensive approach. It has been reported that on the time scales of human history and archaeology, microbiological data can contribute to unraveling human cultural habits, microbe-related human diseases and alterations in cultural artifacts due to biochemical reactions (8).

Studies of microbial biogeography have focused on the microbial communities across spatial distance, and other than revealing a causal relationship between microbes and their environments, provides insights into possible environmental drivers of change in microbial communities (9). The objective of this investigative study is to determine the microbial profile of soil samples from the monument in the University campus.

Materials and method:

Sample collection

Soil samples were collected from four randomly selected points (A, B, C, D) along the edge of the monument. They were obtained from topsoil at a depth of 7.5 cm with a sterile spatula into sterile Bijou bottles according to the method described by Ogunmwonyi et al., (10). The bottles were placed in a beaker and transported back to the microbiology research laboratory of the Department of Biological Sciences, Augustine University, Ilara Epe, Lagos State, Nigeria for microbial analysis.

Determination of pH of soil sample

The pH of the soil samples was determined according to the method described by Ogunmwonyi et al., (10) using a pH meter (Jenway Model 3505, UK). The soil samples were mixed with distilled water in the ratio of 1:2 after which it was stirred for 30 minutes, and allowed to settle before the pH meter probe was inserted into the mixture.

Inoculation of cultures

A 10-fold serial dilution of the soil samples was done and the diluted samples were plated on Nutrient agar (Biolab, UK) for bacteria and Saboraoud Dextrose agar (SDA) for fungi using the pour plate method. NA plates were incubated at 37^{o}C aerobically for 24-48 hours while SDA plates were incubated at 25^{o}C and 37^{o}C for 72 hours.

Bacterial identification

Colonies of bacteria on the NA plates were identified to genus level using cultural morphology (surface appearance, size, shape, pigmentation) (11), Gram staining reaction, and conventional biochemical test scheme for Gram positive (catalase, coagulase, starch hydrolysis) and Gram-negative bacteria (urease, methyl red, Voges Proskauer, and sugar fermentation tests) as previously described by Ajayi et al., (12).

Fungal identification

The identification of the fungal isolates was based on a combination of morphological and cultural characteristics with special reference to the sporulation of the fungal isolates. Macroscopic examination was done by direct observation of the SDA plates and wet mount microscopy of fungal cultures. Physical appearance such as the colony colour, shape and texture (moist mycelia) were examined first as well as abundance of growth. This was carried out using the techniques contained in the illustrated handbook of fungi (13,14). A wet mount of each fungus was prepared by suspending a loopful of the fungal culture in a few drops of lacto-phenol cotton blue solution on a microscope slide, which was then covered with a slip and examined under the light microscope with 40x magnification lens.

Antibiotic susceptibility test of bacterial isolates

Antibiotic susceptibility of each isolated bacteria was determined using the Kirby-Bauer disk diffusion method to selected antibiotics on Mueller-Hinton (MH) agar plates. Antibiotics disks (Rapid Labs CM-12-8PR100, UK) used were; ceftazidime (30µg), cefuroxime (30µg), gentamicin (10µg), ceftriaxone (30µg), erythromycin (5µg), cloxacillin (5µg), ofloxacin (5 µg), and augmentin (30µg).

Inoculum of each isolate was first prepared in 0.9% saline and standardized by comparing with 0.5 MacFarland turbidity standard. Sterile MH agar plate was inoculated with the standardized inoculum using sterile cotton swab, and antibiotic disks were placed on the plate, which was then incubated aerobically at 37^{o}C for 24 hours. The diameter of zones of growth inhibition around each disc was measured and the result was interpreted as sensitive, intermediate or resistant using the Clinical and Laboratory Standards Institute (CLSI) guideline (15).

Results:

The pH of the soil samples from the four sites in Sungbo Eredo Monument on Augustine University are; pH 5.9 (A), pH 6.3 (B), pH 6.0 (C), and pH 6.3 (D). The bacterial and fungal isolates from soil samples obtained from the monument are shown in Tables 1, 2 and 3. A total of twenty-three bacterial isolates in four genera; *Bacillus, Staphylococcus, Cellobiococcus* and *Micrococcus* and nine fungal isolates in three genera; *Saccharomyces*, *Aspergillus* and *Botrytis* were isolated from the monument.

The results of the antibiotic susceptibility test showed that the bacterial isolates were sensitive (>50% sensitivity) to only gentamicin and ofloxacin, with 65.2% and 78.3% sensitivity rates respectively, while they were largely resistant to all other antibiotics such as ceftriaxone, erythromycin, cefuroxime, cloxacillin, ceftazidime and augmentin, with resistance rates of 65.2%, 65.2%, 73.9%, 82.6%, 86.9%, and 91.3% respectively (Table 4).

Table 1: Colonial and morphological characteristics of bacterial isolates from archaeological monument at Augustine University, Ilara Epe, southwest Nigeria

S/N	Isolate code	Colony colour	Colony surface	Gram reaction	Cell shape	Cell arrangement
1	A1	Creamy	Shiny	+	Cocci	Clustered
2	A2	Milky	Shiny	+	Rods	Pairs
3	A3	Milky	Shiny	+	Rods	Chains
4	A4	Creamy	Shiny	+	Rods	Chains
5	A5	Milky	Shiny	+	Cocci	Chains
6	A6	Milky	Shiny	+	Rods	Chains
7	A7	Milky	Moist	+	Cocci	Chains
8	A8	Milky	Slimy	+	Rods	Chains
9	A9	Milky	Viscous	+	Rods	Chains
10	B10	Milky	Viscous	+	Rods	Pairs
11	B11	Milky	Viscous	+	Rods	Chains
12	B12	Milky	Slimy	+	Rods	Slimy
13	B13	Milky	Slimy	+	Cocci	Pairs
14	B14	White	Viscous	+	Rods	Clustered
15	B15	White	Shiny	+	Rods	Chains
16	B16	Milky	Moist	+	Rods	Chains
17	B17	Milky	Shiny	+	Rods	Pairs
18	B18	Creamy	Moist	+	Rods	Clustered
19	C19	Milky	Viscous	+	Rods	Chains
20	C20	Milky	Moist	+	Rods	Clustered
21	C21	Yellow	Shiny	+	Cocci	Chains
22	C22	Orange	Shiny	+	Rods	Chains
23	C23	Orange	Moist	+	Rods	Pairs

Table 2: Biochemical characteristics of bacterial isolates from archaeological monument at Augustine University, Ilara Epe, southwest Nigeria

S/N	Isolate code	Catalase test	Coagulase	Starch hydrolysis	Urease test	Methyl red test	Vogues-Proskauer test	Lactose fermentation	Sucrose fermentation	Most probable bacteria
1	A1	+	-	+	-	-	-	-	-	*Micrococcus* sp
2	A2	+	-	+	-	-	-	AG	AG	*Bacillus* sp
3	A3	+	-	+	-	-	-	-	-	*Bacillus* sp
4	A4	+	-	-	-	-	-	-	A	*Bacillus* sp
5	A5	+	-	+	-	-	-	AG	-	*Cellobiococcus* sp
6	A6	-	-	+	+	-	-	-	-	*Bacillus* sp
7	A7	+	+	+	+	-	-	A	A	*Staphylococcus* sp
8	A8	-	-	+	-	-	-	A	A	*Bacillus* sp
9	A9	+	-	+	+	-	-	-	AG	*Bacillus* sp
10	B10	+	-	+	+	-	-	-	A	*Bacillus* sp
11	B11	-	-	+	+	-	-	-	A	*Bacillus* sp
12	B12	+	-	+	+	-	-	A	AG	*Bacillus* sp
13	B13	+	-	+	-	-	-	A	A	*Cellobiococcus* sp
14	B14	-	-	+	-	-	-	A	AG	*Bacillus* sp
15	B15	-	-	+	-	-	-	A	A	*Bacillus* sp
16	B16	-	-	+	+	-	-	-	-	*Bacillus* sp
17	B17	-	-	+	+	-	-	AG	AG	*Bacillus* sp
18	B18	+	-	+	-	-	-	-	-	*Bacillus* sp
19	C19	-	-	+	-	-	-	A	A	*Bacillus* sp
20	C20	+	-	+	-	-	-	-	A	*Bacillus* sp
21	C21	+	-	+	-	-	-	-	-	*Cellobiococcus* sp
22	C22	+	-	+	+	-	-	-	A	*Bacillus* sp
23	C23	+	-	+	+	-	-	-	-	*Bacillus* sp

Table 3: Morphological and microscopic characteristics of fungal isolates from archaeological monument at Augustine University, Ilara Epe, southwest Nigeria

S/N	Isolate code	Morphological Characteristics	Microscopic Characteristics	Isolate Identity
1	AF1	Dark Green Spores	Possession of colourless hyphae, septate and branched	*Aspergillus* sp
2	AF2	Green Spores with white edges	Possession of colourless hyphae, septate and branched	*Aspergillus* sp
3	AF3	White Spores,	Coenocytic, unicellular, non-septate	*Botrytis* sp
4	CF1	Yellow Growth, Growth on vitamin free medium and YEPG Plates (1% yeast extract, 2% glucose, 1% peptone, 1·5% agar)	Unicellular fungus	*Saccharomyces* sp
5	CF2	Yellow Growth, growth on vitamin free medium and YEPG Plates (1% yeast extract, 2% glucose, 1% peptone, 1·5% agar)	Unicellular fungus	*Saccharomyces* sp
6	CF3	Dark Green Spores	Possession of colourless hyphae, septate and branched	*Aspergillus* sp
7	CF4	Dark-Green Spores	Possession of colourless hyphae, septate and branched	*Aspergillus* sp
8	CF5	White filamentous fungi. Tips turned yellow over time	Septate, filamentous, possession of loose grey material on mycelium surface	*Botrytis* sp
9	DF1	White Spores	Septate, filamentous, possession of loose grey material on mycelium surface	*Botrytis* sp

Table 4: Antibiotic susceptibility of isolated bacteria from archaeological monument at Augustine University, Ilara Epe, southwest Nigeria to selected antibiotics

Antibiotics	Number of isolates tested	Number of sensitive isolates (%)	Number of intermediate isolates (%)	Number of resistant isolates (%)
Ceftriaxone (CTR)	23	7 (30.4)	1 (4.4)	15 (65.2)
Erythromycin (ERY)	23	6 (26.1)	2 (8.7)	15 (65.2)
Cloxacillin (CXC)	23	4 (17.4)	0	19 (82.6)
Ofloxacin (OFL)	23	18 (78.3)	4 (17.4)	1 (4.4)
Augmentin (AUG)	23	2 (8.7)	0	21 (91.3)
Ceftazidime (CAZ)	23	3 (13.0)	0	20 (86.9)
Cefuroxime (CFX)	23	4 (17.4)	2 (8.7)	17 (73.9)
Gentamicin (GEN)	23	15 (65.2)	3 (13.0)	5 (21.7)

Discussion:

The results of this investigative study of the microbiological profile of soil samples from Sungbo Eredo Monument on Augustine University, Ilara Epe campus, Lagos, Nigeria revealed a total of twenty-three bacterial isolates belonging to genera *Bacillus, Staphylococcus, Cellobiococcus* and *Micrococcus* and nine fungal isolates belonging to the genera *Saccharomyces*, *Aspergillus* and *Botrytis*. Realini et al., (16) also reported a variety of organisms including bacteria and fungi in the degradation of monuments, Bhatnagar et al., (17) reported *Bacillus* sp., *Staphylococcus* sp*.,* and *Micrococcus* sp., as part of the bacteria found in monuments and objects of archaeological importance, and Omar et al., (18) reported that microbial swabs taken from infected artefacts from museum objects and rock monuments had microorganisms of the genera *Aspergillus, Penicillium, Acremonium, Rhizopus, Cladosporium, Torula* and *Alternaria* with the genus *Aspergillus* being the dominant genus (49.6% of the total fungal isolates) followed by *Penicillium* and *Acremonium.*

The soil is known to harbor enormous diversity of microorganisms among which are bacteria and fungi that may play pivotal roles for ecosystem functioning such as regulating organic matter decomposition, soil dynamics, and mediating nutrient cycling (2). A large percentage of the world's stone cultural heritage monuments have suffered severe and irreversible degradation and deterioration from microorganisms. This damage and deterioration not only cause loss of aesthetic values but presents challenges to researchers exploring the evolution of ancient civilizations (19).

There have been reports of archaeological observations and analysis of samples from excavations on the Sungbo Eredo monument. However, our study is the first to report bacterial and fungal isolates from the Sungbo Eredo monument, which agrees with the submission of previous researchers on the use of microbiology as a tool for providing information and valuable data for past events. There are ample records of such observations from analysis of samples from excavations and other archaeological sites from different parts of the world. Garg and Shukla (7) reported that the

use of microbiology as a tool outside biological fields provides valuable data to complement and upgrade knowledge about past events. For example, on a geological scale, microbiological data can contribute to the understanding and reconstruction of past climatological, environmental, and sedimentary conditions. Moreover, on the time scales of human history and archaeology, microbiological data can contribute to unraveling human cultural habits, microbe-related human diseases, and alterations in cultural artefacts due to biochemical reactions (9).

The results of the antimicrobial susceptibility test showed that ofloxacin and gentamicin were the two antibiotics the bacterial isolates in our study were susceptible to, with 78.3% and 65.2% sensitivity rates respectively, while they were largely resistant to the other antibiotics. This finding is similar to the study of Owolabi and Hekeu (20) who reported high resistance of three bacteria isolates recovered from polluted soils in Lagos and Ota, Nigeria to amoxicillin, augmentin, cefuroxime, erythromycin and cloxacillin, with sensitivity to only ofloxacin and ceftazidime. This implies that soil samples from the monument and polluted soils may serve as reservoir of antimicrobial resistant (AMR) organisms, that have the potential of being transmitted to humans and animals through contamination of water sources or animal feeds.

The pH of the soil samples collected from different spots on the monument showed values ranging from 5.9 to 6.3. These values are indicative of an acidic soil. Li et al., (19) reported soil pH as the key predictor of soil bacterial community composition and diversity. Neina (21) reported that soil pH is the "master soil variable" that influences myriads of soil biological, chemical, and physical properties and processes that affect plant growth and biomass yield. At very acidic or alkaline levels, organic matter mineralization is reduced severely or stopped due to poor microbial activity. Acidic soil had been identified to support the growth of bacteria poorly while favouring the growth of fungi. Beneficial soil microbes prefer a near neutral pH range of 6 - 7 (10). Cho et al., (22) however reported that results and indices regarding diversity, richness, and evenness examined in their study indicated that pH alone might not play the main role for bacterial diversity in soil.

Conclusion:

The results of our investigative study revealed the presence of antibiotic-resistant bacteria (mainly Gram-positive) and fungi on the soil samples of Sungbo Eredo archaeological monument, adding to the existing data on microbial spectrum of archaeological monuments that could be useful for unraveling human cultural habits and microbe-related human diseases. Microbial communities inhabiting the monument will definitely have significant influences on the terrestrial ecosystem dynamics. However, further studies on molecular identification of these microbial spectrum will be required to ascertain their genetic relatedness and ancestral phylogeny, which will be useful for archaeologists in their study of the Sungbo-Eredo ancestral monument.

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Original Article **Open Access**

Prevalence of symptomatic urinary tract infection and bacterial spectrum of diabetic and non-diabetic patients at the two teaching hospitals in Enugu, Nigeria

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Abstract:

Background: Diabetes mellitus is a group of metabolic disorder characterized by relative or absolute lack of insulin. When this condition is not properly managed, it can lead to complications that make diabetic patients vulnerable to urinary tract infections (UTI). The objectives of this study are to determine the prevalence of microbiologically confirmed UTI and the spectrum of uropathogens in diabetic and non-diabetic patients with clinical features of UTI attending the two tertiary hospitals in Enugu State, Nigeria.
Methodology: Clean catch specimen of single mid-stream urine sample was collected from each of 60 (22 males, 38 females) diabetic and 60 (22 males, 38 females) non-diabetic patients enrolled using stratified random sampling method. The samples were cultured on standard microbiological culture media (MacConkey and Blood agar plates) and incubated aerobically at 37◦C for 24 hours. Plates with significant bacteria growth (>10^5 CFU/ml) were processed further for bacterial identification using conventional biochemical test scheme. Antibiotic susceptibility test (AST) of each isolate to 17 selected antibiotics was performed by the modified disc diffusion method.
Results: Of the total 120 patients enrolled, 101 had bacterial pathogens isolated from their voided urine samples; 51 of 60 (85.0%) diabetics and 50 of 60 (83.3%) non-diabetics (p=0.802). Bacteria were isolated in 59.1% (13/22) of diabetic and 54.5% (12/22) of non-diabetic male patients compared to 100% (38/38) isolation rate in diabetic and non-diabetic female patients. The most frequently isolated bacteria in the diabetic patients were *Proteus* spp (18.6%), *Klebsiella* spp (16.9%) and *Escherichia coli* (15.5%) while the most frequently isolated bacteria among the non-diabetic patients were *E. coli* (30.0%), *Proteus* spp (26.3%) and *Enterobacter* spp (14.0%). Apart from *Klebsiella* spp which was more frequently isolated from the diabetic (16.9%) than non-diabetic patients (6%) (p=0.039), the frequency other bacterial pathogen isolation such as *Proteus* spp, *E. coli*, *Enterobacter* spp, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus* spp was not significantly different between the two population groups (p>0.05). The Gram-positive and Gram-negative bacteria were highly sensitive to imipenem in both diabetic and non-diabetic patients, but the isolates from both study groups exhibited low susceptibility to amoxicillin, nitrofuran-toin, cefixime and cefuroxime.
Conclusion: Although the overall frequency of bacterial pathogen isolation in the diabetic and non-diabetic patients was not significantly different, females had a higher pathogen isolation rate than the males, and diabetic females had a higher frequency of polymicrobial infections compared to non-diabetic females and the male population. The high antimicrobial resistance of the isolated bacteria pathogens underscores the need for clinical microbiology laboratory testings to optimize the management of UTI in diabetic patients.

Keywords: UTI; Diabetes mellitus; Antibiogram; Significant bacteriuria; Pathogen; Enugu

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Prévalence des infections urinaires symptomatiques et spectre bactérien des patients diabétiques et non diabétiques dans les deux hôpitaux universitaires d'Enugu, au Nigeria

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Abstrait:

Contexte: Le diabète sucré est un groupe de troubles métaboliques caractérisés par un manque relatif ou absolu d'insuline. Lorsque cette condition n'est pas correctement gérée, elle peut entraîner des complications qui rendent les patients diabétiques vulnérables aux infections des voies urinaires (UTI). Les objectifs de cette étude sont de déterminer la prévalence des infections urinaires confirmées microbiologiquement et le spectre des uropathogènes chez les patients diabétiques et non diabétiques présentant des caractéristiques cliniques des infections urinaires fréquentant les deux hôpitaux tertiaires de l'État d'Enugu, au Nigeria.
Méthodologie: Un échantillon de capture propre d'un seul échantillon d'urine à mi-jet a été prélevé sur chacun des 60 (22 hommes, 38 femmes) patients diabétiques et 60 (22 hommes, 38 femmes) patients non diabétiques inscrits à l'aide d'une méthode d'échantillonnage aléatoire stratifié. Les échantillons ont été cultivés sur des milieux de culture microbiologiques standard (plaques de gélose MacConkey et Blood) et incubés en aérobie à 37°C pendant 24 heures. Les plaques avec une croissance bactérienne significative ($>10^5$ CFU/ml) ont été traitées davantage pour l'identification bactérienne en utilisant un schéma de test biochimique conventionnel. Le test de sensibilité aux antibiotiques (AST) de chaque isolat à 17 antibiotiques sélectionnés a été réalisé par la méthode de diffusion sur disque modifiée.
Résultats: Sur un total de 120 patients recrutés, 101 avaient des agents pathogènes bactériens isolés de leurs échantillons d'urine évacués; 51 des 60 (85,0%) diabétiques et 50 des 60 (83,3%) des non-diabétiques (p=0,802). Les bactéries ont été isolées chez 59,1% (13/22) des patients diabétiques et 54,5% (12/22) des hommes non diabétiques contre un taux d'isolement de 100% (38/38) chez les femmes diabétiques et non diabétiques. Les bactéries les plus fréquemment isolées chez les patients diabétiques étaient *Proteus* spp (18,6%), *Klebsiella* spp (16,9%) et *Escherichia coli* (15,5%) tandis que les bactéries les plus fréquemment isolées chez les patients non diabétiques étaient *E. coli* (30,0%), *Proteus* spp (26,3%) et *Enterobacter* spp (14,0%). Hormis *Klebsiella* spp qui était plus fréquemment isolé chez les diabétiques (16,9%) que les patients non diabétiques (6%) (p=0,039), la fréquence d'isolement d'autres agents pathogènes bactériens tels que *Proteus* spp, *E. coli*, *Enterobacter* spp, *Pseudomonas aeruginosa*, *Staphylococcus aureus* et *Enterococcus* spp n'étaient pas significativement différents entre les deux groupes de population (p>0,05). Les bactéries Gram-positives et Gram-négatives étaient très sensibles à l'imipénème chez les patients diabétiques et non diabétiques, mais les isolats des deux groupes d'étude présentaient une faible sensibilité à l'amoxicilline, à la nitrofurantoïne, au céfixime et à la céfuroxime.
Conclusion: Bien que la fréquence globale d'isolement des agents pathogènes bactériens chez les patients diabétiques et non diabétiques n'était pas significativement différente, les femmes avaient un taux d'isolement des agents pathogènes plus élevé que les hommes, et les femmes diabétiques avaient une fréquence plus élevée d'infections polymicrobiennes par rapport aux femmes non diabétiques. et la population masculine. La haute résistance antimicrobienne des bactéries pathogènes isolées souligne la nécessité de tests de laboratoire de microbiologie clinique pour optimiser la gestion des infections urinaires chez les patients diabétiques.

Mots-clés: UTI; diabète sucré; Antibiogramme; Bactériurie importante; Agent pathogène; Enugu

Introduction:

Simple urinary tract infection (UTI) is inflammation of the urinary system usually manifesting with frequency, urgency, dysuria, or suprapubic pain in affected patients while complicated UTI occurs in the setting of functional or structural abnormalities of the urinary tracts. Quantitative criterium for establishing a UTI is significant bacteriuria of at least 100,000 colony forming unit (CFU) per ml of urine in a voided midstream clean catch specimen (1).

Evidence has shown that the incidence of UTI in diabetic patients is four times higher than in non-diabetic patients (2). It has also been reported that the occurrence of UTI in diabetic patients is higher in people with low socioeconomic status and the resistant patterns of microorganisms isolated in diabetic patients differs from those from non-diabetic patients

(3). Regrettably, treatment of UTI in patients with diabetes is usually associated with worse outcome compared to those without diabetes, seemingly because diabetics seem to have greater difficulty in handling infection once they occur (4,5). Indeed, several aspects of immune system are altered in diabetic patients; polymorphonuclear leukocyte function is compromised particularly when acidosis is present, and adherence, chemotaxis, phagocytosis and bactericidal activity of the leukocytes may also be depressed (6).

UTI is a serious health challenge affecting millions of people each year. In the United States of America, it is estimated from surveys of office practices, hospital-based clinics and emergency departments that there are over eight million cases of UTI annually (7). Many studies have reported incidence of UTI among patients with diabetes mellitus, for example, an observational study of all patients within the United Kingdom general practice research database reported incidence rate of UTI of 46.9 per 1000 persons among diabetic patients versus 29.9 per persons for patients without diabetes (8). An American database study during 2014 (9) found that a UTI diagnosis was more common in subjects with diabetes compared to those without diabetes (9.4% vs 5.7%). Another study in USA with over 70000 patients with diabetes type 2 reported 8.2% with diagnosis of UTI in one year (10). A Canadian study reported that diabetic females were 6-15 times more frequently hospitalized for acute pyelonephritis than non-diabetic females, and diabetic males were hospitalized 3.4-17 times more than non-diabetic males (11). In a study conducted in Europe, bacteriuria was more prevalent among women with diabetes (26%) than in women without diabetes (6%) (12).

In Nigeria, the prevalence of UTI tend to vary depending on the study population and risk factors. For instance, a prevalence of 61.0% was reported among pregnant women (13) while a prevalence of 46% was reported in diabetic patients in Abakaliki (14). Among HIV-infected children and adolescents, a prevalence rate of 32.5% was reported (15) while 43.6% rate was reported in the general population without reported risks for UTI (16). In the present study, we determined the prevalence of symptomatic UTI and spectrum of uropathogens among 60 diabetic and 60 non-diabetic patients attending the two tertiary hospitals in Enugu State, Nigeria.

Materials and method:

Study area

The study was conducted in the two teaching hospitals in Enugu; University of Nigeria Teaching Hospital (UNTH) and Enugu State University Teaching Hospital (ESUTH), Parklane, Enugu State, Nigeria. The study participants were from out-patient clinics and in-patient general wards of these two hospitals.

Study design and study population

This was a cross-sectional comparative study of the study population that included diabetic and non-diabetic patients with clinical symptoms and signs of UTI attending the out-patient clinics or hospitalized in the general wards of the two hospitals.

Ethical approval

Ethical approval was obtained from the Health Research Ethics committee of the University of Nigeria Teaching Hospital Enugu (UNTH/CSA/329/OL.5).

Subject participants and sampling method

A total of 120 participants with symptoms and signs suggestive of UTI were enrolled into the study, including 60 diabetic (22 males and 38 females), and 60 non-diabetic patients (22 males and 38 females) using stratified random sampling technique. Pooled subjects were divided into groups with similar attributes. Then, simple random sample was taken from each group to ensure that different segments in the population were equally represented. Inclusion criteria were patients of all gender and age groups with symptoms and signs suggestive of UTI, history of diabetes (for the diabetics), and no history of diabetes (for the non-diabetics). Exclusion criteria included pregnancy, history of other underlying illness like HIV/AIDS, and antibiotic usage within 14 days preceding the enrolment

Diabetic patients were enrolled into the study during their regular visits at the endocrinology units of the hospitals through the assistance of their physicians and nurses, while non-diabetics were apparently healthy individuals who were on visit for diabetic status check and confirmed by glucometer measurement of fasting blood glucose (FBS)≤126 mg/dl.

Collection of urine samples

Urine samples (about 10ml) were collected from the study participant who were first

educated on how to collect a 'clean-catch' midstream urine specimen and the importance of avoiding contamination. They were advised on proper hand washing prior to collection and separation of labia (in females). Urine samples were collected into sterile containers with boric acid preservative and transported to the laboratory within 2 hours.

Urine microscopy, culture and bacteria isolation

Each urine sample was cultured on MacConkey agar and Blood agar plates which had been prepared according to manufacturer's instructions. The urine was properly mixed by rotating the container. An inoculating wire loop of 3mm diameter was used to inoculate a loopful (~10µl) of the urine sample onto freshly prepared agar and the plates were incubated aerobically at 37°C for 24 hours (17). The dip stick test on each urine sample was performed using Combi-10 urine test strips to determine urinary nitrite level and protein/glucose as adjunctive in detecting UTI and screening for diabetic nephropathy, respectively. Wet preparation of centrifuged urine was also examined using the 40x objective lens to detect blood, white blood cells (WBC), epithelial cell, casts, and other cells in the voided urine.

Identification of bacterial isolates

Culture plates were examined after 24 hours incubation for colony appearance, size, colour, pigmentation, haemolysis, consistency (mucoid, rough, stringy etc), odor, and evidence of lactose fermentation on MacConkey agar. Only plates with significant bacteriuria (> 10^5 CFU/ml or > 1000 colonies) were processed for bacterial identification. Isolates were identified by their Gram stain reactions and by conventional biochemical tests which included catalase and coagulase tests for Gram-positive bacteria, and oxidases, indole, methyl red, Voges Proskauer, urease, litmus milk decolorization, Kligler iron agar (KIA) and citrate utilization tests for Gram-negative bacteria as previously described (17).

Antibiotic susceptibility testing

Antibiotic susceptibility testing for each isolate was carried out by the Kirby Bauer disc diffusion technique using the Clinical and Laboratory Standards Institute (CLSI) guideline (18). A total of 17 antibiotic discs (HiMedia, Mumbai, India) were employed, which included; ciprofloxacin (10µg), cefuroxime (10µg), amoxicillin (30µg), chloramphenicol (10µg), ofloxacin (10µg), gentamicin (10µg), erythromycin (10µg), ceftriaxone (30µg), streptomycin (30 µg), nitrofurantoin (100µg), levofloxacin (5µg), norfloxacin (10µg), cefixime (5µg), ampicillin/ cloxacillin (30µg), imipenem (10µg), clindamycin (10µg), and pefloxacin (10µg). A suspension of pure colonies of isolated bacteria were inoculated into 0.9% saline solution and standardized by comparing with 0.5 MacFarland turbidity standard. The surface of sterile Mueller Hinton (MH) agar plate was inoculated using sterile cotton swab soaked in bacterial suspension. Antibiotic discs were placed on inoculated agar plates using sterile forcep, and the plates incubated aerobically at 37°C for 24 hours. The diameter of zones of inhibition to each antibiotic disc was measured using a calibrated meter rule and results interpreted as sensitive or resistant according to CLSI guideline (18).

Data analysis

Data were analysed using the Statistical Package for the Social Sciences (SPSS) version 23.0 window-based program. Two tail Chi-square test was used to determine association between two categorical variables and $p < 0.05$ was regarded as significant at 95% confidence interval.

Results:

A total of 120 patients; 60 diabetics (22 males, 38 females) and 60 non-diabetics (22 males, 38 females) with clinical symptoms and signs of UTI were studied. Of the 120 patients, 101 had bacterial pathogens isolated from their voided urine samples; 51 of 60 (85.0%) diabetics and 50 of 60 (83.3%) non-diabetics. The frequency of bacterial pathogen isolation (microbiologically documented UTI) was not significantly different between the two groups (p=0.802) (Table 1).

The most frequently isolated pathogens in the diabetic patients were *Proteus* spp (18.6%), *Klebsiella* spp (16.9%) and *Escherichia coli* (15.5%) while the most frequently isolated pathogens among the non-diabetic patients were *E. coli* (30.0%), *Proteus* spp (26.3%) and *Enterobacter* spp (14.0%). Apart from *Klebsiella* spp which was significantly more frequently isolated from the diabetic (16.9%) than non-diabetic patients (6%) (p=0.039), the frequency of isolation of other bacterial pathogens such as *Proteus* spp, *E. coli*, *Enterobacter* spp, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus* spp

was not significantly different between the two population groups (p>0.05). However, *Salmonella* spp, *Citrobacter* spp and *Providencia* spp were exclusively isolated from diabetic patients while *Corynebacterium* spp was the only pathogen exclusively isolated from one non-diabetic patient.

As shown in Table 2, bacterial pathogens were isolated in only 59.1% (13/22) of diabetic and 54.5% (12/22) of non-diabetic male patients compared to 100% (38/38) isolation rate in the diabetic and non-diabetic female patients. A total of 46 bacterial pathogens were isolated from the 38 diabetic females indicating polymicrobial infections in some of them while there was only one pathogen (monomicrobial infection) isolated from the non-diabetic female patients and from both the male diabetic and non-diabetic patients.

Tables 3a and 3b showed the percentage sensitivity of the bacterial isolates from diabetic and non-diabetic patients to selected antibiotics. The Gram-positive and Gram-negative bacteria were highly sensitive to imipenem in both diabetic and non-diabetic patients, but the isolates from both study groups exhibited low susceptibility to amoxicillin, nitrofurantoin, cefixime and cefuroxime.

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Table 1: Frequency distribution of bacterial isolates from diabetic and non-diabetic patients with urinary tract infections in the two teaching hospitals in Enugu, Nigeria

Bacterial Isolates	**Diabetics**	**Non-diabetics**	**Total**	***p* value**
	n (%)	**n (%)**	**n (%)**	
Proteus spp	11 (18.6)	13 (26.3)	24 (22.0)	0.332
Klebsiella spp	10 (16.9)	3 (6.0)	13 (11.9)	0.039*
Escherichia coli	9 (15.3)	15 (30.0)	24 (22.0)	0.171
Pseudomonas aeruginosa	7 (11.9)	3 (6.0)	10 (9.2)	0.186
Salmonella spp	6 (10.2)	0	6 (5.5)	NA
Enterobacter spp	2 (3.4)	7 (14.0)	9 (8.3)	0.083
Citrobacter spp	1 (1.7)	0	1 (0.9)	NA
Providencia spp	1 (1.7)	0	1 (0.9)	NA
Staphylococcus aureus	6 (10.2)	5 (10.0)	11 (10.1)	0.751
Enterococcus spp	6 (10.2)	3 (6.0)	9 (8.3)	0.298
Corynebacterium spp	0	1 (2.0)	1 (0.9)	NA
Total no of isolates	**59**	**50**	**109**	
No of patients with isolates	51 (85.0)	50 (83.3)	101 (84.2)	0.802
No of patients without isolates	9 (15.0)	10 (16.7)	19 (15.8)	
Total no of patients	**60 (100)**	**60 (100)**	**120**	

n = number of patients; * = statistically significant; NA = not applicable

Table 2: Gender distribution of bacterial isolates from diabetic and non-diabetic patients with urinary tract infection in the two teaching hospitals in Enugu, Nigeria

Bacterial isolates	**Diabetics (n %)**				**Non-diabetics (n %)**			
	Males	**Females**	**Total**	***p* value**	**Males**	**Females**	**Total**	***p* value**
Proteus spp	2 (15.4)	9 (19.2)	11 (18.6)	0.756	4 (33.3)	9 (18.8)	13 (26.3)	0.272
Klebsiella spp	1 (7.7)	9 (19.2)	10 (16.9)	0.326	0	3 (6.3)	3 (6.0)	NA
Escherichia coli	1 (7.7)	8 (17.0)	9 (15.3)	0.376	3 (25)	12 (25.0)	15 (30.0)	0.100
Pseudomonas aeruginosa	2 (15.4)	5 (10.6)	7 (11.9)	0.637	1 (8.3)	2 (4.2)	3 (6.0)	0.553
Salmonella spp	1 (7.7)	5 (10.6)	6 (10.2)	0.754	0	0	0	NA
Enterobacter spp	1 (7.7)	1 (2.1)	2 (3.4)	0.322	1 (8.3)	6 (12.5)	7 (14.0)	0.687
Citrobacter spp	0	1 (2.1)	1 (1.7)	NA	0	0	0	NA
Providencia spp	0	1 (2.1)	1 (1.7)	NA	0	0	0	NA
Staphylococcus aureus	3 (23.1)	3 (6.4)	6 (10.2)	0.075	2 (16.7)	3 (6.3)	5 (10.0)	0.242
Enterococcus spp	2 (15.4)	4 (8.5)	6 (10.2)	0.464	1 (8.3)	2 (4.2)	3 (6.0)	0.553
Corynebacterium spp	0	0	0	NA	0	1 (2.1)	1 (2.0)	NA
Total no of isolates	**13**	**46**	**59**		**12**	**38**	**50**	
No of patients with isolates	13 (59.1)	38 (100)	51 (85)		12 (54.5)	38 (100)	50 (83.3)	
No of patients negative for isolates	9 (40.9)	0	9		10 (45.5)	0	10	
Total no of patients	**22**	**38**	**60**		**22**	**38**	**60**	

n = number of patients; NA = not applicable

Discussion:

The overall prevalence rate of microbiologically confirmed bacterial UTI in this study was not significantly different (p=0.802) among diabetic (85%, 51/60) and non-diabetic patients (83.3%, 50/60). This disagrees with the study of Saleem and Daniel (2) in Bangalore, India which reported that diabetic patients (56.4%) were significantly at increased risk of UTI than non-diabetics (43.6%). However, diabetic patients in our study who had microbiologically confirmed UTI had more bacterial pathogens isolated from their urine (51 patients had 59 pathogens) compared to the non-diabetic patients (50 patients had 50 pathogens), implying that some of the diabetic patients had polymicrobial infections, a situation that frequently occurs in the elderly, immune compromised, and those with indwelling catheters, HIV, malignancies and diabetes (19), the frequency of which may be as high as 39% in UTI (20).

The female patients in our study (both diabetic and non-diabetic) had 100% pathogen isolate rate from their urine samples with some of the female diabetics having polymicrobial infections (38 females with 46 isolated pathogens) while all the female non-diabetics had monomicrobial infection (38 females with 38 isolated pathogens). Comparatively, the diabetic males had 59.1% (13 of 22) pathogen isolation rate while the non-diabetic males had 54.5% (12 of 22) isolation rate.

The anatomical structure of the female urogenital system, with shorter urethra and the close proximity to the anorectal region, may partly contribute to the higher pathogen isolation rates from the female patients as previously established (21). However, the study by Otajevwo (22) in an outpatient setting in Benin City, Nigeria, reported a significantly

higher prevalence rate of UTI in males (58.3%) than females (41.7%), which is contrary to the finding of our current study and those of many others.

Table 3a: Percentage sensitivity of bacterial isolates from diabetic patients to selected antibiotics

Bacterial isolates	**No**	**Percentage (%) of isolates sensitive to antibiotics**										
Gram negative bacteria		**N**	**GN**	**CIP**	**C**	**OF**	**CF**	**PF**	**CT**	**AMX**	**ST**	**IMP**
Proteus spp	11	9.1	63.6	63.6	18.2	45.5	9.1	36.4	36.4	27.3	45.5	90.9
Klebsiella spp	10	0	30.0	20.0	20.0	20.0	0	50.0	30.0	0	0	90.0
Escherichia coli	9	33.3	66.7	44.4	33.3	44.4	22.2	44.4	44.4	22.2	44.4	88.9
Pseudomonas aeruginosa	7	42.9	57.1	42.9	28.6	42.9	28.6	42.9	71.4	42.9	42.9	71.4
Salmonella spp	6	33.3	83.3	50.0	50.0	50.0	50.0	50.0	66.7	50.0	50.0	50.0
Enterobacter spp	2	50.0	50.0	100.0	50.0	50.0	50.0	50.0	100.0	50.0	50.0	100.0
Citrobacter spp	1	0	100.0	100.0	0	100.0	0	100.0	100.0	100.0	100.0	100.0
Providencia spp	1	100.0	100.0	100.0	0	100.0	0	100.0	100.0	100.0	100.0	100.0
Gram positive bacteria		**NB**	**CIP**	**GN**	**OF**	**CD**	**E**	**CT**	**AMP**	**CE**	**LV**	**IMP**
Staphylococcus aureus	6	0	33.3	66.7	33.3	83.3	50.0	50.0	33.3	0	66.7	100.0
Enterococcus spp	6	50.0	83.3	100.0	83.3	83.3	66.7	50.0	33.3	33.3	100.0	100.0

CIP= Ciprofloxacin (10µg), CF= Cefuroxime (10µg), AMX= Amoxicillin, (30µg), C= Chloramphenicol (10µg), OF= Ofloxacin (10µg), GN= Gentamicin (10µg), E= Erythromycin (10µg), CT= Ceftriaxone (30µg), ST= Streptomycin (30µg), N= Nitrofurantoin (100µg), LV= Levofloxacin (5µg), NB= Norfloxacin (10µg), CE= Cefixime (5µg), AMP= Ampicillin/cloxacillin (30µg), IMP= Imipenem (10µg), CD= Clindamycin (10µg), PF= Pefloxacin (10µg)

Table 3b: Percentage sensitivity of bacterial isolates from non-diabetic patients to selected antibiotics

Bacterial isolates	**No**	**Percentage (%) of isolates sensitive to antibiotics**										
Gram negative bacteria		**N**	**GN**	**CIP**	**C**	**OF**	**CF**	**PF**	**CT**	**AX**	**ST**	**IMP**
Proteus spp	13	53.9	30.8	30.8	15.4	30.8	0	23.1	61.5	7.7	30.8	100.0
Klebsiella spp	3	66.7	100.0	100.0	66.7	100.0	33.3	100.0	100.0	33.3	66.7	100.0
Escherichia coli	15	66.7	40.0	33.3	20.0	20.0	0	20.0	73.3	6.7	26.7	93.33
Pseudomonas aeruginosa	3	66.7	33.3	66.8	33.3	33.3	0	33.3	33.3	0	33.3	100.0
Enterobacter spp	7	42.9	57.1	42.9	42.9	42.9	28.6	42.9	71.4	0	42.9	85.7
Gram positive bacteria		**NB**	**CIP**	**GN**	**OF**	**CD**	**E**	**CT**	**AP**	**CE**	**LV**	**IMP**
Staphylococcus aureus	5	0	20.0	0	20.0	60.0	20.0	0	0	0	60.0	100.0
Enterococcus spp	3	0	0	0	0	66.7	0	0	0	0	33.3	100.0
Corynebacterium spp	1	0	0	0	0	100.0	100.0	0	0	0	100.0	100.0

CIP= Ciprofloxacin (10µg), CF= Cefuroxime (10µg), AMX= Amoxicillin, (30µg), C= Chloramphenicol (10µg), OF= Ofloxacin (10µg), GN= Gentamicin (10µg), E= Erythromycin (10µg), CT= Ceftriaxone (30µg), ST= Streptomycin (30µg), N= Nitrofurantoin (100µg), LV= Levofloxacin (5µg), NB= Norfloxacin (10µg), CE= Cefixime (5µg), AMP= Ampicillin/cloxacillin (30µg), IMP= Imipenem (10µg), CD= Clindamycin (10µg), PF= Pefloxacin (10µg)

The most frequently isolated bacteria pathogens among the diabetic patients in our study were *Proteus* spp (18.6%), *Klebsiella* spp (16.9%) and *E. coli* (15.5%), which somewhat agrees with the study by Alebiosu et al., (23) in southwest Nigeria, which reported changing pattern of bacteriuria, with *Klebsiella* spp (42.4%) and *Proteus* spp (38.2%) accounting for the most common cause of bacteriuria among diabetics. However, bacteria isolated from the non-diabetic patients in our study were predominantly *E. coli* (30%) and *Proteus* spp (26.3%), which agrees with the study of Akinnibosun and Iriakpe (24) in Benin City, Nigeria which reported *E. coli* (44.4%) as the predominant bacteria followed by *Proteus mirabilis* (22.2%).

Among the diabetics, gentamicin, ofloxacin, imipenem and ceftriaxone were the most active antibiotics against Gram-positive and Gram-negative bacteria. Generally, resistance to cefuroxime, chloramphenicol, nitrofurantoin, norfloxacin, cefixime, and amoxicillin was high. Among the non-diabetics, imipenem was the most active antibiotics against both Gram positive and Gram-negative bacteria while resistance to norfloxacin, gentamicin, ceftriaxone, ampicillin/cloxacillin, cefixime, and cefuroxime was high. These patterns are similar to those reported by Gangoue et al., (25) in Yaounde, central Cameroon where all Gram-negative isolates were resistant to amoxicillin, with high susceptibility to ofloxacin and imipenem. The high resistance rates reported in our study also agrees with the findings of Tula et al., (26) and Khoshbakht et al., (27).

The high antimicrobial resistance (AMR) rates reported among bacterial pathogens in developing countries often result from overuse and misuse of antibiotics as a result of poor antibiotic prescribing practices by healthcare workers, and self-medication by patients and the general populace. AMR has become a public health challenge around the globe for the last few decades (28). The development of AMR in bacteria population is caused by swift evolution of bacteria genome under selective antibiotic pressure (29), and resistant mutants usually survive in environment such as hospitals where several antimicrobials are usually prescribed and infection prevention and control practices may be suboptimal. Therefore, proper use of antibiotics, especially among the vulnerable populations such as the diabetics, is highly desirable.

Conclusion:

Although the overall frequency of isolation of bacterial pathogens in the two study populations was not significantly different, female population had a higher pathogen isolation rate than the male population, and diabetic females had a higher frequency of polymicrobial infections compared to non-diabetic female and the male population. The high antimicrobial resistance of the isolated bacteria pathogens underscores the need for clinical microbiology laboratory testings to optimize the management of UTI in diabetic patients.

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Original Article **Open Access**

Prevalence of and factors associated with significant bacteriuria among pregnant women attending the antenatal clinic of Adeoyo Maternity Hospital, Yemetu, Ibadan, Nigeria

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Abstract:

Background: Significant bacteriuria is commonly reported in pregnancy which greatly predisposes pregnant women to urinary tract infection (UTI), one of the commonest health challenges in pregnancy worldwide especially in developing countries such as Nigeria. The objectives of this study are to determine the prevalence of and factors associated with significant bacteriuria among pregnant women attending the antenatal clinic (ANC) of Adeoyo Maternity Hospital, Yemetu, Ibadan, Nigeria, as well as determine the bacterial aetiology and antimicrobial susceptibility patterns of the isolates.
Methodology: This is a laboratory-based cross-sectional study of 206 pregnant women between the ages of 15 and 47 years attending the ANC of the hospital, selected by simple random sampling method. Demographic and clinical data were obtained from the subjects using a structured questionnaire. Clean-catch specimen of mid-stream voided urine was collected from each subject participant. Urine samples were processed for culture and isolation of significant bacterial pathogens using standard bacteriological methods, and isolates identified to species level by the combination of colony morphology, Gram reaction, conventional biochemical tests and Analytical Profile Index (API) 20E test kits. Antibiotic susceptibility testing of the isolates to selected antibiotics was performed using the disk diffusion method.
Results: The prevalence of significant bacteriuria in the study population was 8.7% (18/206), with 27.8% (5/18) symptomatic and 72.2% (13/18) asymptomatic. All isolated bacteria were Gram-negative with the most frequent being *Escherichia coli* 9 (50.0%), followed by *Klebsiella pneumoniae* 6 (33.3%), *Pseudomonas aeruginosa* 1 (5.6%), *Acinetobacter haemolyticus* 1 (5.6%) and *Enterobacter aerogenes* 1 (5.6%). The isolates were most sensitive to gentamicin (100%) and nitrofurantoin (94.4%), while they demonstrated highest resistance to amoxicillin-clavulanic acid (33.3%). Significant bacteriuria was associated with pyuria (p=0.01) and past history of UTI (p=0.004).
Conclusions: The high prevalence of asymptomatic significant bacteriuria in this study necessitates the need for screening and treatment of pregnant women for this entity to prevent the subsequent development of UTI that may have grave consequences on pregnancy outcome.

Keywords: urinary tract infection; significant bacteriuria; pregnant women; factors

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Prévalence et facteurs associés à une bactériurie significative chez les femmes enceintes fréquentant la clinique prénatale de la maternité Adeoyo, Yemetu, Ibadan, Nigéria

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Abstrait:

Contexte: Une bactériurie importante est couramment signalée pendant la grossesse, ce qui prédispose grandement les femmes enceintes aux infections des voies urinaires (IVU), l'un des problèmes de santé les plus courants pendant la grossesse dans le monde, en particulier dans les pays en développement comme le Nigéria. Les objectifs de cette étude sont de déterminer la prévalence et les facteurs associés à une bactériurie significative chez les femmes enceintes fréquentant la clinique prénatale (CPN) de l'hôpital de maternité Adeoyo, Yemetu, Ibadan, Nigéria, ainsi que de déterminer l'étiologie bactérienne et les modèles de sensibilité aux antimicrobiens de les isolats.

Méthodologie: Il s'agit d'une étude transversale en laboratoire portant sur 206 femmes enceintes âgées de 15 à 47 ans fréquentant les CPN de l'hôpital, sélectionnées par une méthode d'échantillonnage aléatoire simple. Les données démographiques et cliniques ont été obtenues auprès des sujets à l'aide d'un questionnaire structuré. Un échantillon propre d'urine évacuée à mi-chemin a été recueilli auprès de chaque participant sujet. Les échantillons d'urine ont été traités pour la culture et l'isolement d'agents pathogènes bactériens importants à l'aide de méthodes bactériologiques standard, et les isolats ont été identifiés au niveau de l'espèce par la combinaison de la morphologie de la colonie, de la réaction de Gram, des tests biochimiques conventionnels et des kits de test de l'indice de profil analytique (API) 20E. Les tests de sensibilité aux antibiotiques des isolats aux antibiotiques sélectionnés ont été effectués à l'aide de la méthode de diffusion sur disque.

Résultats: La prévalence de la bactériurie significative dans la population étudiée était de 8,7% (18/206), avec 27,8% (5/18) symptomatique et 72,2% (13/18) asymptomatique. Toutes les bactéries isolées étaient Gram-négatives, la plus fréquente étant *Escherichia coli* 9 (50,0%), suivie de *Klebsiella pneumoniae* 6 (33,3%), *Pseudomonas aeruginosa* 1 (5,6%), *Acinetobacter haemolyticus* 1 (5,6%) et *Enterobacter aerogenes* 1 (5,6%). Les isolats étaient les plus sensibles à la gentamicine (100%) et à la nitrofurantoïne (94,4%), alors qu'ils présentaient la résistance la plus élevée à l'amoxicilline-acide clavulanique (33,3%). Une bactériurie significative était associée à une pyurie (p=0,01) et à des antécédents d'infection urinaire (p=0,004).

Conclusions: La prévalence élevée de bactériurie significative asymptomatique dans cette étude nécessite le dépistage et le traitement des femmes enceintes pour cette entité afin de prévenir le développement ultérieur d'infections urinaires pouvant avoir de graves conséquences sur l'issue de la grossesse.

Mots-clés: infection des voies urinaires; bactériurie importante; femmes enceintes; les facteurs

Introduction:

The urinary system includes the organs involved in the formation, collection, storage, and excretion of urine from the body, which includes two kidneys, two ureters, a bladder and urethra. Urine formed in the kidney is a sterile fluid that can serve as a good culture medium for proliferation of bacteria (1). The urethra however may contain few commensals and also the perineum has a wide variety of Gram–positive and Gram–negative organisms which can contaminate urine during voiding. In the females, urine may become contaminated with organisms from the vagina which is often indicated by the presence of epithelial cells (moderate to many) and a mixed bacterial flora.

Urinary tract infection (UTI) is the inflammation of the urinary tract caused by the presence and multiplication of microorganisms. It is perhaps the single most common bacterial infection of mankind (2). The normal urinary tract is resistant to colonization and subsequent infection by bacteria except for the urethral mucosa and the renal medulla, which appear to be relatively susceptible to colonization and subsequent infection because the urinary tract can efficiently and rapidly eliminate virulent and non-virulent microorganisms. UTI is known to cause short-term morbidity in terms of fever, dysuria, and lower abdominal pain but may result in permanent scarring of the kidneys. All age groups and gender are affected although UTI is commoner in females than males due to the shortness of the female urethra, pregnancy and easy contamination of the urinary tract with faecal flora. Urinary tract infection occurs in both hospitalized and non-hospitalized individuals, with serious impacts on the socio-economic life of the affected individual and the society, and also account for a large proportion of antibacterial drug consumption (3).

Urinary tract infection in pregnancy is among the most common health problems worldwide, especially in developing countries, which can lead to poor maternal and perinatal outcome. Untreated UTI can be associated with serious obstetric complications such as intrauterine growth retardation (IUGR), pre-eclampsia, caesarean section (CS) and preterm delivery. Both symptomatic and asymptomatic UTI are common in pregnancy, and asymptomatic bacteriuria can lead to pyelonephritis later in preg- nancy or during puerperium, with several adverse outcomes both on the mother and the fetus. Yasemi et al., (4) reported that children born by mothers with pyelonephritis are more prone to impairment of mental and motor development. Urinary tract infection in pregnancy commonly starts at week 6 and becomes

most frequent during weeks 22–24 (5). Various physiological, anatomical, and personal factors contribute to this problem during pregnancy such as urethral dilation, increased bladder volume, and decreased bladder tone with urinary stasis. Moreover, a breach of personal hygiene increases the risk of infection. Furthermore, up to 70% of women during pregnancy have glucose in urine, which increases the chances of bacterial growth in the urine.

Investigation of UTI accounts for a significant part of the work load in clinical microbiology laboratories and enteric bacteria, in particular, *Escherichia coli*, remain the most frequent bacterial pathogen causing UTI, although there are changes in the types and distribution of pathogens involved in UTI (6). Bacteriuria (the presence of bacteria in urine) is said to be significant when there are at least 100,000 bacteria colonies of a single pathogen per milliliter in a freshly voided urine, collected by the mid-stream clean catch technique. The original criterion for diagnosis required ≥ 10^5 CFU/ml on two consecutive clean catch samples (7). However, detection of ≥ 10^5 CFU/ml in a single voided mid-stream urine sample is accepted as adequate and a more practical alternative (7). Contaminated cultures usually show fewer than 10^3 CFU/ml of urine. Most voided urine specimens will contain fewer than 10^4 contaminating organisms per ml provided the urine has been collected with care to minimize contamination and the specimen is examined soon after the collection before the commensal microbes significantly multiplied. Colony counts less than 10^5 CFU/ml of urine can however be significant in patients with pyelonephritis, in whom the bacteria are multiplying mainly in the kidneys rather than in the urinary bladder, in patients with symptoms of lower UTI but with low colony counts which may indicate "urethral syndrome", patients with prostatitis and epididymitis, and patients with fungal UTI (8).

Urinary tract infection can be symptomatic or asymptomatic (9). Asymptomatic bacteriuria (ASB), which is sometimes used synonymously with asymptomatic UTI, is defined as the persistent and actively multiplying bacteria within the urinary tract without symptoms or signs (10). There are several factors associated with ASB including socioeconomic status with indigent women having a five-fold greater incidence of bacteriuria compared with non-indigent populations. Other factors reported to be significantly associated with ASB include diabetes, sickle cell disease and trait, multiparity, previous history of UTI, and anatomic or functional urinary tract abnormalities (11). Since ASB and obvious UTI have a close association, screening and treatment of pregnant women with ASB may also help to reduce the adverse outcome such as preterm labour and low birth weight. Globally, the overall prevalence of significant bacteriuria in pregnancy is known to vary from 4%-7%, although a range of 2-11% has been reported (12). However, the prevalence rate among pregnant Nigerian women has been variously reported to be between 1.3%-60% (6,13-23), but the prevalence in our hospital remains unknown. This study was therefore designed to determine the prevalence and factors associated with significant bacteriuria among pregnant women attending the antenatal clinic of Adeoyo Maternity Hospital, Yemetu, Ibadan, Nigeria, as well as to determine the bacteriology and antibiotic susceptibility of the isolates to commonly prescribed antibiotics in pregnancy in our environment.

Materials and method:

Study area

The study was conducted in Ibadan, the capital city of Oyo State, southwest Nigeria. Oyo State is divided into 33 Local Government Areas (LGAs) with an estimated population of 3.5 million. Ibadan, which occupies an area of 828 km^2, lies on Longitude 3°5' East of Greenwich meridian and Latitude 7°23' North of the Equator. There are 11 LGAs in Ibadan metropolitan area consisting of five urban and six semi-urban LGAs. The population is large and heterogeneous with people of different religion, culture and ethnic groups, but predominantly of the Yoruba tribe.

Study population, sample size and sampling

The study population comprises pregnant women attending antenatal clinic of the hospital in January and February, 2020. The sample size was calculated using the Cochran formula; $N = Z/(D)^2\ p\ (1\text{-}p)$, where N = sample size, Z = standard normal value corresponding to 95% confidence interval set at 1.96, D = degree of error tolerance at 5%, P = proportion used for estimation, obtained from previous studies in the study environment (14% was chosen from prevalence range of 1.3% - 60% reported in Nigeria). This gave the calculated sample size (N) of 185, which was corrected for 10% non-response (r) rate using the adjustment formula; Nf = N/(1-r), to give the adjusted sample size of 206.

Pregnant women between the ages of 15 to 47 years were consecutively recruited from the study population by the help of the nurses and midwives in the clinic using simple random sampling technique until the required

sample size of 206 was obtained. Eligibility was based on willingness of the women to participate but those on any antibiotic therapy within two weeks preceding recruitment were excluded from the study.

Ethical considerations

The study proposal was approved by the University of Ibadan/University College Hospital Ethical committee and the Oyo State Ministry of Health. A written informed consent was obtained from each study participant.

Data and sample collection

Socio-demographic information such as age, occupation, and other clinical information such as parity and previous history of UTI were obtained using a structured questionnaire. About 10 ml of clean catch specimen of voided early morning urine were collected from each subject into a sterile, screw-capped wide neck urine container, which had been correctly labeled and given to each subject. Each participant was instructed by the nurses and midwives on the method of urine collection to avoid contamination.

The specimens were transported to the Medical Microbiology and Parasitology laboratory of the University College Hospital (UCH), Ibadan in iced packs, and processed within one hour of collection. However, where immediate processing was not possible, the samples were refrigerated at 4°C to avoid bacteria multiplication.

Microscopic examination of urine

For urinary sediment examination, the urine samples were mixed and aliquots were centrifuged at 5000rpm for 5 min. Deposits were examined using both 10x and 40x objectives of the light microscope. Microscopic findings of pyuria (leukocyte count ≥ 5/HPF) were considered as supportive evidence of UTI. A smear of the urine sample was made on a grease free clean slide, allowed to air dry, Gram stained, and examined microscopically using 100x (oil immersion) objective lens.

Isolation & identification of bacteria from urine

The well-mixed urine sample was inoculated using a sterile calibrated wire loop (0.001 ml) onto Cysteine Lactose Electrolyte Deficient (CLED) agar and 5% sheep blood agar as described in Cheesbrough (24). The culture plates were incubated aerobically at 37^{o}C for 24 hours. Colony counts yielding bacterial growth of 10^5 CFU/ml (≥ 100 colonies on culture plate) was regarded as significant bacteriuria.

Colonies of bacteria on culture plates showing significant growth were characterized and identified to species level by Gram stain, motility test, conventional biochemical test and commercial Analytical Profile Index (24). For identification using API, colonies from overnight cultures were reconstituted in normal saline to 4.0 McFarland standard from which aliquots were taken and inoculated onto API test strips. These strips were incubated at 36^{0}C for 18 hours and then observed for colour reactions and the readings documented. The readings were translated into numbers and the numbers were interpreted into bacteria species using the API software.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed on pure cultures of all significant isolated uropathogens using the disk diffusion method of Bauer et al., (25) against selected antibiotics (Oxoid, UK); amoxicillin/clavulanic acid (20/10µg), ceftriaxone (30µg), gentamicin (10µg), nitrofurantoin (300µg), ceftazidime (30 µg), and cefuroxime (30µg).

Pure colonies of each isolate were suspended in 5 ml normal saline to prepare the inoculum, which was standardized to 0.5 McFarland standard. The surface of Mueller-Hinton agar plates was inoculated with the suspension using sterile swabs, after which antibiotic disks were placed on the inoculated plates equidistant from each other, using sterile forceps and the plates incubated aerobically at 37°C for 24 hours. The diameters of zones of inhibition around each antibiotic disk were measured to the nearest millimeter using a caliper. Isolates were classified as sensitive, intermediate or resistant according to the CLSI (26) guidelines. *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) were used as reference control strains for identification and susceptibility testing.

Data analysis

Data entry and analysis were done using SPSS version 25.0 software package. The frequency of bacteria isolation from urine was presented in percentages. Chi-square test was used to measure association between socio-demographic characteristics and prevalence of significant bacteriuria, and $p<0.05$ was considered to be statistically significant.

Results:

A total of 206 pregnant women attending the antenatal clinic of Adeoyo Maternity Hospital, Yemetu, Ibadan, Oyo State, Nigeria, were studied. The age of the participants ranged from 15 to 47 years with a mean age of 28.64±5.921 years. Of these 206 women, 18

had significant bacteriuria, giving the overall prevalence of 8.7%, 5 (27.8%) of whom were symptomatic and 13 (72.2%) were asymptomatic (Table 1). Table 2 shows the prevalence of significant bacteriuria with respect to socio-demographic and clinical characteristics of the pregnant women. Chi-square test showed that significant bacteriuria was associated with only pyuria (p=0.01) and past history of UTI (p= 0.004), while other factors such as age group, occupation, educational level, parity, gestational age and history of DM were not significantly associated with significant bacteriuria (p>0.05).

From the 18 subjects with significant bacteriuria, 5 bacterial species (all Gram negative and monomicrobial) were isolated, the most prevalent uropathogen was *Escherichia coli* 50% (9/18) followed by *Klebsiella pneumoniae* 33.3% (6/18), and *Pseudomonas aeruginosa* 5.6% (1/18), *Acinetobacter haemolyticus* 5.6% (1/18) and *Enterobacter aerogenes* 5.6% (1/18) (Table 3). Antibiotic susceptibility of the bacterial isolates as presented in Table 4 showed that 100%, 94.4%, 94.4%, 88.9%, 88.2% and 66.7% of isolates were sensitive to gentamicin, nitrofurantoin, ceftriaxone, ceftazidime, cefuroxime, and augmentin respectively. Isolates were least sensitive to augmentin, and none of the isolates, including *P. aeruginosa*, were resistant to gentamicin. *Escherichia coli*, the commonest isolate showed high sensitivity to all tested antibiotics. Conversely, *K. pneumoniae* showed the highest resistance rate to the antibiotics except gentamicin. *Pseudomonas aeruginosa* was sensitive to gentamicin and ceftazidime but resistant to nitrofurantoin and augmentin.

Table 1: Frequency of bacteriuria among pregnant women attending the antenatal clinic of Adeoyo Maternity Hospital, Yemetu, Ibadan

Bacteriuria/symptomatology	**Frequency**	**Percentage (%)**
Significant bacteriuria	18	8.7
Non-significant bacteriuria	90	43.7
Growth of bacterial contaminants	10	4.9
No bacterial growth	88	42.7
Total	**206**	**100**
Significant bacteriuria		
Symptomatic	5	27.8
Asymptomatic	13	72.2
Total	**18**	**100**

Table 2: Socio-demographic and clinical characteristics of pregnant women with urinary tract infections attending the antenatal clinic of Adeoyo Maternity Hospital, Yemetu, Ibadan, Nigeria

Characteristics	No of patients (%)	No positive for bacteria (%)	Chi square or Odds Ratio	95% CI	*p* value
Age group (yrs)					
15-20	17	1	7.26	-	0.29
21-25	54	2			
26-30	66	5			
31-35	41	6			
36-40	21	4			
41-45	6	0			
46-50	1	0			
Educational level					
Primary	25	1	2.01	-	0.57
Secondary	102	8			
Tertiary	76	9			
No formal education	3	0			
Occupation type					
Public	161	13	3.81	-	0.28
Private	18	1			
Self-employed	20	2			
Others	7	2			
Parity					
0	69	6	0.65	-	0.88
1-2	88	8			
3-4	43	3			
>4	6	1			
Gestational age					
1st trimester	25	1	1.534	-	0.46
2nd trimester	142	12			
3rd trimester	39	5			
Pyuria					
Yes	37	18	90.09	0.38-0.70	0.01*
No	169	0			
Past history of UTI					
Yes	84	13	8.08	1.47-12.53	0.004*
No	122	5			
History of DM					
Yes	5	0	0.49	1.05-1.15	0.48
No	201	18			

*= significant at $p < 0.05$' CI = Confidence Interval

Table 3: Frequency distribution of bacteria isolates of significant bacteriuria among pregnant women attending the antenatal clinic of Adeoyo Maternity Hospital, Yemetu, Ibadan, Nigeria

Bacterial isolate	Frequency (%)
Escherichia coli	9 (50.0)
Klebsiella pneumoniae	6 (33.0)
Enterobacter aerogenes	1 (6.0)
Acinetobacter haemolyticus	1 (6.0)
Pseudomonas aeruginosa	1 (6.0)
Total	**18 (100)**

Table 4: Antibiotic susceptibility of uropathogens of significant bacteriuria in pregnant women attending the antenatal clinic of Adeoyo Maternity Hospital, Yemetu, Ibadan, Nigeria

Antibiotics	No of isolates tested	No of isolate sensitive (%)	No of isolates resistant (%)
Nitrofurantoin (F)	18	17 (94.4)	1 (5.6)
Augmentin (AMC)	18	12 (66.7)	6 (33.3)
Gentamicin (CN)	18	18 (100)	0
Ceftriaxone (CRO)	18	17 (94.4)	1 (5.6)
Cefuroxime (CXM)	18	15 (88.2)	2 (11.8)
Ceftazidime (CAZ)	18	16 (88.9)	2 (11.8)

Discussion:

Detection of significant bacteriuria in 206 pregnant women (age range 15-47 years) in this study was based on urine culture, which is currently the "gold standard" test for detection of UTI. Although prevalence of significant bacteriuria/UTI and antibiotic susceptibility of bacterial isolates among pregnant women have been reported at different health facilities in Nigeria (3,6,13-23), such data are lacking in our facility, but antibiotic susceptibility of isolates is known to vary according to regional and geographical location and also changes over time, hence the need for this study. The prevalence of 8.7% for significant bacteriuria in the present study is similar to the rate of 8% reported by Mandara et al., (13) in Zaria, 8% by Oyetunji et al, (15) in Sokoto, 9% by Aminu and Aliyu (23) in Kano, and 9.5% by Demilie et al., (27) in Bahirdar, Ethiopia, but higher than 1.3% rate reported by Yakasai et al., (20) in Kano, Nigeria. However, our rate is far lower than 47.5% reported by Okonko et al., (16) in Ibadan, 54% by Obiogbolu et al., (18) in Akwa metropolis, and 60% by Kolawole et al., (6) in Nasarawa, Nigeria. Based on the high prevalence rate in these studies, it was estimated in 2009 that one in two women will have at least one UTI episode in her lifetime while the National Kidney Foundation (NKF) in 2010 (28) estimated one in five women. However, in our study, we estimated this to be one in ten women.

In this study, the frequency of significant bacteriuria among symptomatic pregnant women was 27.8% compared to 72.2% among asymptomatic pregnant women. These differ from those reported in studies from Akwa Ibom metropolis in Nigeria (0% vs 54%) by Obiogbolu et al., (18), Tanzania (17.9% vs 13%) by Masinde et al., (29), Ethiopia (35.3% vs 22%) by Taye et al., (5), and Uganda (53.5% vs 46.5%) by Martin et al., (30). The differences in these frequencies between study areas might be due to different cultural practices, living standards and category of the study population (31). Our findings indicated that peak prevalence of significant bacteriuria was the second trimester as 66.7% was reported in the pregnant women during this trimester, which agrees with what has been reported in literature (22). This might be due to the fact that UTI in pregnant women usually begins within week 6 and peaks during week 22–24 of pregnancy due to urethral dilation, increased bladder volume and decreased bladder and urethral tone, which encourage bacterial growth in the urine.

Although bacterial aetiology of UTI can show geographic variations and may vary over time within a population, *E. coli* (50%) was the most commonly isolated urinary pathogen in our study. This is in agreement with reports of other studies by Okonko et al., (16), Taye et al., (5) and Jasmin et al., (32). However, our result is contrary to the findings from some other studies from Nigeria which reported *Klebsiella* spp., *Proteus mirabilis* and *Staphylococcus aureus* to be more prevalent than *E. coli* (1,20,26), although we are careful with the interpretation of our findings because of the small sample size. The second most commonly isolated pathogen in our study was *K. pneumoniae* (33%), which agrees with the findings of Obiogbolu et al., (18), Onwuezobe et al., (22), and Taye et al., (5). The high frequency of isolation of *K. pneumoniae* implies that this pathogen is gaining prominence as an important aetiologic agent of ASB and UTI in pregnancy over what has been previously reported in many studies (2,6). We noted that there were no Gram-positive bacteria recovered from the urine in significant number among the pregnant women and all significant isolates were monomicrobial, which is similar to the findings of Onwuezobe et al., (22) and Taye et al., (5). The non-contamination of the urine

observed must have been the result of the urine collection protocol and supervision by nurses and midwives.

Most of the isolated organisms in this study demonstrated a high level of susceptibility to gentamicin (100%), nitrofurantoin (94.4%), ceftriaxone (94.4%) and ceftazidime (88.9%), while high level resistance to amoxicillin-clavulanic acid (33.3%) was observed. The most commonly isolated bacterium, *E. coli*, was sensitive to gentamicin and nitrofurantoin despite the fact that amoxicillin-clavulanic acid is the most commonly used antibiotic for empirical treatment of UTI in our hospital. Our finding is similar to that of Masinde et al., (29) who reported 94% of *E. coli* isolates in their study to be sensitive to nitrofurantoin, and to those of Ajayi et al., (19), Akobi et al., (21), and Jasmin et al., (32). Contrary to our study, Assefa et al., (33) reported high sensitivity (100%) of their *E. coli* isolates to amoxicillin-clavulanic acid in addition to effectiveness of gentamicin and nitrofurantoin. *Klebsiella pneumoniae* isolates in our study demonstrated highest resistance rates to the tested antibiotics except gentamicin. This may be due to the fact that high proportion of clinical *K. pneumoniae* isolates are now known to carry extended spectrum β-lactamase (ESBL) and carbapenemase genes, that may contribute to the high resistance rates to the antibiotics tested in this study. This changing pattern in antimicrobial susceptibility with emergence and spread of resistant pathogens has been reported in previous studies (34,35), and this is attributed to inappropriate antimicrobial use as a result of inadequate knowledge about antimicrobial prescriptions and widespread practice of self-medication common in many African settings, among other factors.

Conclusion:

In view of the high prevalence of asymptomatic significant bacteriuria in pregnancy in this study, routine screening and treatment of symptomatic or asymptomatic pregnant women for this entity should be done in every trimester of the gestational period.

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Original Article **Open Access**

Prevalence of carbapenemase production in *Pseudomonas aeruginosa* isolates causing clinical infections in Lagos University Teaching Hospital, Nigeria

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Abstract:

Background: *Pseudomonas aeruginosa* has been highly associated with carbapenem resistance in which carbapenemases has been suggested to be a major contributory factor. Hence the objective of this study was to phenotypically detect KPC-type carbapenemase, metallo-β-lactamase and OXA-48 carbapenemase production in clinical isolates of *P. aeruginosa* in Lagos University Teaching Hospital (LUTH), Nigeria
Methodology: One hundred and seventy-one *P. aeruginosa* isolates consecutively recovered from clinical specimens of patients with infections at the Medical Microbiology and Parasitology laboratory of the hospital were identified using Microbact™ 24E kit. Preliminary screening for carbapenem resistance was determined by the disc diffusion method on Mueller-Hinton agar using single discs of meropenem and imipenem. Phenotypic detection of carbapenemase production among carbapenem-resistant isolates was performed by the combination disc test of meropenem-phenylboronic acid (MRPBO) and meropenem-dipicolinic acid (MRPDP) as recommended by EUCAST 2013 guideline.
Results: Out of the 171 *P. aeruginosa* isolates, 35 (20.5%) were carbapenem non-susceptible (resistant) while carbapenemase production was detected in 27 (77.1%) of these carbapenem resistant isolates, and no enzyme was detected in 8 (22.9%). Of the 27 carbapenemase producing isolates, 22 (81.5%) produced MBL, 1 (3.7%) produced KPC, while 4 (14.8%) produced both KPC and MBL enzymes.
Conclusion: This study revealed that carbapenem resistance among *P. aeruginosa* clinical isolates in our institution is gradually increasing. The mechanism for this rise is associated with carbapenemases, with MBL being the major carbapenemase involved. There is the need to ensure strict compliance with the LUTH infection control guidelines in order to check the rising incidence of infection caused by carbapenem resistant *P. aeruginosa*.

Keywords: carbapenemase; KPC; MBL; OXA-48; *Pseudomonas aeruginosa*; clinical isolates

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Prévalence de la production de carbapénémases dans les isolats de *Pseudomonas aeruginosa* causant des infections cliniques à l'hôpital universitaire de Lagos, Nigéria

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Abstrait:

Contexte: *Pseudomonas aeruginosa* a été fortement associé à la résistance aux carbapénèmes dans laquelle les carbapénèmases ont été suggérées comme étant un facteur contributif majeur. Par conséquent, l'objectif de cette étude était de détecter phénotypiquement la production de carbapénémase de type KPC, de métallo-β-lactamase et de carbapénémase OXA-48 dans des isolats cliniques de *P. aeruginosa* au Lagos University Teaching Hospital (LUTH), Nigeria.

Méthodologie: Cent soixante et onze isolats de *P. aeruginosa* récupérés consécutivement à partir d'échantillons cliniques de patients infectés au laboratoire de microbiologie médicale et de parasitologie de l'hôpital ont été identifiés à l'aide du kit MicrobactTM 24E. Le dépistage préliminaire de la résistance aux carbapénèmes a été déterminé par la méthode de diffusion sur disque sur gélose Mueller-Hinton en utilisant des disques uniques de méropénème et d'imipénème. La détection phénotypique de la production de carbapénèmes parmi les isolats résistants aux carbapénèmes a été réalisée par le test de disque combiné d'acide méropénème-phénylboronique (MRPBO) et d'acide méropénème-dipicolinique (MRPDP) tel que recommandé par la directive EUCAST 2013.
Résultats: Sur les 171 isolats de *P. aeruginosa*, 35 (20,5%) étaient des carbapénèmes non sensibles (résistants) tandis que la production de carbapénèmes a été détectée dans 27 (77,1%) de ces isolats résistants aux carbapénèmes, et aucune enzyme n'a été détectée dans 8 (22,9%). Sur les 27 isolats producteurs de carbapénémases, 22 (81,5%) produisaient des MBL, 1 (3,7%) produisaient des KPC, tandis que 4 (14,8%) produisaient à la fois des enzymes KPC et MBL.
Conclusion: Cette étude a révélé que la résistance aux carbapénèmes parmi les isolats cliniques de *P. aeruginosa* dans notre institution augmente progressivement. Le mécanisme de cette augmentation est associé aux carbapénémases, la MBL étant la principale carbapénémase impliquée. Il est nécessaire de garantir le strict respect des directives de contrôle des infections LUTH afin de contrôler l'incidence croissante des infections causées par *P. aeruginosa* résistant aux carbapénèmes.

Mots-clés: carbapénémase; CPK; MBL; OXA-48; *Pseudomonas aeruginosa*; isolats cliniques

Introduction:

Pseudomonas aeruginosa is a member of the *Gamma Proteobacteria* class of bacteria, a Gram-negative aerobic bacillus belonging to the family *Pseudomonadaceae* (1). *Pseudomonas aeruginosa* is associated with varying degree of infections ranging from mild to severe infections. These include otitis externa, urinary tract infection, lower respiratory tract infections, folliculitis, keratitis, infection of corneal ulcers, endophthalmitis and opportunistic blood stream infection in immunocompromised patients. It is also an important cause of nosocomial infections such as ventilator-associated pneumonia and wound infections in burn patients, and causes damage to the lung tissue with decreased pulmonary function in patients with cystic fibrosis (2,3). *Pseudomonas aeruginosa* is rarely a part of the microbial flora of healthy individuals, but may colonize the gastrointestinal tract of hospitalized patients, particularly neutropenic patients who have received previous antibiotic therapy (3). *Pseudomonas aeruginosa* is notoriously resistant to a number of antibiotics and only a handful of antibiotics are effective for treating infections caused by *P. aeruginosa*.

Carbapenems are class of beta-lactam antibiotics with a broad spectrum antibacterial activity. They are used in the treatment of severe infections caused by bacteria producing the extended-spectrum beta-lactamases (ESBLs) or multi-drug resistant *Pseudomonas* spp overexpressing chromosomal AmpC β-lactamases (4). Carbapenems approved for clinical use includes imipenem, meropenem, ertapenem and doripenem (5). The widespread occurrence of community-acquired bacterial isolates producing ESBLs capable of hydrolyzing almost all β-lactam antibiotics except the carbapenems (6), led to increased use of the carbapenems, which became very crucial for treating life-threatening infections that are often associated with modern medical techniques and practice such as transplantation, intensive care unit hospitalization and highly technical surgery (7). The subsequent emergence of carbapenem resistance has been largely associated with carbapenemase production. However, the actual prevalence of carbapenemase-producing bacterial strains remains unknown because many countries worldwide do not report rates of antibiotic susceptibility (8).

Carbapenemases are carbapenem-hydrolyzing β-lactamases that are able to hydrolyze almost all β-lactams. Most are resilient against inhibition by commercially viable β-lactamase inhibitors (9). These enzymes are mostly of the *Klebsiella pneumoniae* carbapenemase (KPC), Verona integron-encoded metallo – β - lactamase (VIM), imipenemase (IMP), New Delhi metallo – β - lactamase (NDM) and oxacillinase-48 (OXA-48) types (10), which are enzymes in Ambler classes A, B, and D (9,11,12). The Ambler class A carbapenemases include the KPC (9,13,14), *Serratia marcescens* enzyme family (SME), imipenem-hydrolyzing β-lactamase (IMI), "Not metalloenzyme carbapenemase" (NMC) (9,11) and Guiana extended spectrum (GES) beta-lactamases (15). Although the KPC and GES β-lactamases are predominantly found in *Enterobacteriaceae*, there have been reports of these enzymes in *P. aeruginosa* (16-19). The Ambler class B metallo-β-lactamases (MBLs) include IMP, VIM-1, VIM-2, (20,21), NDM-1, GIM (22), and Seoul imipenemase (SIM) (23). However, VIM and IMP have been reported in *P. aeruginosa* (20). The Ambler class D serine-carbapene- mases include oxacillin-hydrolyzing (OXA) β-lactamases in which *P. aeruginosa* is one of the organisms where it has been mainly implicated (11,24).

There are various strategies that have been proposed for detection of carbapenemases and the genes encoding them. A series of non-molecular tests have also been proposed for the detection of carbapenemase

activity (7,25) such as the inhibitor-based method which depends on demonstrating synergy between an indicator carbapenem and various *in-vitro* β-lactamase inhibitors. MBL inhibitors include ethylenediamine tetra-acetic acid (EDTA) and dipicolinic acid (DPA), while inhibitors of KPC and other class A carbapenemases are boronic acids, usually 3-aminophenylboronic acid (APBA) or phenyl-boronic acid (PBA), although the mechanism of inhibition is not known (26-32). There is no known specific inhibitor for OXA-48-like carba-penemases but the disc diffusion assay using temocillin besides PBA and DPA, is usually utilized since OXA-48 confers high-level temo-cillin resistance. The objective of this study is to determine the prevalence of phenotypic KPC, MBL and OXA-48 carbapenemases prod-uction in clinical isolates of *P. aeruginosa* in Lagos University Teaching Hospital, Nigeria

Materials and method:

Study setting

The study setting is Lagos University Teaching Hospital (LUTH), a 764-bed tertiary care hospital in Lagos, southwest region of Nigeria, which service covers the entire Lagos State and neighboring Ogun State.

Study design, protocol and ethical approval

This is a laboratory-based study of 171 *P. aeruginosa* isolates recovered by stan-dard microbiological methods consecutively from clinical specimens of patients with infec-tions, submitted to the Department of Medical Microbiology and Parasitology of the hospital between November 2014 and October 2015 (33). The isolates were identified to species level using the commercial Microbact™ 24E (Oxoid, UK) kit according to the manufac-turer's instructions. The protocol for the study was approved by the Ethics Review committee of LUTH. Waiver of consent was approved and data were anonymized prior to analysis.

Screening and phenotypic detection of carba-penemase production

Screening for carbapenem resistance was determined by disc diffusion method on Mueller-Hinton agar using commercially avai-lable single discs of meropenem and imi-penem (Oxoid, England, UK) (34). All isolates with zone diameter of inhibition less than the cut-off values for imipenem (<23mm) and/or meropenem (<25mm) (carbapenem resis-tant) were tested for phenotypic production of carbapenemases using the EUCAST 2013 guidelines (35). Phenotypic detection of carb-apenemase production was performed by the combination disc test using commercially avai-lable tablets (Rosco Diagnostica Neo-Sensi-tabs, Rosco, Taastrup, Denmark) (36-37). The KPC/MBL in *P. aeruginosa* Confirm Kit version 2 (98025) was used for testing according to the manufacturer's instructions.

The test was carried out on Mueller Hinton agar using the modified Kirby-Bauer method and EUCAST disk diffusion method 2013 version 3.0 (35). Few morphologically similar colonies of overnight pure culture isolates were inoculated into sterile normal saline and visible turbidity was adjusted to match the turbidity of 0.5 McFarland stan-dards. The inoculum suspension was streaked onto Mueller-Hinton agar plate using a sterile swab stick. Tablets containing Meropenem 10µg (MRP10), Meropenem 10µg + Phenyl-boronic acid (MRPBO) and Meropenem 10µg + Dipicolinic acid (MRPDP) were applied and incubation was done at 35±1°C for 16-20 hours. The zone diameter of inhibition of each isolate to the tablets was measured using a calibrated ruler, and result inter- preted using the EUCAST guideline.

When the zones of inhibition of MRP10, MRPBO and MRPDP were within 2mm of each other, the isolate was reported as not producing KPC and MBL. When the MRPBO showed a zone difference of ≥ 4mm from MRP10, the isolate was reported as demon-strating KPC activity. When MRPDP showed a zone difference of ≥5mm from MRP10, the isolate was reported as demonstrating Metal-lo-β-lactamase activity. Only ceftazidime res-istant isolates were tested, as false positive MBL may be obtained with ceftazidime sensi-tive isolates.

Results:

A total of 171 *P. aeruginosa* were tes-ted for carbapenem resistance and subse-quently tested for carbapenemase production. The isolates were recovered from patients with sepsis (n=3), ocular infections (n=5), oto-laryngeal infections (n=22), chest infections (n=7), intra-abdominal infections (n=2), rep-roductive system infections (n=3), bone/joint infections (n=5), urinary tract infections (n= 39), surgical site infections (n=16) and wound infections (n=69) (Table 1).

A total of 35 (20.5%) isolates were carbapenem non-susceptible (resistant) while carbapenemase production was detected in 27 (77.1%), and no enzyme was detected in 8 (22.9%) of the carbapenem resistant isolates. Of the 27 carbapenemase producing isolates, 22 (81.5%) produced MBL, 1 (3.7%) produced KPC, and 4 (14.8%) produced both KPC and MBL enzymes (Table 2).

Discussion:

In this study, the prevalence of carba-penem resistance among clinical isolates of *P. aeruginosa* in our institution was 20.5%. This

is similar to the findings in Turkey and India where the prevalence of carbapenem resistant *P. aeruginosa* was 20% (38) and 18.2% (39) respectively. This rate is quite high for a drug which is usually reserved for last resort use. Previous studies carried out in our institution reported prevalence rates of carbapenem resistance among *Pseudomonas* isolates to be 4.1% in 2007 (40) and 5.9% in 2013 (33). Comparing the results of the two studies to this present one indicates that the prevalence of carbapenem resistance among *P. aeruginosa* isolates in our institution is increasing.

Carbapenemase production is the most predominant mechanism that confers clinically significant resistance to Gram-negative bacteria (GNB) (41-45), which is why most carbapenem-resistant *P. aeruginosa* isolates in our study (77.1%) produced carbapenemases. This high rate of carbapenemase production was also reported by Ogbolu et al., (46) with a rate of 59.7% and by Mohammed et al., (47) with a rate of 82.1% in other institutions in Nigeria. Also, a study conducted in India (39) using two different types of phenotypic testing methods reported a high rate of carbapenemase production of 93.1% (combined disk synergy test) and 87.2% (double-disk synergy test). It should be noted that 22.9% of the carbapenem resistant *P. aeruginosa* isolates in this study did not produce carbapenemase, which suggests that other resistance mechanisms such as over-production of AmpC cephalosporinase, drug efflux, and deficient production or loss of porins might be responsible for the carbapenem resistance in these isolates (48).

The most common carbapenemases produced by *P. aeruginosa* in this study were MBLs (81.5%) and KPC (3.7 %), which is similar to the findings of Mushi et al., (49) in Tanzania, whereas KPC was reported to be more common than MBL by Vanegas et al., (50) in Colombia.

Table 1: Distribution of *Pseudomonas aeruginosa* isolates from different infection sites in Lagos University Teaching Hospital, Nigeria

Types of infection	**Frequency of *P. aeruginosa***	**Percentage**
Sepsis/Bacteraemia	3	1.8
Eye infections	5	2.9
Otolaryngeal infections	22	12.9
Chest infections	7	4.1
Intra-abdominal infections	2	1.2
Reproductive system infections	3	1.8
Bone/Joint infections	5	2.9
Urinary tract infections	39	22.8
Surgical site infections	16	9.4
Wound infections	69	40.4
Total	**171**	**100**

Table 2: Carbapenem resistant *Pseudomonas aeruginosa* and the carbapenemase types produced

Carbapenemase types	**Frequency (%)**
MBL	22 (62.9)
KPC	1 (2.9)
KPC and MBL	4 (11.4)
No enzyme	8 (22.9)
Total	**35 (100)**

KPC = *Klebsiella pneumoniae* carbapenemase; MBL = metallo-β-lactamase

The KPC/MBL confirmation kit used in this study could not detect OXA-48 enzyme. A more recent phenotypic detection method such as the carbapenem inactivation method (CIM) which detects KPC, MBL and OXA-48 enzymes, may have detected the OXA-48 enzymes if used in this study. However, this method was not available to us at the time of this study. Similarly, molecular methods which are reported to be more sensitive and specific in detecting KPC, MBL and OXA-48 genes, were not available for this study.

Conclusion:

In conclusion, our findings revealed that carbapenem resistance among *P. aeruginosa* clinical isolates in our institution, is gradually on the rise, with carbapenemases being the major contributory mechanism for this, which is line with previous studies in this institution. Also, it is noteworthy to emphasize that *P. aeruginosa* can produce more than one class of carbapenemases as reported in our study, which raises some concern. Our findings also have serious implication for antimicrobial therapy of *P. aeruginosa* infections and infection control measures in our environment, in view of the fact that new antibiotics are not forthcoming. Hence, the need to preserve the antibiotics we have now by embracing antimicrobial stewardship.

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Original Article **Open Access**

Comparative analysis of the phytochemical and antibacterial activity of the root extracts of *Euphorbia heterophylla* and *Vitellaria paradoxa*

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Abstract:

Background: Over time, herbal plants and their various components have been major sources of therapeutic medicine for man. A comparative study was carried out to determine the phytochemical components and antibacterial activities of the different crude extracts of *Euphorbia heterophylla* and *Vitellaria paradoxa* roots on four enteric bacteria; *Salmonella typhi*, *Shigella flexneri, Escherichia coli* and *Proteus vulgaris.*
Methodology: Root samples of *E. heterophylla* and *V. paradoxa* were collected, washed, air dried and processed to fine powder in the microbiology laboratory of Federal University of Technology, Minna, Nigeria. Crude extract of the root samples was done by the cold maceration technique using four solvents (chloroform, methanol, petroleum ether and water). Phytochemical analysis of the extracts was done using previously described technique, and *in vitro* antibacterial activities of different concentrations of the extracts (50-200 mg/ml) and a standard antibiotic (ciprofloxacin) were tested on four enteric bacteria (*S. typhi, S. flexneri, E. coli, P. vulgaris*) by the agar diffusion test. *In vivo* antibacterial activities of the two plants were also tested by daily oral administration of 2000 mg/kg bodyweight (for 7 days) of each extract on inbred mice infected through intraperitoneal inoculation of an infective dose of each of the four enteric bacteria. Data were computed as mean ± standard error and analysed by the Statistical Analysis System (SAS) version 9.4. Associations between variables were determined using analysis of variance (ANOVA), with $p < 0.05$ considered as significant value.
Results: Phytochemical analysis of the crude extracts of both plants revealed the presence of cardiac glycosides, saponins, alkaloids, flavonoids, and tannins but *V. paradoxa* contain more carbohydrates and starch, and less phlobatannins, compared to *E. heterophylla*. *In vitro* assay showed dose-dependent antibacterial activity of the methanol, aqueous and chloroform (but not petroleum ether) extracts of the two plant roots. The *in vitro* antibacterial activities of the different extracts of *V. paradoxica* extracts were significantly higher (higher mean diameters of inhibition zones) than those of *E. heterophylla* ($p<0.05$), and methanol extracts gave the highest antibacterial effects. However, the root extract of *E. heterophylla* gave a higher antibacterial activity with the *in vivo* assay on inbred mice than *V. paradoxa*, and methanol extracts of the two plant extracts gave the highest *in vivo* activity, followed by aqueous extract and least activity was obtained with the chloroform extract.
Conclusion: Crude extracts of *E. heterophylla* and *V. paradoxa* roots produce antibacterial activity against enteric Gram-negative bacteria pathogens involved in diarrhoea illnesses. Further researches should be directed towards isolation and characterization of the active compounds in the crude extracts.

Keywords: Roots; Phytochemicals; *In vitro*; *In vivo*; *Euphorbia heterophylla*; *Vitellaria paradoxa;* Enteric bacteria

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Analyse comparative de l'activité phytochimique et antibactérienne des extraits de racines d'*Euphorbia heterophylla* et de *Vitellaria paradoxa*

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Abstrait:

Contexte: Au fil du temps, les plantes médicinales et leurs divers composants ont été une source majeure de médecine thérapeutique pour l'homme. Une étude comparative a été réalisée pour déterminer les composants phytochimiques et les activités antibactériennes des différents extraits bruts de racines d'*Euphorbia heterophylla* et de *Vitellaria paradoxa* sur quatre bactéries entériques; *Salmonella typhi, Shigella flexneri, Escherichia coli* et *Proteus vulgaris*.
Méthodologie: Des échantillons de racines d'*E. heterophylla* et de *V. paradoxa* ont été collectés, lavés, séchés à l'air et transformés en poudre fine dans le laboratoire de microbiologie de l'Université fédérale de technologie, Minna, Nigéria. L'extraction brute des échantillons de racines a été réalisée par la technique de macération à froid en utilisant quatre solvants (chloroforme, méthanol, éther de pétrole et eau). L'analyse phytochimique des extraits a été effectuée en utilisant la technique décrite précédemment, et les activités antibactériennes *in vitro* de différentes concentrations des extraits (50-200 mg/ml) et d'un antibiotique standard (ciprofloxacine) ont été testées sur quatre bactéries entériques (*S. typhi*, *S. flexneri*, *E. coli*, *P. vulgaris*) par le test de diffusion sur gélose. Les activités antibactériennes *in vivo* des deux plantes ont également été testées par administration orale quotidienne de 2000 mg/kg de poids corporel (pendant 7 jours) de chaque extrait sur des souris consanguines infectées par inoculation intrapéritonéale d'une dose infectieuse de chacune des quatre bactéries entériques. Les données ont été calculées en tant que moyenne ± erreur standard et analysées par le système d'analyse statistique (SAS) version 9.4. Les associations entre les variables ont été déterminées à l'aide d'une analyse de variance (ANOVA), avec $p < 0,05$ considéré comme une valeur significative.
Résultats: L'analyse phytochimique des extraits bruts des deux plantes a révélé la présence de glycosides cardiaques, de saponines, d'alcaloïdes, de flavonoïdes et de tanins mais *V. paradoxa* contient plus de glucides et d'amidon, et moins de phlobatannins, par rapport à *E. heterophylla*. Un essai *in vitro* a montré une activité antibactérienne dose-dépendante des extraits au méthanol, aqueux et au chloroforme (mais pas à l'éther de pétrole) des deux racines des plantes. Les activités antibactériennes in vitro des différents extraits d'extraits de *V. paradoxica* étaient significativement plus élevées (diamètres moyens des zones d'inhibition plus élevés) que celles d'*E. heterophylla* ($p<0,05$), et les extraits au méthanol ont donné les effets antibactériens les plus élevés. Cependant, l'extrait de racine d'*E. heterophylla* a donné une activité antibactérienne plus élevée avec le test *in vivo* sur des souris consanguines que *V. paradoxa,* et les extraits au méthanol des deux extraits de plantes ont donné l'activité in vivo la plus élevée, suivie par l'extrait aqueux et l'activité la plus faible a été obtenu avec l'extrait chloroformique.
Conclusion: Des extraits bruts de racines d'*E. heterophylla* et de *V. paradoxa* produisent une activité antibactérienne contre les bactéries pathogènes entériques à Gram négatif impliquées dans les maladies diarrhéiques. D'autres recherches devraient être dirigées vers l'isolement et la caractérisation des composés actifs dans les extraits bruts.

Mots-clés: racines; phytochimiques; *in vitro*; *in vivo*; *Euphorbia heterophylla*; *Vitellaria paradoxa*; bactéries entériques

Introduction:

Plants basically consist of 32% of the earth surface (1). There are about half a million plants now growing on earth, many of which possess therapeutic and pharmaceutical properties (2). According to an earlier survey, about 25% of modern drugs and medicinal products are derived from secondary metabolites of plant (3). Most of these plants are said to be beneficial in attaining stable health and treatment of most human diseases, and as such, are termed medicinal plants. Medicinal plants can be described as natures' pharmacy for nearly 80% of people living in Africa (4). A medicinal plant has been described as any plant in which one or more of its parts contain substances that can be used for therapeutic purpose or which acts as precursors for the synthesis of useful drugs (5). In Africa, based on cultural acceptability and fewer side effects, herbal medicine still remains the mainstay of treatment in 75-80% of the population at the primary health care (6). In Nigeria, thousands of plant species are known to have medicinal values (5) and the use of different parts of several medicinal plants to cure specific ailments has been in vogue since ancient times (7). In the present scenario of emergence of multiple drug resistance in human pathogenic microorganisms, this has necessitated a search for new antimicrobial substances from plants.

However, the ability of plants to produce many phytochemicals used to perform important biological functions is one of the many characteristics they possess, and the medicinal values of these plants lie in the phytochemicals present in the plants, which in turn produce definite physiological actions in the human body (8,9,10). Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. It is a well-

known fact that plants produce these chemicals to protect themselves, and recent researches have demonstrated that they can also protect humans against various diseases. These chemical substances possess varied activities such as antioxidant, hormonal, stimulatory, antimicrobial, anti-diarrhoeal, anti-histamine, anti-diabetic, anti-malarial and anti-carcinogenic effects. In most cases, due to the presence of these bioactive compounds or phytochemicals, plants may be considered as potential candidates for developing new antimicrobial drugs or alternative treatments of various ailments caused by microorganisms resistant to most commonly available synthetic drugs.

Two efficient medicinal plants that are gradually gaining grounds in the developing countries due to their medicinal benefits are *Euphorbia heterophylla* and *Vitellaria paradoxa*. *E. heterophylla* is one of the numerous plants found in the field, which grows in disturbed localities as a weed of cultivation and waste land, in gardens, and along roadsides from sea-level up to 3000 m altitude (11). It belongs to the family of *Euphorbiaceae*, and is referred to as Mexican fire plant, milk weed and Spurge weed in English, and commonly called *Nonokunchiya* in Hausa, *Egele* in Ibo and *Adimeru* among the Yoruba tribes in Nigeria (12). All the parts of *E. heterophylla* contain latex (11), and the plant is widely used in traditional African medicine and elsewhere in tropical countries. Generally, this plant is regarded as a purgative, anti-asthmatic, anti-inflammatory and an abortifacient (13,14), and has also been reported to be oxytocic (15). In East Africa, the roots are used in the treatment of gonorrhoea and to increase milk production in breast-feeding women (15). *Vitellaria paradoxa* on the other hand, is generally regarded as a deciduous and multipurpose plant, found and used in Africa. The size of the mature tree of *V. paradoxa* varies from 7 to 25m.This plant belongs to the family *Sapotaceae* and is said to produce shea butter as its main product (16). *V. paradoxa* was formerly called *Butyrospermum parkii* (which means butter seed) but could also be called *Butyrospermum paradoxa*. In Nigeria**,** it is locally known as '*emi-emi*' among the Yorubas, '*ka'danya*' among the Hausas and '*okwuma*' among the Igbos (17). The plant is said to possess white latex. The shea tree grows naturally in the wild in the dry savannah belt of West Africa from Senegal in the west to Sudan in the east, and onto the foothills of the Ethiopian highlands. The roots are used as chewing sticks in Nigeria, which is common in savannah areas (17). Roots and root barks are ground to paste and taken orally to cure jaundice, stomach ache and diarrhoea or can be boiled and pound into paste to treat chronic sores (17,18). These plants generally are used for the treatment of diseases caused by enteric bacteria which are known to be associated with high morbidity and mortality in humans.

Enteric bacteria are usually Gram-negative bacteria which are mostly gastrointestinal flora but can cause diarrhoea or other diseases (19). They are also found in various natural habitats aside the intestinal tract. Most enteric bacteria are motile by peritrichous flagella with two major exceptions; *Klebsiella* and *Shigella,* which lack peritrichous flagella (20). Many of the enteric bacteria are facultatively anaerobic in nature, a trait that allows them to thrive in the gut environment, and most produce energy by metabolizing sugars into lactic acid. Some of the enteric bacteria live in the gut without causing diseases in individuals with good host immune system while some almost always cause enteric diseases manifesting with symptoms and signs such as vomiting, diarrhoea, and other related features (19). Based on the health-related threats pose by these enteric bacteria, individuals in the populace tend to overuse and misuse available synthetic drugs, which thereby lead to emergence of resistance in the population of these enteric bacteria. It is therefore imperative to seek other alternative sources of remedy to various enteric diseases and determine the differences between them, which forms the main objective of this study.

Materials and method:

Collection and identification of plant materials

Fresh samples of the roots of both plants (*Euphorbia heterophylla* and *Vitellaria paradoxa*) were collected from Garatu, in a village called Anguwan noma, which lies on longitude 6.44^{o}N and latitude 9.4^{o}E. The plant samples were identified and confirmed at the Department of Biological Sciences, Federal University of Technology, Minna, Nigeria.

Extraction procedure

The roots of both plants were thoroughly washed, air dried at room temperature (28^{o}C), and ground into coarse powder using a sterile mortar and pestle. The dried roots were further ground into a fine powder using an electric blender, which was done to enhance the penetration of the extracting solvent to facilitate the release of the active components (21). One hundred grammes (100 g) of each ground root was macerated successively for three days (with occasional shaking) using cold maceration technique. One liter (1000 ml) each

of distilled water, methanol, chloroform, and petroleum ether were used as the extraction solvents. The macerated samples were sieved with muslin cloth and evaporated to dryness using a steam bath. The dried extracts were weighed and stored in sterile sample bottles and kept in the refrigerator for further studies (21).

Extracts were reconstituted by dissolving different weights of each dried extract (0.05g, 0.1g, 0.15g and 0.2g) in 1 ml of either distilled water or dimethyltetrasulphuric acid ($DMSO_4$), to give concentrations of 50 mg/ml, 100 mg/ml, 150 mg/ml and 200 mg/ml respectively. Similarly, the standard antibiotic (ciprofloxacin) was reconstituted by dissolving different weights of the dry antibiotic powder (0.05 g, 0.1g, 0.15g and 0.2g) in 1 ml of distilled water to produce equivalent concentrations as the extracts.

Phytochemical screening

The phytochemical screenings of the crude extracts of both roots were carried out to detect the presence or absence of some secondary metabolites according to previously described methods (22,23).

Test bacteria and culture media

The test organisms (*Salmonella typhi, Shigella flexneri, Escherichia coli* and *Proteus vulgaris*) were obtained from the stock cultures of the Microbiology Laboratory, General Hospital, Minna, Niger State, Nigeria. MacConkey, Salmonella-Shigella (SS) and Nutrient agar plates were used as differential, selective and for susceptibility testing media of the test bacteria as previously described (24).

***In vitro* antibacterial assay of crude extracts**

A 24-hour well-grown activated cultures of each test bacterium were serially diluted in test tubes with normal saline and standardized with MacFarland turbidity standards to obtain inoculum equivalent to $1.0x10^5$ CFU/ml. The antibacterial assay of the crude extracts of the roots of both plants was done using the agar (punch hole) diffusion method as described by Idu and Igekele (24). The Nutrient agar plates were prepared by dispensing 20ml of sterile molten nutrient agar into sterile Petri plates and allowed to set, and then inoculated with the standardized inoculum of each test bacterium using sterile cotton swab. A 4 mm sterile cork-borer was used to punch five (5) adequately spaced holes in each inoculated agar plates to create wells. Approximately 0.2 ml of the different concentrations (50mg/ml, 100mg/ml, 150mg/ml and 200mg/ml) of the extracts and the standard antibiotic, was introduced into each well. The Petri plates were incubated at a temperature of 37°C for 24 hours, after which diameter of inhibition zones around each well was measured with a calibrated ruler. The diameters of inhibition zones produced by the extracts were compared with that produced by the standard antibiotic (ciprofloxacin) used as control (24). Extracts of each plant that showed little or no antibacterial activity were excluded for the *in vivo* assay.

***In vivo* antibacterial activity of crude extracts**

Experimental animals

Inbred mice within the age of 8-12 weeks with body weight from 18-22 g were acquired from Ibrahim Badamosi Babangida University Lapai, Nigeria. The mice were kept in standard cages with adequate food, water and under hygienic conditions for 2 weeks before inoculation (25).

Preparation of inoculum of test bacteria

A loopful of the test bacteria was inoculated on Salmonella-Shigella agar which was incubated aerobically at 37°C to activate the them. Colonies of the test bacteria were then transferred into test tubes containing 10 ml of sterile nutrient broth and incubated at 37°C for 18-24 hours. The culture was serially diluted in test tubes with normal saline and standardized with McFarland turbidity standards to obtain inoculum equivalent to 1.0×10^5 CFU/ml (26).

Inoculation of mice with test organisms and administration of plant extracts and standard antibiotic

The mice were divided into 28 groups, with each group containing 5 mice (n=5). In each group, 1 ml of the standardized inoculum (containing $1.0x10^5$ CFU as infective dose) of each of the test bacterium (*E. coli, S. typhi, S. flexneri,* and *P. vulgaris*) was introduced into each mouse intraperitoneally (26). After 72 hours, inoculated mice in each group were administered daily with 2,000 mg/kg/body weight of chloroform, methanolic and aqueous root extracts of *E. heterophylla* and *V. paradoxa*, and the control (ciprofloxacin) antibiotic, orally for 7 days (27). The mice were closely observed daily after inoculation and the mortality rate and other physical manifestations were recorded.

Observation of physical manifestations, survival and mortality

The mortality and survival rates of the mice in the groups were calculated as numbers

of mice that died and survived during the course of the experiment in relation to all the mice that were used (26). The animals were observed for frequency, consistency, and the colour of their faecal wastes, and for abnormalities and physical manifestations such as loss of appetite, weight loss and body weakness, during the period of the experiment (27). At the end of the study, infected mice were sacrificed using chloroform and buried, to prevent the spread of the infection associated with enteric pathogens in the environment (27)

Statistical analysis

Data were presented as mean ± standard error of mean, and analyzed using the Statistical Analysis System (SAS) version 9.4. Associations between variables were determined using analysis of variance (ANOVA), with $p < 0.05$ considered as significant value.

Results:

Phytochemical constituents of root extracts of the plants

The phytochemical components of *E. heterophylla* roots were alkaloids, flavonoids, saponins, tannins and cardiac glycosides with all the crude extract solvents while compounds such as carbohydrates and starch were mostly found in *V. paradoxa* (Table 1).

***In vitro* antibacterial activity of extracts and control antibiotic**

Tables 2.1 to 5.2 represent the mean diameters of the inhibition zones of chloroform, methanol, petroleum ether and aqueous extracts of *V. paradoxa* and *E. heterophylla*, and the control (ciprofloxacin) antibiotic, on all the bacterial strains (*S. typhi, S. flexneri, E. coli* and *P. vulgaris*) tested in this study

Table 1: Phytochemical constituents of root extracts of *Euphorbia heterophylla* and *Vitellaria paradoxa*

Phytochemical compound	*Euphorbia heterophylla*				*Vitellaria paradoxa*			
	Chloroform	Methanol	Aqueous	Petroleum Ether	Chloroform	Methanol	Aqueous	Petroleum Ether
Carbohydrates	_	+	+	_	+	+	+	+
Starch	_	+	+	_	+	+	+	+
Cardiac glycosides	+	+	+	+	+	+	+	_
Saponins	+	+	+	+	+	+	+	+
Steroids	_	+	+	_	_	+	+	_
Alkaloids	+	+	+	+	+	+	+	_
Flavonoids	+	+	+	+	_	+	+	+
Phenolics	_	+	_	+	_	+	+	_
Tannins	+	+	+	+	+	+	+	+
Phlobatannins	_	+	_	+	_	_	_	_

+ = presence of the phytochemical compound; - = absence of the phytochemical compound

The tables show dose-dependent antibacterial activity of the root extracts of both *V. paradoxa* and *E. heterophylla* on all the bacterial strains and for all the extracting solvents (except petroleum ether) as shown by the progressive increase in mean diameters of inhibition zones, with increasing doses from 50 mg/ml to 200 mg/ml of the extracts. However, methanol, aqueous and chloroform root extracts of *V. paradoxa* (VPMR, VPAR and VPCR) had significantly higher antibacterial activity (p <0.05) on all the test bacteria as shown by significantly wider mean diameters of inhibition zones from 50 mg/ml to 200 mg/ml extract concentrations, compared to methanol, aqueous and chloroform root extracts of *E. heterophylla* (EHMR, EHAR and EHCR).

The antibacterial activity was highest for methanol root extract of both *E. heterophylla* and *V. paradoxa* followed by aqueous and chloroform root extract, while petroleum ether root extracts of both *V. paradoxa* (VPPR) and *E. heterophylla* (EHPR) showed the least antibacterial activity from 50 mg to 200 mg/ml extract concentrations as shown by the narrowwest diameters of inhibition zones compared to the root extracts of the three other solvents. Hence, petroleum ether root extract was excluded from the *in vivo* experiment. The antibacterial activity of the control antibiotic (ciprofloxacin) was however significantly higher (wider diameters of inhibition zones) than the different solvent root extracts of the two plants at all the concentrations tested, although this was not in a dose-dependent manner.

Table 2.1: Zones of inhibition of extract of *V. paradoxa* at 50mg/ml

Extracts	*S. typhi*	*S. flexneri*	*E. coli*	*P. vulgaris*
VPCR	2.67 ± 0.33^{d}	2.33 ± 0.33^{c}	2.00 ± 0.60^{c}	2.00 ± 0.00^{b}
VPMR	9.33 ± 0.33^{c}	7.00 ± 0.58^{d}	9.33 ± 0.33^{c}	8.67 ± 0.33^{d}
VPAR	9.00 ± 0.58^{c}	5.33 ± 0.58^{b}	8.33 ± 0.67^{c}	7.67 ± 0.68^{d}
VPPR	2.33 ± 0.31^{cd}	1.67 ± 0.34^{bc}	2.00 ± 0.00^{c}	1.67 ± 0.70^{b}
CONTROL	18.00 ± 0.58^{d}	17.33 ± 0.67^{e}	17.00 ± 0.58^{d}	14.33 ± 0.67^{d}

Table 2.2: Zones of inhibition of extract of *E. heterophylla* at 50mg/ml

Extracts	*S. typhi*	*S. flexneri*	*E. coli*	*P. vulgaris*
EHCR	2.00 ± 0.58^{b}	1.67 ± 0.33^{b}	1.67 ± 0.33^{ab}	2.00 ± 0.58^{b}
EHMR	4.33 ± 0.33^{cdef}	4.00 ± 0.58^{cde}	4.67 ± 0.33^{de}	3.67 ± 0.33^{c}
EHAR	3.67 ± 0.33^{bcd}	3.67 ± 0.67^{cd}	5.00 ± 0.58^{de}	3.67 ± 0.66^{c}
EHPR	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
CONTROL	9.00 ± 0.58^{g}	8.00 ± 0.57^{g}	8.67 ± 0.68^{g}	8.67 ± 0.33^{e}

Table 3.1: Zones of Inhibition of the root of *V. paradoxa* at 100mg/ml

Extracts	*S. typhi*	*S. flexneri*	*E. coli*	*P. vulgaris*
VPCR	8.33 ± 0.33^{de}	6.67 ± 0.90^{c}	8.00 ± 0.00^{d}	6.00 ± 1.00^{b}
VPMR	12.67 ± 0.33^{c}	11.33 ± 0.33^{b}	11.33 ± 0.67^{d}	11.00 ± 0.50^{c}
VPAR	11.00 ± 0.58^{c}	10.00 ± 0.58^{b}	10.33 ± 0.31^{cd}	9.67 ± 0.30^{c}
VPPR	7.00 ± 1.00^{cd}	6.00 ± 0.57^{bc}	6.33 ± 0.33^{cd}	5.67 ± 0.70^{b}
CONTROL	24.67 ± 0.33^{d}	23.00 ± 0.00^{c}	24.00 ± 0.58^{d}	20.00 ± 0.58^{c}

Table 3.2: Zones of Inhibition of the root of *E. heterophylla* at 100mg/ml

Extracts	*S. typhi*	*S. flexneri*	*E. coli*	*P. vulgaris*
EHCR	4.00 ± 0.60^{b}	4.33 ± 0.33^{b}	5.00 ± 0.00^{bcd}	3.67 ± 0.33^{b}
EHMR	7.67 ± 0.30^{cde}	7.33 ± 0.33^{defg}	6.33 ± 1.76^{cde}	6.67 ± 1.20^{cd}
EHAR	6.67 ± 0.70^{cd}	6.67 ± 0.30^{cdef}	7.00 ± 00^{cde}	6.33 ± 0.90^{bc}
EHPR	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	2.00 ± 0.00^{ab}	0.00 ± 0.00^{a}
CONTROL	15.00 ± 0.60^{h}	13.33 ± 0.90^{i}	13.33 ± 1.45^{f}	12.33 ± 1.45^{g}

Values are represented as Mean ± Standard Error of Mean of triplicate determinations. Values along the column with different alphabet is significantly ($p < 0.05$)

VPCR = Chloroform root extract of *Vitellaria paradoxa*; VPMR = Methanolic root extract of *Vitellaria paradoxa*; VPAR = Aqueous root extract of *Vitellaria paradoxa*; VPPR = Petroleum ether root extract of *Vitellaria paradoxa*.; EHCR = Chloroform root extract of *Euphorbia heterophylla*; EHMR = Methanolic root extract of *Euphorbia heterophylla*; EHAR = Aqueous root extract of *Euphorbia heterophylla*; EHPR = Petroleum ether root extract of *Euphorbia heterophylla*

Table 4.1: Zones of Inhibition of the root of *V. paradoxa* at 150mg/ml

Extracts	*S. typhi*	*S. flexneri*	*E. coli*	*P. vulgaris*
VPCR	11.00±0.60^{e}	9.67±0.90^{cd}	10.00±0.58^{d}	9.00±0.00^{b}
VPMR	14.67±0.33^{b}	14.33±0.33^{c}	13.00±0.56^{c}	12.00±0.60^{c}
VPAR	13.33±0.58^{ab}	12.00±0.58^{ab}	11.67±0.90^{abc}	10.67±0.86^{bc}
VPPR	8.67±0.67^{a}	7.67±0.70^{bc}	7.67±0.33^{c}	7.33±0.68^{b}
CONTROL	27.00±0.58^{c}	26.00±0.58^{d}	26.33±0.33^{d}	25.67±0.33^{d}

Table 4.2: Zones of Inhibition of the root of *E. heterophylla* at 150mg/ml

Extracts	*S. typhi*	*S. flexneri*	*E. coli*	*P. vulgaris*
EHCR	5.33±0.70^{b}	4.67±0.28^{b}	5.00±0.60^{b}	4.33±0.90^{b}
EHMR	8.33±0.90^{c}	8.00±0.60^{c}	8.33±0.88^{c}	7.67±0.90^{c}
EHAR	8.67±0.30^{c}	7.67±0.90^{c}	8.33±0.33^{c}	8.33±0.30^{c}
EHPR	2.67±0.30^{a}	1.67±0.90^{a}	2.00±0.30^{a}	1.33±0.33^{a}
CONTROL	20.00±0.60^{f}	18.67±0.70^{g}	19.33±0.33^{g}	19.00±0.58^{f}

Table 5.2: Zones of Inhibition of the root of *V. paradoxa* at 200mg/ml

Extracts	*S. typhi*	*S. flexneri*	*E. coli*	*P. vulgaris*
VPCR	12.67±0.33^{d}	11.33±0.88^{g}	12.00±0.60^{e}	11.33±0.70^{g}
VPMR	17.00±0.58^{g}	15.00±0.58^{g}	14.68±0.33^{b}	14.68±0.90^{b}
VPAR	15.68±0.88^{g}	14.67±0.67^{b}	13.67±0.70^{b}	11.33±0.30^{a}
VPPR	9.67±0.33^{c}	8.33±1.20^{bc}	9.00±0.00^{bc}	7.33±1.20^{bc}
CONTROL	30.00±0.58^{c}	29.67±0.33^{c}	29.33±0.67^{c}	28.00±0.58^{c}

Table 5.2: Zones of Inhibition of the root of *E. heterophylla* at 200mg/ml

Extracts	*S. typhi*	*S. flexneri*	*E. coli*	*P. vulgaris*
EHCR	8.33±0.33^{b}	7.00±0.60^{b}	8.33±0.33^{b}	7.33±0.67^{bc}
EHMR	11.00±00de	10.33±0.33^{de}	10.67±0.70^{cd}	9.67±0.30^{de}
EHAR	11.00±0.00^{de}	10.33±0.33^{de}	10.67±0.30^{cd}	10.00±0.60^{de}
EHPR	3.67±0.30^{a}	2.67±0.70^{a}	3.33±0.67^{a}	3.00±0.60^{a}
CONTROL	26.00±0.60^{h}	25.00±0.70^{g}	25.33±0.33^{f}	24.33±0.33^{g}

Values are represented as Mean ± Standard Error of Mean of triplicate determinations. Values along the column with different alphabet is significantly ($p < 0.05$)

VPCR = Chloroform root extract of *Vitellaria paradoxa*; VPMR = Methanolic root extract of *Vitellaria paradoxa*; VPAR = Aqueous root extract of *Vitellaria paradoxa*; VPPR = Petroleum ether root extract of *Vitellaria paradoxa*.; EHCR = Chloroform root extract of *Euphorbia heterophylla*; EHMR = Methanolic root extract of *Euphorbia heterophylla*; EHAR = Aqueous root extract of *Euphorbia heterophylla*; EHPR = Petroleum ether root extract of *Euphorbia heterophylla*

***In vivo* antibacterial activity of the extracts**

Fig 1 represents the *in vivo* effects of chloroform, methanol and aqueous extracts of *V. paradoxa* and *E. heterophylla* on mice infected with *S. typhi* showing antibacterial activity of the extracts in the order E.h/M>V.p/M> E.h/A>V.p/C>V.p/A>E.h/C. This indicates that methanol extracts of *E. heterophylla* demonstrated the greatest *in vivo* antibacterial activity resulting in only 20% of the infected mice having diarrhoea between 1-3 days, and none (0%) manifesting pathological symptoms of diarrhoea, weight loss, loss of appetite and body weakness after 4 days of extract administration. Comparatively, chloroform extract of *E. heterophylla* demonstrated the least *in vivo* activity as shown by high percentage (40-60%) of infected mice showing pathological symptoms of diarrhoea, weight loss, loss of appetite and body weakness after 4 days, and up till the 7th day of extract administration.

Fig 2 presents the *in vivo* effects of the extracts of *V. paradoxa* and *E. heterophylla* on mice infected with *S. flexneri* showing antibacterial activity of the extracts in the order E.h/M>V.p/M>V.p/A>E.h/C>E.h/A>V.p/C. The methanol extracts of *E. heterophylla* demonstrated the highest *in vivo* antibacterial activity with only 40% and 20% of the infected mice manifesting diarrhoea symptoms between 1-3 days and 4-6 days respectively, and none (0%) showing symptoms of weight loss, loss of appetite and body weakness after 5 days of extract administration. Conversely, chloroform extracts of *V. paradoxa* demonstrated the lowest *in vivo* activity as shown by the high percentage (80 %) of infected mice showing symptoms of diarrhoea in 1-3 days and 4-6 days, and 40% showing symptoms of weight loss, loss of appetite and body weakness after 5 days and up till the 7th day of extract administration.

Fig 3 represent the *in vivo* effects of the extracts of *V. paradoxa* and *E. heterophylla* on mice infected with *E. coli* showing antibacterial activity of the extracts in the order E.h/M>V.p/M>E.h/A>V.p/A>V.p/C>E.h/C. The methanol extracts of *E. heterophylla* demonstrated the highest *in vivo* activity with only 60% and 20% of infected mice showing pathological symptoms of diarrhoea in 1-3 days and 4-6 days respectively, while none (0%) showed symptoms of diarrhoea, weight loss, loss of appetite and body weakness after 5 days of extract administration. However, chloroform extracts of *E. heterophylla* demonstrated the lowest *in vivo* activity as shown by the high percentage of infected mice (100%) manifesting diarrhoea symptoms in 1-3 days, and 40% with diarrhoea, weight loss, loss of appe-

tite and body weakness after 4 days and up till the 7th day of extract administration.

Fig 4 represent the *in vivo* effects of the extracts of *V. paradoxa* and *E. heterophylla* on mice infected with *P. vulgaris* showing anti-bacterial activity of the extracts in the order E.h/M>V.p/M>E.h/A>V.p/A>V.p/C>E.h/C. The methanol extracts of *E. heterophylla* demonstrated the highest *in vivo* activity with only 60% and 20% of infected mice showing pathological symptoms of diarrhoea in 1-3 days and 4-6 days respectively, and none (0%) showed symptoms of diarrhoea, weight loss, loss of appetite and body weakness after 5 days of extract administration. However, chloroform extract of *E. heterophylla* demonstrated the lowest *in vivo* activity as shown by the high percentage of infected mice with diarrhoea (60%) having diarrhoea in 1-3 days and 40% with diarrhoea, weight loss, loss of appetite and body weakness after 4 days and up till the 7th day of extract administration.

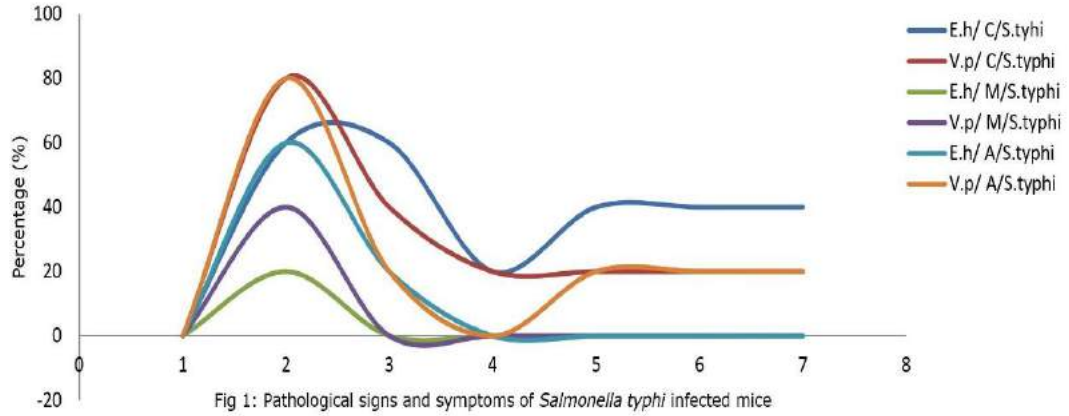

Fig 1: Pathological signs and symptoms of *Salmonella typhi* infected mice

1 = Mortality; 2 = Watery diarrhoea (1 – 3 days); 3 = Watery diarrhoea (4 - 6 days); 4 = Watery diarrhoea (> 7 days); 5 = Weight loss; 6 = Loss of appetite; 7 = Body weakness; E.h/C = Chloroform root extract of *Euphorbia heterophylla*; V.p/C = Chloroform root extract of *Vitellaria paradoxa*; E.h/M = Methanolic root extract of *Euphorbia heterophylla*; V.p/M = Methanolic root extract of *Vitellaria paradoxa*; E.h/A = Aqueous root extract of *Euphorbia heterophylla*; V.p/A = Aqueous root extract of *Vitellaria paradoxa*

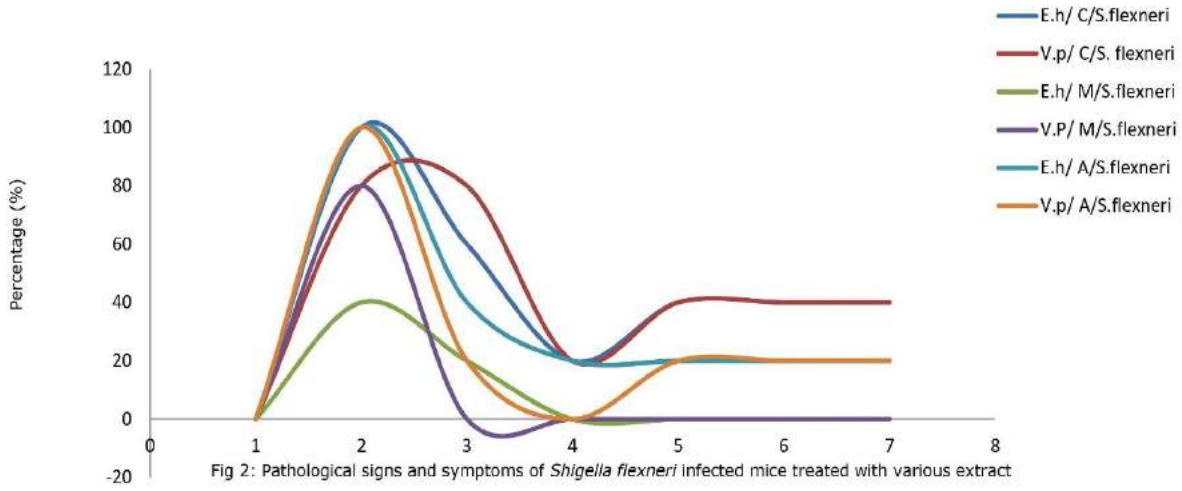

Fig 2: Pathological signs and symptoms of *Shigella flexneri* infected mice treated with various extract

1 = Mortality; 2 = Watery diarrhoea (1 – 3 days); 3 = Watery diarrhoea (4 - 6 days); 4 = Watery diarrhoea (> 7 days); 5 = Weight loss; 6 = Loss of appetite; 7 = Body weakness; E.h/C = Chloroform root extract of *Euphorbia heterophylla*; V.p/C = Chloroform root extract of *Vitellaria paradoxa*; E.h/M = Methanolic root extract of *Euphorbia heterophylla*; V.p/M = Methanolic root extract of *Vitellaria paradoxa*; E.h/A = Aqueous root extract of *Euphorbia heterophylla*; V.p/A = Aqueous root extract of *Vitellaria paradoxa*

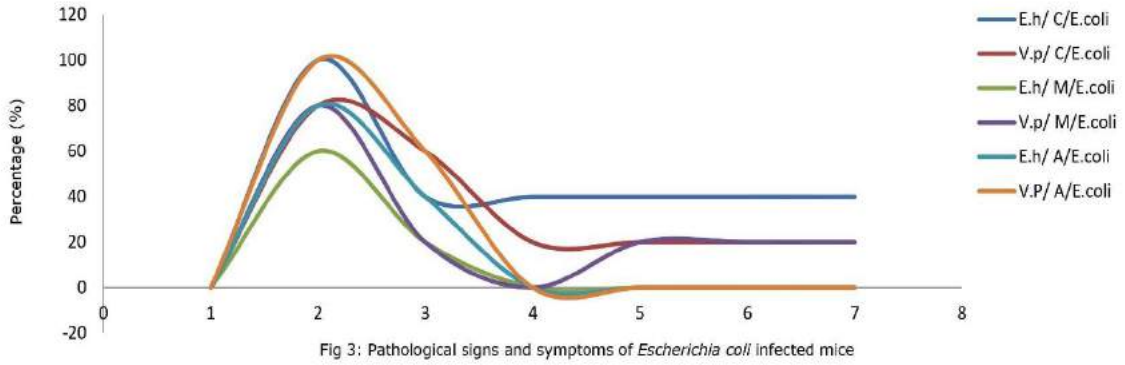

Fig 3: Pathological signs and symptoms of *Escherichia coli* infected mice

1 = Mortality; 2 = Watery diarrhoea (1 – 3 days); 3 = Watery diarrhoea (4 - 6 days); 4 = Watery diarrhoea (> 7 days); 5 = Weight loss; 6 = Loss of appetite; 7 = Body weakness; E.h/C = Chloroform root extract of *Euphorbia heterophylla*; V.p/C = Chloroform root extract of *Vitellaria paradoxa*; E.h/M = Methanolic root extract of *Euphorbia heterophylla*; V.p/M = Methanolic root extract of *Vitellaria paradoxa*; E.h/A = Aqueous root extract of *Euphorbia heterophylla*; V.p/A = Aqueous root extract of *Vitellaria paradoxa*

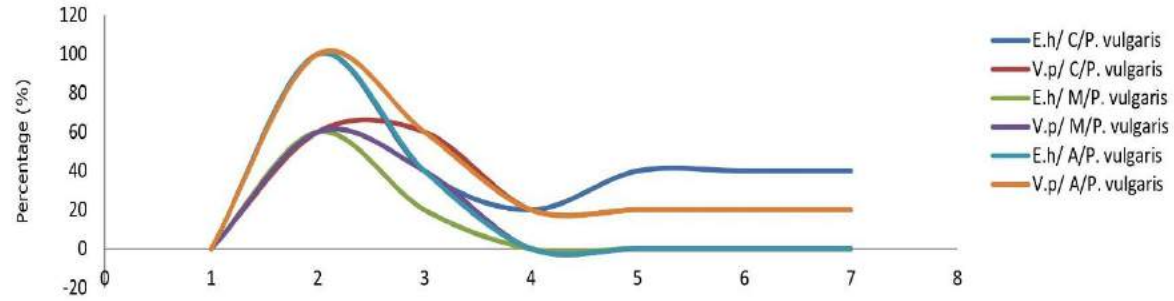

Fig 4: Pathological signs and symptoms of *Proteus vulgaris* infected mice

1 = Mortality; 2 = Watery diarrhoea (1 – 3 days); 3 = Watery diarrhoea (4 - 6 days); 4 = Watery diarrhoea (> 7 days); 5 = Weight loss; 6 = Loss of appetite; 7 = Body weakness; E.h/C = Chloroform root extract of *Euphorbia heterophylla*; V.p/C = Chloroform root extract of *Vitellaria paradoxa*; E.h/M = Methanolic root extract of *Euphorbia heterophylla*; V.p/M = Methanolic root extract of *Vitellaria paradoxa*; E.h/A = Aqueous root extract of *Euphorbia heterophylla*; V.p/A = Aqueous root extract of *Vitellaria paradoxa*

Discussion:

The basis of the therapeutic activities of plants lies on the phytochemical components contained in them (28), which implies that variations observed among the phytochemicals of the different plants will result in differences in their antibacterial activities. Thus, in this study, different phytochemicals were extracted in varying proportions by the solvents from the roots of the plants. Crude extracts of the roots of both *E. heterophylla* and *V. paradoxa* by the different solvents (chloroform, methanol, petroleum and water) contain cardiac glycosides, saponins, alkaloids, flavonoids, and tannins but *V. paradoxa* contain more carbohydrates and starch, and less of phlobatannins, compared to *E. heterophylla*. This is similar to the findings of Onwuliri (29), who observed the presence of phytochemicals such as phlobatannins, alkaloids, saponins, phenolics, tannins, and cardiac glycosides in tropical plants growing in Nigeria, some of which have been shown to exhibit varying biological activities, and medicinal properties (30). Mensah et al., (31) reported the importance of alkaloids, saponins and tannins in production of antibiotics used in treating common pathogenic microorganisms, which have also been known to produce definite physiological actions in the human body (32). The presence of cardiac glycosides indicates that they may act as good sedatives and antispasmodics (33). Schneider and Wolfhing (34) reported the therapeutic effects of some phytochemical constituents such as tannins and cardiac glycoside against cardiovascular diseases and digestive problems. Therefore, the presence of some of these phytochemicals in different proportions in the roots of the two plants in this study may be responsible for the observed *in vitro* and *in vivo* antibacterial activities.

In this study, there was a great disparity in the results obtained from the anti-

bacterial activities of the root extracts of both *E. heterophylla* and *V. paradoxa* in the *in vitro* and *in vivo* assays, and with regards to the different solvents used for extraction. The *in vitro* study showed that root extract of *V. paradoxa* had higher antibacterial activity than *E. heterophylla* for all the extracting solvents and on all the test bacteria, while the *in vivo* study showed that the root extract of *E. heterophylla* had higher antibacterial activity against infected mice than *V. paradoxa*, especially with methanol as solvent. The reason for this disparaging observation is not apparent because both plants had comparatively similar contents of phytochemicals except that *V. paradoxa* had more carbohydrates and starch, and less phlobatannins, which cannot explain the difference in the conflicting antibacterial activity. However, the study by Calixto (35) reported that differences in antibacterial activity of plants could be due to differences in geographical location, season of plant, age of the plant, and method of extraction, all of which can affect the yield and the active constituents of medicinal plants. Similarly, differences observed in the antibacterial activities of both root extracts could be attributed to the penetrating effects of the different solvents. Nevertheless, methanol appeared the best of all the four extracting solvents in this study for both plant extracts, and although the antibacterial activities of the methanol root extracts of both plants were lower compared to the standard antibiotic (ciprofloxacin), the finding in our study is significant if one considers that only crude extraction was done while the probable active antibacterial components of the plants were not characterized or purified.

We noted that 40% of mice infected with *S. typhi, E. coli* and *P. vulgaris* treated with chloroform root extract of *E. heterophylla*, and 40% of mice infected with *S. flexneri* treated with root extract of *V. paradoxa* showed pathological symptoms of watery diarrhoea, weight loss, loss of appetite and body weakness after 4 days, and up till the 7th day of extract administration, as compared to the groups of infected mice treated with methanol and aqueous extracts. The presence of diarrhoea (caused by the inoculated bacteria) in the infected mice after 4 days implies that chloroform extract produced poorer yield of the antibacterial components required for *in vivo* efficacy of the two plants, compared to the methanol and aqueous extracts. Aside this, the presence of weight loss, loss of appetite and body weakness, which though may be consequences of diarrhoea in the infected mice, could also be attributed to the physiological adaptation of the mice to chloroform, which can lead to low appetite and lower caloric intake by the animals. This condition has been previously reported by Rhiouani et al., (36). Furthermore, chloroform and some other extraction solvents can trigger abnormal behavioral changes in test animals, as reported by Pillai et al., (37), who observed reversible treatment-related behavioral signs such as apathy and reduced locomotor behavior in mice during toxicity test with methanolic leaf extract of *Plectranthus amboinicus.*

Conclusion:

Crude extracts of *E. heterophylla* and *V. paradoxa* produce *in vitro* antibacterial activity against the enteric Gram-negative bacteria pathogens involved in diarrhoea illnesses, in a dose-dependent manner. Methanol extract produced the best *in vitro* and *in vivo* antibacterial activities for both plants, while chloroform extracts produced the least antibacterial effects, and may also have been responsible for the physiological adaptations in the mice resulting in body weakness, loss of appetite and weight loss. Further researches should be directed towards isolation and characterization of the active compounds in the crude extracts

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Original Article **Open Access**

The role of *Escherichia coli* in the etiology of piglet diarrhea in selected pig producing districts of central Uganda

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Abstract:

Background: Pig production in Uganda is highly constrained by rampant piglet mortalities with diarrhea being a key feature. The present study was conducted to determine possible involvement of *Escherichia coli* (*E. coli*) as agents of diarrhea in piglets and elucidate the factors for their spread and virulence, towards development of mitigation strategies in the smallholder pig value chains in Uganda.
Methodology: This was a cross-sectional study carried out from January to August 2020 on pre- and post-weaned piglets from households in Kayunga and Mityana districts of Central Uganda, selected by snowballing method to redundancy. Data about herd management and risk factors for colibacillosis were collected from selected farmers in the two districts. A total of 179 faecal samples were collected from randomly selected neonatal and pre-weaning piglets for bacteriological isolation of *Escherichia coli*. Virulence (enterotoxin and fimbrial) genes from the isolates were detected by multiplex polymerase chain reaction (PCR) assay.
Results: From the 179 faecal samples, a total of 158 (88.3%) *E. coli* isolates were obtained. Virulence gene markers were detected in 18.4% (29/158) of the isolates. Among the investigated genes encoding for enterotoxin production, ST_b was the most prevalent (16/158, 10.13%), followed by ST_a (12/158, 7.59%), while gene for LT was not detected. The gene coding for F4 adhesin was the only one detected while F18 adhesin was not detected from the isolates. On multiple logistic regression analysis, only tertiary educational level (OR=0.141; 95% CI=0.30-0.666; p=0.013) and infrequent use of antibiotics (OR=0.231, 95% CI=0.062-0.859; p=0.029) among the farmers, were the two factors significantly protective of the piglets from diarrhoea.
Conclusion: This study reports a high prevalence of enterotoxin gene markers among *E. coli* isolates in piglets and revealed the potential role of these bacteria in the aetiology of piglet diarrhoea and mortalities in Uganda. Additionally, this study identified risk factors that can be useful in formulating treatment and control strategies of infection caused by these bacteria. Further studies are needed to identify more adhesins these *E. coli* isolates employ for intestinal colonization, a step that will help inform vaccine development.

Keywords: Antibiotic resistance; *Escherichia coli*; piglet diarrhea; virulence factors; Uganda

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Le rôle d'*Escherichia coli* dans l'étiologie de la diarrhée des porcelets dans certains districts producteurs de porcs du centre de l'Ouganda

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Abstrait:

Contexte: La production porcine en Ouganda est fortement limitée par la mortalité généralisée des porcelets, la diarrhée étant une caractéristique clé. La présente étude a été menée pour déterminer l'implication possible

d'*Escherichia coli* (*E. coli*) en tant qu'agents de diarrhée chez les porcelets et élucider les facteurs de leur propagation et de leur virulence, vers le développement de stratégies d'atténuation dans les chaînes de valeur des petits producteurs de porcs en Ouganda.
Méthodologie: Il s'agit d'une étude transversale réalisée de janvier à août 2020 sur des porcelets pré- et post-sevrés issus de ménages des districts de Kayunga et Mityana du centre de l'Ouganda, sélectionnés par la méthode boule de neige jusqu'à la redondance. Les données sur la gestion du troupeau et les facteurs de risque de colibacillose ont été recueillies auprès d'éleveurs sélectionnés dans les deux districts. Au total, 179 échantillons de matières fécales ont été prélevés sur des porcelets néonatals et en pré-sevrage sélectionnés au hasard pour l'isolement bactériologique d'*Escherichia coli*. Les gènes de virulence (entérotoxine et fimbrial) des isolats ont été détectés par une amplification en chaîne par polymérase (PCR) multiplex.
Résultats: À partir des 179 échantillons de matières fécales, un total de 158 (88,3%) isolats d'*E. coli* ont été obtenus. Des marqueurs du gène de virulence ont été détectés dans 18,4% (29/158) des isolats. Parmi les gènes étudiés codant pour la production d'entérotoxines, ST_b était le plus répandu (16/158, 10,13%), suivi de ST_a (12/158, 7,59%), tandis que le gène de la LT n'a pas été détecté. Le gène codant pour l'adhésine F4 était le seul détecté alors que l'adhésine F18 n'a pas été détectée dans les isolats. Sur l'analyse de régression logistique multiple, seul le niveau d'enseignement supérieur (OR=0,141; IC à 95%=0,30-0,666; *p*=0,013) et l'utilisation peu fréquente d'antibiotiques (OR=0,231, IC à 95 %=0,062-0,859; *p*=0,029) parmi les éleveurs, étaient les deux facteurs de protection significative des porcelets contre la diarrhée.
Conclusion: Cette étude rapporte une prévalence élevée de marqueurs génétiques d'entérotoxines parmi les isolats d'*E. coli* chez les porcelets et a révélé le rôle potentiel de ces bactéries dans l'étiologie de la diarrhée et de la mortalité des porcelets en Ouganda. De plus, cette étude a identifié des facteurs de risque qui peuvent être utiles dans la formulation de stratégies de traitement et de contrôle de l'infection causée par ces bactéries. D'autres études sont nécessaires pour identifier plus d'adhésines que ces isolats d'*E. coli* utilisent pour la colonisation intestinale, une étape qui aidera à éclairer le développement de vaccins.

Mots-clés: Résistance aux antibiotiques; *Escherichia coli*; diarrhée de porcelet; facteurs de virulence; Ouganda

Introduction:

Pork is one of the most consumed meat products worldwide (1) and the demand for pig meat is expected to increase with the growth in global population and economy. After China, the European Union (EU) is the second biggest producer of pig meat with a yearly production of 22.3 million tons (2). Therefore, maintenance of good health of pigs is very important. Uganda is among sub-Saharan Africa countries where pig production is increasing rapidly. The pig prolificacy is helping provide food security, however, it is constrained by low post-natal piglet survival (3). A study to quantify and classify pre-weaning causes of piglet mortality and how mortality relates to piggery management was conducted in peri-urban areas of Kampala, and out of 681 piglets born, 222 piglets died pre-weaning, representing 32.6% mortality (4). This is very high mortality considering that efficiency of piggery depends on the surviving litter size. In that study, infectious diseases were among the major causal factors reported. Moreover, during the smallholder pig value chain assessment in Uganda conducted by International Livestock Research Institute (ILRI) (3), among the major complaints' farmers put forward was rampant diarrhea. Similarly, in another study conducted in Gulu and Soroti, 38% of the pig keeping households reported diarrhea as a problem (5).

Globally, poor piglet survival is mainly due to *Escherichia coli*-induced diarrhea (6) or due to other bacterial pathogens (7). However, the causes of diarrhea/mortalities in the piglets have not been determined in Central Uganda, the major pig-producing region of the country. *Escherichia coli* infections seriously affect pig productivity through diarrhea, growth retardation, high morbidity and mortality among diseased pigs (6). Two distinct diarrhea syndromes are majorly recognized; neonatal diarrhea in piglets from 1-7 days of life, and post-weaning diarrhea that manifests about a week after weaning (8). The objectives of this study are to establish the involvement of *E. coli* pathogens in diarrhea among Ugandan piglets and to characterize their virulence genes, which is aimed towards adoption/development of appropriate vaccines for application to prevent piglet diarrhea and related mortalities in the small holder pig value chains in Uganda.

Materials and method:

Study area and design

This was a cross-sectional study carried out from January to August 2020 on pre- and post-weaned piglets from selected households in Kayunga and Mityana districts of Central Uganda. The location of Mityana district is between 00 27N and 32 03E while the coordinates for Kayunga district are 01 00N and 32 52E.

Sample size determination

The sample size of households included in the study was arrived at using the Cochran formula; $n = Z\alpha^2 (P*Q)/E^2$ (9), where, "n" is the sample size and "P" is the proportion of households keeping pigs. In this study, "P" was taken

as 6%, which is the percentage of households rearing pigs in Kayunga (10) and "Q" is the proportion of households not keeping pigs (1-P). The "E" is the level of acceptable error and was taken to be 5% while the "Zα" is the 95% confidence interval taken to be 1.96. Therefore, total number of households keeping pigs' targeted for the study was 87.

Ethical consideration

Ethical approval was obtained from the Ethical Review Committee of the School of Biosecurity, Biotechnical and Laboratory Sciences, College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University. Pig farmers were informed about the research and their consent to participate in the study was obtained before start of data collection.

Sampling, administration of questionnaire and collection of faecal samples

There was no information available regarding the households' keeping pigs in Uganda, therefore identification of the households was done using the Snowballing method to redundancy (11,12). Pig keeping households were visited to administer questionnaire and collect stool samples from diarrheic and non-diarrheic piglets. The first farmer was identified by the help of the extension agents and subsequent farmers were identified by the help of fellow farmers. A questionnaire was administered to selected farmers after obtaining their consent verbally in order to ascertain the factors that may underlie the spread of *E. coli* diarrhea in their piggeries. The roles of various household members in relation to pig care and management were also assessed particularly in relation to their gender.

Subsequently faecal samples were collected randomly from 2 piglets in each litter and before any antibiotic treatment was given in case of diarrhea, to ensure optimum recovery of *E. coli*. During the sample collection, each piglet was scored as being diarrheic or not. The faecal samples from each piglet were collected per rectum using sterile swabs (BD, Loveton, Maryland, USA) and were immediately placed into 5 ml of sterile chilled peptone water medium. The samples were then transported on ice in a cool box to the Microbiology laboratory at the College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, Uganda within 48 hrs of sampling for bacteriological culture and isolation.

Bacteriological isolation and identification

Each rectal swab was streaked out on sterile MacConkey agar (Condalab, Madrid, Spain) for the isolation of *E. coli*. Inoculated plates were incubated aerobically at 37°C for 18-24 hr. Preliminary identification of isolates was based on colonial morphology on all the media used. Lactose-fermenting colonies on MacConkey agar were biochemically confirmed as *E. coli* using tryptophan broth for indole test, methyl red for MR/VP test and citrate agar for citrate utilization test. Biochemically confirmed *E. coli* were stored in Brain heart infusion broth (Oxoid Ltd, Hampshire, United Kingdom) with 20% glycerol in duplicate at -20°C pending DNA extraction.

Antibiotic susceptibility tests

Antimicrobial susceptibility testing of *E. coli* isolates was performed by the standard Kirby-Bauer disk diffusion method against selected antibiotics (Oxoid Ltd, Hampshire, United Kingdom) which includes; kanamycin (K 30 µg), nalidixic acid (NA 30µg), tetracycline (TE 30 µg), ampicillin (AMP 10µg), chloramphenicol (C 30 µg), erythromycin (E 15µg), gentamicin (CN 10 µg) and ciprofloxacin (CIP 5µg) on Mueller-Hinton agar (Oxoid Ltd, Hampshire, United Kingdom). The 2018 Clinical and Laboratory Standards Institute (CLSI) guidelines (13) were used for the interpretation and categorization of the test strains as sensitive (S), intermediate (I) or resistant (R). Control *E. coli* strain ATCC 25922 was also set along with positive isolates during susceptibility testing for each antibiotic used.

Extraction of DNA from *E. coli* isolates

DNA was extracted from each isolate using the boiling method (5). Briefly, the stored isolates were thawed thoroughly before vortexing to ensure uniformity of the bacterial cell suspension. One ml of the sample was transferred into a correspondingly labelled 1.5 ml Eppendorf tubes, and centrifuged at 15,000 × g for 15 min in a microcentrifuge. The supernatant was discarded and remaining pellets were washed twice (by resuspending in 1 ml nuclease free water) followed by centrifugation at 15,000 × g for 10 min in a microcentrifuge. The pellets were then re-suspended in 100 µl of nuclease free water and boiled at 100°C for 10 min in a water bath. The tubes were thereafter frozen at -20°C for 10 min in a freezer and after thorough thawing, were centrifuged at 15,000 × g for 10 min in a microcentrifuge. Finally, 50 µl of the supernatant was transferred into correspondingly labelled Eppendorf tubes and stored at -20°C pending analysis by PCR.

Amplification of fimbrial and enterotoxin genes by multiplex PCR assay

Multiplex PCR assay was used to amplify the fragments of genes encoding enterotoxins

(ST_a, ST_b and LT) and fimbriae (F4 and F18) of each *E. coli* isolate. The primers used are as shown in Table 1. Each reaction consisted of 1× PCR buffer II, 3 mM $MgCl_2$, 200 µM each of dATP, dTTP, dCTP and dGTP and 1.5U of Ampli*Taq* Gold DNA polymerase (Applied Biosystems, ThermoFisher Scientific Corporation, Massachusetts, USA). The cycling conditions were set as follows; 95°C for 5 min, followed by 35 cycles of 94°C for 60 sec, 59°C for 60 sec and 72°C for 60 sec, followed by a final extension at 72°C for 5 min. DNA from *E. coli* strains 853/67; O149 ($F4^+$, $F6^+$, LT^+, ST_a^+, ST_b^+ and $EAST1^+$) (Uppsala, Sweden) and a blank sample without DNA were used as positive and negative controls, respectively.

Agarose gel electrophoresis

Eight microliters of each of the PCR amplicons were mixed with 2 µL of the loading buffer and resolved on 2% agarose gel in 1×TBE buffer at 125 V for 45 min using mupid-exu submarine electrophoresis system. The gels were stained by ethidium bromide, visualized by UV-transillumination and gel pictures captured by Smartphone camera.

Statistical analysis

Data obtained from the questionnaires were entered into a Microsoft Excel spreadsheet and descriptive statistics was performed. A univariate analysis using Chi-square test or Fisher's exact test was used to analyze the laboratory data and also test the associations between the independent variables and the outcome variable. Variables with $p \leq 0.25$ were then tested for confounding by cross tabulation of two variables. If the variables were confounding ($p \leq 0.05$), only one was taken for multivariate logistic regression analysis based on biological plausibility. The model fitness was assessed by Hosmer and Lemeshow (HL) test.

Results:

Socio-demographics of the farmers involved in the study.

In total, samples were collected from 74 households; 35 and 39 in Mityana and Kayunga districts, respectively (Table 2). Male gender dominated pig farming in the both districts, with 19 (52.3%) and 28 (71.8%) from Mityana and Kayunga, respectively. Majority of farmers in Mityana were in age group of 31-40 years (34. 3%), while in Kayunga, piggery was practiced mostly by farmers who were between 51-60 years of age (33.3%), which indicated that the youth were less frequently involved in piggery in the two districts (Table 3).

Table 1: PCR primers for amplifying specific DNA fragments of enterotoxin and fimbrial adhesin genes

Gene target	Primer sequence	Amplified product (bp)	Reference
LT	F – TAG AGA CCG GTA TTA CAG AAA TCT GA	282	(29)
	R – TCA TCC CGA ATT CTG TTA TAT ATG TC		
ST_a	F - GGG TTG GCA ATT TTT ATT CTG TA	183	(29)
	R – ATT ACA ACA AAG TTC ACA GCA GTA		
ST_b	F - ATG TAA ATA CCT ACA ACG GGT GAT	360	(29)
	R - TAT TTG GGC GCC AAA GCA TGC TCC		
F4	F - ATC GGT GGT AGT ATC ACT GC	601	(30)
	R - AAC CTG CGA CGT CAA CAA GA		
F18	F - GTG AAA AGA CTA GTG TTT ATT TC	510	(27)
	R - CTT GTA AGT AAC CGC GTA AGC		

F = Forward primer; R = Reverse primer; bp = base pair

Table 2: Number of households and sub counties selected in two districts of central Uganda

District	Sub-county	No of selected households
Mityana	Butayunja	12
	Kakindu	6
	Zigoti Town Council	8
	Mityana Municipality	9
Kayunga	Kayunga Town council	11
	Kangulumira	10
	Kitimbwa	8
	Kayunga	10

Table 3: Demographic characteristics of the household heads, pig owners, breeds and pig herd sizes in selected households in Mityana and Kayunga districts of Central Uganda

Demographic variables		Mityana n (%)	Kayunga n (%)	Total n (%)	*p* value
Gender	Male	19 (54.3)	28 (71.8)	47 (63.5)	0.118
	Female	16 (45.7)	11 (28.2)	27 (36.5)	
Age group (Years)	10-20	2 (5.7)	2 (5.1)	4 (5.4)	0.90
	21-30	1 (2.9)	4 (10.3)	5 (6.8)	
	31-40	12 (34.3)	4 (10.3)	16 (21.6)	
	41-50	9 (25.7)	8 (20.5)	17 (23.0)	
	51-60	5 (14.3)	13(33.3)	18 (24.3)	
	>61	6 (17.1)	8 (20.5)	14 (18.9)	
Education level	None	5 (14.3)	5 (12.8)	10 (13.5)	0.113
	Primary	14 (40.0)	9 (23.1)	23 (31.1)	
	Secondary	7 (20.0)	18 (46.2)	25 (33.8)	
	Tertiary	9 (25.7)	7 (17.9)	16 (21.6)	
Occupation	Farmer	18 (51.4)	18 (46.2)	36 (48.6)	0.438
	Civil Servant	2 (5.7)	4 (10.3)	6 (8.1)	
	Self employed	13 (37.1)	16 (41.0)	29 (39.2)	
	Student	2 (5.7)	0	2 (2.7)	
	Builder	0	1 (2.6)	1 (1.4)	
Ownership	Husband	9 (25.7)	18 (46.2)	27 (36.5)	0.016*
	Wife	17 (48.6)	15 (38.5)	32 (43.2)	
	Male child	2 (5.7)	5 (12.8)	7 (9.5)	
	Girl child	0	1(2.6)	1 (1.4)	
	Family	7 (20.0)	0	7 (9.5)	
Breed of pigs	Local	22 (62.9)	8 (20.5)	30 (40.5)	0.01*
	Exotic	8 (22.9)	23 (59.0)	31 (41.9)	
	Local & Exotic	5 (14.3)	8 (20.5)	13 (17.6)	
Pig herd size	(1-10)	26 (74.3)	19 (48.7)	45 (60.8)	0.153
	(11-20)	4 (11.4)	10 (25.6)	14 (18.9)	
	(21-30)	4 (11.4)	7 (17.9)	11 (14.9)	
	>30	1 (2.9)	3 (7.7)	4 (5.4)	
Pig Caretaker	House Wife	13 (37.1)	11 (28.2)	24 (32.4)	0.025*
	Husband	2 (5.7)	9 (23.1)	11 (14.9)	
	Male Child	4 (11.4)	4 (10.3)	8 (10.8)	
	Female Child	3 (8.6)	0	3 (4.1)	
	Hired Person	3 (8.6)	10 (25.6)	13 (17.6)	
	Family	10 (28.6)	5 (12.8)	15 (20.3)	

"n" denotes the number of selected households; * = statistically significant at $p < 0.05$

In Mityana, 14/35 (40%) farmers had primary level of education only, while in Kayunga, 18/39 (46.2%) farmers were educated up to secondary level. These statistics show that 13.5% of all those involved in keeping pigs did not have formal education. The difference in the

education level of pig farmers in the two districts was however not statistically significant (p= 0.113). However, 48.6% of those engaged in piggery in both districts were involved in farming as their major occupation. Wife owned pigs in 32 of the 74 households (43.2%) while in Mityana alone, 48.6% (17/35) of pigs were owned by wife. There was significant difference (p=0.016) in the ownership of pigs in both districts, with wife owning more pigs than husband. In both Kayunga and Mityana, housewives were most frequently involved in caring for the pigs (32.4%). Hired labourers were more frequently involved in pig care in Kayunga (25.6%) compared to Mityana (8.6%). On the other hand, husbands were less frequently involved in pig care (14.9%) (Table 3).

Fifty-nine percent of farmers in Kayunga reared exotic pig breeds, but Mityana farmers mostly reared local breeds (62.9%). In both districts, farmers were mostly involved in small scale pig rearing of about 1-10 per household. Additionally, 5.4% of farmers in this study were keeping more than 30 pigs per herd. However, the differences in herd size in both districts were not significant (p > 0.05).

***E. coli* strains isolated**

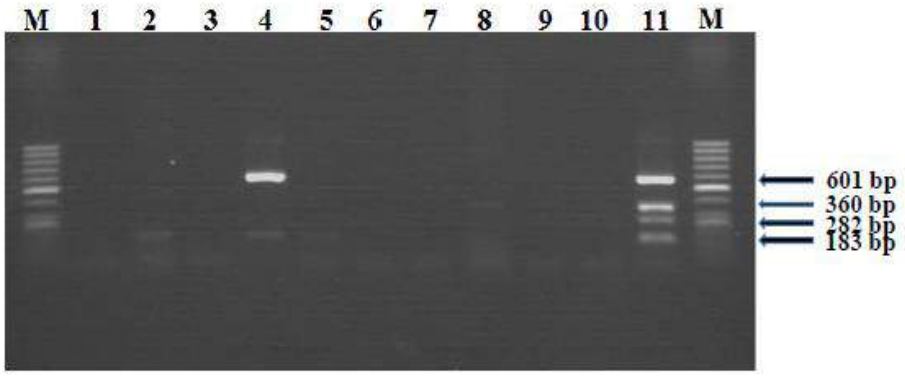
From the total of 179 faecal samples collected, 158 (88.3%) *E. coli* isolates were obtained following bacteriological culture. Majority of the *E. coli* isolates (85, 53.8%) were from Kayunga while 73 (46.2%) were from Mityana district (Table 4).

Virulence genes detected

Fig 1 shows a representative electrophoretic gel of virulence gene detection by PCR. Multiplex PCR assay revealed that 18.4% (29/158) of the isolates harbored virulence genes. Among the investigated genes coding for toxin production, ST_b was the most prevalent (16/158, 10.13%), followed by ST_a (12/158, 7.59 %), while gene for LT was not detected. Gene for fimbriae was present in 0.63% (1/158) of the isolates. The F4 was the only fimbrial gene detected in this study while F18 was not detected in any of the isolates. The one *E. coli* strain carrying F4 gene also carried enterotoxin STa gene, and no fimbrial adhesin was detected in the rest of 157 (99.4%) *E. coli* isolates (Fig 2).

Table 4: Frequency of *Escherichia coli* isolation from the 179 faecal samples

Isolates	Mityana	Kayunga	Total (%)
Escherichia coli isolated	73	85	158 (88.3)
No *Escherichia coli* isolated	10	11	21 (11.7)
Total	**83**	**96**	**179 (100)**



Lanes M = Molecular weight standard (200 base pair ladder); Lanes 1 to 9 = 10 *Escherichia coli* isolates tested; Lanes 10 = Negative control: Lane 11 = Positive control. Top arrow indicates the position of migration expected for the amplified fragment produced by the amplification protocol for F4 (601 bp), followed by STb (360 bp), LT (282 bp) and STa (183 bp)

Fig 1**:** Gel electrophoresis of PCR products of fimbrial and enterotoxin genes of selected *Escherichia coli* isolates

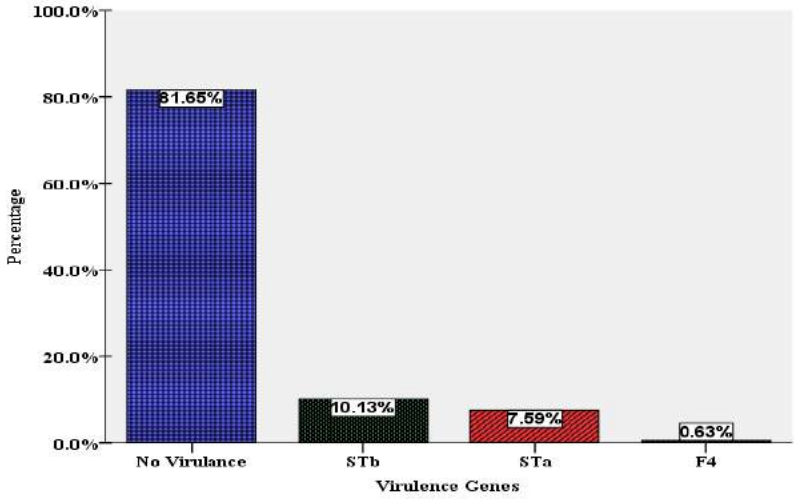


Fig 2: Frequency distribution of virulence genes in the 158 *Escherichia coli* Isolates

Table 5: Univariate analysis of statistically significant factors associated with piglet diarrhea

Factors		Diarrheic	Non-diarrheic	*p* value
Occupation	Farmer	13	23	0.027
	Civil Servant	0	6	
	Self-employed	16	13	
	Student	2	0	
	Builder	1	0	
Education level	None	3	7	0.020
	Primary	8	15	
	Secondary	17	8	
	Tertiary	4	12	
Breed of pigs kept	Local	9	21	0.029
	Exotic	19	12	
	Both	4	9	
District	Mityana	10	25	0.020
	Kayunga	22	17	
Frequent use of antibiotics	Yes	27	19	0.01
	No	5	23	

Risk factors associated with diarrhea in piglets

Sixteen independent variables; sex of household head, education level, age of household head, occupation, ownership of the farm, district, breed of pigs, breeding method, pig herd size, pigs keeping methods, house provided for neonate, pig care taker, source of water, weaning period, diarrhea common in the farm and common use of antibiotics, were analyzed as potential risk factors in the causation of diarrhea in piglets in the total of 74 farms in Mityana and Kayunga districts. Univariate analysis shows that occupation, education level, breed of pigs, district and frequent use of antibiotics were significantly associated ($p<0.05$) with diarrhea in the piglets (Table 5). At multivariate analysis, two factors (education level and frequent use of antibiotics) were significantly associated with diarrhea in the piglets (Table 6). Tertiary education (OR=0.141, 95% CI=0.30-0.666, $p=0.013$) and infrequent use of antibiotics (OR=0.231, 95% CI=0.062-0.859, $p=0.029$) among the farmers were the two factors significantly protective of the piglets from diarrhoea.

Results of antimicrobial susceptibility test

The antibiotic to which the *E. coli* isolates exhibited the most resistance in both districts was erythromycin (Fig 3), followed by tetracycline. In general, the *E. coli* isolates from Kayunga district showed higher resistance to the antibiotics compared to those from Mityana district (Table 7). The most effective antibiotic drug was kanamycin, followed by ciprofloxacin and gentamicin.

Table 6: Risk factors associated with piglet diarrhea in multivariate logistic regression model

Factors	Coefficient (B)	SE	OR	95% CI	*p* value
Education level	-1.960	0.793	0.141	0.30 - 0.666	0.013*
Breed of pigs	0.128	0.861	1.137	0.210 - 6.148	0.882
House provided for neonate	-0.195	0.625	0.823	0.242 - 2.802	0.756
Frequently use antibiotics	-1.466	0.670	0.231	0.062 - 0.859	0.029*
Constant	2.882	1.008	17.859	-	0.004

Hosmer and Lemeshow Test *p*= 0.003; Model = 0.257; * = statistically significant; OR = Odds Ratio; SE = Standard Error; CI = Confidence Interval

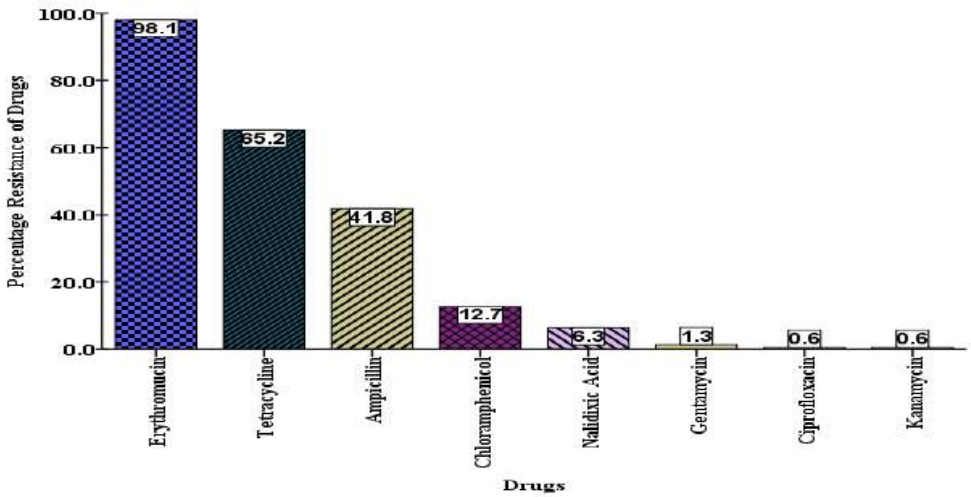


Fig 3: Antimicrobial resistance of 158 *Escherichia coli* isolates obtained from the piglets

Table 7: Comparison of resistance rates of different isolates between Mityana and Kayunga districts of Uganda

Antibiotics	Mityana	Kayunga	Total	*p* value
	n (%)	n (%)	n (%)	
Kanamycin	0	1 (1.2)	1 (0.6)	1.000
Nalidixic acid	6 (8.3)	4 (4.7)	10 (6.3)	0.514
Tetracycline	38 (52.8)	65 (75.6)	103 (65.2)	0.004*
Ampicillin	28 (38.9)	38 (44.2)	66 (41.8)	0.521
Chloramphenicol	8 (11.1)	12 (14.0)	20 (12.7)	0.638
Erythromycin	69 (95.8)	85 (100)	154 (98.1)	0.092
Gentamycin	1 (1.4)	1 (1.2)	2 (1.3)	1.000
Ciprofloxacin	1 (1.4)	0	1 (0.6)	0.456

* = statistically significant at $p < 0.05$

Discussion:

This was the first study to elucidate the role of *E. coli* in the aetiology of piglet diarrhea as well as the risk factors and antimicrobial resistance of these pathogens in Central Uganda. The finding of 18.4% of the *E. coli* with virulence factors (fimbriae and/or enterotoxin genes) is significant since it confirms the possible involvement of these bacteria in the etiology of diarrhea and piglet mortalities in Uganda. In the present study, the only adhesin detected in *E. coli* was F4, which is in agreement with the previous study conducted in Northern and Eastern Uganda by Ikwap et al., (5) but differ from developed countries (14) where F4 was most predominant. In our study, F18 adhesin was not detected, however, F18 adhesin was recently reported in diarrheic weaners from large commercial farms in Central Uganda (15). Since F18 adhesin is associated with post weaning diarrhoea (PWD), the prevalence of PWD could be very low in weaners from small holder herds, since this condition is mainly related to intensive rearing systems with high infectious load, stress caused by early weaning, moving and mixing of pigs (5,16) Secondly, the non-detection of F18 could be due to the low number of diarrheic piglets tested in our study. Interestingly, no adhesins were detected in 99.4% of the *E. coli* isolates tested in this study, although we investigated only F4 and F18 fimbriae. There is the need to therefore conduct more research on other adhesins in order to determine the key adhesins used by these bacteria to colonize piglet intestines, which can subsequently lead to diarrhea. This step will enable identification of appropriate vaccines for disease prevention.

In our study, the prevalence of piglet diarrhea was 16.8%. This is similar to the finding of a study conducted in India by Vinodh-Kumar et al., (17) which reported a prevalence of 14.29%, although slightly higher than 10% rate reported in a similar study conducted in Spain by Mesonero-Escuredo et al., (18). Worldwide, *E. coli* expressing enterotoxins (ETEC) are well known to cause severe diarrhea often with high mortality rates in both neonatal and post-weaning piglets (19). The ETEC strains associated with piglet diarrhea normally produce LT, ST_a or ST_b toxins or their combinations (20). In this present study, ST_a and ST_b were the only toxin genes detected, and in agreement with previous studies that reported high prevalence of ST_b in *E. coli* isolates from neonatal and weaned piglets (21,22), the most prevalent enterotoxin detected in *E. coli* from piglets in our study was ST_b (10.13%). However, the gene for LT was not detected in our isolates, suggesting that *E. coli* harboring genes encoding for this toxin are not yet widespread. The absence of the gene for LT in all of the *E. coli* isolates in our study is in agreement with previous study in Northern and Eastern Uganda by Ikwap et al., (5) where none of the LT gene was detected. This suggests that ETEC diarrhea in Central Uganda could be largely contributed by ST_b and ST_a in neonatal and post-weaning piglets. In order to cause colibacillosis, an ETEC strain needs to simultaneously carry at least one fimbria and one toxin gene (23). Considering this criterion, less than 1% of the isolates in our present study could be classified as ETEC since there was only one ST_a/F4 combination detected in this study. The other isolated strains might have been non-pathogenic or belonged to other pathotypes such as EPEC and the fimbrial type F5, F6 or F41, which were not investigated in this study.

Two factors (tertiary education level and infrequent use of antibiotics) were significantly protective of the pigs from diarrhea from our study analysis. This may probably indicate that highly educated farmers (with tertiary level education) consider hygiene and other disease control measures more importantly than less educated farmers (with secondary level education) and therefore adhere to the required processes to control and prevent disease. However, if a farmer with formal education lacks training and experience in animal husbandry, it can lead to flaw in pig production as knowledge uptake itself does not necessarily guarantee an improvement in the everyday practices (24). Pig farmers in the study area were also involved in other farming activities, self-employed or were civil servants. This picture gives the impression that pig farming alone does not make farmers to make a living within the study area.

Infrequent use of antibiotics was significantly associated with lower frequency of diarrhea in the piglet probably because of the adherence to disease control measures such as environment hygiene and proper feeding. Farmers who frequently use antibiotics tend to assume that these drugs are a panacea to proper rearing of pigs, yet not all antibiotics are effective against bacteria pathogens. For treatment to be effective against disease-causing bacteria, antibiotics must reach the site of infection at sufficient concentrations and for sufficient amount of time. Inappropriate use of these antibiotics may not only promote antimicrobial resistance but also aggravate the disease. Furthermore, commensal beneficial bacteria provide a natural barrier and defense system against pathogenic bacteria, treatment with antibiotics results in killing of these beneficial bacteria, thereby rem-

oving the natural defense system of the animals and making them more vulnerable to disease.

Antimicrobial resistance profiles of *E. coli* isolates in the present study revealed high resistance to erythromycin and tetracycline. This is highly concerning considering that the tetracyclines are the most frequently used antibiotics for treatment of infections in animals and poultry in Uganda, compared to other antimicrobial agents (25). Therefore, the high resistance levels in our study might be the result of widespread and frequent use of tetracycline to treat colibacillosis in the field. Interestingly one of the risk factors detected associated with piglet diarrhea was frequent use of antimicrobial agents against infections in the pig herds. This is attributable to probable emergence of antimicrobial resistance, hence the inability to control infections using the commonly available antimicrobials in these households.

Resistance to ampicillin, a commonly prescribed antibiotic for the treatment of colibacillosis, was relatively high with overall resistance of 41.8% in this study. A study done in Korea by Kyung-Hyo et al., (26) showed higher resistance of 84.9%. Overuse of ampicillin in Uganda to treat piglets against diseases such as clostridiosis and colibacillosis has probably led to such high degree of resistance of *E. coli* to this drug. Withdrawing such antibiotic from use by making it unavailable in the market and/or restricting its use may allow it to recover its potency, and may then subsequently be re-introduced, through a process of antibiotic cycling.

Majority of *E. coli* isolates in this study were susceptible to kanamycin, gentamicin, ciprofloxacin, nalidixic acid, and chloramphenicol. These findings are similar to that of a previous study on *Salmonella* spp in piglets and weaners from Northern and Eastern Uganda (27). Apart from gentamicin and kanamycin, the other three antimicrobials (chloramphenicol, ciprofloxacin and nalidixic acid) are mainly used in humans in Uganda. Furthermore, some of these antibiotics are very expensive and therefore not commonly used. Possibly this could be the reason for the high susceptibility of the piglet derived isolates of *E. coli* to these drugs in the present study. However, high levels of resistance to gentamicin were reported among *E. coli* isolated from diseased pigs in Belgium, Poland and Spain with rates of 46%, 45%, and 20% respectively (28). There is therefore the need for continuous monitoring, restrictions and judicious use of antimicrobials to ensure the future availability of effective antimicrobial drugs for use in both humans and veterinary medicine in Uganda.

Conclusion:

This study reports a high prevalence of enterotoxin gene markers in *E. coli* in tested piglets therefore highlighting the potential role of these bacteria in the aetiology of piglet diarrhea and mortalities in Uganda. Additionally, this study identified risk factors that can be useful in formulating treatment and control strategies for infections caused by these bacteria. Since no adhesins were identified in most of the *E. coli* isolates, further studies are needed to identify the adhesins these *E. coli* isolates employ for intestinal colonization, a step that will help inform vaccine development in Uganda settings.

Conflict of interest:

Authors declare no conflict of interest.

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3. Bakare, R. A., Oni, A. A., Okesola, A. A., et al. Efficacy of pefloxacin on acute uncomplicated gonococcal urethritis. Nig Qt J Hosp Med. 1996; 6: 335

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