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Review Article **Open Access**

Microbiome: pharmacokinetics, pharmacodynamics and drug/xenobiotic interactions

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Abstract:

The participation of microbiota in myriads of physiological, metabolic, genetic and immunological processes shows that they are a fundamental part of human existence and health maintenance. The efficiency of drugs' absorption depends on solubility, stability, permeability and metabolic enzymes produced by the body and gut microbiota. Two major types of microbiota-drug interaction have been identified; direct and indirect. The use of antibiotics is a direct means of targeting intestinal microbes and short-term use of antibiotic can significantly alter the microbiome composition. It is noteworthy that not every microbial drug metabolism is of benefit to the host as some drugs can shut down microbial processes as observed in the co-administration of antiviral sorivudine with fluoropyridimide resulting in a toxic buildup of fluoropyridimide metabolites from blockade of host fluoropyridimide by the microbial-sorivudine metabolite. It has been reported that many classes of drugs and xenobiotics modify the gut microbiome composition which may be detrimental to human health. Microbiome-drug interaction may be beneficial or detrimental resulting in either treatment success or failure which is largely dependent on factors such as microbial enzymes, chemical composition of candidate drug, host immunity and the complex relationship that exists with the microbiome. The effects of microbiota on pharmacology of drugs and *vice versa* are discussed in this review.

Keywords: microbiome; pharmacokinetic, pharmacodynamic, drug, xenobiotic

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Microbiome: pharmacocinétique, pharmacodynamique et interactions médicamenteuses/xénobiotiques

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Abstrait:

La participation du microbiote à des myriades de processus physiologiques, métaboliques, génétiques et immunologiques montre qu'ils sont un élément fondamental de l'existence et du maintien de la santé de l'être humain. L'efficacité de l'absorption des médicaments dépend de la solubilité, de la stabilité, de la perméabilité et des enzymes métaboliques produites par le corps et le microbiote intestinal. Deux types principaux d'interaction microbiote-médicament ont été identifiés; direct et indirect. L'utilisation d'antibiotiques est un moyen direct de cibler les microbes intestinaux et une utilisation à court terme d'antibiotique peut modifier de manière significative la composition du microbiome. Il est à noter que tous les métabolismes de médicaments microbiens ne sont pas bénéfiques pour l'hôte, car certains médicaments peuvent arrêter les processus microbiens observés lors de l'administration concomitante d'antiviral sorivudine et de fluoropyridimide, ce qui entraîne une accumulation toxique de métabolites de fluoropyridimide résultant du blocage du fluoropyridimide par l'hôte. métabolite microbien-sorivudine. Il a été rapporté que de nombreuses classes de médicaments et de xénobiotiques modifiaient la composition du microbiome intestinal, ce qui pourrait nuire à la santé humaine. Une interaction médicamenteuse-microbiome peut être bénéfique ou préjudiciable, entraînant le succès ou l'échec du traitement,

qui dépend en grande partie de facteurs tels que les enzymes microbiennes, la composition chimique du médicament candidat, l'immunité de l'hôte et la relation complexe qui existe avec le microbiome. Les effets du microbiote sur la pharmacologie des médicaments et inversement sont discutés dans cette revue.

Mots-clés: microbiome; pharmacocinétique, pharmacodynamique, médicament, xénobiotique

Introduction:

The understanding of the facts that microbiota/microbiome play an important role in the lives of humans dictates how much microorganisms matter. Their participation in myriads of physiological, metabolic, genetic, and immunological processes shows that they are a fundamental part of humans' existence and health maintenance. The customized genetic differences in the microorganisms exceeding the human genome also have significant consequences for drug reactions in humans. This is as a result of microbiota involvement in its metabolism through the action of enzymes and a complex host-microbiota interaction as the host genome can influence the microbiome in terms of phenotypes and function (1,2).

Most drugs are designed for oral administration. Drugs movement through the gut makes them come in direct contact with other extra-intestinal organ such as the liver. Also, they encounter intestinal environmental conditions such as the stomach acids as well as coming in contact with myriads of gut microorganisms from small and large intestines. The efficiency of drugs' absorption depends on solubility, stability, permeability and metabolic enzymes produced by the body and gut microbiota (3, 4). Furthermore, parenterally administered drugs, as well as their metabolites, also reach the intestines through biliary secretion making the gut a central station for drug metabolism prior to absorption thereby the bioavailability and distribution of the drug is altered (1,3). However, little is known about microbiome and drug interactions (4,5).

Two major types of microbiota-drug interaction have been identified; direct and indirect.Indirect interaction may involve competition between microbiota-derived metabolites and administered drugs for the same host metabolizing enzyme; microbiome reactivation of secreted inactive drug metabolite; effect on the immune system and effect on the overall level of the metabolizing enzymes in the liver and intestine. Direct interactions include a partial or complete biochemical transformation of a candidate drug into more or less active metabolites by microbially-derived enzymes known as Microbiome-Derived Metabolism (MDM) (4). Although MDM has been observed about 50 years ago it was overlooked in drug development. Studies that are investigating this process focused on specific bacterium against a specific drug rather than a systematic assessment of human gut microbiome's capability to metabolize several oral drugs. This problem is due to the microbiome's huge complexity and the overwhelming technical challenge of testing several drugs against several isolate cultures under multiple conditions. More so, the unavailability of the global standard has hampered the reliability to predict undesired microbiome effect on pharmacodynamics and pharmacokinetics of drugs (4).

Methodology:

A review of microbioata as it relates to pharmacology of drugs was conducted. The materials used for the review included relevant published articles in journals spanning the period 2001 to 2019, using google and google scholar search engines. The keywords used for the search were 'xenobiotic', 'microbiome', 'microbiota', 'pharmacology' and 'gut microbioata'. A total of 150 articles were initially identified but following assessment, 53 were selected for the review.

Microbiome effects on drugs

The use of antibiotics is a direct means of targeting intestinal microbes and short-term use of the antibiotic drug is enough to significantly alter the microbiome composition (6). A population-based study involving deep sequencing of gut microbiomes of 1135 subjects showed an association between microbial species and 19 drug groups (7). The degree to which the gut microbiome is affected may depend on the type and number of drug taken either combined or singly by an individual. While drugs such as Non-Steroidal Anti-inflammatory Drugs (NSAIDs), and Proton Pump Inhibitors (PPIs) showed differences in the individual gut microbiome composition, drugs such as PPIs, antidepressants and antipsychotics showed significant association with single bacteria abundance (6, 8). Conversely, in a study, gliclazide (anti-diabetic) pre-treated with probiotics showed

higher blood levels of gliclazide in treated rats compared with non-treated rats revealing that gut microbiota may mediate the degree to which a drug is absorbed (9). Furthermore, another study reported a 3-day administration of *Lactobacillus reuteri* (K8) to have reduced orally administered acetaminophen in mice while another variant of the same species (K9) showed no observable effect; this effect may be mediated by probiotic-induced-modulation of the gut microbial enzyme activity as sulfatase and arylsulfate transferase were increased by the probiotic and β-glucuronidase enzyme decreased (10).

Microbiome modifications of drug absorption

The bioavailability and absorption rate of candidate drugs administered orally pose a major obstruction to drug efficiency as about 40% of these oral drugs are no more in use as a result of poor bioavailability and/or pharmacokinetics in the 1990s (11). The governing principles of absorption totally lies in the chemical properties of the drug and how the host can actively import or export the molecule. Early studies implied that drug transport may be influenced by microbiome in a composition-dependent manner, and multiple animal studies also demonstrated that the microbiome modulates absorption of drugs (2).

Among the various methods of *invitro* screening of drug absorption parameters, cell-based drug absorption assays are one of the most common and readily adopted for high-throughput screening (12). The Caco-2 cell line (ATCC HTB-37) is an ideal model for these assay types as it has the ability to differentiate into a monolayer homologous to the enterocytes of the small intestines with characteristic expression of transporters which include p-glycoprotein and OATP-A/B (2, 12). When the Caco-2 cells are grown on a semi-permeable membrane such as the Transwells, a polarized membrane is produced with an apical and basolateral chamber which is functionally homologous to the intestinal lumen and underlying circulation respectively. The apical to basolateral and vice versa are measured for a candidate drug, or suitable surrogate compounds such as p-glycoprotein model substrates digoxin, calcein-AM, or rhodamine 123 (13, 14). Complex Caco-2 cell models such as the gut-on-a-chip and HuMiX models allow for 3D structures development including the villi, and divergent cell types with a relative enhancement of CYP3A4 activity while facilitating co-culture with bacterial cultures (2).

Several modes through which the microbiome regulatess host transport have been suggested, but they either include controlling host gene expression, llosteric regulators, substrate competition, or binding of microbial products acting as inhibitors (2). Through the use of quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) panels or RNA sequencing, examination of host expression can be achieved in a targeted manner. Meanwhile, the examination of co-differentially regulated genes may give more insight into the pathways through which modulation occurs (2). For instance, p-glycoprotein is under the control of FXR, PXR, AP-1 and NFκB pathways, but the examination of co-regulated transcripts employing the pathway analysis tools may help detect the active specific pathway. It has been established that indole metabolites and other microbial-derived compounds function as PXR ligands (15, 16).

The intestinal lumen contains a variety of cell types, many of which do not have an absorptive function. To be able to target absorptive enterocytes therefore, laser capture microdissection or single-cell RNA sequencing may be employed to limit differential expression analyses to target cell types of interests. This may be essential in the case of genes which have functions in other cell types such is the case of P-glycoprotein expressed in phagocytes and T cells (17).

Microbiome-drug metabolism

It is noteworthy that not every microbial drug metabolism is of benefit to the host as some drugs can shut down microbial processes as observed in the co-administration of antiviral sorivudine with fluoropyridimide resulting in a toxic build up of fluoropyridimide metabolites as a result of blockade of host fluoropyridimide by the microbial-sorivudine metabolite (2). About fifty known drugs have been shown to be metabolized by bacteria, however neither the microbial species nor the genetic determinants responsible for these drug metabolisms are known (18). Moreso, since there are no observable pieces of evidence for bacterial metabolism to have taken place, the number of drugs metabolized by the microbiome is likely to be higher (19).

The discovery of high-throughput culturing has made screen collections and bacteria selection possible, representing extensive panels of strains for drug metabolism that are human-associated. It is possible to observe substrate loss or new metabolite production by the co-incubation of

each microbial strain or collection (synthetic community) with each of the desired drugs. While high-throughput isolate screens have the merit of detecting bacteria capable of drug metabolism with direct interpretation, high-density plate-based culturing and robotics provide a superficial sampling of microbial genetic variation as development of analytical method for the drug is a primary limitation (2). Large chunk of the genetic variations within a species is achieved via horizontal gene transfer which is usually associated with resistance to antibiotics (2, 20).

Many phenotypes such as the ability to inactivate the drug digoxin, a cardiac drug, occurs periodically within species (21). The presumptive selection of representative strains may likely not have the genes of interest due to superficial sampling at the species level; however, this approach has proven successful in the identification of multiple drug metabolizing bacterial species (22). The use of *ex vivo* human stool samples incubations may be an alternative to culture-collection screens as this method has the advantage of identifying many of the drugs that are susceptible to microbial metabolism. Nonetheless, inter-strain antagonism may obscure metabolism when testing a community of microorganisms collectively and this may be true especially if these metabolizing microbes are impeded by antimicrobial products such as bacteriocins or organic acids of metabolically inactive strains (23). In addition, using media formulations, some microbes may be favored, conferring dominance on them over the entire culture. One method to overcome this is to perform *ex vivo* incubations through manifold media formulations with broad selection pressures ready for aerobic spore-forming Gram-positive, strictly anaerobic, and Gram-negative microbes (2).

Another methodology for investigating microbial biochemical changes from a mixed community is the use of faecalase preparation, which is a cell-free faeces extract including microbial enzymes (2). This method has been used to assess gut microbial enzyme activity against herbal glycosides generating genotoxic aglycone products (24). Also, this method has been used to demonstrate that lovastatin, a cholesterol-lowering drug can be metabolized by the gut bacteria (25). However, the presence of, or competition for, essential cofactors and membrane-bound coenzymes may lead to false-negative results in this methodology; these may or may not be present in the faecalase preparation. Furthermore, the secretion of cytosolic enzymes which may not be active in buffer conditions and the lack of constant generation of NAD(P)/FAD in the cell may inhibit metabolic activity and consequently, the sensitivity of this methodology can be problematic to define (21).

A number of approaches may be employed to unearth the molecular mechanisms responsible for detecting metabolism. Since it has been established that a specific microbial strain is capable of metabolism, multi-omics can be especially informative (2). In the bacterial genome, genes that are functionally related are typically restricted and transcriptionally regulated as operons. After stimulated by a ligand, there is the induction of specific effector gene expression and this can be exploited for the identification of candidate effector enzymes and molecules through gene expression analysis such as RNA sequencing (RNA-seq) by differentiating expression between drug-exposed and vehicle controls (2). However, not all enzymes are subject to substrate-mediated transcriptional regulation and there is need for caution to syncronize the the drug-exposed cultures and growth phase of control prevent false-positive results stemming from changes in cellular physiology (19).

Lastly, many non-antibiotic drugs may have either bactericidal or bacteriostatic effects *invitro*, potentially causing serious changes in gene expression due to stress responses. As soon as specific genes are identified, their induction can be readily confirmed by quantitative reverse transcriptase PCR (qRT-PCR) on independent cultures. Nonetheless, follow-up studies are very paramount to make causal links between the identified genes and specific biochemical reactions (2). Nuclear receptors especially CAR, PXR, FXR and AHR regulate the expression levels of transporters and enzymes for drug-metabolizing (26,27). The CAR, PXR and FXR are classified members of the steroid receptor superfamily that regulate their associated genes through the formation of a DNA-binding heterodimer with retinoid X receptor (27). The CAR receptor regulates the following drug-metabolizing enzymes; CYP2B6 and CYP2C9 (phase 1), UGTB1 and SULT1E1 (phase 2) and organic anionic transport protein OATP1B3 (phase 3). Meanwhile PXR regulates CYP3A, CYP3B, CYP2B, CYP2C and GSTA1, UGT1A3, as well as UGT1A6 in phase 1 and phase 2 metabolizing enzymes respectively (27). Both CAR and PXR regulate overlapping groups of genes involved in phase 1, 2 and 3 metabolism (27).

Impacts of xenobiotics and drugs on gut microbiome

It has been reported that many classes of drugs and xenobiotics modify the composition of gut microbiome which may be detrimental to human health. Infection by *Clostridium difficile* has been reported to be associated with the use of proton pump inhibitors by patients (28). Several drugs such as sulfasalazine, antibiotics, digoxin and phenacetin have been investigated to have the greatest impact on the functionality of gut microbiome but the degree to which antibiotic treatment modulates the metabolism of orally administered drugs is subject to further perusal (27). Additionally, many xenobiotics have been reported to modify the gut microbiota and most notable xenobiotic is arsenic which has been demonstrated in a mouse model to significantly decrease the abundance of *Firmicutes* which are mainly producers of butyrate thereby altering the composition of indole and glucuronide metabolites (27).

Microbiome impacts on phytochemical metabolism and bioavailability

The most notable function of host-microbial co-metabolism is the conversion of dietary plant substances into bioactive molecules. This role has attained accelerated importance with traditional medicine and herbal supplements now widespread (29). Phenolics and flavonoids are the most common phytochemicals most disposed in the human colon to microbial metabolism (27). More over, microbiome impact on phytochemicals includes metabolic changes involving esterases, demethylations, glycosidases, dehydroxylations and decarboxylations. Curcumin is the best-studied naturally occurring phenolics due to its anti-oxidant and anti-inflammatory activities. Its pharmacologic activity is thought to be as a result of tetrahydrocurcumin, a metabolite produced by gut microbiota. *Escherichia coli* from human faeces exhibits the highest curcumin metabolizing activities (27, 30).

Microbiome impacts on drug/xenobiotic metabolism and pharmacokinetics

The gut microbiome uses several mechanisms to modify the nature, potency and toxicity of both drugs and xenobiotics. These mechanisms are achieved through; (i) expression of enzymes that either inactivate or activate drugs metabolically, seen in the case of sulfasalazine for the treatment of gut inflammation being converted to 5-amino 5-salicylic acid (its active form) by microbial enzymes while on the other hand, digoxin is being inactivated by a cardiac glycoside produced by the bacterium *Eggerthella lenta*; (ii) the drug may be separated in order to store its component by binding directly to the bacterial organism as in the case of L-DOPA sequestered by *Helicobacter pylori*; (iii) the drug may be reactivated metabolically by expressed enzymes produced by microbes and an example of this mechanism is irinotecan (CPT-11), a drug that was previously inactivated in the liver via glucuronidation, being reactivated by bacterially expressed beta-glucuronidase resulting in diarrhoea within the intestines; (iv) metabolites that function as intermediates may be generated by the microbiota and an example is the toxicity in melamine as a result of the microbial formation of cyanuric acid (its metabolite); and (v) microbial p-cresol and metabolites produced by the host from a candidate drug acetaminophen may directly compete for a host enzyme SULT1A1 (29,31,32). Metabolic reactions of microbiota known to largely affect the bioactivity of xenobiotics and drugs comprise reduction, proteolysis, hydrolysis, acetylation, dihydroxylation, deacetylation, deconjugation and deglycosylation processes (27).

Several enzymes expressed by gut microbes have been identified. These include azoreductases which have been reported to metabolize the drug prontosil and neoprontosil. When injected intravenously, the drugs gain entry into the intestine and are converted to sulfanilamide by gut microbiota. Another drug, balsalazide is metabolized into 5-amino-salicylic acid by microbially expressed enzymes (azoreductases). However, antibiotic treatment suppresses the conversion of orally administered balsalazide into 5-aminosalicylic acid, prontosil and neoprontosil to sulfanilamide in rat respectively (3).

Among many studies that provide insight into the upcoming role of gut microbiome in the metabolism and pharmacokinetics of drugs, bacterial nitroreduction reactions are of keen interest as they can largely affect the pharmacologic activity of nitroaromatic drugs such as chloramphenicol, 2-chloro-5-nitro-N-phenoxybenzamine (GW9662), nitrobenzodiazepine and 5-(aziridine-1-y1)-2,4-dinitrobenzamide (CB1954) (27). Chloramphenicol, a substrate of bacterial nitroreductase, GW9662 is an antagonist of peroxisome proliferator which is activated by receptor g and a chemo-preventive agent, while the principal plasma metabolite of GW996 is an amine metabolite. The nitroreduction of this aimine metabolite by bacterial nitroreductases

can largely change its mutagenicity. The study of both nitroreduction of nitrobenzodiazepine (an addictive sedative in the treatment of anxiety and sleep disorders) resulted in further characterization of bacterial nitroreductases and its metabolism (27).

Nitroreductase being identified as NfsB is expressed by *E. coli* and it has been suggested that NfsB may be suitable for development of anti-addictive agents as nitroreduction leads to the inactivation of nitrobenzodiazepine. CB1954, an anticancer drug which is a dinitrobenzamide prodrug has been developed to target cancer cell through the delivery of NfsB transgene (33). Furthermore, other enzymes expressed by *E. coli* species capable of nitro and azo reductions under aerobic conditions comprise NfsA and AzoR. Other organisms capable of expressing nitroreductases include *Bacillus* spp., *Mycobacterium*, *Enterobacter* and *Staphylococcus* (34). Although nitroreductases are involved in antibiotic resistance, their role in the metabolism of currently prescribed drugs is yet undetermined.

N-oxide reduction by bacteria lies at the central relationship between microbial enzymes and the host in the metabolism of an inhibitor (BILR355) of the human immunodeficiency virus (HIV). BILR355 is metabolized by CYP3A, nonetheless, a study in which simultaneous administration of BILR355 with ritonavir occurred, unravelled a distinct role of aldehyde oxidase and gut bacteria (35). The biotransformation of BILR355 involves a two-step process; the first step involves the reduced form of N-oxide generated by the gut bacteria while the second step involves bacterially derived metabolite subject to the further host enzymes metabolism either by CYP3A or aldehyde oxidase. Nevertheless, the action of CYP3A is inhibited while the bacterial enzyme or aldehyde oxidase takes over (27, 35).

The relationship between the gut bacterial enzymes and host cytochrome P450 is also required in fostamatinib (tyrosine kinase inhibitor) metabolism. Fostamatinib is a prodrug oxidatively metabolized by CYP3A4 upon cleavage of alkaline phosphatases. Furthermore, a metabolite believed to have been formed in faeces via O-demethylation and dihydroxylation by gut anaerobes has been recognized. An *invitro* colon model supported with metabolomics has shown that cholesterol dissolving drug, simvastatin can be metabolized by the colon microbiota where anaerobes are prevalent. Simvastatin, designed to inhibit 3-hydroxyl-3methylglutaryl coenzyme A in the liver to reduce cholesterol level is hydroxylated and subjected to b-oxidation, glutathione conjugation, and glucuronidation (36, 37). Other drugs metabolized by the colon anaerobic bacteria include ranitidine (H_2 antagonist) and prednisolone, a glucocorticoid agonist and anti-inflammatory agent (27).

Arsenic and polyaromatic hydrocarbons are xenobiotics that have been subjected to microbial metabolism in the gut (27). A study using a simulator of the microbiota (human gut) showed that colon microbiota can be involved in the extensive metabolism of arsenic (38). Sulphate reducing bacteria such as the *Desulfovibrio desulfuricans* are notable for their H_2S production which converts monomethyl arsenic acid to monomethyl monothioarsenic which is a more noxious form of arsenic (38). The metabolism of polyaromatic hydrocarbons involves oxidation reactions by the cytochrome P450, followed by phase 2 conjugation with either glucuronic acid gluthathione or sulphate. Report from the simulation of human microbiota shows that colonic human bacteria are also capable of biotransforming polyaromatic compounds such as naphthalene, phenanthrene, pyrene, and benzo[a]pyrene. *Micrococcus luteus* has been identified to metabolize benzo[a]pyrene and the most likely enzyme involved was DszA/NtaA-like oxygennase (27, 39).

Microbiome, detoxification and excretion

Detoxification and excretion of drugs in the human system are intrinsic to drug distribution and are basically propelled by hepatocytes for most medications (2). The proximity of the liver to the gut makes it close relatives linked by the biliary tract and the portal vein allowing for metabolite exchange derived by microbes and host as well as other compounds (2). There are evidences pointing to the gut-liver axis in which the microbiome and the liver interact through biliary excretion, recycling, signalling and regulation of gene expression (40). Furthermore, evidence also point to the gut-liver axis in relation to drug excretion. A study on differential expression of xenobiotic metabolism in the livers of germ-free or conventionally raised mice suggested that microbial metabolites of tryptophan act as aryl hydrocarbon receptor (AHR) activators (2, 41).

Microbiome and drug interactions in anticancer therapies

Anticancer drugs are specially designed to target malignant cells but sometimes not without being toxic to the host cells which may

result in life-threatening adverse effects. Anticancer drugs are also faced with challenge of resistance which is a major cause of chemotherapeutic treatment failure in human tumours and this failure can be partially due to genetic factors. However, efforts have been channelled into the development of more specific anticancer therapeutic approaches with lesser host cell toxicity. Immunotherapy has been a new paradigm in oncology where drugs are targeting immune cells rather than cancer cells, that is, the drugs are aimed at stimulating the host antitumor immune response (42).

The microbiome can interfere with the efficacy of anticancer treatment by metabolizing the drugs, inactivating or activating them. Also, they can modulate the immune system, interfere with side effects or the therapy modulates the microbiome (43). Both gut microbiota and intra-tumour bacteria can modulate chemotherapy and mediate its toxic effects. *Mycoplasma* infections, especially from *Mycoplasma hominis* and its presence in tumour tissues, have been reported in some types of cancers (44, 45, 46) as they are found to express nucleoside analogue-catabolizing enzymes that impairs drug efficacy (44). In a study involving simultaneous injection of *M. hominis,* infected colon cancer cells showed resistance to gemcitabine (2′,2′-difluorodeoxycytidine) due to deamination to its inactive metabolite (2′,2′-difluorodeoxyuridine) in the liver (46). Other organisms, especially Gammaproteobacteria were reported to also induce gemcitabine resistance due to the expression of a bacterial long-form enzyme, cytidine deaminase. In a colon cancer mouse model, resistance to gemcitabine-induced by Gammaproteobacteria was reversed by co-administration of ciprofloxacin, hence confirming the role of these bacteria in anticancer treatment failure (41,46). *Invivo* studies showed that *E. coli* impairs the action of gemcitabine as shown by increased tumour volume and reduced survival. Meanwhile, cytotoxicity of the drug, CB1954, was increased by the action of nitroreductase.

Evidence that bacteria may influence the value of chemotherapy regimen, decreasing certain drug activities while enhancing the activity of others (42,45) was observed in microbiota response for platinum compounds where mice dosed with combination of cisplatin and *Lactobacillus* showed better treatment response. These perceived effects were associated with the modulation of VEGFA, BAX and CDKN1B genes expression in the tumour and to the bacterial enhancement of the T cell immunity (47). Furthermore, cyclophosphamide (CTX) treatment in tumour-bearing mice caused translocation of Gram-positive bacteria such as *Lactobacillus johnsonii*, *Lactobacillus plantarum, Lactobacillus murinus*, *Barnesiella intestinihominis* and *Enterococcus hirae* into the mesenteric lymph node and spleen where they were involved in stimulation of Th1 and Th17 immune response. Germ-free mice treated with antibiotics against Gram-positive bacteria failed to generate this immune response and induced resistance to CTX in the study (43, 48).

Selective Estrogen Receptor Modulators (SERMs) such as tamoxifen can modulate microbiome composition as they can be toxic for organisms such as *Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumannii, Porphyromonas gingivalis, Streptococcus mutans, Enterococcus faecium* and *Bacillus stearothermophilus*. No bacterial drug metabolism has been related to SERMs, but resistance to tamoxifen has been shown to be a reason for changes to cancer cell metabolism modulated by the microbiome. Furthermore, tranxes may be subject to microbial metabolism and interfere with bacterial LPS in activating the immune system (43). Poly ADP ribose polymerase (PARP) inhibitors are potential drugs in the future treatment of breast cancer as they have been shown to increase the diversity of gut microbiome. There has been no report on the relationship between aromatase inhibitors, navelbine, GnRH-analogs and the microbiome (43).

Microbiome and anti-hypertension drugs

A study examined 52 common drugs of which 5 were anti-hypertension medications (β-blockers, α-blockers, ACE inhibitors, Sartan, and calcium channel blockers) and the microbiota (5). β-blockers in relation to *Firmicutes* and α-blockers in relation to Proteobacteria were linked to gut dysbiosis while no association was found between the microbiome and other three medications. In another population-based metagenomic analysis, there was positive correlation between the use of ACE inhibitors/β-blockers and microbiota composition. Similarly, hypertensive rats administered ACE inhibitors captopril showed reduced gut dysbiosis including decreased intestinal permeability, fibrosis and improved villi length (5). Consequently, it can be deduced that drugs alter the gut microbiome, probably removing opportunistic and adverse pathogenic bacterial species (5).

Incorporating microbiome and drug interaction into clinical setting

The influence of the microbiome on the effect and toxicity of drugs such as irinotecan (anti-cancer) and digoxin (cardiac drug) has been well recognized (49). In patients diagnosed with metastatic colorectal cancer receiving irinotecan (CPT-11), the build-up of metabolites of CPT-11 known as SN-38 glucuronide produced from microbial β-glucuronidases caused epithelial damage resulting into severe diarrhoea in some patients (31). In the application of shotgun metagenomics and targeted metabolomics, there was report of a characterized phylogenetically diverse group of bacterial β-glucuronidases and transporter proteins associated with high production of SN-38 glucuronide as well as possible higher risk of irinotecan-dependent toxicity (50). Investigating metabolomics and metagenomic basis of diversification in drug metabolism employing *exvivo* drug incubations with human faecal samples may offer putative biomarkers of drug effectiveness or risk of treatment failure (49).

Metabolic phenotyping studies of microbiome-drug interaction using RNA/DNA high throughput sequencing with metabolomics have shown that the degree of complexity of gut microbiome related to drug metabolism differ between drugs (50,51,52). The main impediment is the comprehension of what microbiome features identified through preclinical studies. Using model systems or human faecal samples as an alternative for the gut microbiome will translate into accurate surrogate endpoints for clinical studies. An example is the fact that the presence or absence of a candidate microbe or enzyme in a sequenced faecal sample may lack the ability to forecast drug metabolism (49). A way to overcome this barrier is to combine features using machine learning such as random forest method to detect the combinations of features most predictive of drug metabolism as this method can combine chemical and molecular features (49).

There are several clinical trials investigating the efficiency of probiotics to modify microbiome-dependent adverse drug responses. A study of the randomized double-blind design set up to explore the probiotic potential to minimize CPT-11 induced toxicity where patients were randomized into probiotic and placebo groups respectively showed that 39% of participants in the probiotic group had grade 3 to 4 diarrhoea while 61% in placebo group had diarrhoea (53). Another way to reduce CPT-11 toxicity is the targeted inhibition of microbial enzymes that changes the inactive form of the drug to its active form. β-glucuronidase inhibitors from *E. coli* has been identified to significantly reduce CPT-11 induced toxicity in mice but not on the orthologous mammalian enzyme (31). A clinical trial taking the safety and efficiency of this method into cognizance in human population has the possibility of producing useful insight into the efficiency of targeted, small molecule modulators of specific microbiota functions (49).

Conclusion:

The evident involvement of microbiome especially of the gut community in drug metabolism has been established. Also, microbiome-drug interaction may be beneficial or detrimental, resulting in either treatment success or failure, which is largely dependent on factors such as the microbial enzymes, chemical composition of the candidate drug, host immunity and the complex relationship that exists with the microbiome. Despite the fact that several research studies have been conducted on this topic, there is still a large gap to the full understanding of the microbiome involvement in drug metabolism that will lead to improve treatment regimen in patients.

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Original Article **Open Access**

Serological evidence of association between *Helicobacter pylori* infection and coronary artery disease

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Abstract:

Background: Studies have reported relationship between chronic *Helicobacter pylori* infection and coronary artery disease (CAD). The cytotoxin-associated gene A product (CagA) is an immunodominant protein which indicates infection with virulent *H. pylori* strains. Significant associations of CagA-positive *H. pylori* strains with coronary artery disorders have been widely reported. *H. pylori* is also known to produce different heat shock proteins (HSPs) which can stimulate the production of specific antibody against microbial proteins and capable of eliciting autoimmune reaction against human tissue expressing HSPs such as vascular endothelial cells. The objectives of this study are to investigate the association between *H. pylori* and CagA with coronary atherosclerosis and CAD, and to determine the possible role of *H. pylori* HSP60 protein in increasing the risk of CAD development.
Methods: This study included 70 patients with stable angina and 70 age and gender-matched controls. Each group was evaluated by clinical history, physical examination, cardiac echocardiography (ECHO) and electrocardiography (ECG) with and without exercise. Fasting blood glucose, total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides (TG) were estimated by automated enzymatic methods. *H. pylori* IgG, CagA IgG and HSP60 IgG were measured by enzyme-linked immunosorbent assay (ELISA) for both groups.
Results: The seroprevalence of *H. pylori* infection was high in both groups; 75.7% in case and 68.6% in control (p=0.346). Serum IgG levels were significantly higher for CagA (p=0.028) and HSP60 (p<0.001) in cases than in controls. There was significant association between *H. pylori* and CagA IgGs in cases (p=0.007) but no association in controls (p=0.700). Higher HSP60 IgG level was significantly associated with both positive *H. pylori* IgG (p<0.001) and CagA IgG (p<0.001) in cases but no significant association was found with *H. pylori* (p=0.815) or CagA (p=0.332) IgG levels in the control group. Serum values were significantly higher for TC (p<0.001), TG (p<0.001) and LDL (p=0.004) while value for HDL was significantly lower (p<0.001) in *H. pylori* IgG-positive subjects (case and control).
Conclusion: There is serological evidence that *H. pylori* infection may pose a significant risk factor for CAD. Since *H. pylori* can be eliminated by specific treatment, this may be a good preventive approach for CAD.

Key words: *H. pylori*, coronary artery disease, CagA, HSP60, serology.

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Preuve sérologique d'association entre l'infection à *Helicobacter pylori* et la maladie coronarienne

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Abstrait:

Contexte: Des études ont montré un lien entre l'infection chronique à *Helicobacter pylori* et la maladie coronarienne. Le produit du gène A associé à la cytotoxine (CagA) est une protéine immunodominante qui indique une infection par des souches virulentes de *H. pylori*. Des associations significatives de souches de *H. pylori* CagA-positives avec des troubles coronariens ont été largement rapportées. *H. pylori* est également connu pour produire différentes protéines de choc thermique (HSP) capables de stimuler la production d'anticorps spécifiques contre les protéines microbiennes et capables de provoquer une réaction auto-immune contre les HSP exprimant le tissu humain, telles que les cellules endothéliales vasculaires. Les objectifs de cette étude sont d'étudier l'association entre *H. pylori* et CagA avec l'athérosclérose coronarienne et la coronaropathie, et de déterminer le rôle possible de la protéine *H. pylori* HSP60 dans l'augmentation du risque de développement de coronaropathie.
Méthodes: Cette étude a inclus 70 patients présentant une angine de poitrine stable et 70 témoins de même âge et de même sexe. Chaque groupe a été évalué par antécédents cliniques, examen physique, échocardiographie cardiaque (ECHO) et électrocardiographie (ECG) avec et sans exercice. La glycémie à jeun, le cholestérol total (TC), les lipoprotéines de basse densité (LDL), les lipoprotéines de haute densité (HDL) et les triglycérides (TG) ont été estimés par des méthodes enzymatiques automatisées. Les IgG anti-*H. pylori*, IgG CagA et IgG HSP60 ont été mesurées par dosage immuno-enzymatique (ELISA) pour les deux groupes.
Résultats: La séroprévalence de l'infection à *H. pylori* était élevée dans les deux groupes; 75,7% en cas et 68,6% en contrôle (p=0,346). Les taux sériques d'IgG étaient significativement plus élevés pour CagA (p=0,028) et HSP60 (p<0,001) chez les sujets témoins. Il y avait une association significative entre les IgG anti-*H. pylori* et CagA dans les cas (p=0,007), mais aucune association chez les témoins (p=0,700). Un taux plus élevé d'IgG HSP60 était associé de manière significative à la fois aux IgG positives pour *H. pylori* (p<0,001) et aux IgG anti-CagA (p<0,001), mais aucune association significative n'a été constatée avec H. pylori (p = 0,815) ou CagA (p=0,332). Taux d'IgG dans le groupe témoin. Les valeurs sériques étaient significativement plus élevées pour le CT (p<0,001), le TG (p<0,001) et le LDL (p=0,004), tandis que les valeurs pour le HDL étaient significativement plus basses (p<0,001) chez les sujets positifs pour *H. pylori* IgG.
Conclusion: Il existe des preuves sérologiques que l'infection à *H. pylori* peut constituer un facteur de risque significatif de coronaropathie. Étant donné que *H. pylori* peut être éliminé par un traitement spécifique, cela peut constituer une bonne approche préventive pour la coronaropathie.

Mots clés: *H. pylori*, maladie coronarienne, CagA, HSP60, sérologie.

Introduction:

Helicobacter pylori infection is one of the most widespread infections worldwide, affecting half the population of the world. The bacterium causes chronic gastritis, peptic ulcer and gastric cancer (1). The infection stimulates both cell mediated and humoral immune system with elevation of basophils and polymorphs (2), and increased concentration of local and systemic vasoactive cytokines (3). These reactions are not restricted to the digestive tract (4) but also involved many extra gastrointestinal manifestations including haem-atological disorders (idiopathic thrombocyto-penic purpura and unexplained iron deficiency anemia), neurological disorders (stroke, Par-kinson and Alzheimer's diseases), obesity and skin diseases (5,6).

Several studies have reported relation-ship between chronic *H. pylori* infection and coronary artery disease (CAD), highlighting its role in the pathogenesis of coronary vascular disorders (7). The cytotoxin-associated gene A (CagA) product is an immunodominant protein which indicates infection with virulent *H. pylori* strains. Some studies have reported significant associations of CagA-positive *H. pylori* strains with coronary artery disorders (8).

Helicobacter pylori have been detected in human atherosclerotic plaques by immuno-histochemistry and polymerase chain reaction (9), and particularly in patients undergoing coronary bypass grafting (10). Chronic *H. pylori* infection is believe to induce low-grade constant inflammatory response with release of mediators that causes vascular endothelial damage through recruitment of monocytes and T-lymphocytes to the vascular wall, even in the absence of the pathogen. Therefore, chronic *H. pylori* infection does not only induce coronary atherosclerosis but can activate acute coronary rupture (11).

A variety of systemic effects including atherosclerosis can be induced by chronic infection in several different manners through increasing circulating cytokine (interleukins 1 and 6) production and formation of the acute-phase products such as C-reactive protein and white blood cells. Also, chronic infection can stimulate the immune system with production of antibodies against the infecting pathogen (12). Furthermore, pathogens can stimulate proliferation and migration of smooth muscle

cells, accumulation of lipid, and formation of several pro-coagulants, with inhibition of endothelial cell apoptosis (13).

Heat shock proteins (HSPs) represent well conserved protein families sharing wide sequence homology amongst various species, from bacteria to humans (14). Exposure to stressful stimuli such as sudden increase in temperature, hypoxia, infection, inflammation, mechanical stress, and oxidizing agents can induce or up-regulate production of HSPs (14). These proteins play essential roles in the bacterial growth at different temperatures and protection against a variety of injurious factors (15). *H. pylori* are known to produce different HSPs that stimulate the production of specific antibodies (16). Due to high sequence homology of HSPs, autoimmune reaction directed against bacterial HSPs as well as human (such as vascular endothelium) HSPs, could occur (17).

The objectives of this study are to; (i) determine association between *H. pylori* and CagA protein with CAD; (ii) investigate the possible role of *H. pylori* in CAD by estimating its specific HSP60 IgG levels in patients with CAD and healthy subjects and (iii) determine association between *H. pylori* and known risk factors for CAD.

Methodology:

Subjects (case and control)

This case control study was conducted over a period of six months (January to June 2019), and included 70 patients (cases) with stable angina selected by stratified random sampling among patients admitted to the cardiology unit or attending the cardiology outpatient clinic of Mansoura University Hospital, Egypt. Clinical history, physical examination, cardiac echocardiography (ECHO), and electrocardiography (ECG) with and without exercise, were done for each subject. The cases had signs and symptoms of angina at exercise ECG with more than 2mm ST segment depression. The controls were 70 age and gender-matched patients randomly selected from units other than cardiology who had no feature of CAD (negative tread mill test).

Exclusion criteria

The exclusion criteria for both case and control subjects were; (i) significant kidney insufficiency (creatinine more than 1.5 mg/dL), (ii) significant hypothyroidism/hyperthyroidism (serum thyroid stimulating hormone level more than 4.5IU/mL or free thyroxine more than 2ng/dL), (iii) significant C-reactive protein elevation (more than 10mg/L) and (iv) persons with any history of intake of antibiotics for *H. pylori* infection during the last 3months.

Ethical approval

The study was approved by Institutional Review Board of the Faculty of Medicine, Mansoura University, Egypt. All subject participants gave informed consent for the study.

Clinical and laboratory evaluations of case and control subjects

Subjects in both groups were evaluated for hypertension (indicated by systolic blood pressure ≥140mmHg, diastolic blood pressure of ≥90mmHg (or antihypertensive medication), body mass index (BMI), and smoking (patients who had stopped smoking for 10years or less were classified as smokers). Approximately 10ml of venous blood was collected from each subject with the serum separated and stored in aliquots at -80°C until specific tests were done.

Determination of serum glucose and lipids

Fasting blood glucose (FBG), total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides (TG) were measured by enzymatic methods in an automated chemistry analyzer (Toshiba TBA-120 FR, Toshiba Medical Systems, Japan)

Determination of *H. pylori* infection IgG

The level of serum IgG to *H. pylori* O antigen was determined by a commercial enzyme-linked immunosorbent assay (ELISA) (Euroimmun, Germany). The value of upper limit of normal range for *H. pylori* specific IgG level for the assay kit is 22units/mL.

Determination of *H. pylori* CagA IgG

The level of serum IgG to *H. pylori* CagA protein was serologically detected by a commercial ELISA assay (Radim Diagnostics, Germany). The value of upper limit of normal range for *H. pylori* CagA specific IgG levels for the assay kit is 15units/mL.

Determination of *H. pylori* HSP60 IgG

The level of serum IgG to *H. pylori* HSP60 was serologically detected by a commercial ELISA assay (Elabscience Biotechnology, China). The detection range of the kit was from 78.13-5000 pg/mL.

Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences software version 17.0 (SPSS Inc., Chicago, IL, USA). The association between two variables

was evaluated using Chi-square or Fisher Exact tests for categorical variables and Student *t* tests for continuous variables, with $p<0.05$ considered significant.

Results:

This study was conducted on 70 patients who had stable angina, with mean age of 58.9 years, and 70 controls with a comparable mean age of 57.7 years ($p=0.426$). The cases (36 males, 34 females) were gender-matched with the controls (40 males, 30 females) ($p=0.497$). The mean BMI of cases and controls were 30.4 and 29.8 respectively ($p=0.251$). There were no significant differences between the case and control subjects regarding history of smoking ($p=0.319$), hypertension ($p=0.122$), diabetes mellitus ($p=0.231$) or fasting blood glucose ($p=0.074$).

The prevalence of *H. pylori* infection was 75.7% in the case and 68.6% in control subjects ($p=0.346$). On the other hand, CagA IgG ($p=0.028$) and HSP60 IgG ($p<0.001$) values were significantly higher in cases than in controls (Table 1).

Table 1: Comparison of demographic and clinical data of case and control subjects

Parameter		Control (n=70)	Case (n=70)	*p* value
Age (years)	Mean ± SD	57.7 ± 9.2	58.9±9.6	0.426
Age range (years)		41 – 72	45 – 75	
Gender				
Male	n (%)	40 (57.1)	36 (51.4)	0.497
Female	n (%)	30 (42.9)	34 (48.6)	
BMI (kg/m2)	Mean ± SD	29.8 ±3.0	30.4±3.5	0.251
Smoking	n (%)	14 (20.0)	19 (27.1)	0.319
DM	n (%)	14 (20.0)	22 (31.4)	0.122
FBG (mg/dL)	Median (range)	107.5 (78 -256)	110.5 (85-350)	0.074
Hypertension	n (%)	26 (37.1)	33 (47.1)	0.231
Helicobacter pylori IgG	n (%)	48 (68.6)	53 (75.7)	0.346
CagA IgG	n (%)	31 (44.3)	44 (62.9)	0.028*
HSP60 IgG	Median (range)	839 (550-4837)	3365 (1187-5298)	<0.001*

* = significant difference

Table 2: Association of CagA IgG with *Helicobacter pylori* IgG in case and control subjects

			Control (n=70)		*p value*	Case (n=70)		*p value*
			H. pylori IgG negative (n=22)	*H. pylori* IgG positive (n=48)		*H. pylori* IgG negative (n=17)	*H. pylori* IgG positive (n=53)	
CagA IgG	Negative	n (%)	13 (59.1)	26 (54.2)	0.700	11 (64.7)	15 (28.3)	0.007*
	Positive	n (%)	9 (40.9)	22 (45.8)		6 (35.3)	38 (71.7)	

* = significant difference

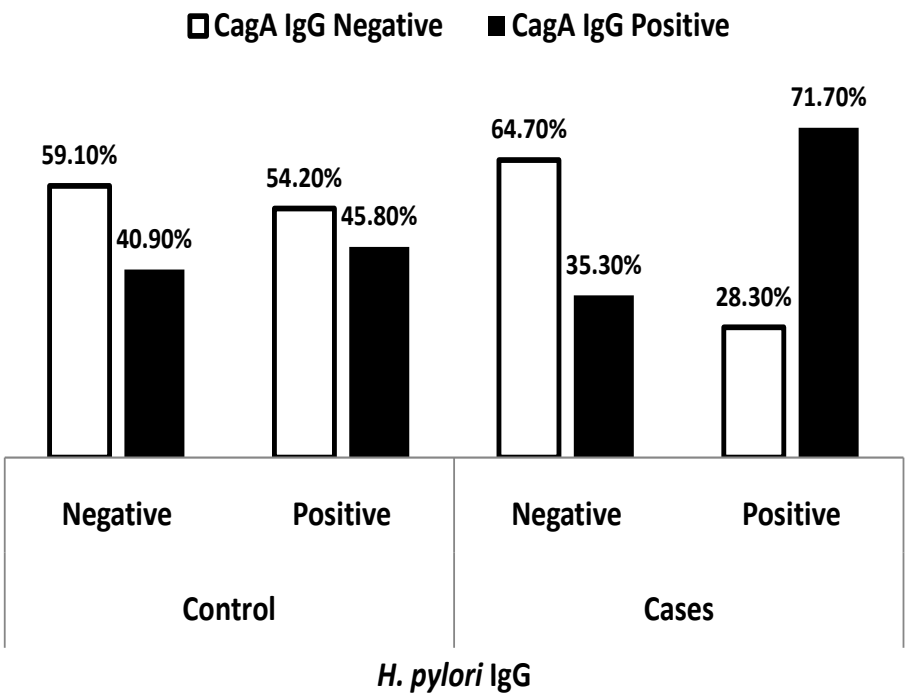

Fig. 1: Association of CagA IgG with *H. pylori* IgG in case and control subjects

There was significant association of *H. pylori* and CagA IgGs in the case (p=0.007) but no such association in the control (p=0.700) (Table 2 and Fig 1). Higher anti-HSP60 antibodies level was significantly associated with positive *H. pylori* IgG (p<0.001) and CagA IgG (p<0.001) in the case but no significant association was found with *H. pylori* IgG (p=0.815) or CagA IgG (p=0.332) in the control group (Table 3, Figs 2 and 3). The serum levels were significantly higher for TC (p<0.001), TG (p<0.001), and LDL (p=0.004) in *H. pylori* IgG-positive than *H. pylori* IgG-negative subjects (case and control) while HDL value was significantly lower (p<0.001) in *H. pylori* IgG-positive subjects (Table 4).

Table 3: Association of HPS60 IgG with *H. pylori* and Cag A IgGs in case and control subjects

		Control (n=70)		*p value*	Case (n=70)		*p value*
		H. pylori IgG negative (n=22)	*H. pylori* IgG positive (n=48)		*H. pylori* IgG negative (n=17)	*H. pylori* IgG positive (n=53)	
HSP60 IgG	Median	962.5	794	0.815	2698	3658	<0.001*
	Range	550-4398	550-4837		1187-3827	1187-5298	
		CagA IgG negative (n=39)	CagA IgG positive (n=31)	*p value*	CagA IgG negative (n=26)	CagA IgG positive (n=43)	*p value*
HSP60 IgG	Median	839	1445	0.332	2765	3876	<0.001*
	Range	550-4398	560-4837		1187-3265	1576-5298	

* = significance difference

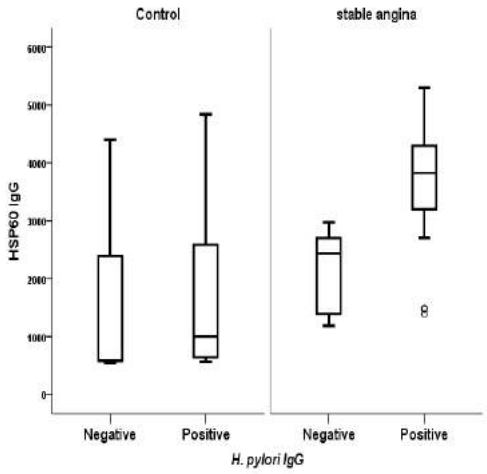

Fig. 2: Association of HPS60 IgG with *H. pylori* IgG in case and control subjects

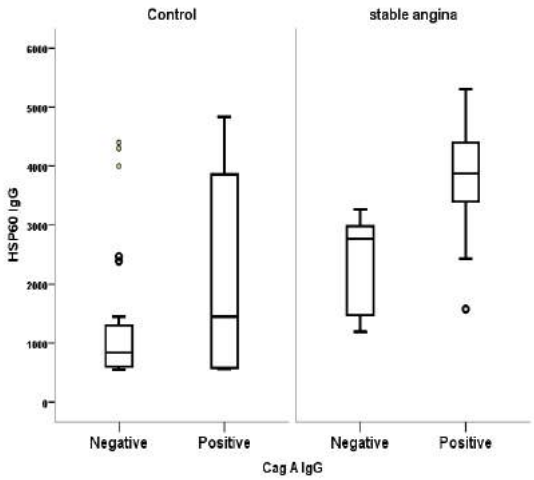

Fig 3: Association of HPS60 IgG with CagA IgG in case and control subjects

Table 4: Association of *Helicobacter pylori* IgG with lipid profiles in case and control subjects

Parameter	Control (n= 70)					Case (n= 70)				
	H. pylori IgG negative (n= 22)		*H. pylori* IgG positive (n= 48)		*p value*	*H. pylori* IgG negative (n=17)		*H. pylori* IgG positive (n=53)		*p value*
	median	range	median	range		median	range	median	range	
TG (mg/dL)	50	42-76	125	76-234	<0.001	49	42-102	130	50-145	<0.001
TC (mg/dL)	116	94-195	160	94-220	<0.001	129	50-166	182	135-330	<0.001
LDL (mg/dL)	23.5	12-154	75.5	16-165	<0.001	200	160-272	230	124-340	0.004
HDL (mg/dL)	55	52-59	50	44-52	<0.001	55	48-57	47	38-55	<0.001

Discussion:

CAD has been considered one of the extra gastrointestinal diseases associated with *H. pylori* infection (18). In this study designed to determine, by serology, association between *H. pylori* and CAD, the cases were carefully matched for age, gender and important risk factors for CAD such as history of smoking, hypertension and diabetes mellitus, with the controls. In our study, *H*. *pylori* IgG was high in both case and control groups (p=0.346). Studies done on the association between *H*. *pylori* infection and cardiovascular diseases have been mostly carried out with the serological *H*. *pylori* IgG test because it is rapid, inexp- ensive and non-invasive (19, 20). However, there have been contradictory reports between serology and occurrence of CAD. While some researchers have reported non-significant association between *H*. *pylori* seropositivity and CAD (21,22), others have reported sign- ificant association (19,20). These conflicting reports may be explained by the fact that antibody testing cannot differentiate recent and past infection (23). Therefore, serological test is usually performed to study only association between the occurrence of *H. pylori* infection and CAD (19).

In this study, CagA IgG was significantly detected in cases more than controls (p=0.028), and there was significant association of *H. pylori* and Cag A IgGs in the cases (p=0.007) but not in the controls (p=0.700). It has been reported that patients with CAD are more prone to infection by CagA positive *H. pylori* strains with more vigorous clinical manifestations (24). This may be due to the exaggerated inflammatory reaction in CagA-positive *H. pylori* infection from increased systemic levels of interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor-alpha (TNF-α), which tend to exert injurious damage to the vascular endothelial cells (11). In addition, elevated levels of thrombin factor and prothrombin subunits F1+2 in patients with both CAD and CagA positive *H. pylori* strains have been reported (25). Moreover, antibodies against CagA show cross-reactivity with vascular wall antigens, providing a potential role of CagA in vascular wall inflammation (26).

The serum HSP60 IgG level in our study was significantly higher in cases than in controls (p<0.001) and higher anti-HSP60 antibodies level was significantly associated with both *H. pylori* and CagA IgGs positivity in the case but no such significant association in the control group. This may suggests an association between *H. pylori* HSP60 and development of CAD. Consistent with the hypothesis of Wick and coworkers (27), atherosclerosis can be associated with high levels of HSP antibodies because of an autoimmune response directed against endothelial tissue expressing high levels of HSP in response to traumatic stimuli such as local infections, cytokines, elevated LDL, or other stressful conditions. Another explanation is that both bacterial toxic metabolites and the concomitant inflammatory response can change the epithelial HSP such that immune tolerance to self HSP is lost, with production of autoantibodies which cross-react with *H. pylori* HSPs (11). Latif et al., (28) found a strong similarity between HSP60 and heavy chain of cardiac myosin, therefore, cross-reaction between related epitopes could result in autoimmunity.

In our study, *H*. *pylori* IgG-positive subjects had significantly elevated TG, TC and LDL levels and significantly lower HDL level than those with *H*. *pylori* IgG-negative ones. Some researchers have reported that chronic *H. pylori* infection changes the lipid profile. Rahman et al., (29) and Lee et al., (30) reported significantly lower levels of HDL in patients with *H. pylori* infection with and without CAD. Jia et al., (31) reported that *H. pylori* infection may lower HDL levels with predisposition to coronary atherosclerosis. Kim et al., (32) also reported that LDL cholesterol level was significantly higher in *H. pylori* infected patients, and increasing *H. pylori* severity increased LDL levels, which is the most important risk factor for atherosclerosis. Therefore, atherogenic lipid (elevated LDL and decreased HDL) profile has been reported in *H. pylori* infected compared to un-infected patients (33). However, other researchers have reported that lipid profile was not affected by *H. pylori* infection and therefore not considered as a risk factor for CAD (34).

Conclusion:

There is serological evidence that *H. pylori* infection may pose a significant risk factor for CAD. Since *H. pylori* can be eliminated by specific treatment, this may be a good preventive approach for CAD.

Conflicts of interest:

Authors declare non financial conflicts of interest in this study

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Original Article **Open Access**

Prevalence and factors associated with *Helicobacter pylori* infection among treatment naïve dyspeptic adults in University of Benin Teaching Hospital, Benin City, Nigeria

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Abstract:

Background: Infectious diseases such as malaria, HIV/AIDS and tuberculosis have received tremendous attention globally but inspite of the widespread nature of infections caused by *Helicobacter pylori*, little attention has been paid to it especially in the developing countries. The objective of this study was to determine the prevalence of and factors associated with *H. pylori* infection among dyspeptic patients attending the University of Benin Teaching Hospital, Benin City, Nigeria.
Methodology: This was a hospital based descriptive cross-sectional study of 354 treatment naive dyspeptic patients aged 18 to 44 years, recruited consecutively after obtaining institutional ethical approval and subjects' informed consent. A pre-tested interviewer administered questionnaire was used to obtain subjects' data. The stool antigen test was used to detect *H. pylori* infection.
Results: The prevalence of *H. pylori* infection among the subject participants was 34.2% (38.4% in female, 24.0% in male, p=0.009). The age group < 20 years had the highest prevalence of 40%. Gender, occupation, increased body mass index, high number of household occupants, and rural dwelling, were significantly associated with *H. pylori* infection (p<0.05) and alcohol intake was inversely related to *H. pylori* infection (p<0.05).
Conclusion: The prevalence of *H. pylori* infection in University of Benin Teaching Hospital is high and factors such as gender, obesity, occupation with risk of contact, low socio-economic status, and poor hygiene, may be responsible for this.

Keywords: Dyspepsia, *Helicobacter pylori*, infection, stool antigen test

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Prévalence et facteurs associés à l'infection à *Helicobacter pylori* chez les adultes dyspeptiques naïfs de traitement à l'hôpital universitaire de Bénin, Benin City, Nigéria

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Abstrait:

Contexte: Les maladies infectieuses telles que le paludisme, le VIH / sida et la tuberculose ont fait l'objet d'une attention considérable dans le monde, mais malgré la nature généralisée des infections causées par *Helicobacter pylori*, peu d'attention y a été accordée, en particulier dans les pays en développement. L'objectif de cette étude était de déterminer la prévalence et les facteurs associés à l'infection à *H. pylori* chez les patients dyspeptiques fréquentant l'hôpital universitaire de Benin, à Benin City, au Nigéria.

Méthodologie: Il s'agissait d'une étude descriptive transversale réalisée à l'hôpital auprès de 354 patients dyspeptiques naïfs de traitement, âgés de 18 à 44 ans, recrutés de manière consécutive après avoir obtenu l'approbation éthique de l'établissement et le consentement éclairé des sujets. Un questionnaire pré-testé administré par l'intervieweur a été utilisé pour obtenir les données des sujets. Le test d'antigène dans les selles a été utilisé pour détecter une infection par *H. pylori*.
Résultats: La prévalence de l'infection à *H. pylori* chez les sujets était de 34,2% (38,4% chez les femmes, 24,0% chez les hommes, p=0,009). Le groupe d'âge <20 ans avait la prévalence la plus élevée de 40%. Le sexe, la profession, l'augmentation de l'indice de masse corporelle, le nombre élevé d'occupants et l'habitation rurale étaient significativement associés à l'infection à H. pylori (p<0,05) et la consommation d'alcool était inversement liée à l'infection à *H. pylori* (p<0,05).
Conclusion: La prévalence de l'infection à *H. pylori* à l'hôpital universitaire de Bénin est élevée et des facteurs tels que le sexe, l'obésité, l'occupation avec risque de contact, le faible statut socio-économique et l'hygiène médiocre peuvent en être la cause.

Mots-clés: Dyspepsie, *Helicobacter pylori*, infection, test antigène dans les selles

Introduction:

Infections caused by *Helicobacter pylori* const itute a global health challenge with diverse consequences. Infections occur in every geographical area among different ethnic and age groups but with certain observed factors influencing their distribution. The discovery of *H. pylori* by Warren Robin (a biologist) and Marshall Barry (a clinician) in 1983 was a major breakthrough in the management of dyspepsia (1). *H. pylori* was first cultured from the dental plaque of a 29 year old patient in 1989 (2). This discovery had major implications in the management of dyspepsia, peptic ulcer disease and gastric cancer (3). The 2005 Nobel Prize in Physiology and Medicine was awarded to the two discoverers (4).

Dyspepsia is a common clinical problem seen by primary care physicians and gastroenterologists, and is the usual presentation of symptomatic patients infected with *H. pylori* (5), although infection may remain asymptomatic. An un-investigated dyspeptic patient may have functional (non-ulcer) dyspepsia or develop chronic active gastritis, duodenal ulcer, gastric ulcer, gastric carcinoma or gastric MALT lymphoma (5). Studies have documented a higher prevalence of *H. pylori* infection in Africa (6, 7), and in Nigeria, even though most data are from paediatric age groups and specialized centers, it is said to be hyper-endemic, with prevalence affected by socio-economic status, being more common in poorer sections of the society (8). Although geographical and sociodemographic prevalence of human *H. pylori* infections varies, prevalence does not parallel the incidence of morbidity caused by the infection, especially amongst Africans (9).

A number of diagnostic tests are available ranging from invasive to non-invasive. Invasive tests are performed during endoscopy with biopsy taken for histology, culture, rapid urease test, polymerase chain reaction and florescence in-situ hybridization, while non-invasive tests include serology, stool antigen test, urea breath test, salivary, and urinary antibody tests (10). The non invasive tests are important in primary care because they are cost effective, and the stool antigen test (HpSA) seems to be more promising.

The increasing incidence of *H. pylori* infection in developing countries as a result of overcrowding, low socio-economic status and poor sanitation, is particularly worse in rural and poor resource settings (11). In indust-rialized countries, the rate of acquisition of *H. pylori* has decreased substantially over recent decades with availability of various treatment options and higher socio-economic status (12). In Nigeria, literatures on the prevalence of *H. pylori* infection among treatment naïve dyspeptic adult patients attending primary care settings are scarce, because most studies are conducted in specialized centers where endoscopies are performed. Also, most of these studies are based on invasive methods for detection of *H. pylori*.

In Nigeria where antibiotic use is rampant (13), most patients in the specialized units are often not treatment naïve. This current study aimed to determine the prevalence of *H. pylori* infection among treatment naïve patient in a primary care setting using non-invasive method, and associated factors for *H. pylori* infection. This is to enable focused attention towards preventing its spread and eradication of the infection.

Materials and methods:

Study area

The study was carried out in the General Practice/National Health Insurance Scheme (NHIS) clinic of University of Benin Teaching Hospital, Benin City, Nigeria, tertiary

hospital that offers both inpatient and outpatient services. The clinic is managed by the Department of Family Medicine on outpatient basis.

Study design and protocol

This was a hospital based descriptive cross sectional study. The study group consisted of 354 subjects, age range 18 to 44 years, drawn from the General Practice Clinic (GPC) and Staff/NHIS clinic. The sample size was calculated using the Leslie and Kish formula; $n=z^2pq/d^2$ (14), where 'n' is the sample size, 'z' is normal variate at 5% type 1 error, 'p' is prevalence of disease in a population from previous study, and 'd' is absolute precision with 5% error margin. Assuming a 90% response rate, the formula for adjusted non-response sample size, $n_s = n/0.90$, gave a sample size of 393. Recruitment of subjects was done over a period of two years and 354 of 393 subjects completed the questionnaires administered in the study.

Inclusion and exclusion criteria

The inclusion criteria were subjects in age range of 18 to 44 years who were treatment naïve and had complaint of dyspepsia lasting for at least two weeks. A naïve subject was taken as any participant with dyspepsia who had not taken antibiotics in the last 4 weeks, PPIs in the last 2 weeks, and H_2 receptor blockers in the last 24 hours (15). Subjects who were critically ill, pregnant or have evidence of upper gastro-intestinal bleeding were excluded.

Ethical clearance

Approval for the study was obtained from the Hospital's Ethical Committee (Protocol number: ADM/E22/A/VOL.VII/762). A written informed consent was obtained from each participant. Appropriate management was given to the participants and confidentiality was maintained throughout the study.

Data collection instruments

The data was collected using a pre-tested semi-structured, interviewer administered questionnaire adapted from a previously validated Dutch Gastrointestinal Symptom Questionnaire (16), as well as from questions drawn from the literature. This was modified in line with the study objectives.

The questionnaire had four sections; section A assessed the demographic characteristics of the subjects including age, sex, religion, educational status, ethnicity, socio-economic status, residence, height and weight (BMI) and family size; section B identified dyspeptic symptoms in the subjects as well as duration of symptoms; section C collected information on associated factors for *H. pylori* infection, such as number of household occupants, number of room occupants, and past sources of drinking water, as well as duration of symptoms, smoking and alcohol consumption. Household occupants were taken as number of persons living in a house, room occupants were taken as number of person sharing a room, smoking status was taken as present smokers, past smokers or never smoked cigarette, alcohol consumption was taken as current consumption of beer, wines, whisky or local gin. Past sources of water was taken as sources of water during subjects childhood days, family size was taken as ≤4 children or > 4 children. Section D was for determination of the presence of *H. pylori* in the subjects stool samples using *H. pylori* stool antigen test (HpSA).

Measurement of weight, height and body mass index

The weight and height of each subject were measured free from pocket objects, wristwatch, jewelries, shoes, and head dressing using a mechanical weighing and height scale (RGZ-160; Shanghai Maney Medical Technical Ltd, China). The BMI was calculated for each subject using the formula: BMI (kg/m^2) = w/h^2, where 'w' is weight in kg and 'h' is height in metres. They were classified using the World Health Organization's classification of obesity (17). Obesity was taken as BMI greater than or equal 30 kg/m^2, overweight was regarded as BMI between 25 and 29.9 kg/m^2, while normal weight was taken as BMI between 18.5-24.9 kg/m^2 and underweight as BMI less than 18.5 kg/m^2.

Stool antigen test for *H. pylori*.

A commercial validated Stool Antigen Test kit, Bioline SD Rapid Test (Standard Diagnostics, Inc, Lot number 164006, cat number 04FK20) was used. The test is based on immunochromatographic principle with the test kit impregnated with antibodies specific to *H. pylori* antigen in human faecal specimens. The kit, as indicated by the manufacturer, has specificity of 91.9% and sensitivity of 92.4% compared to *H. pylori* stool culture as 'gold standard'.

Stool antigen test, according to the manufacturers' instructions, was done within 24 hours of sample collection. The test was performed by a laboratory scientist trained

during the pilot test, during which inter-rater reliability was assessed and confirmed. A sterile swab stick from the stool kit was used to collect faeces from the container, which was then inserted into the sample collection tube containing the assay diluents. The swab stick was swirled about 10 times inside the diluents until the stool sample dissolved. The sample collection tube was capped and left for about 5 minutes. Three drops of prepared stool sample was placed in the sample well of the test device and result interpreted in 15 minutes. Two red lines showing control (C) and test (T) on the test kit indicate positive while a single red line showing control indicates negative result. Result is indeterminate if there is no line or only T line, in which case the test is repeated.

Data management and analysis

Data were checked for completeness and consistency, and then analysed using the Statistical Package for Social Sciences (SPSS version 16.0, Chicago Illinois). Results were presented as percentages and tables. Pearson's Chi square test was used to compare association between prevalence of *H. pylori* infection and socio-demographic variables as well as associated factors. Risk estimate odds ratio was also used to test the association between gender and risk of *H. pylori* infection, and *p* value less than 0.05 was considered significant.

Results:

Sociodemographic characteristics of subjects and *H. pylori* infection

As shown in Table 1, a total of 354 subjects were studied; 59.9% were in age range of 40-49 years, 104 (29.4%) males, and 250 (70.6%) females (M: F ratio of 1: 2.4). Of the 354 subjects, 121 were positive for *H. pylori* by the stool antigen test, given a prevalence rate of 34.2%. The prevalence was significantly higher in females (38.4%) than males (24.0%) (p=0.009). Although most of the subjects in this study were unskilled (53.7%), the prevalence of *H. pylori* infection was significantly higher among professionals (87.5%) than among unskilled and other categories of subjects (p=0.009). The prevalence of *H. pylori* infection was also significantly higher in obese (61.1%) and overweight (37.0%) than normal weight subjects (16.5%) (p<0.001). Although, majority of the subjects (78.5%) resided in urban centers, the prevalence of *H. pylori* infection was significantly higher in rural dwellers (48.7%) than in subjects from urban areas (30.2%) (p=0.003).

There were no significant differences in the prevalence of *H. pylori* infections with respect to age groups (p=0.313), marital status (p=0.168), socioeconomic status (p=0.142), ethnicity (p=0.611), religion (p=0.520) and level of education (p=0.266).

Factors associated with *H. pylori* infection

Table 2 shows that subjects whose number of household occupants ranged from 1 to 5 persons had *H. pylori* prevalence of 29.5%, which was significantly lower than 39.6% in subjects whose occupants were above 5 (p=0.044). The prevalence of *H. pylori* infection was also significantly higher in subjects who do not consume alcohol (38.3%) than in subjects who consume less than 3 units of alcohol (33.6%) and those who consume more than 3 units (18.6%) of alcohol (p=0.049).

There was no significant difference in the prevalence of *H. pylori* infections between subjects who had one roommate (33.6%) and those who had more than one roommate (36.0%) (p=0.683). Similarly, there were no significant differences in the prevalence of *H. pylori* infections with respect to past sources of water (p=0.210). Though not statistically significant (p=0.124), the prevalence of *H. pylori* infection was lower in cigarette smokers (21.9%) than non smokers (35.4%).

Table 1: Socio-demographic and clinical characteristics of subjects in relation to *H. pylori* infection

Variables	No of subjects (n=354)	No positive (%) (n=121)	X^2	95% CI	*p* value
Gender					
Male	104	25 (24.0)	6.733	1.175 - 3.303	0.009
Female	250	96 (38.4)			
Age group (years)					
< 20	5	2 (40.0)	3.563	0.309 - 0.409	0.313
20-29	65	16 (24.6)			
30-39	72	28 (38.9)			
40-49	212	75 (35.4)			
Marital status					
Single	75	21 (28.0)	5.052	0.151 - 0.233	0.168
Married	224	78 (34.8)			
Widowed	44	20 (45.5)			
Separated/divorced	11	2 (18.2)			
Occupation					
Professional	8	7 (87.5)	13.620	0.000 - 0.013	0.009
Skilled	39	9 (23.1)			
Semi-skilled	44	12 (27.3)			
Unskilled	190	69 (36.3)			
Unemployed	73	24 (32.9)			
Socioeconomic status					
High	55	19 (34.5)	3.901		0.142
Middle	120	33 (27.5)			
Lower	179	69 (38.5)			
Body mass index					
Normal weight	91	15 (16.5)	25.07	0.000 - 0.008	< 0.001
Over weight	227	84 (37.0)			
Obese	36	22 (61.1)			
Ethnicity					
Bini	171	60 (35.1)	3.580	0.594 - 0.694	0.611
Esan	53	20 (37.7)			
Etsakor	26	10 (38.5)			
Igbo	62	20 (32.3)			
Yoruba	15	2 (13.3)			
Others	27	9 (33.3)			
Religion					
Christianity	314	106 (33.8)	2.239	0.522 - 0.625	0.524
Islam	19	5 (26.3)			
Traditional religion	19	9 (47.4)			
Other religion	2	1 (50.0)			
Level of education					
No formal	83	34 (41.0)	3.963	0.260 - 0.356	0.266
Primary	96	35 (36.5)			
Secondary	118	33 (28.0)			
Tertiary	57	19 (33.3)			
Subjects' residence					
Rural	76	37 (48.7)	9.049		0.003
Urban	278	84 (30.2)			

X^2 = Chi square, CI = Confidence Interval

Table 2: Factors associated with *H. pylori* infections in treatment naïve dyspeptic patients in University of Benin Teaching Hospital

Variables	No of subjects (n=354)	No positive (%) (n=121)	X^2	95% CI	*p* value
Household occupants					
1-5persons	190	56 (29.5)	4.039		0.044
>5persons	164	65 (39.6)			
Number of roommates					
1 roommate	265	89 (33.6)	0.166		0.683
>1 roommate	89	32 (36.0)			
Past sources of water					
River	93	29 (31.2)	8.408	0.146 - 0.227	0.210
Stream	30	7 (23.3)			
Water tanker	3	0			
Well	84	35 (41.7)			
Bore hole	10	2 (20.0)			
Tap water	91	36 (39.6)			
Rain water	43	12 (27.9)			
Smoking of cigarettes					
Yes	32	7 (21.9)	2.368		0.124
No	322	114 (35.4)			
Alcohol consumption					
Non consumers	180	69 (38.3)	6.037		0.049
≤3units	131	44 (33.6)			
>3units	43	8 (18.6)			
Duration of symptoms					
≤6 months	98	28 (28.6)	1.895		0.169
>6 months	256	93 (36.3)			

X^2 = Chi square; CI = Confidence Interval

Discussion:

This study aimed at determining the prevalence of *H. pylori* and factors associated with infection caused by this bacterium in treatment naïve dyspeptic patients attending primary care unit of a tertiary hospital in Nigeria. We report a high prevalence of 34.2% using stool antigen test, which agrees with a similar study by Smith et al., in southwest Nigeria (18), although this was done in a tertiary healthcare referral center with facility for endoscopy compared to our present study in a primary care setting. This further confirmed the high prevalence of *H. pylori* infection regardless of the level of care. Also, Yucel et al., (19) reported a high prevalence of *H. pylori* among students who were not classified as dyspeptic, as distinct from our current study on dyspeptic patients. This confirms that high prevalence of *H. pylori* infection occurs in both symptomatic and asymptomatic populations.

However, reports of other studies that determined *H. pylori* infection by other methods (20, 21, 22, 23, 24) aside from stool antigen test are at variance with our study. Most of these studies were conducted in healthcare centers with endoscopic facility for invasive procedures, and these centers tend to receive patients referred from primary care setting following unresolved dyspeptic symptoms after empirical treatment. One of the criteria for referrer of dyspeptic patients is unresolved symptoms following treatment (25).

The prevalence of *H. pylori* infection in our study was significantly higher in females. A number of studies have reported that female gender had higher prevalence than males with odds ratio of 1.97. Tanih et al., (26) in South Africa using histological method reported significantly higher prevalence in females (69.5%) compared to males (60.0%). Most females from our present study were market women with higher tendency for indiscriminate eating habit and poor hygiene. This is critical as they may eventually become carriers and a host for the spread of *H. pylori* infection. However, a meta-analysis involving 18

different adult population studies by Martel et al., (27) showed that male gender was significantly associated with *H. pylori* infection, even though some of the individual studies showed otherwise. Another study by Ugwuja et al., (11) from Abakaliki, Nigeria, showed that the prevalence of *H. pylori* infection was similar in males (28%) and females (25%).

Similar to findings from other studies, the prevalence of *H. pylori* was significantly higher among respondents living in rural settlements and those with high number of household occupants. Gunaid et al., (28) reported higher prevalence of *H. pylori* infection among family whose size is equal or greater than 6 members. Similarly, Santos et al, (29) found a higher prevalence of 69.7% in families with more than 4 children or relatives as compared to 58.5% in families with less than 4 children or relatives. Rural dwelling in our study area is typified by overcrowding and poor hygiene as found in other developing countries, which is at variance with rural settings from developed countries (30). This suggests that a higher number of persons per household may increase infection transmission rate. A similar association was seen in the study by Aguemon et al., (30) in which subject participants with 1 roommate and those with 2 or 3 roommates had a prevalence of 51.0% and 77.6% respectively. This present study further supports the notion that *H. pylori* infection is a disease of overcrowding and poor hygiene.

In our study, the prevalence of *H. pylori* infection was associated with body mass index (BMI), with obese and overweight patients having significantly higher prevalence than normal weight patients. This agrees with the study by Al-Akwaa (31) in Saudi Arabia, which showed high *H. pylori* prevalence of 85.5% in a group of morbidly obese Saudi patients. The reason for this may be bidirectional as persons with dyspepsia from *H. pylori* infection may eat more frequently, in order to alleviate dyspeptic symptoms, and so are more likely to become overweight. On the other hand, obese people may be less discriminatory of where they eat. This may impact on the food hygiene, predisposing them to *H. pylori* infection. However, many studies in children and adult populations have shown that *H. pylori* infection is not associated with BMI or overweight (32, 33, 34).

Although most studies have found no significant relationship between occupation and *H. pylori* infection, our study showed a high prevalence of *H. pylori* infection of 87.5% among professionals (majority are health workers), which was significantly different from other occupational groups. Some studies have shown various pathways of agent transmission, favoring person-to-person transmission early in life, and oro-oral, faeco-oral and gastro-oral transmission have been proposed (35, 36). Our finding supports possible person-to-person mode of spread of *H. pylori* infection among health workers hence the need for implementation of standard precautions among high risk professionals.

Studies on association of alcohol consumption and *H. pylori* infection are conflicting however, our study showed low prevalence of *H. pylori* infection among subjects who consume alcohol. This may suggest that dyspepsia, which is common among alcoholics, may be more of functional origin than *H. pylori* infection related. Some studies have shown that alcohol has an antibacterial effect against *H. pylori* and the increase in acid secretion caused by alcohol has a bactericidal effect. While this may explain the inverse association between *H. pylori* infection and alcohol (37, 38), others have found no significant association (39, 40). The effects of alcohol on *H. pylori* infections require further research.

Conclusion:

The prevalence of *H. pylori* infection in treatment naïve dyspeptic patients in our area is high. Gender and factors related to poor hygiene, increase faeco-oral transmission, and low socio-economic status are related to the high prevalence. Alcohol consumption was inversely related to *H. pylori* infection hence dyspepsia among subjects who consume alcohol needs to be thoroughly investigated, as organic origin might be responsible. Consequently the test and treat approach as recommended by the European *Helicobacter pylori* group; Maastricht 111 (updated in 2005) is invaluable in our environment. Improving personal hygiene remains an efficient means of curtailing the menace of *H. pylori* infections and the attendant sequelae.

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Original Article **Open Access**

Green synthesis of Ag, Zn and Cu nanoparticles from aqueous extract of *Spondias mombin* leaves and evaluation of their antibacterial activity

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Abstract:

Background: Nanotechnology offers an advantage as a green route for synthesis of metal nanoparticles (NPs) with plant extracts as capping agent. *Spondias mombin* is a fruit-bearing tree and its leaf extracts have been reported to possess anxiolytic, hypoglycaemic, antiepileptic, antipsychotic, sedative, antioxidant, and antimicrobial properties. The objective of the study is to determine the antibacterial potential of a simple non-toxic product of green synthesis of metallic (Ag, Zn and Cu) nanoparticles using the leaf of *Spondias mombin* aqueous extracts (SMAE) as a reducing and capping agents of the metal ions.
Methodology: Nanoparticles were characterized by UV visible spectrophotometeric analysis, Fourier Transform Infra-Red (FT-IR) spectrophotometer and scanning electron microscope (SEM). Antimicrobial activities of synthesized NPs against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were determined by agar well diffusion technique.
Results: The synthesized NPs varied in colour from dark brown to green and appears dominantly spherical, occasionally rod or triangular shaped with size ranging from 65-90 nm. UV spectroscopy absorption spectra of Ag, Zn and Cu NPs had absorbance peak at 267, 262 and 765 nm respectively. FT-IR spectrometry of Zn NP, Cu NP, and SMAE gave wave number ranging from 895.71-3320.67, 747.02-3225.45 and 658.25-3674.49 respectively. FT-IR analysis showed that SMAE acted as reducing and stabilizing agent while the NPs exhibited lower energy absorption band when compared to the plant extract. The NPs demonstrated higher antimicrobial activities against *S. aureus* than *Ps. aeruginosa* and *E. coli.* The antimicrobial activity was higher with copper NP than Ag and Zn NPs, and also higher than SMAE.
Conclusion: The result from this study presents an indication for an alternative means for development of novel antimicrobial agents for clinical and biotechnological applications.

Keywords: synthesis, nanoparticles, FT-IR, UV-visible spectrophotometry, antibacterial activities

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Synthèse verte de nanoparticules d'Ag, de Zn et de Cu à partir d'extrait aqueux de feuilles de *Spondias mombin* et évaluation de leur activité antibactérienne

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Abstrait:

Contexte: La nanotechnologie offre un avantage en tant que voie verte pour la synthèse de nanoparticules métalliques (NP) avec des extraits de plantes comme agent de coiffage. Spondias mombin est un arbre fruitier et ses extraits de feuilles possèdent des propriétés anxiolytiques, hypoglycémiques, antiépileptiques, antipsychotiques, sédatives, antioxydantes et antimicrobiennes. L'objectif de l'étude est de déterminer le potentiel antibactérien d'un simple produit non toxique de synthèse verte de nanoparticules métalliques (Ag, Zn et Cu) à l'aide de la feuille d'extraits aqueux de Spondias mombin (SMAE) en tant qu'agent réducteur et coiffant de la ions métalliques.
Méthodologie: Les nanoparticules ont été caractérisées par analyse spectrophotométrique UV visible, spectrophotomètre à transformée de Fourier infrarouge (FT-IR) et microscope électronique à balayage (MEB). Les activités antimicrobiennes de NP synthétisées contre *Escherichia coli*, *Staphylococcus aureus* et *Pseudomonas aeruginosa* ont été déterminées par une technique de diffusion sur puits d'agar.
Résultats: Les NP synthétisés ont une couleur allant du brun foncé au vert et apparaissent principalement sphériques, parfois en bâtonnets ou en triangles, avec des tailles allant de 65 à 90 nm. Les spectres d'absorption par spectroscopie UV des NP Ag, Zn et Cu présentaient des pics d'absorbance à 267, 262 et 765 nm respectivement. La spectrométrie FT-IR de Zn NP, Cu NP et SMAE a donné un nombre d'onde allant de 895,71 à 3320,67, 747,02 à 3225,45 et 658,25 à 3674,49 respectivement. L'analyse FT-IR a montré que le SMAE agissait en tant qu'agent réducteur et stabilisant, alors que les NP présentaient une bande d'absorption d'énergie inférieure à celle de l'extrait de plante. Les NP ont démontré des activités antimicrobiennes plus élevées contre *S. aureus* que *Ps. aeruginosa* et *E. coli*. L'activité antimicrobienne était plus élevée avec les NP en cuivre que dans les NP Ag et Zn, et également supérieure à celle du SMAE.
Conclusion: le résultat de cette étude présente une indication d'un autre moyen de développement de nouveaux agents antimicrobiens pour des applications cliniques et biotechnologiques.

Mots-clés: synthèse, nanoparticules, FT-IR, spectrophotométrie UV-visible, activités antibactériennes

Introduction:

Nanotechnology is unfolding as a highly advanced multidisciplinary technology involving chemistry, physics, material science, biology and medicine. The synthesis of nano-particles (NPs) has been the most important stride in the area of nanotechnology (1). In the subject of biology, NPs have diverse use in drug delivery systems, antibacterials, minerals and many others. There are wide spans of chemical and physical methods being utilized for the synthesis of NPs. Nonetheless, these methods have few limitations such as the use of dangerous solvents, high energy utilization, and dangerous byproducts.

The use of plant extracts in the green synthesis of NPs is attaining significance over chemical synthesis. Plant extracts (possessing intrinsic ability) as surface stabilizing agents, may act as bio-template for the synthesis of NPs with potential for better crystal growth control and stabilization. This class of NPs synthesis plays a vital role in diverse nano-technological applications (2, 3).

Spondias mombin is a fruit-bearing tree which belongs to the genus Spondias, a flowering plant family, Anacardiaceae, home to the lowland slightly wet forest of the Amazon (4, 5). The dispersal of this plant reach all of tropical America, Brazil, Nigeria, West Indies and other tropical rain forests worldwide (5, 6). *S. mombin* enamate gum is used as adhesives, its bark and root decoctions are used as purgatives and medicament for dysentery, diarrhea, and haemorrhoids, while leaf extracts of the tree have been reported to possess anxiolytic, antiepileptic, anathematic, sedative, antipsychotic, antioxidant, and anti-microbial properties (7-11).

The biomolecules existing in plants can act as capping and reducing agents and thus increase the rate of reduction and stabilization of NPs. Biosynthesized metal NPs are more stable in nature and their synthesis rate is faster than other procedures. Some degree of antibacterial and antifungal activities have been demonstrated in green synthesized NPs of zinc and silver oxide of aqueous extract of some plants (12, 13, 14). In this study, the antibacterial activity of *Spondias mombin* leaf extract synthesized silver (Ag), zinc (Zn) and copper (Cu) NPs was determined against our laboratory stocks of *Escherichia coli*, *Pseudo-monas aeruginosa* and *Staphylococcus aureus* clinical isolates.

Materials and methods:

Collection and preparation of plant materials

Spondias mombin leaf was collected from Ijegun, Lagos State, Nigeria. Identi-fication was done at the Botany department, University of Lagos, Akoka, Lagos with voucher number LUH 7667. Healthy leaves were hand-picked and isolated from the rest. The freshly collected leaf was carefully washed with distilled water, dried at room temperature and

cut into fine pieces. 20g of finely cut leaf were kept in a separate beaker containing 100 ml deionized water (0.2g/ml) and boiled for 60 mins. The extract was cooled and filtered with Whatman filter paper and stored at 40^0C till further use.

Preparations of Ag, Zn and Cu nanoparticles

Preparations of silver (Ag) NP

A 1mM of $AgNO_3$ was prepared by weighing 0.017g of $AgNO_3$ and dissolved in deionized water. The solution was then transferred into a volumetric flask and filled up to 1 liter mark. Five milliliter (1g) of the *Spondias mombin* leaf extract was added to 45ml (0.02g/ml) of 1mM $AgNO_3$. The composition was incubated in a dark chamber to decrease photo-activation of $AgNO_3$ at room temperature.

Preparations of zinc (Zn) NP

A 4mM solution of zinc sulphate heptahydrate solution ($ZnSO_4.7H_2O$) was prepared by dissolving 0.5751g of $ZnSO_4.7H_2O$ in deionized water. The solution was transferred into a volumetric flask and filled up to 500 ml mark. Ten milliliter (2g) of the aqueous *Spondias mombin* leaf extract was added to 200ml of 4mM $ZnSO_4.7H_2O$ solution (0.01g/ml) and stirred for 5 minutes at room temperature. Then, 2M NaOH was added to the mixture dropwise with constant stirring at room temperature, and pH sustained at 12. The resulting white precipitate was centrifuged at 10,000rpm for 15mins. The solution was filtered with Whatman filter paper and washed twice with sterile distilled water, after which the precipitate was dried at 60^0C for 6 hrs.

Preparation of copper (Cu) NP

A 0.1M copper acetate [$Cu(CH_3COO)_2$] solution was prepared by dissolving 9.0815g of $Cu(CH_3COO)_2$ in deionized water and making it up in a 500ml volumetric flask. Five milliliter (1g) of *Spondias mombin* leaf extract was added to 45ml of 0.1M aqueous $Cu(CH_3COO)_2$ solution (0.02g/ml) for the reduction of Cu^{2+} ions. The solution was stirred for homogenous mixing, a change was observed in the colour solution from blue to dark green. The resultant solution was filtered with Whatman filter paper and the precipitate was dried in the oven at 60^0C.

Characterization of synthesized nanoparticles

Scanning electron microscope (SEM)

SEM analysis was done by using Vega 3 Tescan SEM machine coupled to Scandium 4.0 software. Thin films of Ag, Cu, and Zn NPs of SMAE each sample was prepared by dropping a very small amount of the sample on a carbon-coated copper grid and the film was then allowed to dry by putting it under a mercury lamp for 5 min.

UV-visible spectroscopy analysis of NPs

The reduction of the pure metal ions and the formation of the nanoparticles were ascertained using double beam UV-visible spectrometer (PG Instruments Ltd, T80 +, S/N 15-1885-01-0094) to measure the absorbance of solution at a resolution of 1 nm from 180 to 900 nm, using deionized water as blank.

FT-IR spectroscopy analysis of NPs

Approximately 0.02g of NP solutions was dissolved in 20ml distilled water and filtered to obtain a clear solution. The FTIR spectra were then recorded with detector at 4000-400 cm^{-1} resolution and 20 scans per sample.

Antimicrobial activities of synthesized NPs

Antimicrobial activities of the synthesized NPs were investigated against clinical isolates of *E. coli, S. aureus* and *Ps. aeruginosa* using agar well diffusion to determine the antimicrobial activity, and agar dilution to determine the MICs of each of the synthesized NPs in comparison with aqueous plant extract and ciprofloxacin as control.

Standardization of inoculum of test organisms

The overnight cultures of *E. coli, S. aureus* and *Ps. aeruginosa* in Tryptone soya broth (TSB) were streaked onto Tryptone soya agar (TSA) which were incubated at 37°C for 24 hours. Colonies of the organisms were emulsified in a bottle containing 20ml sterile normal saline, and their turbidity was adjusted to match that of 0.5 McFarland standards.

Preparation of samples

A 100%, 50%, 25% and 12.5% concentrations of the different NP solutions of Ag (0.02 g/ml, 0.01 g/ml, 0.005 g/ml, 0.0025 g/ml), Zn (0.01 g/ml, 0.005 g/ml, 0.0025 g/ml, 0.00125 g/ml) and Cu (0.02 g/ml, 0.01 g/ml, 0.005 g/ml, 0.0025 g/ml) and similar concentrations for the plant extract were used for the assay. The MIC working concentrations of ciprofloxacin used were 20µg/ml, 15µg/ml, 10µg/ml and 5µg/ml.

Antibiotic susceptibility test by agar diffusion

Different sections of Mueller Hinton (MH) agar plates used were assigned to the various concentrations of *Spondias mombin*

extract and the synthesized Ag, Cu, and Zn NPs. 1 ml of the standardized inoculum of each organism was pipetted onto the surface of its assigned MH agar plates and allowed to spread on the surface. A sterile cork borer of size 7mm was used to create 4 wells on each plate. The wells were filled with 150 μL of the test and control samples as assigned on each plate. The plates were left on the bench for 4 hrs to allow for diffusion of samples, after which they were incubated for 24 hrs at 37°C. The zones of inhibition were measured with a calibrated ruler. The assay for each sample was done in duplicates.

Determination of minimum inhibitory concentration (MIC) by agar dilution

Ten distinct stock concentrations of the different samples of the plant extract, and Ag, Zn and Cu NP solutions were prepared as 0.1% (0.0196875 mg/ml), 0.2% (0.039375 mg/ml), 0.4% (0.07875mg/ml), 0.8% (0.1575 mg/ml), 1.6% (0.315 mg/ml), 3.2% (0.630 mg/ml), 6.4% (1.260.0 mg/ml), 12.8% (2.52 mg/ml), 25.6% (5.1 mg/ml) and 51.2% (10.2 mg/ml). One milliliter of each stock concentration was made up to 20 ml on molten MH agar to give final concentrations of 0.984375 µg/ml, 1.96875 µg/ml, 3.9375 µg/ml, 7.875 µg/ml, 15.75µg/ml, 31.5 µg/ml, 63 µg/ml, 126 µg/ml, 255 µg/ml, and 510 µg/ml in the MH agar plates. For ciprofloxacin, 13 different concentrations were prepared; 0.001 µg/ ml, 0.002 µg/ml, 0.004 µg/ml, 0.008 µg/ml, 0.016 µg/ml, 0.032 µg/ml, 0.064 µg/ml, 0.128 µg/ml, 0.256µg/ml, 0.512 µg/ml, 1.024 µg/ml, 2.048 µg/ml

Approximately 0.1 ml of standardized inoculum of each organism was plated on the MH agar containing respective concentrations of the extract, synthesized NPs and ciprofloxacin. The plates were left on the bench for 4 hours to allow diffusion of the inoculum into the agar, after which they were incubated for 24 hours at 37°C. The MICs were recorded as the least concentration that produced no growth of the organism on the plate.

Results:

As shown by the SEM, the NPs varied in colour from dark brown to green and appears dominantly spherical, occasionally rod or triangular shaped with the size ranging from 65-90 nm. Complete reduction of the metals to metal ions was confirmed by the colour change on addition of the *Spondias mombin* extract to the metal salt solution. Silver and zinc solution changed from pale yellow to brown while copper solution changed from blue to dark green. UV-visible spectroscopy absorption spectra of Ag, Zn and Cu NPs had an absorbance peak at 267, 262 and 765 nm (Table 1).

The Fourier-Transform Infrared (FT-IR) spectroscopy for the synthesized Zn NP, Cu NP, and *Spondias mombin* aqueous extract (SMAE) showed wave number ranging from 895.71-3320.67, 747.02-3225.45, and 658.25-3674.49 respectively (Table 2).

The zones of inhibition for *Spondias mombin* aqueous extract and synthesized Ag, Cu, and Zn NPs against *E. coli, S. aureus* and *Ps. aeruginosa* are shown in Table 3. The highest zone of inhibition was observed at 20 mg/ml with SMAE zone of inhibition of 26 mm against *E. coli*, and Cu NP of 40 mm and 36 mm against *Ps. aeruginosa* and *S. aureus* respectively.

The MICs of *Spondias mombin* aqueous extract and the synthesized Ag, Zn and Cu NPs against *E. coli*, *Ps. aeruginosa* and *S. aureus* are shown in Table 4. The MICs of ciprofloxacin are 0.002µg/ml for *E. coli,* 0.008µg/ml for *S. aureus* and 1.024µg/ml for *Ps. aeruginosa* (Table 5). Antimicrobial effect was observed to be higher in Gram positive bacteria (*S. aureus*) than Gram negative bacteria (*E. coli* and *Ps. aeruginosa*) and most especially for Cu NP.

Table 1: UV-visible absorbance of Ag NP, Cu NP, Zn NP and SMAE

Sample	Peak (nm)	Absorbance
SMAE	490, 655, 581, 267, 363, 405	0.1822, 0.1765, 0.1503, 1.1113, 0.2923, 0.1290
$AgNO_3$	198, 273	1.0470, 0.1121
Ag NP	489, 598, 647, 267, 922, 655, 546, 429, 463, 438, 579	0.0163, 0.0241, 0.0142, 0.7306, 0.1507, 0.1560, 0.0071, 0.0149, 0.0818, 0.0447, 0.0209
$ZnSO_4$	252	0.0344
Zn NP	262, 489, 590, 358, 372	0.6209, 0.6372, 0.3664, 0.2269, 0.0886
$[Cu(CH_3COO)_2]$	764, 743, 780, 797, 819, 848	0.2056, 0.0330, 0.0165, 0.0333, 0.0154, 0.0148
Cu NP	765,490,585, 598, 427, 358,397, 366, 372	0.8897, 0.6841, 0.3823, 0.3671, 0.4620, 0.3366, 0.2158, 0.0779, 0.0651

NP = nanoparticle; Ag = silver; Cu = copper; Zn = zinc; SAME = *Spondias mombin* aqueous extract

Table 2: Fourier Transform Infra-Red (FT-IR) spectrophotometry of synthesized Cu NP, Zn NP and SMAE

SMAE		Zn NP		Cu NP	
Wave no (cm^{-1})	Functional group	Wave no (cm^{-1})	Functional group	Wave no (cm^{-1})	Functional group
3674.49, 3278.52	OH of alcoholic and phenolic group	3320.67	OH of alcoholic and phenolic group	3225.45	OH of alcoholic and phenolic group
2919.75, 2851.23	C-H stretching vibration of an alkyl group	1581.52	N–H band of primary amines	1708.33	C=O carbonyl stretching vibration
1976.79	C=C=C	1493.16	N-H stretching of aromatic amino group	1692.12	C=C stretching vibration of an aromatic or alkene
1726.44	C=O carbonyl stretching vibration	1481.91	N-H stretching of aromatic amino group	1582.84	N–H band of primary amines
1602.88	C=C stretching vibration of an aromatic or alkene	1467.88	N-H stretching of aromatic amino group	1479.44, 1440.84	N-H stretching of aromatic amino group
1439.52, 1372.49-1323.19	N-H stretching of aromatic amino group	895.71	C-H deformed of alkenes	1343.13	O-H bending vibration
1222.13-1160.03	C-O stretching vibration)			1179.98	C-C
1030.72	C-N bending vibration			1033.22	C-N bending vibration
817.34, 774.41-717.34	C-H deformed of alkenes			966.55	
658.25	Alkanes			747.02	Alkanes

Table 3: Zones of inhibition for synthesized Ag NP, Cu NP, Zn NP and SMAE against *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli*

	Zones of inhibition											
	Escherichia coli				*Pseudomonas aeruginosa*				*Staphylococcus aureus*			
Conc. mg/ml	SMAE	Ag NP	Zn NP	Cu NP	SAME	Ag NP	Zn NP	Cu NP	SMAE	Ag NP	Zn NP	Cu NP
20	26mm	16mm	ND	16mm	24mm	32mm	ND	40mm		20mm	ND	36mm
10	20mm	10mm	16mm	12mm	20mm	-	14mm	38mm		16mm	26mm	30mm
5	20mm	-	12mm	-	20mm	-	10mm	16mm		12mm	24mm	24mm
2.5	12mm	-	-	-	12mm	-	10mm	14mm		10mm	-	14mm
1.25	ND	ND	-	ND	ND	ND	-	ND		ND	-	ND

ND = not done; (-) = no zone of inhibition; SMAE - *Spondias mombin* aqueous extract; NP = nanoparticle; Ag = silver; Zn = zinc; Cu = copper

Table 4: Minimum inhibitory concentration of SMAE, Ag NP, Zn NP and Cu NP against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*

Minimum inhibitory concentration												
Conc	*Escherichia coli*				*Pseudomonas aeruginosa*				*Staphylococcus aureus*			
µg/ml	SMAE	Ag NP	Zn NP	Cu NP	SMAE	Ag NP	Zn NP	Cu NP	SMAE	Ag NP	Zn NP	Cu NP
0.98	+	+	+	+	+	+	+	+	+	+	+	+
1.97	+	+	+	+	+	+	+	+	+	+	+	+
3.94	+	+	+	+	+	+	+	+	+	+	-	+
7.88	+	+	+	+	+	+	+	+	+	+	-	+
15.75	+	+	+	+	+	+	+	+	+	+	-	-
31.5	+	+	+	-	+	+	-	-	+	-	-	-
63	+	+	+	-	+	+	-	-	-	-	-	-
126	+	+	+	-	-	+	-	-	-	-	-	-
255	+	+	+	-	-	+	-	-	-	-	-	-
510	-	-	-	-	-	-	-	-	-	-	-	-
MIC µg/ml	510	510	510	31.5	126	510	31.5	31.5	63	31.5	3.94	15.75

(+) = growth of organism; (-) = no growth of organism; SMAE = *Spondias mombin* aqueous extract; NP= nanoparticle; Ag = silver; Zn = zinc; Cu = copper

Table 5: Minimum inhibitory concentration of ciprofloxacin against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*

Concentration (µg/ml)	*Escherichia coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
0.001	+	+	+
0.002	-	+	+
0.004	-	+	+
0.008	-	-	+
0.016	-	-	+
0.032	-	-	+
0.064	-	-	+
0.128	-	-	+
0.256	-	-	+
0.512	-	-	+
1.024	-	-	-
2.048	-	-	-
4.046	-	-	-
MIC	0.002 µg/ml	0.008 µg/ml	1.024 µg/ml

(+) = growth of organism; (-) = no growth of organism

Discussion:

Spondias mombin is a fruit-bearing tree. The leaf extracts have been reported to possess anxiolytic, hypoglycaemic, antiepileptic, antipsychotic, sedative, antioxidant, and antimicrobial properties (7-11). In this study, green synthesized metallic NPs were identified by UV–visible and Fourier Transform Infra-Red spectrophotometer. While the generation and steadiness of the reduced metal NPs in the colloidal solution were scanned by UV–visible spectrophotometer, the FT-IR showed the feasible biomolecules for the reduction of metallic NPs.

The NPs varied in colour from dark brown to green color and were rod shaped, with the size ranging from 65-90 nm as revealed by the SEM. The appearance of colour change from pale yellow to brown for Ag and Zn solution, and from blue to dark green for Cu showed the formation of the respective NPs. Metal NPs such as Ag, Zn and Cu have free electrons, which give rise to surface plasmon resonance absorption band. The excitation of surface plasmon vibration in the metal NPs

gave rise to the colour reaction (15, 16). Subhankari and Nayak (17) have also reported that the biomolecules confirmed to be present in the aqueous extract of plant origin reduced the metal ions in addition, and also stabilized the metal NPs by blocking them from being oxidized after synthesis.

The zones of inhibition exhibited with *Spondias mombin* aqueous extract synthesized Cu NP in this study agrees with those of Pragati et al., (12), Palaniselvam et al., (13), Logeswari et al., (14), and Thatoi et al., (18). The Cu NP gave the highest antimicrobial activities against *Ps. aeruginosa* and *S. aureus* followed by Ag NP against *Ps. aeruginosa.* The SMAE alone had higher activity than Ag and Zn NPs. Although the ciprofloxacin standard gave much lower MIC values which were also well within the 2016 CLSI breakpoints of ≤1 µg/ml for susceptibility to ciprofloxacin (19) for the isolates used, these cannot be compared with the SMAE extract and synthesized NPs. This is because the actual compounds in the extract responsible for the antimicrobial activity were not determined in our study.

Metallic NPs are being reported to have desired antibacterial activity because of their large surface area to volume ratio (20). It is quite noteworthy that all bacterial species tested in this study showed appreciable level of susceptibility towards the green synthesized NPs.

Conclusion:

A simple development of Ag, Zn and Cu NPs by biological method using *Spondias mombin* aqueous leaf extracts was established by UV-visible spectrophotometer and FT-IR spectroscopy analysis. From this study, synthesized Ag, Zn and Cu NPs were found to possess antibacterial potential against *E. coli, S. aureus,* and *Ps. aeruginosa* though with comparatively less activity than ciprofloxacin that was used as standard.

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Original Article **Open Access**

Genotypic identification of coliforms isolated from cases of subclinical mastitis among pastoral herds in parts of Kaduna State, Nigeria

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Abstract:

Background: Mastitis caused by *Staphylococcus aureus* was initially considered the major problem in dairy herds, but over the last few decades, the incidence of coliform mastitis has increased among the pastoral herds in Nigeria due to poor environmental and milking hygiene. Hence, this study was aimed at genotypic identification of coliform bacteria isolated from cases of bovine mastitis among pastoral herds in parts of Kaduna State, Nigeria.
Methods: A cross-sectional survey of 30 herds of cows across 7 Local Government Areas of Kaduna State, Nigeria, was conducted. One hundred and forty seven cows were proportionately selected by purposive sampling technique. The milk samples were aseptically collected and bacteriologically screened for coliform bacteria following standard bacteriological techniques. Nine out of 12 coliforms identified phenotypically were selected for PCR amplification and sequencing of their 16S rRNA gene. The Basic Local Alignment Search Tool (BLAST) analysis of the sequences obtained was done on the National Centre for Biotechnology Information (NCBI) data base, and isolates confirmed based on similarity to 16S rDNA sequences in the Gen Bank
Results: Five of the 9 coliforms were confirmed to be *Klebsiella pneumoniae* (prevalence rate, 3.4%) and 4 were confirmed to be *Escherichia coli* (prevalence rate, 2.7%).
Conclusion: This study shows that raw milk of mastitic cows can serve as a vehicle for the spread of pathogens such as *K. pneumoniae* and *E. coli* which, according to the Department of Health and Human Services of the United States Public Health Services, are potential threats to public health and safety of humans, animals and plant products.

Keywords: pastoral herds, subclinical mastitis, cows, PCR, 16s rRNA, sequencing

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Identification génotypique de coliformes isolés à partir de cas de mammites subcliniques parmi des troupeaux pastoraux dans certaines parties de l'État de Kaduna, au Nigéria

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Abstrait:

Contexte: La mammite causée par *Staphylococcus aureus* était à l'origine considérée comme le principal problème des troupeaux laitiers, mais au cours des dernières décennies, l'incidence de la mammite à coliformes a augmenté dans les troupeaux pastoraux du Nigéria en raison de la mauvaise hygiène de l'environnement et de la traite. Par conséquent, cette étude visait l'identification génotypique de bactéries coliformes isolées à partir de cas de mammite bovine parmi les troupeaux pastoraux dans certaines parties de l'État de Kaduna, au Nigéria.
Méthodes: Une enquête transversale sur 30 troupeaux de vaches dans 7 zones de gouvernement local de l'État de Kaduna au Nigéria a été réalisée. Cent quarante sept vaches ont été sélectionnées proportionnellement par une technique d'échantillonnage raisonné. Les échantillons de lait ont été collectés de manière aseptique et soumis à un dépistage bactériologique des bactéries coliformes selon les techniques bactériologiques classiques. Neuf des 12 coliformes identifiés phénotypiquement ont été sélectionnés pour l'amplification par PCR et le séquençage de leur gène ARNr 16S. L'analyse par l'outil de recherche d'alignement local de base (BLAST) des séquences obtenues a été effectuée sur la base de données du Centre national d'information sur la biotechnologie (NCBI), et des isolats ont été confirmés sur la base de la similarité avec les séquences d'ADNr 16S de la banque de gènes.
Résultats: *Klebsiella pneumoniae* a été confirmé chez cinq des neuf coliformes (taux de prévalence de 3,4%) et dans 4 cas chez *Escherichia coli* (taux de prévalence de 2,7%).
Conclusion: cette étude montre que le lait cru de vaches mastitiques peut servir de vecteur à la propagation d'agents pathogènes tels que K. pneumoniae et E. coli qui, selon le ministère de la Santé et des Services sociaux des États-Unis, sont: menaces potentielles pour la santé publique et la sécurité des personnes, des animaux et des produits végétaux.

Mots-clés: troupeaux pastoraux, mammite subclinique, vaches, PCR, ARNr 16s, séquençage

Introduction:

The genera of coliforms mostly implicated in cases of bovine mastitis are *Klebsiella*, *Escherichia*, and *Enterobacter*. However, the most frequently isolated species of coliform bacteria are *Klebsiella pneumoniae* and *Escherichia coli* (1, 2). Furthermore, coliforms are not natural inhabitants of the mammary gland, but many strains are capable of surviving and multiplying there. Hence, intramammary infections caused by coliforms and other Gram-negative bacteria typically result from the bacteria that traverse the teat canal and multiply in the gland (1, 2, 3).

Klebsiella pneumoniae is one of the known causes of primarily environment derived *Klebsiella* mastitis and has been the subject of numerous studies (4, 5). *K. pneumoniae* is an opportunistic human pathogen mainly affecting immunocompromised or elderly patients. Recently a hyper-virulent *K. pneumoniae* strain was reported to be capable of causing fatal infections in healthy individuals (6). *K. pneumoniae* has been reported in different countries in serious outbreaks or in isolated cases of per-acute or acute bovine mastitis, predominantly in the first two weeks of lactation. Mammary infections are often associated with wood or sawdust contamination used in the environment of the animals (4, 7, 8).

Since the 1980s there has been a presence of individuals with community acquired *K. pneumoniae* infections resulting in primary liver abscesses. These liver abscesses are caused by the capsular serotype K1 and the gene *mag*A (9). This gene is believed to be involved in exo-polysaccharide biosynthesis which can protect the bacteria and contribute to its virulence. All isolates containing the *mag*A gene were all serotype K1 which leads researchers to believe that the K1 capsular serotype is an important virulence factor (9).

Escherichia coli are parts of the normal microbiota of the gut, and can benefit their hosts by producing vitamin K_2 (10) and preventing colonization of the intestine by other pathogenic bacteria, having a symbiotic relationship (11, 12). *E. coli* is released into the environment within fecal matter. The bacterium grows massively in fresh fecal matter under aerobic condition for three days and declines gradually afterwards (13). *E. coli* is one of the main causative agents of bovine mastitis which is responsible for significant losses on dairy farms that is of great economic interest (14, 15, 16). Most *E. coli* strains do not cause disease, but virulent strains can cause gastroenteritis, urinary tract infections, neonatal meningitis, and haemorrhagic colitis (17). Common signs and symptoms include severe abdominal cramps, diarrhoea, haematochezia, vomiting, and sometimes fever. In rare cases, virulent strains are also responsible for bowel necrosis and perforation without progressing to hemolytic-uremic syndrome, peritonitis, mastitis, septicemia, and Gram-negative pneumonia (18). Young children are more vulnerable to develop severe illness, such as hemolytic uremic syndrome; however, healthy individuals of all ages are at

risk to the severe consequences that arises as a consequence of being infected with *E. coli* (17, 18).

In the 1960s, Dubnau *et al*., (19) noted conservation in the 16S rRNA gene sequence relationships in *Bacillus* spp. The extensive use of this gene sequence for bacterial identification and taxonomy followed a body of pioneering work by Woese, who defined important properties. Foremost is the fact that it seems to behave as a molecular chronometer, as pointed out in an excellent review article by Woese (20). Furthermore, no gene has shown as broad applicability over all the taxonomic groups as the 16S rRNA gene. Thus, if the goal is to identify an unknown organism on the basis of no prior knowledge, the 16S rRNA gene sequence is an excellent and extensively used choice (21). The 16S rRNA gene sequence has been determined for a large number of bacterial strains. Gen Bank, the largest databank of nucleotide sequences, has over 20 million deposited sequences, of which over 90,000 are of 16S rRNA gene (21). The 16S rRNA gene is universal in bacteria, and so relationships can be measured among all bacteria (22, 20, 21). Hence, this study was carried out to confirm some coliform bacteria isolated from cases of subclinical mastitis by PCR amplification of the 16S rRNA gene and direct nucleotide sequencing.

Materials and method:

Study area and population

The study area, which is same as in a previous study by Makolo et al., (23), involved settlements in each of 7 Local Government Areas of Kaduna State, Nigeria; Chikun, Igabi, Soba, Birnin Gwari, Sabongari, Zaria and Giwa (Fig. 1). One hundred and forty seven lactating cows were proportionately selected by purposive sampling technique among 309 within 30 pastoral herds (Table 1).

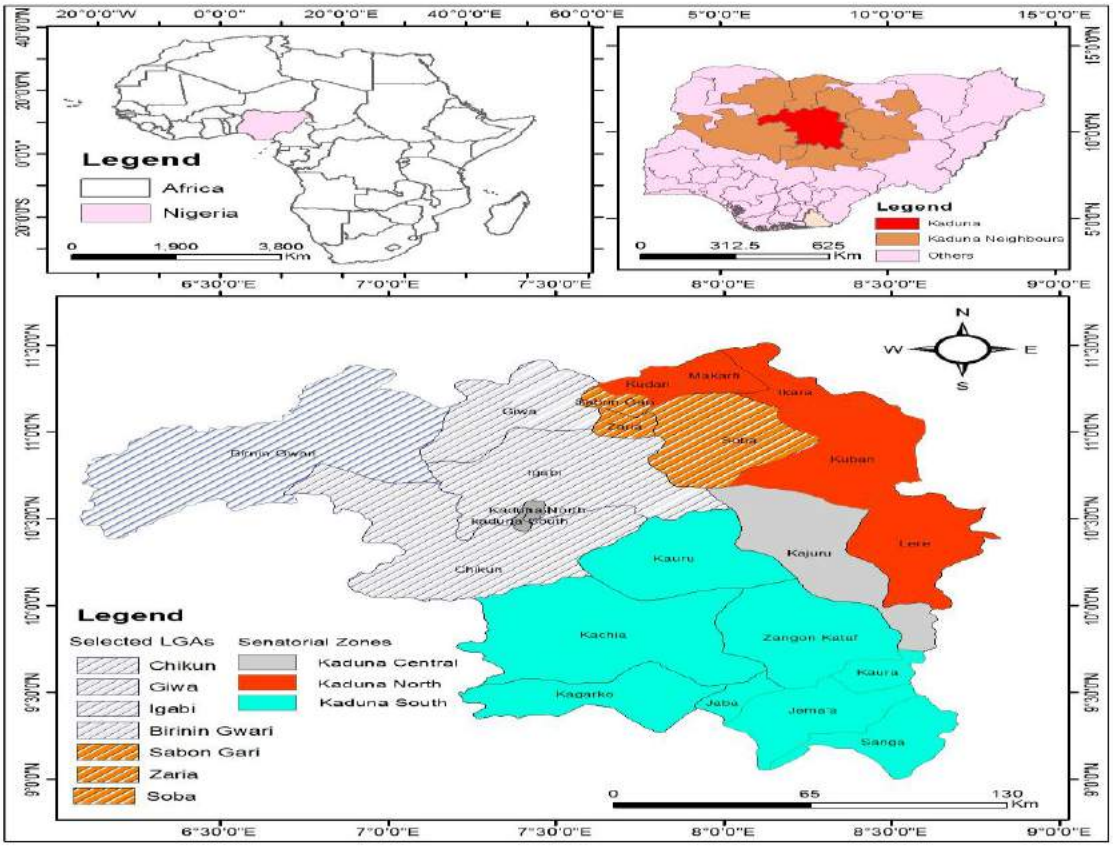

Fig 1: Map of Africa, Nigeria and Kaduna State showing the study area
Source: Makolo et al. (23)

Table 1: Proportionate distribution of samples across the study population

S/N	Local Government Area/Settlements	No. of Herds	No. of Lactating Cows	No. of Cows Examined
1.	Giwa (Settlement A)	5	50	24
2.	Igabi (Settlement B)	3	30	15
3.	Chikun (Settlement C)	4	39	19
4.	Soba (Settlement D)	3	27	12
5.	Zaria (Settlement E)	5	55	26
6.	Sabongari (Settlement F)	4	40	19
7.	Birnin Gwari (Settlement G)	6	68	32
	Total	30	309	147

Source: Makolo et al., (23)

Bacteria isolates

Nine coliform isolates recovered from cows with mastitis in the previous study and stored in the Microbiology Laboratory of Ahmadu Bello University, Zaria were selected for the study. These isolates have been previously identified as coliform bacteria using the conventional biochemical tests and Microgen identification kit (23).

DNA extraction from coliform isolates

Genomic DNA was extracted using DNA Miniprep; 100mg (wet weight) of bacterial cells were re-suspended in a tube containing 200µL of isotonic buffer (Applied Biosystems, USA), and 750µL lysis solution was added to the tube. This was secured in a bead fitted with 2ml tube holder assembly and processed at maximum speed for 5 minutes. The mixture in the ZR Bashing Bead™ Lysis Tube was centrifuged in a microcentrifuge at 10,000xg for 1minute. Exactly 400µL of the supernatant was transferred to a Zymo-Spin™ IV Spin Filter (orange top) in a collection tube and centrifuged at 7,000xg for 1 minute. Then, 1,200µL of bacterial DNA binding buffer was added to the filtrate in the collection tube after which 800µL of the mixture was transferred to a Zymo-Spin™ IIC Column in a collection tube and centrifuged at 10,000xg for 1 minute.

The flow through from the collection tube was discarded and 200uL DNA pre-wash buffer was added to the Zymo-Spin ™ IIC Column in a new collection tube and centrifuged once again at 10,000xg for 1 minute. Then, 500µL bacterial DNA wash buffer was added to the Zymo-Spin™ IIC Column and centrifuged at 10,000xg for 1 minute. The mixture from Zymo-Spin™ IIC Column was transferred to a clean 1.5 ml microcentrifuge tube and 100µL DNA elution buffer was added directly to the column matrix. This was centrifuged at 10,000xg for 30 seconds to elute the genomic DNA for PCR and sequencing.

DNA quantification

The extracted genomic DNA was quantified using the NanoDrop 1000 Spectrophotometer at the wavelength of 260/280nm. Briefly, the upper and lower optical surfaces of the micro volume spectrophotometer sample retention system was cleaned by pipetting 3µL of clean deionized water onto the lower optical surface. The lower arm was closed, ensuring that the upper pedestal came in contact with the deionized water. The lower arm was lifted and both optical surfaces were wiped off with a clean, dry, lint-free lab wipe. Then, the NanoDrop software was opened and 'nucleic acid application' was selected.

Using a small volume calibrated pipettor, 1 µL of buffer was measured onto the lower optical surface. The lever arm was lowered and 'blank' was selected in the nucleic acid application. Constant of 260-280nm was selected for the DNA samples to be measured. Then, 1µL of nucleic acid sample was dispensed onto the lower optical pedestal and the lever arm was closed. The 'measure' was selected in the application software which automatically measured the nucleic acid and purity ratios.

PCR assay

A 10µL reaction mixture was used for DNA amplification by PCR which contained 1.0µL of PCR buffer, 1.0µL of $MgCl_2$, 0.5µL of forward primer (5'-GTGCCAGCAGCCGCGCTAA-3'), 0.5 µL of reverse primer (5'-AGACCCG GGAACGTATTCAC-3'), 1.0µL of DMSO, 0.8µL of dNTPs (2.5Mm), 0.1µL of Taq DNA polymerase (5U/ µL), 2.0µL of template DNA (10ng/µL) and 3.1µL distilled water. DNA amplification was carried out in a thermocycler (ABI-

PRISM®3700 Genetic Analyzer) with a pre-cycle at 94°C for 5 minutes, 36 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 45 sec, and final extension at 72°C for 7min with expected amplicon size of 850bp (24).

Agarose gel electrophoresis of PCR products

The amplified PCR products of the coliform bacteria were loaded on 1.5% agarose gel and electrophoresed at 120V for 15 minutes along with a 100bp ladder (Bioline, USA) and negative control (nuclease free water). The gels were stained with ethidium bromide and observed UV transillumination, and photographed.

PCR product purification

The PCR product was purified by adding 20µL of absolute ethanol to the PCR product and incubating at room temperature for 15minutes. The product was spun at 1,000 rpm for 15 minutes. With the supernatant decanted and spun again at 1,000rpm for 15minutes. Then, 40µL of 70% ethanol was added supernatant was decanted and air dried. About 10µL of ultrapure water was added and the amplicon was checked on 1.5% agarose.

Sequencing of PCR products

The PCR products were sequenced using the Big Dye Terminator Kit on ABI-PRISM 3700 Genetic Analyzer (Applied Biosystems, USA) at the Biosciences Centre, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria.

Basic Local Alignment Search Tool (BLAST) analysis of gene sequences

The BLAST analysis was carried out on the gene sequences obtained using the online database of National Centre for Biotechnology Information: http://blast.ncbi.nlm.nih.gov./Blast.cgi. Briefly, the gene sequences per organism were copied from the FASTA file format and paste in the Blast Window online where Nucleotide Sequence Blast was selected. The result showed the lineage report on the organism identified and the sequences producing significant alignments with those deposited in the Gene Bank. Interpretation of results was done following the instructions of NCBI.

Results:

Table 2 shows the result of quantitative analysis of the extracted DNA of the coliform isolates using spectrophotometer at the wavelength of 260/280nm. The result revealed that the DNA extraction of all the isolates fell within the acceptable standard (1.6-1.9) of quality and purity for PCR and sequence analysis. All the 9 coliform isolates amplified on PCR of 16S rRNA genes (Fig 2). The 16S rRNA PCR sequencing confirmed the isolates to be *Klebsiella pneumoniae* (n=5) and *Escherichia coli* (n=4) (Table 3).

Table 2: Quantification of the extracted DNA from coliform isolates by Spectrophotometer at the wavelength of 260/280nm

Sample identity	Concentration	Purity
E1	87.7	1.76
E3	47.9	1.85
E4	40.7	1.77
K1	87.7	1.81
K2	31.9	1.86
K4	119.9	1.8
K6	59.2	1.8
K7	36.5	1.86
P1	90.6	1.83

E1-E4 and P1 = *Escherichia coli* isolate; K1-K7 = *Klebsiella pneumoniae* isolates

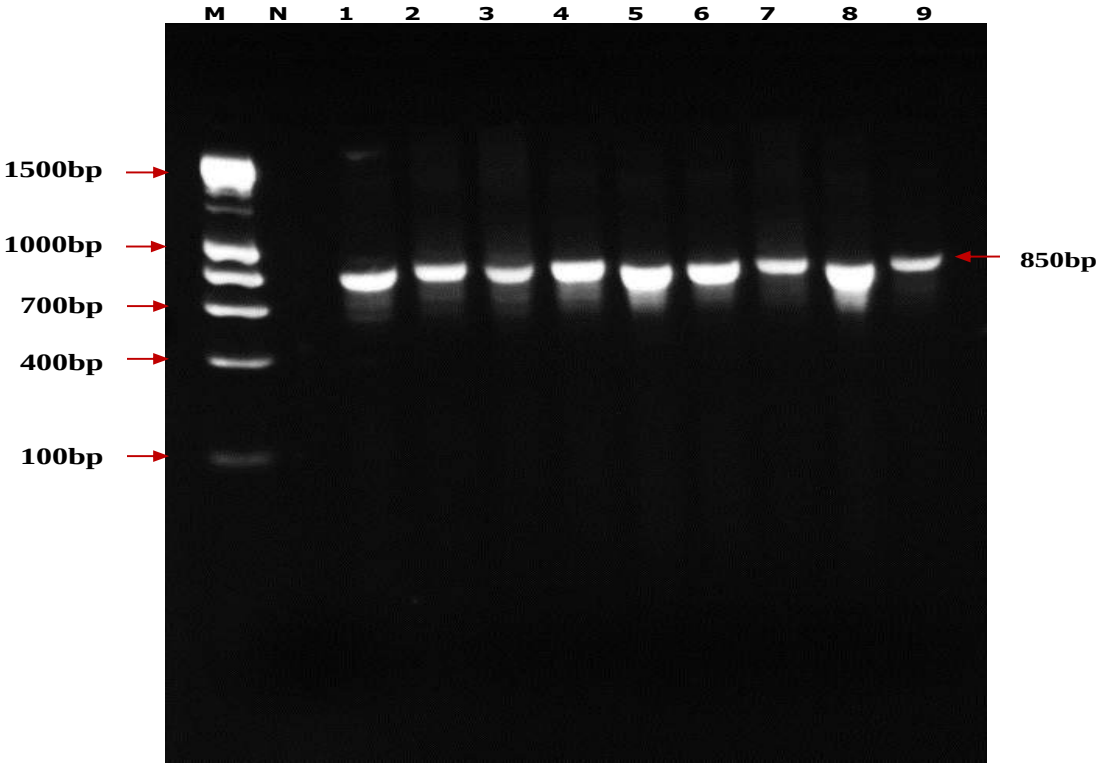

Fig 2: Amplified fragment of 16S rRNA gene of coliform isolates (850bp)
Lane M (Molecular Marker of 100bp); Lane N (Nuclease free water as negative control); Lanes 1, 4, 5, 6, and 8 (*Klebsiella pneumoniae* isolates); Lanes 2, 3, 7, and 9 (*Escherichia coli* isolates)

Table 3: BLAST analysis of 16S rRNA sequences of coliforms with the GenBank database of National Centre for Biotechnological Information

Isolates Code	Genotypic identification	Sequence with significant alignment	E-value	QC	ID
E1	*Escherichia coli*	*Escherichia coli* strainASRM93	0.0	95%	100%
E 3	*Escherichia coli*	*Escherichia coli* strain AMUM1	0.0	97%	99%
E 4	*Escherichia coli*	*Escherichia coli* strain NF113	0.0	97%	94%
K1	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* strain MH18	0.0	97%	99%
K2	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* strain NF16	0.0	97%	99%
K4	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* strain NF15	0.0	95%	100%
K6	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* strain Ma19	0.0	97%	99%
K7	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* strain NF82	0.0	97%	98%
P1	*Escherichia coli*	*Escherichia coli* strain 244	0.0	96%	99%

E-value= Expected value; QC = Query Cover; ID = Identity

Discussion:

This study has established that coliform bacteria were associated with cases of subclinical mastitis among the investigated cows from pastoral herds in parts of Kaduna State, Nigeria. This is similar with the report of Mbuk et al., (25) in Kaduna State, Nigeria, Junaidu et al., (26) in Sokoto, Nigeria, Agunbiade et al. (27) in Kaduna State, Nigeria, Hempen et al., (28) in Gambia, Senegal and Guinea, and Lingathurai and Vellathurai, (29)

in Madurai, India who isolated coliforms from cases of bovine mastitis in similar studies.

The species of coliforms isolated in this study based on the genotypic identification were *K. pneumoniae* and *E. coli* with *K. pneumoniae* being the more frequent species associated with bovine mastitis. This is in agreement with the study of Mbuk et al., (25) who isolated similar species of these organisms in Kaduna State with *K. pneumoniae* being predominant and non *E. coli* was isolated. These findings also agree with the report of Hogan and Smith (1) and Podder et al., (30). Generally, the presence of these coliform bacteria in milk is an indication of feacal and environmental contamination resulting from poor hygienic practices of rearing and milking the animals as they are no established mastitis control practices employed among the herdsmen. This affirms the reports of the Centre for Disease Control (31), Food and Agricultural Organization (32), and other regulatory agencies around the world which reported that pathogens from raw milk including potential agents of diarrhoea, tuberculosis, diphtheria, typhoid and strepto-coccal infections make raw milk unsafe for consumption (33). Similarly, Claeys et al., (34) reported that consumption of raw milk poses a realistic health threat due to a possible contamination with human pathogens.

Furthermore, from epidemiological point of view, the presence of these organisms in the milk of cows reared for commercial purpose is of significant public health threat. This assertion is affirmed by the report of United States Public Health Services Depart-ment of Health and Human Services which listed all species and serotypes of *Klebsiella*, *Proteus*, *Escherichia*, *Citrobacter*, *Enterobacter*, *Pantoea* and *Serratia* as dangerous biological agents with potential threat to public health and safety of humans, animals and plant products (35). Consequently, the coliform species isolated in this study are part of this list. Therefore, consumption of raw milk from bovines with mastitis may pose serious health challenges to the populace.

The results of BLAST alignment revealed that the best match of the organisms isolated in this study based on sequence similarity with those in GenBank was within the range of 94-100%. This significant correlation between these sequences implies close similarity in the gene sequences of the isolated coliform with their best match in GenBank. Therefore, from epidemiological point of view, the measure used in the control of the best match of these isolates in their various countries of origin may also be effective in controlling them within the current study population on the ground of homology (those with 100% similarity) which makes these strains to share the same structure and function. The BLAST results also revealed that the majority of these organisms that showed significant sequence alignments with those isolated in this study were from human sources. This affirms the findings of Hogan and Smith (1) that coliforms are not natural inhabitants of the bovine mammary gland, but are usually transmitted from humans and contaminated environment through poor hygienic process of milking and rearing the animals. Generally, this study affirms the previous reports that identifying bacteria by DNA sequencing instead of phenotype provide better identification of poorly described, rarely isolated, or biochemically aberrant strains.

Conclusion:

In conclusion, all 9 coliform bacteria previously identified by phenotypic methods were confirmed to be *K. pneumoniae* and *E. coli* based on significantly high similarity and best match with NCBI GenBank data. Consequently, the presence of *K. pneumoniae* and *E. coli* in raw milk samples of cows reared for commercial purposes poses a potential public health threat to the study population. Therefore, periodic investigations on the bacteriological quality of milk from pastoral herds and the health status of the cows should be given a priority to avoid the epidemics of food borne and zoonotic disease.

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Authors contributions:

ABS and DM conceived and designed the study; ABS, MB and OSO provided critical intellectual content; DM and IA performed the

laboratory analysis; DM and ABS analyzed the gene sequence data; IA contributed reagents, materials and analysis tools; and DM wrote the manuscript. All authors agreed to the final version of the manuscript.

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Seroprevalence of *Toxoplasma gondii* infection and associated risk factors among pregnant women attending antenatal clinic at the Bamenda Regional Hospital, Cameroon

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Abstract:

Background: *Toxoplasma gondii* is a ubiquitous, coccidian parasite that causes toxoplasmosis. This infection, if acquired during pregnancy may result in severe damage. It affects a third of the world's population. In many developing countries, its prevalence is unknown, and data concerning its seroprevalence among pregnant women is scarce in our study area. The objective of this study is to determine the seroprevalence of *T. gondii* infection and the associated risk factors among pregnant women attending the antenatal clinic (ANC) at the Bamenda Regional Hospital in Cameroon. The results obtained will be useful in giving an estimate of the prevalence among pregnant women thus informing policy on preventive measures.
Methodology: This is a descriptive cross-sectional study of pregnant women recruited between January and April 2018 using systematic random sampling technique. Socio-demographic data of participants and predisposing factors to toxoplasmosis were collected using a pretested structured questionnaire administered to them. Five milliliters of blood were collected and the serum screened for IgG and IgM antibodies against *T. gondii* using the cassette and buffer immunochromatographic method. The positive IgG cases were tested further by ELISA technique. Data were analyzed using SPSS version 20. Associations between variables were tested by Chi square and p value < 0.05 was considered statistically significant.
Results: Of 127 women tested, 44 were seropositive for IgG *T. gondii* infection (34.6%) by cassette and buffer method and only 1 with both IgG and IgM antibodies (0.8%) were found among them with Elisa test. Pet ownership and handling of their litters were risk factors significantly associated with toxoplasmosis (p=0.013 and 0.006 respectively). Although the frequencies of consumption of raw dried meat and farming among the subjects were high, their associations with toxoplasmosis were not statistically significant.
Conclusion: The overall seroprevalence of *T. gondii* antibodies among the pregnant women is still high compared with previous finding in the same area (34.6 % for IgG and 0.8% for IgG and IgM). Pet ownership and handling of their litters were risk factors significantly associated with toxoplasmosis in this study. Screening of pregnant women during ANC and treatment of positive cases, are necessary to prevent congenital infections in the newborn. Health education on how to minimize exposure to the risk factors should be given.

Keywords: Risk factors, toxoplasmosis, congenital transmission, serological diagnosis.

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Séroprévalence de l'infection à *Toxoplasma gondii* et des facteurs de risque associés chez les femmes enceintes en visites prénatales à l'Hôpital Régional de Bamenda au Cameroun

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Résumé:

Contexte: *Toxoplasma gondii* est un parasite coccidien omniprésent qui cause la toxoplasmose. Cette infection, si elle est contractée pendant la grossesse, peut entraîner de graves dommages. Elle affecte un tiers de la population mondiale. Dans de nombreux pays en développement, sa prévalence est inconnue, et les données concernant sa séroprévalence chez les femmes enceintes sont rares dans notre zone d'étude. L'objectif de cette étude est de déterminer la séroprévalence de l'infection à *T. gondii* et les facteurs de risque associés chez les femmes enceintes fréquentant la clinique prénatale de l'hôpital régional de Bamenda au Cameroun. Ceci dans l'optique de donner des estimations sur sa prévalence chez ces femmes enceintes et de générer des stratégies à prendre pour des mesures préventives.
Méthodologie: Il s'agissait d'une étude descriptive transversale effectuée de janvier à avril 2018 à l'aide d'une technique d'échantillonnage systématique. Les données sociodémographiques et certains comportements qui prédisposaient les participants à l'infection ont été recueillis à l'aide d'un questionnaire préétabli. Cinq millilitres d'échantillon sanguin ont été prélevés et le sérum a été dépisté pour détecter les anticorps IgG et IgM contre *T. gondi* en utilisant la méthode cassette et tampon. Les cas positifs d'IgG ont été soumis à la technique ELISA. Les données ont été analysées à l'aide du logiciel SPSS version 20. Les résultats étaient jugés statistiquement significatifs lorsque la valeur p était inférieure à 0,05
Résultats: Des 127 femmes testées, 44 avaient des anticorps IgG pour l'infection à *T. gondii* à 34,6 % et seulement 1 avait des anticorps IgG et IgM à 0,8. Les facteurs de risque associés à l'infection étaient la possession et la manipulation des fèces des animaux de compagnie (p=0,013 et 0,006 respectivement). Les proportions concernant la consommation de viande crue séchée et l'agriculture été élevées, mais les associations n'étaient pas statistiquement significatives.
Conclusion: La séroprévalence globale des anticorps *T. gondii* chez les femmes enceintes reste élevées dans la région (soit 34,6 % pour l'IgG et 0,8% pour IgG et IgM). La possession et la manipulation des animaux de compagnie étaient les facteurs de risque importants associés à l'infection. Le dépistage précoce et systématique de l'infection devrait être envisagé pendant la consultation prénatale, et le traitement pour tout cas positif. Une éducation sanitaire sur les façons de réduire au minimum l'exposition aux facteurs de risque devrait être fournie.

Mots clés: Facteurs de risques, toxoplasmose, transmission congénitale, diagnostic sérologique.

Introduction:

Toxoplasma gondii, the causative agent of toxoplasmosis*,* infects warm-blooded animals including humans. Seroprevalence studies show that the organism is found in almost every country with different climates and social conditions (1). Globally, about one third of the world's human population is estimated to carry *T. gondii* parasite (2). Bradyzoites, tachyzoites and the sporozoites in oocysts are the three infectious stages of the coccidium, parasite to all hosts member of the cat family as its definitive host and has a wild range of intermediate hosts including humans (3). Humans usually acquire *T. gondii* infection through consumption of raw or undercooked meat, of improperly washed vegetables and fruits as well as drinking of water which contains oocysts. Moreover, transplacentally tachyzoites infect the fetus in pregnant women (4,5).

Toxoplasmosis is normally asymptomatic in healthy individuals but can cause maternal-fetal transmission in women who acquire primary *Toxoplasma* infection during pregnancy (6,7). The risk of vertical transmission and associated problems are determined by the gestational age at which the primary infection is acquired. Transmission to the fetus increases from the first trimester (10 to 24%) to the third trimester (60 to 90%), but the potential of congenital defect is more severe with earlier infections (8,9). Most pregnant women with acute acquired infection do not experience obvious symptoms or signs. A minority may experience malaise, low-grade fever, and lymphadenopathy (10). Severe clinical signs in the infected infant commonly observed in offspring of women whose infection was acquired early in gestation (6,11). A minority may experience malaise, low-grade fever, and lymphadenopathy (5). In the immunocompetent subjects, *Toxoplasma* infection is often asymptomatic, and frequently results in the chronic persistence of cysts within host tissues, that probably lie dormant for life. In contrast, in immunocompromised subjects, it is always life threatening.

Diagnosis of toxoplasmosis in humans is performed using different techniques. Acute and latent *T. gondii* infections during pregnancy are mostly diagnosed by serological tests including detection of anti-*T. gondii* specific IgM and IgG antibodies (12). Latex agglutination (LA) testing, enzyme-linked immunosorbent assay (ELISA), and/or indirect fluorescent antibody testing (IFAT) are some of the tests that used to detect the antibodies (13). In our context, most studies on prevalence of *T. gondii* infections have relied on antibody detection, although this is associated with false positives. A more reliable way of

diagnosing active infection is the use of molecular techniques such as PCR using blood, brain biopsy, and liver biopsy materials (14). However, this is very expensive and not easily available in most developing countries making its use in routine diagnosis not feasible. It is for this reason that diagnosis of toxoplasmosis in most health facilities in these countries still largely rely on serology, hence the use of this method in the current study

There is uneven distribution of *T. gondii* prevalence among pregnancy and childbearing age from different parts of the world. In Europe, the prevalence of Toxoplasmosis varies between 20-50% in the South and between 50-70% in the West (15). Seroprevalence of 10.3% and 69% have been reported from Japan (16) and North-eastern Brazil (17). In Africa, data on *T. gondii* infection during pregnancy is scanty and its burden in pregnant women is an under-estimated public health concern. In these countries, higher (up to 92.5%) seroprevalence has been reported. However, the prevalence of infection varies widely between countries (from 10 to 80%) and often within a given country or between different communities in the same region (14). Pregnant women are not routinely investigated for *T. gondii* during pregnancy, and follow-up hardly exists (18,19). In Cameroon, Mbouo-Bandjoun in particular, the seroprevalence of *T. gondii* infection is 45.5% (20) which represents a real public health problem as there are limited researches that have been conducted about risk factors associated with such high rate.

At the Bamenda Regional Hospital (BRH) measurement of *T. gondii* immunoglobulins is part of the routine tests prescribed by consulting gynaecologists, however due to ignorance of the importance of this test and sometimes financial constraints, pregnant women often fail to have this test performed during the entire period of their pregnancies. Also, the diagnosis of *T. gondii* infection is solely based on a qualitative test, and only women with positive IgM are treated while those with positive are usually not tested further to confirm their diagnosis, and are therefore not treated. In addition, information is scarce on the seroprevalence of *T. gondii* infection in pregnant women particularly in the study area. It is for these reasons that the present study was undertaken, with the aim of determining the seroprevalence of *T. gondii* infection, and identifying associated risk factors among pregnant women attending the antenatal clinic (ANC) of the BRH, Cameroon.

Materials and method:

Study design and setting

This was a descriptive cross-sectional study involving pregnant women attending the antenatal clinic (ANC) at the Bamenda Regional Hospital (BRH) located in the capital city of North West Region of Cameroon. The ANC unit is part of the Obstetric and Gynaecologic department, one of the 21 departments that make up the second degree referral hospital. Its services include: reception/registration room, laboratory, two examination rooms and general conference hall where antenatal consultation and education of pregnant women take place. ANCs take place from Monday to Friday every week. This study was carried out for a period of four months (1st January to 31st April 2018).

Sample size and sampling technique

All participants, attendees of the ANC service, who met the inclusion criteria, were recruited over the study period, using a systematic random sampling method. After identification, the potential study participants were taken through the informed consent process whereby the study objectives, risks, benefits and study procedures were explained. A total of 127 pregnant women were enrolled among 243 who attended the ANC clinic during the study period.

Data collection

A pre-tested questionnaire was used to collect information by face-to-face interview on socio-demographic and economic status as well as epidemiological risk factors of *T. gondii* infection. Data was collected on the following variables: age, ethnic group, marital status, level of education, occupational status, age of pregnancy, area of residence, history of miscarriage and still births, owning of pets or domestic animals, types of pets, handling of pets litter, farm or gardening work, history of children with eye or head malformations, habit of consumption of undercooked meat, raw dried meat commonly known as 'Kilichi', raw vegetables, drinking water sources, and history of recent blood transfusion. The level of knowledge regarding toxoplasmosis and sources of information on *T. gondii* infection were also evaluated.

Sample collection and processing

Approximately 5 ml of venous blood was collected aseptically from each participant into a plain dry. The blood sample was allowed to clot and centrifuged at 3,000 rpm for 5 minutes, and the serum was separated into another plain dry. The sera were tested for anti-*T. gondii* IgM and IgG antibody using the cassette and buffer technique (an immunochromatographic test also named Rapid Diagnostic Test (RDT)). For the samples with a positive IgG but negative IgM, the quantitative testing was then done using

Enzyme Linked Immuno-Sorbant Assay (ELISA) test kit at the biochemistry lab, strictly following the manufacturer's instruction.

Rapid immunochromatographic test for toxoplasmosis

The sera were tested by the rapid ICT (cassette and buffer) test. During the period of collection, the test components were allowed to get to room temperature every morning. When the sera were ready to test, a test cassette was removed from the pouch, placed on a clean surface and labeled with the corresponding patient's code. One drop (about 30-45µL) of the specimen was placed in the sample well using a plastic dropper. One drop of specimen diluent was then added to the specimen and the timer was set. After 15mins the cassette was examined for result. The ***c***assette and buffer ICT test contains a build in control (C) line which develops after adding the specimen and sample diluent. If the 'C' line does not develop, the whole procedure is reviewed and the test repeated with a new cassette. The result was negative or non-reactive if only the 'C' line was present with the absence of any burgundy line in both the test lines (M and G) indicating that no anti-*T. gondii* Ig was detected in the specimen. The result was considered positive or reactive for; (i) anti-*T. gondii* IgM if only the M line develops in addition to the 'C' line; (ii) anti-*T. gondii* IgG if only the 'G' line develops in addition to the 'C' line; and (iii) anti-*T. gondii* IgG and IgM if both the G and M lines develop, in addition to the 'C' line.

ELISA test for toxoplasmosis

Serum samples positive for IgG but negative for IgM in the rapid ICT were further tested using the ELISA Toxo (DRG kit, Germany) test. For this second testing, all specimens were refrigerated at -10°C. On test days, both the specimens and test components were allowed to thaw to room temperature. The different steps of the ELISA technique were then performed. Each patient specimen was diluted with the sample diluents (1 in 100) into new tubes and allowed to stand for 15 mins before the start of the assay. The required number of microliter strips or wells were labeled and inserted into the holder, with the first six corresponding to the blank, negative control, standards 1-3, and the positive control.

Briefly, 100µL each of the negative control, standards and positive control was dispensed in the corresponding well, while making sure the blank is always empty and dry. 100µL of each specimen was then dispensed into the corresponding wells as labeled, making sure to use each disposable tip only once. The wells were covered with the foil paper provided in the kit and allowed to incubate for 1 hour at 37ºC. The content of the wells was then briskly shaken out and rinsed 5 times with the diluted wash solution (300µL per well). The wells were stroked against an absorbent paper to make sure all the residual droplets and air bubbles were removed, as the precision of the result is directly affected by the quality of the washing. 100µL of the enzyme conjugate was then dispensed into each well except the blank and incubated at room temperature, making sure not to expose to direct sunlight. The content of the wells was briskly shaken out, the washing repeated as above, and the wells stroked against an absorbent paper. 100µL of substrate solution was then added to all the wells and incubated for 15mins at room temperature in the dark. 100µL of the stop solution was then added to each well and any blue color that developed during the incubation turned yellow and highly positive samples at times got dark precipitates. The wells were then inserted into the ELISA reader which has been pre-programmed and a graph of absorbance value (mean) of the negative control, standards and their respective concentrations. This was then used to calibrate the absorbance of each of the test specimens and gave corresponding values.

The normal value range for ELISA was established by the BRH laboratory, based on its patient's population in the geographical area; negative when <45 IU/mL, cut-off value at 50 IU/mL, grey zone (equivocal) between 45-55 IU/mL and positive when >55 IU/mL. Patients with equivocal results were called 2 weeks later (according to diagnostic principles of *T. gondii*) and blood samples were recollected for confirmation of test results, either the negative (memory immunity) or positive serology (active infection).

Data management and analysis

All data from the questionnaire and laboratory record were analyzed using SPSS version 20.0 software package. Descriptive statistic was performed to describe demographic profile of the study participants. The univariate analysis involved frequency distributions for categorical variables, and descriptive statistics for continuous and discrete variables was done to give an understanding of the characteristics of the sample, as well as description of the response variables (*T. gondii* infection). Bivariate analysis was used to investigate association between the response variable (*T. gondii* infection) and sociodemographic and other variables of interest (risk factors). The χ^2 test was used to test association between 2 categorical variables and p value <0.05 was considered as statistically significant.

Ethical considerations

Ethical clearance (N°2017/0051H/UBa/IRB) was obtained from the Institutional Review Board at the Faculty of Health Science of the University of Bamenda. The administrative authorizations were obtained from the Delegation of Public Health of the North West Region and the General Supervisor of the Bamenda Regional Hospital. Moreover, written informed consent was obtained from all study participants prior to interview and blood collection. Confidentiality of the collected information and laboratory test results was maintained, and used solely for research purposes and neither for stigmatization nor to generate profit. The results of the study were made available for all participants as they desire with the attending physician for further management of the cases.

Results:

A total of 127 pregnant women age 14 to 50 years (27.4 ± 6.21 years) out of 243 who attended BRH ANC clinic were included and tested for anti-*T. gondii* IgG and IgM antibody. Of this, 43 (33.9%) were IgG seropositive and 1 (0.8%) was positive for both IgG and IgM. Table 1 shows the socio-demographic characteristics and obstetric history of the study participants with regard to seroprevalence of *T. gondii* infection. The age group 24 to 34 years had the highest seroprevalence (48.0%). Majority of the participants (91.3%) were Grassfield ethnic group and the least represented ethnic groups were the Beti and others (such as Sawa and Bakossi) with 1.6% each. No statistically significant association between *T. gondii* infection and the various ethnic groups was observed.

Among the 127 women who took part in this study, the grassfield was the ethnic group more represented and more infected with 90.9% of positive cases (40/44). The majority (59.0%) were married and represented 61% (27/44) of positive cases. There was no significant association between *T. gondii* infection and marital status of the participants. There was also no significant association between age of pregnancy, area of residence, history of miscarriage and stillbirth and *T. gondii* infection ($p>0.05$). With respect to gestational age, most of the participants were in their first trimester (50.4%) among which 54.5% (24/44) were positive for *T. gondii* infection. Regarding the area of residence, 79.5% were living in urban areas among which 75 % (33/44) were positive to *T. gondii* infection. Women with history of miscarriage represented 38.6% and 40.9% of positive cases, while those with a history of still birth (11.8%) represented 11.4% of positive cases.

Table 2 depicts the seroprevalence of study participants and factors associated with *T. gondii* infection. Among the total respondents, 32 (37.0%) own a pet and 21 (16.5%) handled pet litters. There was a significant association between *T. gondii* infection and owing a pet ($p=0.013$), and handling pet litters ($p=0.006$). There was no significant association between *T. gondii* infection and farming or gardening, and source of drinking water ($p>0.05$). However, among the 127 participants who practiced farming or gardening, 64 (61.36%) were positive for *T. gondii* infection. With respect to sources of drinking water, 96/127 had tap water, and among these, 68.2% were positive (30/44) for *T. gondii* infection. Ninety nine of 127 pregnant women responded that they consume raw fruits/vegetables, of which 81.8% were positive for *T. gondii* infection; 9 of 127 responded that they consume undercooked meat, of which 11.4% were positive to *T. gondii* infection; while 52.3% of those who consume raw dried meat were postive for *T. gondii* infection. There was no significant association between *T. gondii* infection and habit of consuming raw fruits/vegetables, undercooked meat, and raw dried meat. The pregnant women who had had blood transfusion in the year before their pregnancy represented 81.8% of positive cases but there was no significant association between *T. gondii* infection and history of blood transfusion.

Table 1: Socio-demographic and obstetric characteristics of study participants in relation to seroprevalence of *Toxoplasma gondii* infection

Variables	Seroprevalence		Total (%) n = 127	OR (95% CI)	*p value*
	Positive (%) n = 44	Negative (%) n = 83			
Age groups (years)					
14-24	14 (11,0)	30 (23,6)	44 (34.6)	0.766 (0.347-1.687)	0.507
24-34	21 (16.5)	40 (31.5)	61 (48.3)	1.026 (0.491-2.144)	0.945
34-44	6 (4.7)	11 (8.7)	17 (2.4)	0.890 (0.304-2.604)	0.831
44-50	3 (2.4)	2 (2.4)	5 (3,9)	2.963 (0.476-18.441)	0.244
Ethnic group					
Mbororo	1 (0.8)	3 (2.4)	4 (3.1)	0.020 (0.063-6.145)	0.683
Grassfield	40 (31.5)	76 (59.8)	116 (91.3)	0.921 (0.254-3.335)	0.900
Beti	1 (0.8)	1 (0.8)	2 (1.6)	1.907 (0.116-31.244)	0.651
Bayangi	1 (0.8)	2 (1.6)	3 (2.4)	0.942 (0.083-10.686)	0.961
Others	1 (0.8)	1 (0.8)	2 (1.6)	1.907 (0.116-31.244)	0.651
Marital status					
Single	13 (10.2)	25 (19.7)	38 (29.9)	0.973 (0.437-2.164)	0.946
Married	27 (21.2)	48 (37.8)	75 (59.0)	1.158 (0.549-2.44)	0.700
Divorced	1 (0.8)	0	1 (0.8)	5.894 (0.235-147.799	0.280
Cohabiting	3 (2.4)	10 (7.9)	13 (10.2)	0.562 (0.146-2.161)	0.401
Level of education					
FSLC	12 (9.4)	18 (14.2)	30 (23.6)	1.354 (0.582-3.150)	0.481
Ordinary	11 (8.7)	17 (13.4)	28 (22.0)	1.294 (0.544-3.076)	0.556
Advance	9 (7.1)	19 (15.0)	28 (22.0)	1.031 (0.423-2.513)	0.946
Tertiary	12 (9.4)	29 (22.8)	41 (32.3)	0.721 (0.32-1.612)	0.425
Employment status					
Unemployed	11 (8.6)	17 (13.4)	28 (22.0)	1.294 (0.544-3.076)	0.559
Public sector	6 (4.7)	16 (12.6)	22 (17.3)	0.671 (0.242-1.859)	0.443
Private sector	9 (7.1)	15 (11.8)	24 (18.9)	1.166 (0.464-2.929)	0.744
Self employed	11 (8.7)	23 (18.1)	34 (26.8)	0.869 (0.377-2.004)	0.743
Student	7 (5.5)	12 (8.8)	29 (14.3)	1.119 (0.406-3.0827)	0.827
Trimester of pregnancy					
1st trimester	24 (18.9)	40 (31.5)	64 (50.39)	1.290 (0.619-2.685)	0.496
2nd trimester	18 (14.2)	38 (29.9)	56 (44.1)	0.819 (0.391-1.718)	0.599
3rd trimester	2 (1.6)	5 (3.9)	7 (5.5)	0.761 (0.141-4.095)	0.750
Residence					
Urban	33 (26.0)	68 (53.5)	101 (79.5)	0.662 (0.274-1.599)	0.359
Rural	10 (7.9)	15 (11.8)	25 (19.7)	1.333 (0.542-3.279)	0.531
Unknown	1 (0.8)	0	1 (0.8)	0.215 (0.026-1.749)	0.150
History of miscarriage					
Yes	18 (14.2)	31 (24.4)	49 (38.6)	1.161 (0.549-2.452)	0.695
No	22 (17.3)	52 (41.0)	74 (58.4)		
History of still birth					
Yes	5 (3.9)	10 (7.9)	15 (11.8)	0.936 (0.299-2.931)	0.909
No	39 (30.7)	73 (57.5)	112 (88.2)		

OR: Odd ratio, CI: Confidence interval, n: Frequency, %: Percentages, FSLC: First School Living Certificate

Table 2: Seroprevalence rate and risk factors for *Toxoplasma gondii* infection among study participants

Variables	Seroprevalence		Total (%) (n = 127)	OR (95% CI)	*p* value
	Positive (%) (n = 44)	Negative (%) (n = 83)			
Owning pet					
Yes	17 (13.4)	15 (11.8)	32 (37.0)	2.854 (1.25-6.51)	0.013
No	27 (21.3)	68 (53.5)	85 (74.8)		
Contact with cat					
Yes	8 (6.3)	6 (4.4)	14 (10.7)	2.851 (0.921-8.829)	0.069
No	36 (28.1)	77 (60.6)	113 (88.8)		
Handling pet litter					
Yes	13 (10.2)	8 (6.3)	21 (16.5)	3.931 (1.48-10.42)	0.006
No	31 (24.4)	75 (59.1)	106 (83.5)		
Farming/gardening					
Yes	27 (21.3)	37 (29.1)	64 (50.4)	1.975 (0.937-4.161)	0.075
No	17 (12.4)	46 (36.2)	63 (48.6)		
Source of drinking water					
Tap	30 (21.9)	66 (52.0)	96 (73.9)	0.552 (0.241-1.264)	0.159
Spring	7 (5.5)	7 (5.5)	14 (11.0)	2.054 (0.671-6.289)	0.207
Well	5 (3.9)	6 (4.7)	11 (8.7)	1.645 (0.472-5.730)	0.434
Bottle	2 (1.6)	3 (2.4)	5 (3.9)	1.413 (0.227-8.776)	0.712
Tap and stream	0	1 (0.79)	1 (0.79)	0.618 (0.025-15.488)	0.769
Habit of eating raw fruits/vegetables					
Yes	36 (28.8)	63 (49.6)	119 (78.0)	1.429(0.571-3.572)	0.446
No	8 (5.9)	20 (15.7)	28 (21.9)		
Habit of eating undercooked meat					
Yes	5 (3.94)	4 (3.15)	9 (7.09)	2.532(0.644-9.961)	0.184
No	39 (30.7)	79 (62.2)	118 (90.7)		
Habit of eating raw dried meat					
Yes	23 (18.1)	30 (23.6)	53 (41.7)	1.935(0.921-4.063)	0.081
No	21 (16.5)	53 (41.7)	74 (58.3)		
History of blood transfusion					
Yes	6 (4.7)	8 (6.3)	14 (11.1)	1.480(0.479-4.574	0.495
No	38 (29.9)	75 (59.1)	113 (89.0)		

OR: Odd ratio, CI: Confidence interval, n: Frequency, %: Percentages

Discussion:

The present study reports the seroprevalence of *T. gondii* infection among pregnant women in the BRH to be 35.4% (34.6% for IgG and 0.8% for both IgG and IgM. This rate is similar to the those reported in Nigeria by Umar et *al.,* (21) and Bello *et al.,* (2), but lower than rates previously reported by Guemgne *et al*., (20), Nguefack *et al.,* (21) and Njunda *et al.,* (22) in Cameroon and Yohanes *et al*., (23) in Ethiopia. In the study by Njunda *et al.,* (22), the rate was 70% in pregnant women, however, the study looked at a metropolitan population in a third degree referral health facility (Douala General Hospital) whereas in the present study, it was conducted in a second degree referral unit (BRH). Also Douala is a town with a more precarious living condition which could also explain the higher prevalence reported in that study. In the study by Yohanes *et al*., (23) on 232 pregnant women, the overall seroprevalence of *T. gondii* infection was 79.3% with 175 (75.43%) positive for IgG, 9 (3.9%) for IgM, 2 of which were positive for both IgG and IgM. Another study carried out by Wams *et al.,* (24) in Njinikom-Cameroun reported a prevalence of 54.5%, which is also higher than the rate in our study.

The observed differences in the seroprevalence rate of anti-*T. gondii* infection may be due to differences in the two study populations and the sample size. In fact, the present study was confined to an urban population in Bamenda, as opposed to the rural population in the study by Wams *et al.,* (24). As such there is perceived higher level of awareness, and preventive measures about *T. gondii* in our study population. The variation in seroprevalence of *T. gondii* infection may be due to differences in geographical distribution of the parasite, socioeconomic, personal hygienic practices, and feeding habit of the study participants. In addition, differences in test methods may also account for the variation.

The presence of IgM antibodies during pregnancy indicates acute *T. gondii* infection with high risk of maternal-fetal transmission (25). A previous study has estimated that in the absence of treatment, the risk of congenital infection from acute *T. gondii* infection during pregnancy is about 50% (26). Early diagnosis of infections in pregnant mothers is of great importance so that measures that can reduce the risk of transmission and possible sequelae in the newborn are promptly initiated. Therefore, screening of pregnant women for *Toxoplasma* infection should be considered as a part of the routine investtigation during ANC follow up.

Our study did not show any statistically significant association between the trimester of pregnancy and *Toxoplasma gondii* infection, which agrees with studies from Egypt by Mandour *et al.,* (27) in 2017 and Yemen by Saif *et al.,* (28) in 2014 but disagrees with those of Khan *et al.,* (29), Alanyande *et al*., (30) and Bello *et al*., (2) who reported significant association. A significant association between pet ownership and *T. gondii* infection was found. Similarly, Khan *et al.,* (29) in 2011 and Yohanes *et al*., (23) in 2017 showed coherent associations. Contrarily, studies by Pal *et al*., (31) and Saif *et al*., (28) showed different results. There was no significant association between cat ownership and *T. gondii* infection, similar to what has been reported by Ebrahimzadeh *et al.,* (32), Shao *et al.,* (15), Murebwayire *et al*., (14), Jumaian (33), Mandour *et al*., (18), Makiani *et al*., (34), Njunda *et al*., (22), Wam *et al*., (24)] and Saif *et al*., (28). However, many studies among which Moura *et al*., (1), Dwinata *et al*., (3), Umar *et al.,* (25), Yohanes *et al*., (23), Agmas *et al*., (36), Nissapatorn *et al*., (37), Duan *et al*., (38) and Nguefack *et al.,* (21) demonstrated the contrary. This may be explained by the fact that the presence of cat in the house is not enough to cause zoonosis but rather handling of cats' litter is of more importance. Our study demonstrated a significant association between handling of pets' litter and *T. gondii* infection, a finding that agrees with that of Dwinata *et al*., (3) in 2016.

There was no significant association between farming or gardening and *T. gondii* infection, which is similar to studies by Moura *et al*., (1), Agmas *et al*., (36), Makiani *et al*., (34), Nissapatorn *et al*., (37), and Yohanes *et al*., (23) but contradicts the studies by Jumaian *et al.,* (33) in 2005 and Mandour *et al.,* (18) in 2017. The findings of these studies appeared different because contamination of soil occurs principally after defecation of cats, and contamination rate largely depend on cat density, with urban areas where cat density is low tending to have low soil contamination rate, and therefore low prevalence of *T. gondii* infection.

Conclusion:

This study reports seroprevalence of *T. gondii* infection in pregnancy to be 34.6%. The risk factors associated with *T. gondii* infection include pets' ownership and handling of their litters. There is need for routine screening of pregnant women for *T. gondii* infection during ANC and treatment of cases. Education on hygiene and awareness of risk exposures regarding *T. gondii* infection to minimize its effects among pregnant women and the general population are imperative.

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Authors' contributions:

NNF and FP were involved in the design of the study, drafted the protocol with input from other authors. NNF, DWP and KFHL monitored laboratory work and analyzed the data. GBM performed the laboratory analysis and collected the results. NNF and KFHL drafted and finalized the manuscript for publication. DWP and TWA edited the manuscript. All authors contributed to the writing of the paper and approved the final version.

Conflict of interest:

Authors declared no conflict of interest

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Original Article **Open Access**

Biochemical and immunological characterization of haemolysin produced by *Pseudomonas aeruginosa* PAO1 isolated from burn wounds

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Abstract:

Background: Infection of burn wounds by multidrug-resistant (MDR) *Pseudomonas aeruginosa* (*P. aeruginosa*) is a leading cause of morbidity and mortality and remains one of the most challenging concerns for the burns unit. The aim of this study is purify and characterize the haemolysin produced by multidrug resistant *P. aeruginosa* PAO1 isolated from burn wounds.
Methods: Isolation and identification of *P. aeruginosa* from burns was done by standard bacteriological methods. *P. aeruginosa* PAO1 was identified by PCR amplification and sequencing of the 16S rRNA gene. The haemolysin of *P. aeruginosa* PAO1 was purified by 70% ammonium sulphate precipitation followed by gel filtration on Sephadex G-100, and separation by SDS-Poly Acrylamide Gel Electrophoresis. *In vivo* toxicity of the purified haemolysin was determined by intraperitoneal injection of Swiss albino mice, and *in vitro* toxin-antitoxin neutralization test was performed as previously described.
Results: The pure haemolysin had a molecular weight of 37 kDa, with maximum activity at 25°C for 30 minutes and stable within pH range of 4-9 (maximum activity at pH 7). The haemolysin was activated by Ca^{2+}, Fe^{3+} and Cu^{2+}. Intraperitoneal injection of mice with 0.5ml of haemolysin (128 HU/ml) caused 100% mortality while 0.5 and 0.1 ml of haemolytic titer (64 HU/ml) of the heated haemolysin (toxoid) caused 50% and 0% mortality respectively. *In vitro* toxin-antitoxin neutralization test revealed that anti-haemolysin antitoxin was present in the serum of the mice that were previously vaccinated with heated toxin.
Conclusion: This study concluded that haemolysin can be a potential vaccine component for prevention of haemolysis caused by multidrug resistant *P. aeruginosa* in burn patients.

Keywords: haemolysin, *Pseudomonas aeruginosa*, multidrug resistant organism

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Caractérisation biochimique et immunologique de l'hémolysine produite par *Pseudomonas aeruginosa* PAO1 isolée de brûlures

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Abstrait:

Contexte: L'infection des plaies par brûlures par *Pseudomonas aeruginosa* (*P. aeruginosa*) multirésistante (MDR) est l'une des principales causes de morbidité et de mortalité et demeure l'une des préoccupations les

plus difficiles pour l'unité des brûlures. Le but de cette étude est de purifier et de caractériser l'hémolysine produite par *P. aeruginosa* PAO1 multirésistante isolée de brûlures.
Méthodes: L'isolement et l'identification de *P. aeruginosa* des brûlures ont été effectués par des méthodes bactériologiques standard. *P. aeruginosa* PAO1 a été identifié par amplification par PCR et séquençage du gène d'ARNr 16S. L'hémolysine de *P. aeruginosa* PAO1 a été purifiée par une précipitation au sulfate d'ammonium à 70% suivie d'une filtration sur gel sur Sephadex G-100 et d'une séparation sur électrophorèse par gel SDS-Poly Acrylamide. La toxicité *in vivo* de l'hémolysine purifiée a été déterminée par injection intrapéritonéale de souris albinos suisses et un test de neutralisation *in vitro* toxine-antitoxine a été effectué comme décrit précédemment.
Résultats: L'hémolysine pure avait un poids moléculaire de 37 kDa, avec une activité maximale à 25°C pendant 30 minutes et stable dans une plage de pH de 4 à 9 (activité maximale à pH 7). L'hémolysine a été activée par Ca^{2+}, Fe^{3+} et Cu^{2+}. L'injection intrapéritonéale de souris avec 0,5 ml d'hémolysine (128 HU/ml) a causé une mortalité de 100% tandis que 0,5 et 0,1 ml de titre hémolytique (64 HU/ml) de l'hémolysine chauffée (anatoxine) ont causé respectivement 50% et 0% de mortalité. Un test de neutralisation *in vitro* toxine-antitoxine a révélé que l'antitoxine anti-hémolysine était présente dans le sérum des souris préalablement vaccinées avec de la toxine chauffée.
Conclusion: Cette étude a conclu que l'hémolysine peut être un composant vaccinal potentiel pour la prévention de l'hémolyse causée par *P. aeruginosa* multirésistante chez les patients brûlés.

Mots-clés: hémolysine, *Pseudomonas aeruginosa*, organisme multirésistant

Introduction:

Infection in burn patients is a leading cause of morbidity and mortality and remains one of the most challenging concerns for the burns unit (1). Multidrug-resistant (MDR) *Pseudomonas aeruginosa* (*P. aeruginosa*) is an emerging cause of mortality and morbidity in burn patients, which is estimated to cause 4-60% of nosocomial infections in different parts of the world (2).

Numerous *P. aeruginosa* virulence factors contribute to the pathogenesis of wound infections in patients with burns. Pili and flagella are essential for the organism ability to persist in burns wound and cause disseminated infections (3). *P. aeruginosa* elaborate many toxic products and enzymes such as catalase, lipase, lecithinase, elastase, proteases and haemolysins, which enable the organism to invade and destroy the host tissues (4-6). In particular, haemolysin contributes to virulence of *P. aeruginosa*, and decreased haemolytic activity has been associated with decrease virulence (7).

The aim of current study is to purify and characterize the haemolysin produced by MDR *P. aeruginosa* PAO1 isolated from burn patients as a preliminary step to producing toxoid from it.

Materials and method:

Study setting

This study was conducted in the Medical Microbiology and Immunology Department, Faculty of Medicine, and Botany Department Faculty of Science, Zagazig University, Egypt. Approval for performing the study was obtained from Institutional Review Board of Faculty of Medicine, Zagazig University. Consent was obtained from each patient enrolled in the study. All experiments were carried out in compliance with the relevant laws and guidelines.

Collection of specimens

Forty five swab specimens were collected using sterile cotton swabs from patients with burn wound infections in Central Hehia Hospital, Hehia city, El-Sharkia, Egypt during the period July to September 2013. All samples were transported to the Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, within two hours of collection.

Isolation and identification of *Pseudomonas aeruginosa*

The swabs were cultured on nutrient, MacConkey and blood agar plates, and incubated aerobically at 37°C for 24 hours. Colonies were identified as *P. aeruginosa* on culture plates using standard bacteriological methods (8). Antibiotic susceptibility of *P. aeruginosa* isolates was determined by the CLSI disk diffusion method (9). The identity of the MDR *P. aeruginosa* was confirmed by PCR amplification and sequencing of the 16S rRNA gene at the Sequence Unit, Sigma Company, Giza, Egypt as *P. aeruginosa* PAO1 (NR 074828 1).

Determination of haemolysin level

The haemolysin level of *P. aeruginosa* PAO1 (NR 074828 1) was determined by the modified disk diffusion method (10). The overnight broth culture of the isolates was diluted in sterile saline to 0.5 McFarland standards. Sterile filter paper disk was immersed in each bacterial suspension to absorb 0.01ml. The disks were placed on the surface of blood agar medium and incubated overnight at 37°C. Diameters of haemolysis (H) and growth (G) zones were measured and the H/G ratio was determined.

Haemolysin purification

P. aeruginosa PAO1 (NR 074828 1) was grown in nutrient broth medium at optimum conditions for maximum haemolysin produc-

tion. The medium was optimized in presence of glucose as carbon source and yeast extract as nitrogen source at pH 7.0 for 2 days. The culture supernatant was mixed with 70% ammonium sulphate concentration, with constant stirring at 4°C overnight. The precipitate was then separated by centrifugation at 4000 rpm for 20 minutes and re-suspended in small amount of phosphate buffer (pH 7.0). The re-suspended pellets were applied to a Sephdex G-100 column pre-equilibrated with the same buffer. The active fractions were collected, pooled and concentrated by dialysis against the same buffer. For each fraction, protein content and haemolytic activity were determined by methods previously described (11,12).

Haemolytic titer

The titer of *P. aeruginosa* PAO1 haemolysin was determined using two fold dilution method (13). 0.5ml of haemolysin of was diluted by two fold serial dilutions in 0.5ml phosphate buffered saline (pH 7.4). 0.5ml of 1% suspension of red cells was added to tubes containing the dilutions of the haemolysin. The final readings were made visually after 2hours of incubation at 37°C in a water bath. The reciprocal of the highest dilution showing complete haemolysis was taken as the number of haemolytic units per ml (HU/ml) present in the preparations

Determination of molecular weight

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to determine the purity and molecular weight of the protein, as described by Laemmli (14) using 12% separating acrylamide gel.

Amino acid analysis

Amino acid composition of the purified protein was determined by applying the GLC hydrolytic technique (15) with a Beckman amino acid analyzer system (Sykam-S 7130 Amino Acids, Reagent Orgaizer).

Biochemical properties of purified haemolysin

Thermal stability of the haemolysin was studied after pre-incubation at various temperature (25, 37, 45, 55 and75°C) using 0.01M phosphate buffer (pH 7.0) for different periods of time (30, 60, 90 and 120 minutes). A 1% RBCs suspension was added to the haemolysin (0.5 ml/0.5 ml v/v). The thermal inactivation rate (Kr min) was described by the first-order kinetic model (16)**;** Ln (A_t/A_0) k_tT, where A_o and A_t are the specific activity at zero and 't' time respectively. The $T_{1/2}$ (time at which the haemolysin loses 50% of its activity) was calculated from the linear equation for each temperature. The temperature at which the haemolysin loses 50% of its activity (Tm) was calculated from the linear equation of different pre-incubation temperature at 60 minutes.

The stability of the haemolysin was examined after pre-incubation for 30 minutes at different pH values (4, 5, 6, 7, 8 and 9). Acetate (0.2M), phosphate (0.2M) and Tris HCl (0.2M) buffers were used to create pH range 4-5, 6-7 and 8-9 respectively. After adding 1% RBCs suspension and incubating at 37°C for 2 hours, the haemolytic activity was determined for each pH

To assay the metal ions effect, the purified haemolysin was pre-incubated in 1mM EDTA at 37°C for 10 minutes and then dialyzed against 0.01M phosphate buffer (pH 7.0). The haemolysin was incubated with each metal ion separately for 30 minutes before adding 1% RBCs suspension. Metal ions in form of FeSO4, $CuSO_4$, $MgSO_4$, $ZnSO_4$, $CaCO_3$, Iodine (5mM) as well as EDTA (1 and 5mM) were used. The haemolytic activity was determined after incubation at 37°C for 2 hours.

Mice toxicity of fresh and heated haemolysin

The purified haemolysin was heated for 15minutes in a boiling water bath to prepare the toxoid (17). The haemolytic titer of the fresh (toxin) and heated (toxoid) haemolysin was determined as described previously (13). Six groups of four male Swiss albino mice (6-8 weeks old) were injected intra-peritoneally with doses of 0.5, 0.3 and 0.1ml of the purified toxin (groups A, B and C) and toxoid (groups D, E and F). The toxicity reflected by the mortality rate in each group was measured using the following equation (18);

$$\text{Mortality rate} = \frac{\text{Number of animals that died}}{\text{Total number of animals per group}}$$

***In vitro* toxin-anti-toxin neutralization test**

Toxin neutralization test was performed to detect the presence of serum antibodies that can neutralize the haemolytic properties of the haemolysin. Blood sample was obtained from five Swiss albino mice previously immunized by repeatedly injecting them with 0.1ml toxoid after which their sera were separated. Fifty, 45, 40, 35 and 30µL of serum from the immunized mice were added to 0, 5, 10, 15 and 20µL of haemolysin respectively. 25µL of 1% RBC suspension was added to each tube and incubated at 37°C for 6 hours. All tubes were centrifuged for 2 minutes and the supernatants were examined for haemolysis. The last tube with no haemolysis was the endpoint of the test (19), which contains the least concentration of the antibody that neutralizes the haemolysin.

Results:

Purification of haemolysin

As shown in Table 1, the haemolytic activity was 32, 64 and 128 HU/ml for the crude broth culture filtrate, after precipitation with 70% ammonium sulphate and following gel filtration chromatography on Sephadex G-100 respectively. Compared to the crude culture broth filtrate, the haemolysin was more purified with purification factor of 29.7 and 10.7 fold using chromatography and 70% ammonium sulphate precipitation resp-ectively. The haemolysin was purified as an extracellular enzyme from the liquid cultures of *P. aeruginosa* PAO1 growing in broth medium (pH7). The purification profile of tested haemolysin using sephadex-G100 showed a sharp peak containing the active haemolysin in fractions 9 to 11 with specific activity 196.9 Umg^{-1} proteins compared to 6.6 Umg^{-1} of the crude protein (Table 1).

Molecular weight of purified haemolysin

Fig 1 shows the SDS-PAGE of the purified haemolysin of *P. aeruginosa* with one major band of approximately 37 KDa.

Amino acids of purified haemolysin

As shown in Table 2, the haemolysin was composed of 40.2% neutral amino acids (glycine, valine, leucine, isoleucine and alanine), 20.7% acidic amino acids (glutamic acid and aspartic acid), 19.7% basic amino acids (lysine and arginine), and 60.9% oxy amino acids (serine, threonine and proline). The haemolysin has high concentration of glycine (26.7%) among other detected amino acids.

Table 1: Purification of the haemolysin from *Pseudomonas aeruginosa* PAO1 using different methods

Purification step	Haemolytic activity (HU/ml)	Protein (mg/ml)	Specific activity (HU/mg protein)	Purification factor (folds)	% Recovery
Culture broth (crude)	32	4.83	6.62	1.0	100
70% ammonium sulphate	64	1.12	70.90	10.7	20.9
Sephadex G-100	128	0.65	196.92	29.7	15.6

HU = haemolytic units

Table 2: Amino acids composition of *P. aeruginosa* PAO1 haemolysin

Amino acid name	Concentration (mg/100ml)	Ratio (%)
Aspartic	2.32	5.76
Threonine	1.21	3.00
Serine	1.31	3.25
Glutamic	6.00	14.92
Proline	5.42	13.47
Glycine	10.74	26.7
Alanine	1.48	3.68
Valine	1.30	3.23
Isoleucine	0.94	2.33
Leucine	1.72	4.27
Histidine	2.54	6.31
Lysine	2.81	6.98
Arginine	2.42	6.01

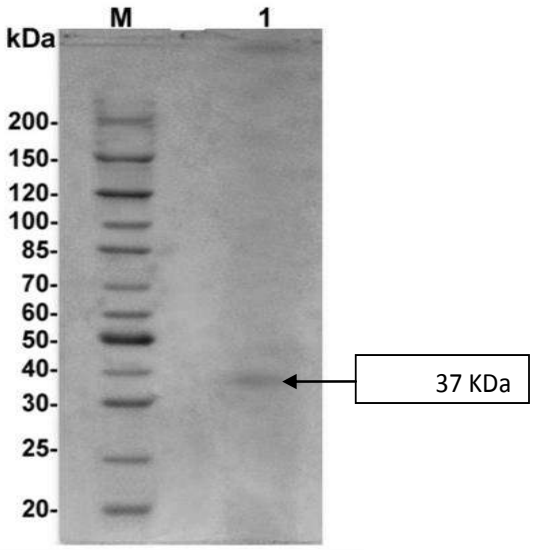

Fig.1: SDS-PAGE profile of purified haemolysin of *Pseudomonas aeruginosa* PAO1 shows that molecular weight of the purified enzyme was 37 KDa (Lane 1) when compared to the protein markers (Lane M)

Table 3: Biochemical characters of *Pseudomonas aeruginosa* haemolysin

Temperature (°C)	**Time (minute)**	**Hemolytic activity (HU/ml)**	**Relative activity %**
25	30	127.4	100
	60	120.9	94.8
	90	115.6	90.7
	120	112.8	88.5
37	30	107.4	84.3
	60	104.9	82.3
	90	95.4	74.8
	120	93.9	73.7
45	30	87.3	68.5
	60	85.8	67.3
	90	84.7	66.4
	120	83.0	65.1
55	30	75.9	59.6
	60	68.1	53.4
	90	64.0	50.2
	120	63.1	49.5
70	30	55.3	43.4
	60	53.7	42.1
	90	52.8	41.4
	120	48.9	38.4

Temperature (°C)	**$T_{½}$ (min)***	**Kr min^{-1}****	**Tm*** (°C)**
25	196.96	0.162	61
37	230.78	0.145	
45	129.41	0.305	
55	109.79	0.379	
70	85.06	0.499	

Effect of pH on haemolysin activity		**Effect of ions on haemolysin activity**	
pH values	Hemolytic activity (HU/ml)	Metal ion (mM)	Hemolytic activity (HU/ml)
4	112.8 ± 0.1	Fe^{3+} (5 mM)	126.1 ± 0.2
5	114.5 ± 0.4	Cu^{2+} (5 mm)	120.7 ± 0.7
6	116.8 ± 0.1	Ca^{2+} (5 mm)	135.1 ± 0.2
7	126.7 ± 0.2	Mg^{2+} (5 mM)	82.5 ± 0.6
8	115.6 ± 0.4	Zn^{2+} (5 mM)	74.0 ± 0.3
9	100.3 ± 0.7	Iodine (5 mM)	59.9 ± 1.0
		EDTA (5 mM)	61.3 ± 0.3
		EDTA (1 mM)	104.8 ± 0.8

*Half-life times; **Thermal inactivation rate; *** Half-life temperature

Table 4: *In vivo* toxicity of the fresh and heated haemolysin in mice

Injected material	Hemolytic titre (HU/ml)	Dose (ml)	Mortality of mice after days									
			1	%	2	%	3	%	4	%	5	%
Fresh (Toxin)	128	0.5	0/4	0	1/4	25	2/4	50	3/4	75	4/4	100
		0.3	0/4	0	0/4	0	0/4	0	0/4	0	¾	75
		0.1	0/4	0	0/4	0	0/4	0	0/4	0	2/4	50
Heated (Toxoid)	64	0.5	0/4	0	0/4	0	0/4	0	0/4	0	2/4	50
		0.3	0/4	0	0/4	0	0/4	0	0/4	0	¼	25
		0.1	0/4	0	0/4	0	0/4	0	0/4	0	0/4	0

HU = haemolytic units

Biochemical properties of purified haemolysin

As shown in Table 3, maximum activity of the haemolysin was at 25°C for 30 minutes and decline of activity with increased exposure time. Also increasing temperature decreased enzymatic activity. With regards to the thermal kinetic parameters of the haemolysin, the half-life ($T_{1/2}$) was longest (230.78 min) at 37°C and half-life temperature (Tm) was estimated to be 61°C. The haemolysin was approximately stable within pH range of 4-9 and maximum activity was at pH 7.0. The activity of the haemolysin was increased by the divalent ions Ca^{2+} followed by $Fe2^{+}$, Cu^{2+} and 1mM EDTA in that order. The haemolysin was inhibited in the presence iodine followed by 5mM EDTA, Mg^{2+} and Zn^{2} in that order.

Mice toxicity of fresh and heated haemolysin

As shown in Table 4, intraperitoneal (IP) injection of 0.5, 0.3 and 0.1 ml of fresh toxin (128 HU/ml) caused mortality rate of 100%, 75%, and 50% respectively in the injected mice. The haemolysin lost its toxicity after heating for 15 minutes in boiling water. The unprotected mice survived intraperitoneal (IP) injection of 0.1 ml heated haemolysin. The haemolysin partially lost its toxicity at a dose of 0.5 and 0.3 ml, causing mortality rate of 50% and 25% respectively in the mice. With respect to the *invitro* toxin-antitoxin neutralization test shown in Table 5, three samples (1, 2, 3) showed no haemolysis with different endpoints while the last 2 samples (4, 5) showed haemolysis.

Table 5: *In vitro* toxin-anti-toxin neutralization test

Sample	Haemolysis	Endpoint
1	-ve	35 µl
2	-ve	40 µl
3	-ve	35 µl
4	+ve	--
5	+ve	--

Discussion:

Infection by multidrug resistant *P. aeruginosa* is a big challenge in patients with burn wounds with tendency to development of septicaemia and high risk of death. The organism is naturally resistant to a significant number of antimicrobials, and infections caused by it is therefore associated with high mortality rate because treatment is difficult due to reduced availability of effective antimicrobials (20). Research into novel therapies aside conventional antimicrobials is recommended to combat these multi-drug and sometimes pan-drug resistant organisms (21).

The anti-virulence approach is one of the new strategies to disarm *P. aeruginosa* infective arsenals by inhibiting the expression and activity of its virulence factors. This has the tendency to reduce its invasiveness and avoid emergence of resistance since proliferation is not affected (22). Haemolysin is one of the virulence factors of many organisms. *P. aeruginosa* produces haemolysin to sense and sequester iron from its environment, which also helps in survival of the pathogen by inhibiting host factors (23). Alpha-haemolysin of uropathogenic *Escherichia coli* can induce apoptosis of target host cells including neutrophils, T-lymphocytes and renal cells, which promotes exfoliation of epithelial cells of the bladder, contributing to nephropathogenicity (24).

Cultures of *P. aeruginosa* considered to be of proven virulence have been reported to have higher titers of extracellular haemolysin than cultures of less virulence ones. Intra-corneal injection of purified haemolysin produced extensive corneal opacification with extensive leukocytic infiltration of the tissue (25), and it was suggested that *P. aeruginosa* haemolysin played a role in the pathogenesis by effecting lysis of host cells and/or subcellular organelles, leading to the release of enzymes destructive to corneal tissue (25). Therefore targeting the haemolysin of *P. aeruginosa* can be a new non-antibiotic approach in the therapy of MDR strains of this organism in burn patients.

In this study, we purified, and chemically and immunologically characterized haemolysin of MDR *P. aeruginosa* PAO1 as a preliminary step to producing a vaccine for combating the effect of this virulence factor in burn patients. The haemolysin was more purified following chromatography with a factor of 29.7 fold. The purification profile using sephadex-G100 shows a sharp peak containing the active haemolysin in fractions 9 to 11 with specific activity 196.9 U mg^{-1} proteins compared to 6.6 U mg^{-1} of the crude protein. Our result agrees with another study on *P. florescens* (26) which reported that high performance liquid chromatography (HPLC) gives 32.6 fold increases in haemolysin purification. HPLC increased the purity of haemolysin of *Bacillus mycoides* by 40.8 folds with the assumption that filtration step is the key to the homogeneity observed. HPLC increases the purity of haemolysin because it polymerizes with other impurities (27).

Our study showed that using SDS-PAGE, purified haemolysin of *P. aeruginosa* had one major band with molecular weight of approximately 37 kDa. Amino acid analysis of the haemolysin also showed that it is a hetero polymer. One study estimated the molecular weight of haemolysin produced by *P. aeruginosa* to be 78 kDa using both SDS-PAGE and Sephacryal S-200 column chromatography while another one estimated it to be 76 kDa with high-performance size exclusion chromatography (28). The molecular weight of haemolysins of other organisms reported includes 12 kDa for *Actinobacillus actinomycetemcomitans* (29) and 45 kDa for *Porphyromonas gingivalis* (30). Similarly, extracellular protein showing haemolysin with SDS-PAGE profile of approximately 50 kDa was characterized from a pathogenic *Aeromonas hydrophila* strain An4 isolated from marine catfish (31).

The amino acid composition of the haemolysin makes it a good immunogen (33), which can induce antibody formation. We investigated whether this haemolysin can be converted to toxoid by heating it for 15 minutes in boiling water to prepare the toxoid (17)**.** The lack of haemolysis in some tubes in the *in vitro* toxin-antitoxin neutralization test can be attributed to presence of antitoxin in the serum obtained from a previously immunized (injected with heated

toxin) mice. This serum is believed to contain antitoxin which neutralized the haemolysin. The samples that showed haemolysis may be due to the fact that the titer of the antitoxin was too low to neutralize the toxin (haemolysin). Other toxins of *P. aeruginosa* have been found to be immunogenic and mounted protective immune response in mice. Passive immunization of mice with antitoxin A gamma globulin protected mice against the lethality of intraperitoneal infection with *P. aeruginosa* (34). Pavlovskis et al., (35) and Snell et al., (36) observed that passive immunization of mice with specific antitoxin increased the survival of burned mice infected with toxigenic strains (but not with non-toxigenic strains) compared to control mice. Combining antibiotic therapy (gentamicin) with passive antitoxin therapy significantly improved the survival of the burned infected mice compared to infected mice receiving only antitoxin (37).

Conclusion:

This study concluded that haemolysin of *P. aeruginosa* PAO1 is a protein of 37kDa with 27.7% glycine content. The crude haemolysin has a haemolytic activity of 32 HU/ml and 128 HU/ml after purification with gel filtration on Sephadex G-100, with maximum activity at 25^{o}C for 30 minutes at pH7 with half life temperature ($T_{1/2}$) of 61^{o}C. The haemolysin activity is enhanced by Ca^{2+} and Fe^{3+} and inhibited by iodine and 5mM EDTA. The haemolysin lost its activity by heating for 15 minutes in boiling water and induces neutralizing antibodies in vaccinated mice. It is recommended that haemolysin of *P. aeruginosa* PAO1 be investigated further as a potential component of vaccine for prevention of haemolysis caused by multi-drug resistant strains of *P. aeruginosa* in burn patients.

Conflict of interest:

No conflicts of interest is declared

Authors' contributions:

AAA and WEH were responsible for concept and design of the study. All authors contributed to data collection. AME and EHAH undertook acquisition and interpretation of data and drafting of the manuscript. AAA undertook critical review of the manuscript. All authors agreed to the final draft.

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Original Article **Open Access**

Lactobacillus sp and some fungi from termite nests on kolanut trees had mild antagonistic effects against pathogens isolated from paediatric patients

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Abstract:

Background: Residents in a rural suburb of Akure jettisoned antibiotic treatment; sought alternative cure to rising incidence of paediatric infections in 2017 from local herbal dealers, with many residents claiming of better treatment response. We investigated these claims since the local herbal formula included kola nut barks and ground termites.
Methodology: Microorganisms associated with termite nests on kola nut trees in the affected community were characterized and identified using standard techniques. The Kirby Bauer disk diffusion was used to evaluate the susceptibility of the bacterial isolates to selected antibiotics. Plasmid profile of multiple antibiotic resistant bacterial isolates (MDRIs) was determined by the Birnboim and Doly method while post plasmid curing antibiotic susceptibility was performed on the MDRIs against the same selected antibiotics. The microorganisms were also evaluated for possible antagonistic effects against *Salmonella* sp, *Staphylococcus aureus* and *Streptococcus pyogenes* isolated from paediatric patients during the period of study using previously described methods.
Results: Bacteria (*Corynebacterium* sp, *Streptococcus* sp, *Acinetobacter* sp and *Lactobacillus* sp) and fungal (*Geotrichum condidum, Aspergillus niger, Fusarium oxysporum* and *Fusarium fujikuroi*) were isolated from the termite nests. The antibiotic susceptibility revealed that *Corynebacterium* sp and *Streptococcus* sp were multiply antibiotic resistant, and this was confirmed to be plasmid mediated based on plasmid analysis and curing. The *Lactobacillus* sp, *Aspergillus niger, Fusarium fujikuroi* and *Geotrichum condidum* exhibited mild antagonisms against *Staphylococcus aureus, Salmonella* sp and *Streptococcus pyogenes* isolated from paediatric patients.
Conclusion: This study suggests that termite nests on kola nut trees contain microbes that possess antagonistic actions against pathogens from paediatric patients and that some bacteria associated with termite guts may pose significant risk of increased antibiotic resistance if implicated in human infections.

Keywords: Termite nests, Resistance, Antagonistic microbes, Termites, Plasmid, Kola nut tree

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Lactobacillus sp et certains champignons provenant de nids de termites sur les arbres de kolanut ont eu de légers effets antagonistes contre les agents pathogènes isolés de patients pédiatriques

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Abstrait:

Contexte: Les résidents d'une banlieue rurale d'Akure ont abandonné le traitement antibiotique; ont recherché un remède alternatif à l'augmentation de l'incidence des infections pédiatriques en 2017 auprès des vendeurs de plantes locales, de nombreux résidents affirmant une meilleure réponse au traitement. Nous avons étudié ces allégations, car la formule à base de plantes locales comprenait des écorces de noix de kola et des termites moulus.
Méthodologie: Les micro-organismes associés aux nids de termites sur les arbres de noix de kola dans la communauté affectée ont été caractérisés et identifiés à l'aide de techniques standard. La diffusion sur disque de Kirby Bauer a été utilisée pour évaluer la sensibilité des isolats bactériens aux antibiotiques sélectionnés. Le profil plasmidique de plusieurs isolats bactériens résistants aux antibiotiques (MDRI) a été déterminé par la méthode de Birnboim et Doly tandis qu'une sensibilité aux antibiotiques après polymérisation du plasmide a été réalisée sur les MDRI contre les mêmes antibiotiques sélectionnés. Les micro-organismes ont également été évalués pour d'éventuels effets antagonistes contre *Salmonella* sp, *Staphylococcus aureus* et *Streptococcus pyogenes* isolés de patients pédiatriques pendant la période d'étude en utilisant les méthodes décrites précédemment.
Résultats: Des bactéries (*Corynebacterium* sp, *Streptococcus* sp, *Acinetobacter* sp et *Lactobacillus* sp) et fongiques (*Geotrichum condidum, Aspergillus niger, Fusarium oxysporum* et *Fusarium fujikuroi*) ont été isolées des nids de termites. La sensibilité aux antibiotiques a révélé que Corynebacterium sp et Streptococcus sp étaient multi-résistants aux antibiotiques, et cela a été confirmé comme étant médié par les plasmides sur la base de l'analyse et du durcissement des plasmides. Les *Lactobacillus* sp, *Aspergillus niger*, *Fusarium fujikuroi* et *Geotrichum condidum* présentaient de légers antagonismes contre *Staphylococcus aureus*, *Salmonella* sp et *Streptococcus pyogenes* isolés de patients pédiatriques.
Conclusion: Cette étude suggère que les nids de termites sur les arbres de noix de kola contiennent des microbes qui possèdent des actions antagonistes contre les agents pathogènes des patients pédiatriques et que certaines bactéries associées aux intestins des termites peuvent poser un risque significatif d'augmentation de la résistance aux antibiotiques si elles sont impliquées dans des infections humaines.

Mots-clés: nids de termites, résistance, microbes antagonistes, termites, plasmidique, arbre à noix de Kola

Introduction:

Termites are social insects that live in enclaves known as termite nests or "termitaria" (1,2). They belong to the order Isoptera with more than 1800 species in 200 genera (3,4). They form their nests using mud, gut exudates, secretions and faeces (5,6). In various rural settlements of western Nigeria, the termite specie *Odontotermes badius* is predominant and serves as food supplement for local residents (6). Certain bacteria form part of termite gut flora and aid the digestion of food in termite guts (1,2). However, fungi are also present in termite guts either through ingestion of infested food or propagating fungal spores (1,5). Conversely, studies have recently linked interactions existing between termites and microorganisms inhabiting their guts with respect to the tree environment on which they live (1,6). This has necessitated investigation into the termite microbiota and the types of microbes associated with their nests (3,4).

Kola nuts (*Cola* sp) are widely cultivated in West Africa because they serve as natural stimulants that suppress fatigue (7). In Western Nigeria, the species, *C. nitida* is widely cultivated in various farm settlements for various purposes and this tree species harbor vast reserves of termites, since kola nut is an excellent source of soft wood (7).

Our field correspondence with local residents in a suburb of Akure, Nigeria in 2017 revealed that due to rising incidence of paediatric infections, the inhabitants jettisoned known antibiotics and sought alternative medical help from local herbal concoction dealers, with claims of better treatment response from the use of these concoctions in affected infants. These claims from use of local medicinal formula which included kola nut barks and ground termites were widely uncorroborated scientifically. In view of this, our study sought to identify microorganisms from termite nests on kola nut trees in the affected community, evaluate possible antagonistic effects of these microbes against clinical isolates of paediatric importance, and raise public health awareness of associated risks with use of these herbal concoctions.

Materials and methods:

Description of study location

The study area is Ipogun, a very small community with dispersed settlements. It is a

suburb of Akure metropolis, Nigeria at 7°11′ N 7°12′ N/ 5°4′ E 5°9′ E coordinates (8).

Sample collection and processing

We designated kola nut plantations in the study area as sampling points for sample collection in September 2017. A total 20 dead termite aggregate samples from termite nests on kola nut trees in the study location were collected using guidelines described by Barreto et al., (9). The samples were stored under airtight ice packs and analyzed micro-biologically within 4hrs of collection (10).

Isolation of microorganisms from prepared samples

Sample preparation and isolation of microorganisms was according to Afolami et al. (1). Sterile normal saline was used as diluent and 1.0g of sample stocks was weighed into 1.0ml of the diluent for a serial dilution process and four dilutions were obtained for a pour plate technique. Thereafter, 1ml of the last dilution was used to inoculate already prepared nutrient agar and potato dextrose agar plates containing 250mg chloramphenicol (for total filamentous fungi count). Bacterial cultures were incubated at 37°C for 24hrs and fungi at 26±2°C for 3-5 days (1,2).

Identification of bacterial isolates

Identification of bacterial isolates was done using methods of Afolami et al., (1) and Aribisala et al., (2). Subcultures of distinct colonies were identified by gram reaction and biochemical tests such as catalase, motility, sugar fermentation (glucose, sucrose, lactose and mannitol), triple sugar iron, methyl red/voges proskauer test, oxidase and spore staining tests. The identified isolates were freshly subcultured on MacConkey and Bile Esculin agar plates, and incubated at 37°C for 24hours (11).

Identification of fungi isolates

The authors used methods described in Samson et al., (12) and Onifade et al., (13) for identification of fungi isolates and compared the cultural and microscopic characteristics of fungal mycelia with the available literature using the Compendiums for Air, Soil, Food and Indoor fungi (12,13). The cultural and micro-morphological properties of isolated fungi were obtained through microscopy of stained mycelia with cotton blue in Lactophenol dye (13). Photomicrographs of different mycelium clones were juxtaposed with matching information contained in the available literature for air and soil fungi as described in Onifade et al., (13).

Antibiotic susceptibility of bacterial isolates

The Kirby Bauer disk diffusion test was used to evaluate *invitro* activity of selected antibiotics against the bacterial isolates on Mueller Hinton (MH) agar. An 18h old broth culture of each isolate was standardized with 0.5McFarland standards and plated on MH agar using sterile swabs as previously described (14). The selected antibiotic disks (AB Biodisk, Solna, Sweden) from seven different antibiotic classes used were amoxicillin (25µg), eryth-romycin (15µg), ofloxacin (5µg), ciprofloxacin (5µg), pefloxacin (5µg), cotrimoxazole (25µg), ceftriaxone (30µg), gentamycin (10µg), strep-tomycin (10µg) and chloramphenicol (15µg).

The antibiotic disks were gently placed on the plates and incubated for 24hours at 37°C. The diameter of zone of inhibition of each isolate was measured with a calibrated ruler and interpreted as sensitive, intermediate or resistant according to the guidelines of the National Committee for Clinical Laboratory Standards (15) while *Staphylococcus aureus* ATCC 25923 was used as control strain. Test isolates with resistance to more than two classes of antibiotics were designated as multiple antibiotics resistant (14, 16).

Plasmid profile analysis of MDRIs

Plasmid extraction and analysis from the multiple antibiotic resistant bacterial isolates (MDRIs) was carried out using alkaline lysis method (Zymogen, UK) as previously described by Akingbade et al., (16). Plasmid extraction solution containing 20.0mM sodium acetate and 2.0mM EDTA adjusted to pH 7.8 using 10% acetic acid was prepared with a sample buffer containing 25% sucrose, 5.0mM sodium acetate and 0.05% bromophenol blue. Electrophoresis of plasmid DNA was done on 0.9% agarose gel stained with gr-Green dye (1µl/ml) at room temperature while pBR322 DNA/BsuRI (HaeIII) was used as marker. After the run time, gel was observed under UV transillumination and analyzed using a photo documentation system.

Plasmid curing of MDRIs

The curing of resistant plasmids from the MDRIs was done overnight by using the methods of Birnboim and Dolly (17) and Vivyan et al., (11). Antibiotic susceptibility testing of MDRIs post-plasmid curing was carried out with the same set of antibiotic disks (AB Biodisk, Solna, Sweden).

Antagonistic assays

All the isolates (bacteria/fungi) were evaluated for possible antagonistic effects against clinical pathogens (*Salmonella* sp, *Staphylococcus aureus* and *Streptococcus pyogenes*) isolated from paediatric patients and obtained from the Department of Laboratory Services, Ondo State Specialist Hospital, Akure, Nigeria. These pathogens were adjudged to have had direct impact on the rising incidence of paediatric infections in Akure suburbs at the time of the research.

The Fokkema and Heuvel (18) method was used in evaluating antagonistic potentials of fungal isolates while the methods described by Afolami et al., (1) and Aribisala et al., (2) were used to evaluate antagonistic potentials of bacterial isolates against the clinical pathogens. The susceptibility cutoffs in the assays were determined by susceptible-dose-dependent criteria of the 2012 Clinical and Laboratory Standards Institute (19) and as described by Jabeen et al., (20). The inhibition zones were denoted as positive (+ve) at ≥16.00mm, intermediate (I) at 11.00-15.00 mm and negative (-ve) at ≤10.00mm (1,27). *Candida parapsilosis* ATCC 22019 and *Haemophilus influenzae* ATCC 49247 were used as control strains in the antagonistic assays.

Data analysis

The diameters of zone of inhibition for the antagonistic assays were obtained by means of triplicate values and separated using Duncan's New Multiple Range test at $p < 0.05$ level of significance (and 95% confidence interval) to determine whether they were significant or not.

Results:

The bacterial isolates across sampling points are shown in Tables 1 and 2. A total of forty-three bacteria isolates; *Corynebacterium* sp (n=9), *Lactobacillus* sp (n=17), *Acinetobacter* sp (n=10) and *Streptococcus* sp (n=7), and 22 fungi isolates; *Aspergillus niger* (n=8), *Fusarium oxysporum* (n=6), *Fusarium fujikuroi* (n=5) and *Geotrichum condidum* (n=3) were recovered from the samples.

The highest zone diameter of inhibition to antibiotics by the bacterial isolates was 20.35±1.28mm while the lowest zone was 3.20±1.08mm. The susceptibility patterns of the isolates are denoted as susceptible (S), resistant (R) and intermediate (I) in Tables 3 - 6. As shown in Table 3, two of the four bacteria genera/species (*Corynebacterium* sp, and *Streptococcus* sp) were multiple antibiotic resistant isolates (resistant to more than two classes of antibiotics tested). A representative isolate from each genus was profiled for plasmid analysis as shown in Fig 1.

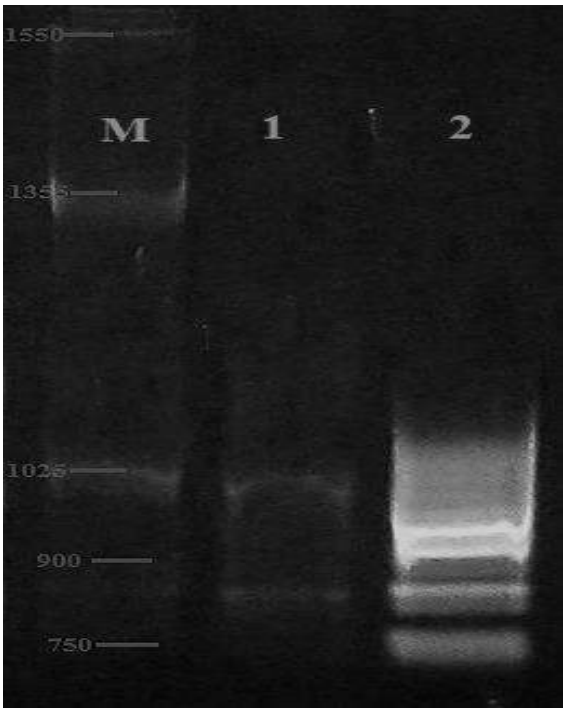

Fig 1: The electropherogram of plasmids of the MDRIs
1 = *Corynebacterium* sp, 2 = *Streptococcus* sp, M = molecular marker in kilobase pair

The antagonistic features (inhibitory effects) of the bacterial isolates against the pathogens obtained from paediatric patients (*Salmonella* sp, *Staphylococcus aureus*, and *Streptococcus pyogenes*) are shown in Table 4 while the antagonistic features (inhibitory effects) of the fungi isolates against these pathogens are shown in Table 5**.** Only *Lactobacillus* sp among the bacterial isolates had mild antagonism against the pathogens with inhibition zone ranging from 12.82±1.68 to 16.19±1.21mm, while the fungi (*Fusarium fujikuroi* and *Geotrichum condidum*) had antagonistic activity against the pathogens with inhibition zones ranging from 11.91±1.66 to 18.91±1.33mm.

The zones of inhibition and susceptibility patterns of the MDR isolates after plasmid curing in Table 6 showed the two MDR bacteria genera (*Corynebacterium* sp, and *Streptococcus* sp) to be susceptible, which confirms that their multidrug resistance property was plasmid mediated.

Table 1: Morphological and biochemical identification of bacterial isolates from samples analyzed

Isolates	Gram Stain	Sugar Fermentation					O/C	M.t./ Sp. T	MR/VP	Growth on Media			No of isolates
		Lac	Glu	Suc	Mann	TSI				NA	Mac A	YEA	
A. sp	+ve (bacilli rods)	-ve	+ve	+ve	+ve	A/G	+ve/ +ve	+ve/ +ve	-ve/+ve	Cream/ raised	-ve	-ve	10
St. sp	+ve (bacilli rods)	+ve	+ve	+ve	-ve	A/G	+ve/ +ve	+ve/ +ve	+ve/+ve	Milky/lobate	+ve (pale yellow)	-ve	7
Cy. sp	+ve (cocco-bacilli rods)	-ve	+ve	+ve	-ve	A/G	+ve/ +ve	-ve	-ve/-ve	Cream/ raised	+ve (pink)	-ve	9
Lac. sp	+ve (bacilli)	+ve	+ve	+ve	-ve	K/NF	+ve/ +ve	-ve/ -ve	-ve/+ve	Cream/ lobate	-ve	-ve	17

Keys: I = Isolates, *A.* sp = *Acinetobacter* sp, *St.* sp = *Streptococcus* sp, *Cy*. sp = *Corynebacterium* sp, *Lac*. Sp = *Lactobacillus* sp, Lac = Lactose, Glu =Glucose, Suc = Sucrose, Mann = Mannitol, TSI =Triple Salt Iron, O/C = Oxidase/Catalase test, M.t./Sp. T = Motility test/Spore test, MR/VP = Methyl red/Voges Proskauer, NA = Nutrient Agar, Mac A = MacConkey Agar, YEA = Yeast Extract Agar, -ve = negative, +ve = positive, A/G = Acid/ Gas, K/NF = Alkaline slant/ No fermentation

Table 2: Morphological and cultural characteristics of fungi isolates from the samples

RIC	Characteristics		Fungi species	No of isolates
	Cultural	Morphological		
1	Fastidious white flattened mycelium which becomes brownish grey with age having tall and short projection centers	Sporangiophores branched in simple monopodial forms, appearance of non-septate hyphae with smooth edged zygospores	*Fusarium fujikuroi*	5
2	Fastidious stained white mycelium with brownish black centers that spreads rapidly	Long thin walled hyaline conidiophores with globose radiate heads appear smooth with black bars; conidiophores are branched and lumped with cylindrical phalides	*Aspergillus niger*	8
3	Fastidious white fluffy mycelium with dark green velvety centers	Globose smooth walled, non-septate conidia observed with biserate radiate head	*Geotrichum tropicum*	3
4	Peachy velvety spreading aerial mycelium with brown edges that appear dark yellowish as culture ages	Long thin walled conidiophores with fusiform conidia that appears convex at the apex. Hyphae is hyaline with branched monophalides	*Fusarium oxysporum*	6

Keys: RIC- Representative Isolate Clones from different sampling points

Table 3: Zones of inhibition and antibiotic resistance patterns of characterized bacterial isolates

A.t.	Zones of inhibition (mm)					Antibiotic class	Resistance patterns			
	Cy. sp.	*A*. sp.	*St*. sp.	*Lac*. sp.	*S. aureus* Control		*Cy*. sp.	*A*. sp.	*St*. sp.	*Lac*. Sp
ERY	3.20±1.08[a]	5.33±1.46[a]	3.34±1.02[a]	9.24±1.28[a]	22-30	Macrolide	R	R	R	I
CPX	6.23±1.26[b]	8.48±1.31[a]	9.42±1.21[b]	13.44±1.28[b]	22-30	Fluoroquinolone	R	I	R	I
COT	8.44±1.22[b]	5.08±1.23[a]	5.81±1.09[a]	7.28±1.19[a]	24-32	Cotrimoxazole	R	R	R	R
AMX	3.87±0.24[a]	11.26±1.82[b]	4.51±1.05[a]	10.72±1.48[b]	-	Penicillin	R	I	R	I
OFL	19.29±1.22[e]	18.46±1.70[c]	11.43±1.81[c]	20.59±1.22[c]	24-28	Quinolone	S	S	I	S
STR	12.13±1.28[d]	14.23±1.59[b]	6.52±1.28[a]	18.35±1.14[c]	14-22	Aminoglycoside	I	I	R	S
CHL	7.83±1.81[b]	16.55±1.31[c]	12.99±1.01[c]	11.23±1.71[b]	19-26	Chloramphenicol	R	S	I	I
CEF	9.28±1.22[b]	13.36±1.29[b]	6.41±1.66[a]	6.67±1.31[a]	22-28	Cephalosporin	R	I	R	R
GEN	5.29±1.48[a]	13.79±1.52[b]	14.55±1.05[c]	14.51±1.39[b]	19-27	Aminoglycoside	R	I	I	I
PEF	18.10±1.34[e]	19.26±1.58[c]	20.35±1.28[d]	19.21±1.21[c]	-	Fluoroquinolone	S	S	S	S

The isolates; *Cy*. sp. and *St*. sp. are resistant to more than 2 antibiotics classes (MDRIs)

Keys: *Cy*. sp=*Corynebacterium* sp; *A*. sp=*Acinetobacter* sp; *St*. sp=*Streptococcus* sp; *Lac*. sp=*Lactobacillus* sp; Control *S. aureus* ATCC 25923; A.t.=Antibiotics; COT=Cotrimoxazole; CPX=Ciprofloxacin; ERY= Erythromycin; AMX= Amoxycillin; OFL= Ofloxacin; STR= Streptomycin; CHL=Chloramphenicol; CEF=Ceftriaxone; GEN=Gentamycin; PEF= Pefloxacin; R = Resistant; I= Intermediate; S=Susceptible; MDRIs=Multiple antibiotic resistant isolates; values with the same letter as superscript are not significantly different ($p > 0.05$)

Table 4: Antagonistic patterns of bacteria isolates against selected clinical pathogens

Bacteria isolate	Observed antagonistic interaction (mm)			Deduced antagonistic patterns			
	Salmonella sp	*Staphylococcus aureus*	*Streptococcus pyogenes*	*Control strain	*Salmonella* sp	*Staphylococcus aureus*	*Streptococcus pyogenes*
Acinetobacter sp.	00±00[a]	9.36±1.45[b]	13.23±1.54[c]	+ve	-ve	-ve	I
Streptococcus sp.	2.12±0.29[a]	5.58±1.64[b]	00±00[a]	-ve	-ve	-ve	-ve
Corynebacterium sp.	00±00[a]	00±00[a]	6.84±1.23[b]	I	-ve	-ve	-ve
Lactobacillus sp.	14.34±1.53[b]	12.82±1.68[c]	16.19±1.21[d]	+ve	I	I	+ve

I= Intermediate; -ve= Negative antagonism or no antagonistic interaction; +ve= Positive antagonism; values with the same letter as superscript are not significantly different ($p > 0.05$). **Haemophilus influenzae* ATCC 49247

Table 5: Antagonistic patterns of fungal isolates against selected clinical pathogens

Fungal isolates	Observed antagonistic interaction (mm)			Deduced antagonistic patterns			
	Salmonella sp	*Staphylococcus aureus*	*Streptococcus pyogenes*	*Control strain	*Salmonella* sp	*Staphylococcus aureus*	*Streptococcus pyogenes*
A. niger	14.01±1.03[c]	9.23±1.76[a]	18.21±1.48[c]	I	I	-ve	+ve
F. oxysporium	8.42±1.48[b]	12.08±1.82[b]	11.28±1.56[b]	I	-ve	-ve	I
F. fujikuroi	00±00[a]	13.25±1.74[b]	17.56±1.67[c]	+ve	-ve	I	+ve
G. condidum	11.91±1.66[c]	18.91±1.33[c]	00±00[a]	+ve	I	+ve	-ve

Keys: *A. niger=Aspergillus niger*; *F. oxysporium=Fusarium oxysporium*; *F. fujikuroi=Fusarium fujikuroi*; *G. condidum=Geotrichum condidum;* I= Intermediate; -ve= Negative antagonism or no antagonistic interaction; +ve= Positive antagonism; values with the same letter as superscript are not significantly different ($p > 0.05$). **Candida parapsilosis* ATCC 22019

Table 6: Zones of inhibition and antibiotic susceptibility patterns of MDRIs post plasmid curing

Antibiotics	Zones of inhibition (mm)		Susceptibility patterns	
	Corynebacterium sp	*Streptococcus* sp	*Corynebacterium* sp	*Streptococcus* sp
ERY	17.15±1.82[a]	18.15±1.82[a]	S	S
CPX	19.31±1.61[a]	17.43±1.19[a]	S	S
COT	18.33±1.28[a]	19.21±1.48[a]	S	S
AMX	15.78±1.09[a]	17.44±1.29[a]	S	S
OFL	19.85±1.45[a]	16.89±1.75[a]	S	S
STR	18.67±1.46[a]	19.27±1.84[a]	S	S
CHL	19.59±1.08[a]	19.78±1.33[a]	S	S
CEF	20.18±1.84[a]	21.18±1.24[a]	S	S
GEN	18.58±1.42[a]	18.47±1.96[a]	S	S
PEF	19.29±1.71[a]	19.46±1.84[a]	S	S

Keys: COT=Cotrimoxazole; CPX=Ciprofloxacin; ERY= Erythromycin; AMX= Amoxycillin; OFL= Ofloxacin; STR= Streptomycin; CHL=Chloramphenicol; CEF=Ceftriaxone; GEN=Gentamycin; PEF= Pefloxacin; R- Resistant; I- Intermediate; S- susceptible; MDRIs- Multiple antibiotic resistant isolates; values with the same letter as superscript have no significant difference at $p \leq 0.05$ level of significance.; R- Resistant; I- Intermediate; S- susceptible. MDRIs- Multiple drug resistant isolates, values with the same letter as superscript have no significant difference at ($p > 0.05$)

Discussion:

The microorganisms identified from this study showed that termite nests have high microbial diversity. Some of the organisms obtained in this study such as *Corynebacterium* sp*, Acinetobacter* sp*, Streptococcus* sp, *Aspergillus niger* and *Geotrichum condidum* have been investigated in studies on termite nest micro flora, most especially by Afolami et al,. (1) and Aribisala et al., (2) where authors recently embarked on a similar work. Fungi isolates obtained in this study such as *Fusarium oxysporium* and *Aspergillus niger* produce mycotoxins which are of pathological importance to kola nut trees and termites alike, although recent reports by Afolami et al., (1) and Aribisala et al,. (2) may suggest that these fungi would also directly impact economic losses and effects on crop plants, animals and humans.

A previous report by Bignell et al., (21) suggested that termites may harbor micro-aerophilic bacteria that aid cellulose digestion and this underscores the benefit the mutual-listic relationship between termites and bacteria inhabiting their guts. Our study underscored this claim since the bacteria *Acinetobacter* sp and *Corynebacterium* sp obtained from the study are micro-aerophilic (5). The study by Lo and Eggleton (22) also affirmed that termites use faecal matter to make internal ventilating structures (combs) which act as substrates for the growth of the symbiotic fungi in their nests. This corroborate the presence of soil borne fungi, *A. niger*, *F. oxysporum*, *F. fujikuroi* and *G. condidum* obtained from our study.

Earlier reports by Afolami et al., (1) and Aribisala et al., (2) observed that termites might develop defensive strategies against invading pathogens by producing antimicrobial substances in defensive glandular secretions which allow them resist termite borne pathogens over an extensive period of time. It might be safe to suggest that these previous reports explain why all the bacterial isolates obtained from termite nests have varying degrees of antibiotic resistance since they may have been exposed previously to wide range antimicrobial substances from termite secretions and insecticide sprays prior to this study. This may also partly give a scientific explanation to uncorroborated claims made by local respondents about the potency of termite based herbal concoction in treating paediatric infections. However, the results also presented showed that these locally made herbal

concoctions used by local dwellers may pose serious health risks since bacteria in this study showed stellar multiple antibiotic resistance and respondents risk ingestion of these MDRIs.

Consequently, the susceptibility patterns of the bacteria isolates observed in our study might have resulted from exposure of termite nests on kola nut barks to active components in many herbicides used by local farmers, which may be similar to the compounds present in the antibiotics used in our study. This may trigger a form of acquired antibiotic resistance in the isolates. Similar observations have been made by Brune and Dietrich (23), Afolami et al., (1) and Aribisala et al., (2). As observed in our study, plasmid-mediated antibiotic resistance mechanisms might have been responsible for resistance in the MDRIs, which could manifest as antibiotic efflux, gene mutation, and aberrant protein expressions that reduce permeability of bacteria cell envelopes to many antibiotics, resulting in resistance (24).

Local residents from Ipogun community have described clinical symptoms suggestive of acute typhoid fever and infantile food poisoning in children, attributed to *Salmonella* sp, *S. aureus* and *S. pyogenes* (personal communication). Meanwhile, similar infection patterns caused by pathogens used in our study have also been previously reported by the local health authorities of the affected area, which informed the testing of isolates we obtained from termite tubes in our study against these pathogens. Rabasa et al*.,* (25) described the clinical presentations of acute salmonellosis in infants as a case study in western Nigeria (same region of the affected community) while Shittu et al*.,* (26) and Odigwe et al., (27) suggested that infants (below age 9) may be more susceptible to haemolytic infections and food poisoning in Nigerian communities where basic life amenities are inadequate. Rural communities such as Ipogun (the study area) lack basic amenities. Hence, it is not surprising that the rising incidence of paediatric infections in this community had defied antibiotic interventions. This explains why the local residents affected sought for traditional indigenous medical interventions.

Since this study aims at corroborating claims made by local residents, the authors cited the report of Holt and Leepage (4) suggesting that microorganisms inhabiting the termite guts are capable of exhibiting overt antagonisms against other pathogens as part of their adaptation. Hence, *Lactobacillus* sp, *F. fujikuroi* and *G. condidum* showed mild antagonism against the pathogens tested. The antagonisms exhibited by *Lactobacillus* sp may be due to the production of antimicrobials such as bacteriocins and lactic acid derivatives that can hinder the metabolism of the pathogens, while the fungal isolates (*F. fujikuroi* and *G. condidum*) are producers of lytic enzymes and toxic exudates that can lyse cell walls of pathogens (24).

Although previous reports by Afolami et al., (1) and Aribisala et al., (2) described antagonistic patterns of isolated organisms from Mango and Cocoa trees in Ibule-soro, the findings presented in this current study showed antagonisms of *Lactobacillus* sp and certain fungi (*F. fujikuroi* and *G. condidum*) from termite nests on kola nut trees in a different community (Ipogun). This made the current study better defined in scope for a new study area than previous studies. Hence, the antagonisms observed in this study clearly establish termite tubes to contain certain microbes that may aid inhibitory properties of the local medicine formula adopted by rural dwellers in this affected community against pathogens causing infantile infections in their community.

Conclusion:

This study has shown that *Lactobacillus* sp and certain fungi contained in termite nests on kola nut trees possess antagonistic potentials against pathogens obtained from paediatric patients and that some bacteria present in termite guts may possess overt multiple drug resistances due to their carriage of resistant plasmids.

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Original Article **Open Access**

Salinity induced apoptosis in food spoilage yeast *Zygosaccharomyces bisporus*

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Abstract:

Background: Food spoilage is one of the most serious challenges in agriculture, and food and beverage industry, which can lead to worldwide food economic loss. The crucial organoleptic species, *Zygosaccharomyces bisporus,* is a highly resistant yeast fungus that can escape industrial quality check. They survive high salt environments by undergoing immediate programmed cell death (PCD), which plays an important role in mediating adaptive responses to adverse environmental conditions. Production of reactive oxygen species (ROS) prompted by salt stress is an early event in apoptosis, which in later stage is associated with prime genomic degradation.
Methodology: In this study, the tolerance mechanism to salt of *Z. bisporus* MTCC 4801 cells was investigated by serial dilution of exponential growth phase of the cells in 1.0M sodium chloride (NaCl) as salt stressor, and spotting on Yeast Peptone Dextrose Agar (YPDA) plates with incubation at 28°C for growth assessment and colony count. Transmission electron microscopy (TEM) was used to demonstrate characteristic ultrastructural hallmark features of apoptosis on *Z. bisporus* cells exposed to 1.0M NaCl at three different stress interval periods; 60, 90, and 120 minutes.
Results: Growth of *Z. bisporus* cells on the YPDA plates was observed after 16 hours incubation period. Comparing the growths, *Z. bisporus* tolerated salt concentration below 1.0M NaCl but no growth was observed at 1.0M NaCl concentration indicating 1.0M NaCl to be limiting concentration for *Z. bisporus* growth. TEM analyses showed that treatment of *Z. bisporus* with 1.0M NaCl resulted in nuclear and cytoplasmic condensation, membrane blabbing, cytoskeletal distortion, and formation of apoptotic bodies. However, on prolonged stress span (90 and 120 minutes), the fungal cells were able to osmoadapt and repaired the damaged cells, resulting in lowering of the apoptotic ratio.
Conclusion: These qualitative analyses contribute more insights regarding stress adaptive mechanisms in moderately halotolerant food spoilage yeast.

Key words: Apoptosis; salt stress; food spoilage yeast; TEM; cell survival

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Apoptose induite par la salinité chez la levure altérant les aliments *Zygosaccharomyces bisporus*

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Abstrait:

Contexte: La détérioration des aliments est l'un des problèmes les plus graves dans l'agriculture et l'industrie de l'alimentation et des boissons, ce qui peut entraîner des pertes économiques dans le monde de l'alimentation. L'espèce organoleptique cruciale, *Zygosaccharomyces bisporus*, est un champignon de levure très résistant qui

peut échapper au contrôle de la qualité industrielle. Ils survivent aux environnements riches en sel en subissant une mort cellulaire programmée (PCD) immédiate, qui joue un rôle important dans la médiation des réponses adaptatives aux conditions environnementales défavorables. La production d'espèces réactives de l'oxygène (ROS) provoquées par le stress salin est un événement précoce de l'apoptose, qui est associé à un stade avancé de dégradation génomique.
Méthodologie: Dans cette étude, le mécanisme de tolérance au sel des cellules MTCC 4801 de *Z. bisporus* a été étudié par dilution en série de la phase de croissance exponentielle des cellules dans du chlorure de sodium (NaCl) à 1,0M en tant que facteur de stress salin, et par repérage sur la gélose de levure peptone Dextrose Agar (YPDA) avec incubation à 28°C pour l'évaluation de la croissance et le nombre de colonies. La microscopie électronique à transmission (MET) a été utilisée pour démontrer les caractéristiques de l'apoptose ultrastructurales des cellules de *Z. bisporus* exposées à 1,0M de NaCl à trois périodes d'intervalle de stress différentes; 60, 90 et 120 minutes
Résultats: La croissance des cellules de *Z. bisporus* sur les plaques de YPDA a été observée après une période d'incubation de 16 heures. En comparant les croissances, une concentration en sel tolérée par *Z. bisporus* inférieure à 1,0M de NaCl, mais aucune croissance n'a été observée à une concentration de 1,0M en NaCl, ce qui indique que NaCl 1,0M était la concentration limite pour *Z. bisporus* croissance. Les analyses TEM ont montré que le traitement de *Z. bisporus* avec 1,0M de NaCl entraînait une condensation nucléaire et cytoplasmique, un blabbing membranaire, une distorsion cytosquelettique et la formation de corps apoptotiques. Cependant, sur une période de stress prolongée (90 et 120 minutes), les cellules fongiques ont pu s'osmoadapter et ont réparé les cellules endommagées, ce qui a entraîné une diminution du rapport apoptotique.
Conclusion: ces analyses qualitatives apportent davantage d'informations sur les mécanismes d'adaptation au stress de la levure altérante modérément halotolérante.

Mots clés: apoptose; stress salin; levure d'altération des aliments; TEM; survie cellulaire

Introduction:

Despite satisfactory sanitation practices, large proportions of processed foods such as juices, pickles, jams and beverages are lost to due to spoilage by microbial contamination such as bacteria and yeasts. This has the potential to cause serious health hazards and substantial economic loss to food and beverage industries. Although, bacteria have extensively been studied for the evolved and conserved halotolerant mechanisms, yeasts have received more attention as key players responsible for food deterioration and spoilage. Among these, the major problematic genus *Zygosaccharomyces*, notably *Z. bisporus*, is typically osmotolerant and moderately halotolerant which ensures its remarkable survival in high salinity, a unique adaptation of *Z. bisporus* (1-4).

Salinity produces osmotic stress by decreasing the chemical activity of water and loss of turgor pressure, and ionic stress by cellular accumulation of high salt concentration (5-6). Under such conditions, strong plasma membrane depolarization due to Na^+ uptake favors K^+ efflux via depolarization-activated outward-rectifying K^+ TOK1 channels. The resultant elevated cytoplasmic Na^+/K^+ ratio and osmotic stress induces mitochondrial oxidative burst which is known to be crucial for triggering the complex apoptotic cascade in stressed cells (7-9). As the wobbled redox status implicates over production of oxygen radicals ($HO^{\cdot}$, $\bullet O_2^-$, H_2O_2), the predominant death signaling molecule in stimulating *bax*-linked ancestral apoptotic pathway, shorten the chronological life span (CLS) of yeast (9-11). A number of studies on evaluation of salt induced osmotic stress in yeasts have focused on cell physiology, morphology and genetic of the networks of cell division inhibition, decreased cell vitality, DNA damage, and increased cell death particularly due to apoptosis.

Programmed cell death (PCD) is widely associated with characteristic hallmark features such as nuclear fragmentation, chromatin condensation, cell shrinkage, and cytoplasmic condensation with clustering of apoptotic bodies (12-16). Although cytoplasmic condensation occurs, typical changes in the nucleus provide the most important means of identifying apoptosis (13, 14). Previous studies on *Zygosaccharomyces rouxii*, *Debaryomyces nepalensis*, *Cryptococcus laurentii* and other halotolerant fungi species had described physiological mechanisms underlying extreme salt tolerance in relation to apoptosis (17,18), even as the capacity of these halotolerant food spoilage yeasts, to survive, grow, and metabolize actively under such hyperosmotic challenged conditions is essentially an important study in food industry.

In particular, identification of plausible mechanisms and physiological responses that shape the halostress response downlinks in *Zygosaccharomyces* food spoilage yeasts are crucial. However, the cellular mechanisms of salt injury in these yeasts, and the early events during induction of PCD by salt stress are scarcely known. Hence, this research was conducted to study the effects of salt stress on induction of PCD by determining cyto-

morphological response of a moderately halotolerant yeast, *Z. bisporus* in an experimental model.

Materials and Methods:

Inoculum preparation & culture of yeast strain

The yeast strain *Z. bisporus* MTCC 4801 used in this study was procured from the Department of Biotechnology, Himachal Pradesh University, Shimla, India, and maintained at 4°C on pre autoclaved Yeast Peptone Dextrose Agar plates containing (w/v) 2% dextrose, 1% yeast extract, 2% peptone, and 2% agar. Cells were first grown on agar plates (pH 5.5) and incubated at 28°C for 48 hours. One loopful of the growth on the plate was transferred to 10 mL Yeast Peptone Dextrose (YPD) medium for inoculum preparation at incubated at 28°C for 16 hours at 200rpm in a rotary shaker. Cell growth was measured as an increase in optical density to ~0.5 (mid log phase cells) at 600nm with UV spectrophotometer (1800 Shimadzu, Japan). From this pre-culture, 1.0% (v/v) of inoculum containing 0.7×10^6 cells/ml was transferred to YPD medium in Erlenmeyer flasks under similar growth conditions to obtain exponential phase yeast cells.

Stress treatment of yeast strain

In order to determine salt stress response, the cells survival, of exponentially growing yeast cells were serially diluted (10^5 factor) in 1.0M sodium chloride stressor, spotted on YPD agar plate, and incubated at 28°C for 48 hours. Cultures that yielded colonies of *Z. bisporus* were counted and further analyzed according to standard microbiological procedures (18).

Transmission electron microscopy examination of yeast strains

Yeast cells in exponential growth phase were exposed to 1.0M NaCl, and incubated at 28°C in a rotary shaker (200 rpm) for periods of 60, 90 and 120 minutes. Both control (without stress exposure) and salt (NaCl) treated yeast cells at three different time intervals were first fixed in 3.0% glutaraldehyde in Sorensen's phosphate buffer (pH 7.2) for 4-6 hours at 4ºC and rinsed twice with same buffer for 15 minutes. This was followed by secondary fixation with 1.0% Osmium tetroxide in Millonin's phosphate buffer (pH 7.2) for 90 minutes at 4°C. The cells were again rinsed twice in Millonin's buffer for 15 minutes and dehydrated using increasing alcohol concentrations of 70%, 90%, and 100% at room temperature, followed by rinsing in propylene oxide once for 15 minutes on the rotator for complete dehydration. The fixed specimens were then put in an epon mixture of 50% propylene oxide and 50% resin for at least 2 hours or overnight at room temperature before embedding in Taab-812 embedding medium.

Ultrathin sections of 60nm thickness were cut on Leica EM UC-6 ultramicrotome (Leica Mikrosysteme GmbH, Wien, Austria) and taken on Nickel grids. The sections were stained with uranyl acetate and lead citrate and examined at 80kV accelerating voltage Transmission Electron Microscope (JEM-1400 Plus, JEOL, Tokyo, Japan) equipped with XR81M-B Camera (Advanced Microscopy Techniques Corp, Woburn, MA, United States). Digital electron micrographs were obtained using AMT Image Capture Engine V602 software (Advanced Microscopy Techniques Corp, Woburn, MA, United States) with resolution of 500nm and magnification power of 8000X (19).

Results:

Effect of salt on growth of *Z. bisporus*

Growth of the yeast cells on YPD medium supplemented with salt concentration of 1.0M NaCl was observed after 16 hours incubation period by spotting assay. Comparing the growths, *Z. bisporus* tolerated salt concentration below 1.0M NaCl, but no growth was observed at 1.0M NaCl concentration indicating 1.0M NaCl to be limiting concentration for *Z. bisporus* growth. In the control (without NaCl), maximum cell growth was observed (Fig 1.).

Morphological analysis by TEM

The apoptotic cells observed under transmission electron microscope exposed to 1.0M NaCl at different stress intervals, established characteristic ultrastructure features known to be hallmarks of apoptosis (Fig 2). Yeast cells without salt stress treatment (control) were assessed along with cells treated with 1.0M NaCl at three different stress imposition time intervals. The control cell exhibited characteristic ultrastructural morphology of a normal cell with uniform nucleus composed of regular dispersed chromatin and two nucleoli, and cytoplasm rich in rough endoplasmic reticulum (filled with ribosomes, polyribosomes), and mitochondria. The well-developed Golgi complex was located in the cytoplasm in perinuclear areas, and scarce, dense bodies and occasional

autolysosomes were present in the cytoplasm (Fig 2: A).

Cytological observations in cells exposed to 1.0M NaCl for 60 minutes included deeply invaginated, irregular nucleus with dense chromatin margination along the nuclear envelope and nuclear condensation (pyknosis), nuclear fragmentation (multi-nucleation), cytoplasmic extrusion, cell shrinkage, abnormalities of mitochondrial cristae (ruptured and condensed), cell membrane blabbing, holes and formation of apoptotic bodies, increased cellular granularities (dark condensed cytoplasm), and dilated nuclear membranes. The cells exhibited intense vacuolization filled with heavy granulated electron dense materials (Fig 2: B, C).

Cells exposed to stress for 90 minutes (Fig 2: D, E) and 120 minutes (Fig 2: F, G) showed low toxicity to both stress phases compared to control (Fig 2: A). Stress exposure for 120 minutes produced least toxicity compared to 90 and 60 minutes. These features collectively suggest that NaCl-treated *Z. bisporus* demonstrated apoptotic-like cell death (ALCD) mechanism.

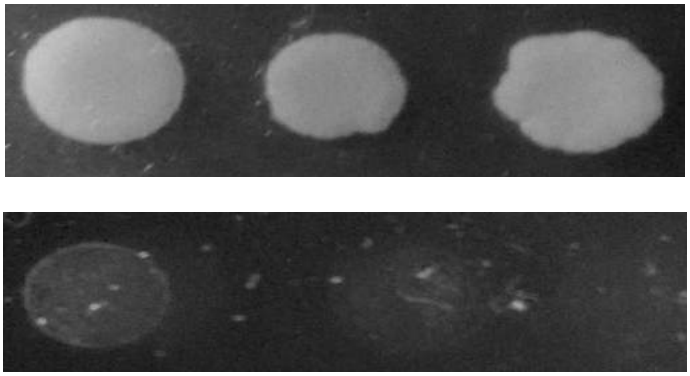
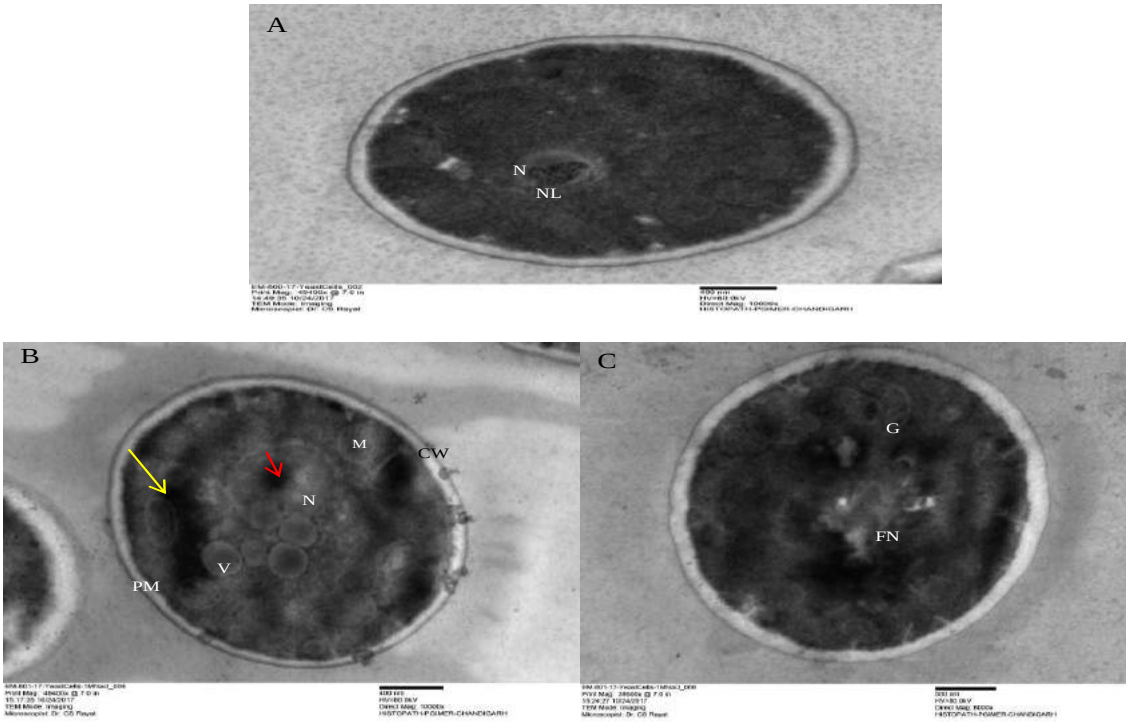


Fig 1: Serial-dilution (SD) colony spotting assay for *Zygosaccharomyces bisporus* sensitivity to NaCl in representative control (upper panel) and stressed (lower panel) cells on Yeast Peptone Dextrose Agar (YPDA) plates



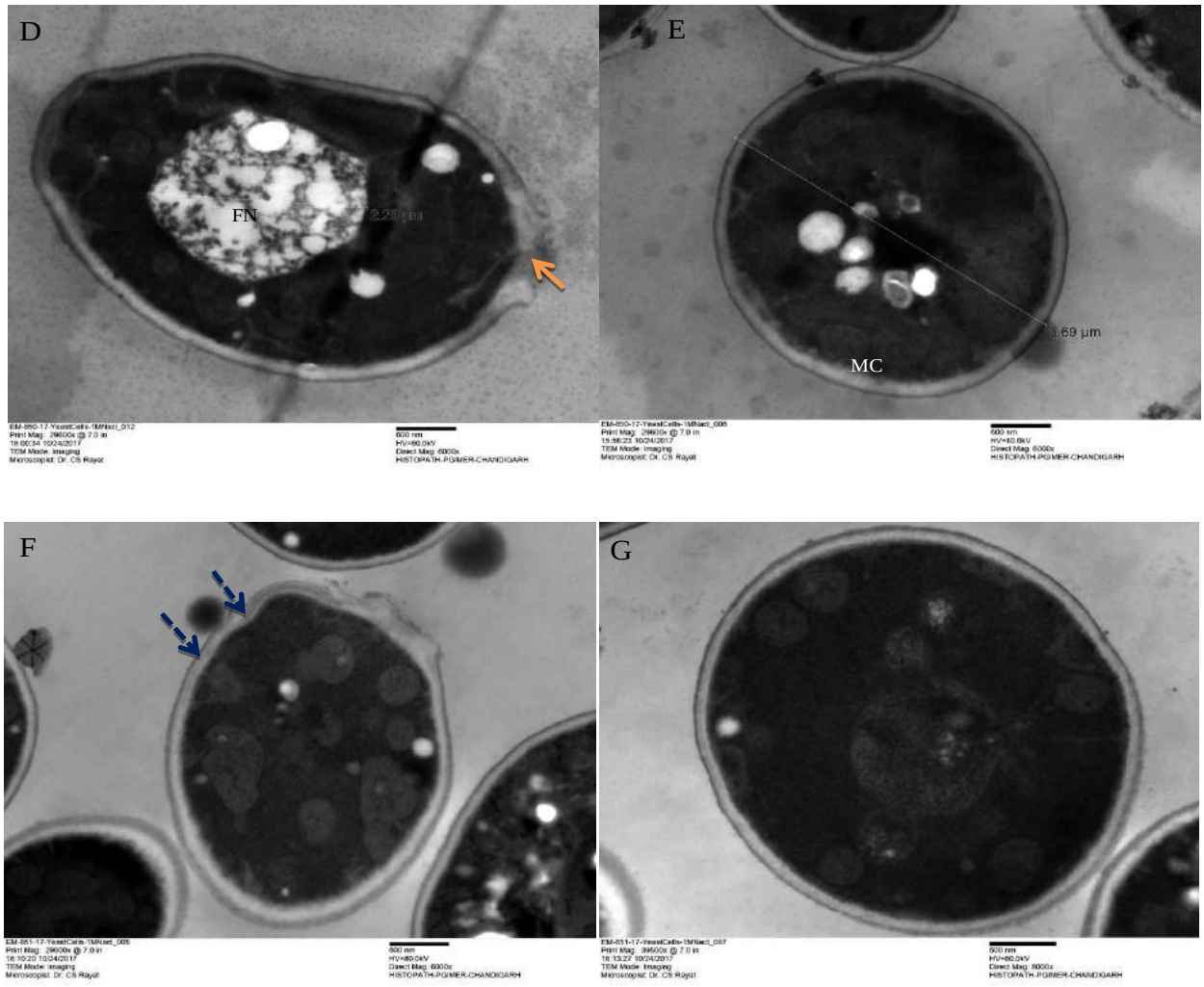


Fig 2: Transmission electron microscopy analysis (TEM) was performed to detect autophagic and apoptotic structures in; Control (A); 1.0 M NaCl treated cells for 60 minutes (B, C); 1.0 M NaCl treated cells for 90 minutes (D, E); and 1.0 M NaCl treated cells for 120 minutes (F, G) depicting time dependent effects of sodium chloride (NaCl) on cytology of *Z. bisporus* cell. Scale bars, 2 µm. Electron micrograph of untreated *Z. bisporus* cells demonstrates normal structure of cell with Nucleus (N), nucleolus (NL) and cytoplasm appearing normal without abnormal changes (A). B and C demonstrated morphological features of early apoptosis; cell shrinkage, chromatin condensation (red arrow), clustering of dysfunctional mitochondria (encircled dotted), and cytoplasmic condensation (yellow arrow). D and E demonstrated the condensed cristae of mitochondria (MC), fragmented nuclei (FN), and cell membrane disintegration as typical morphological features of intermediate apoptosis (orange arrow). F and G demonstrated morphological feature of late apoptosis; continuing blebbing (blue dotted arrow), and little cytoplasmic condensation.

Discussion:

Apoptosis is a well known mechanism of removing senescence and damaged cells as well as a means of adaptation in which yeast cell survives biotic and abiotic stresses. The biochemical changes involving ROS elevation, increased Na^+/K^+ ratio and proteolysis are found to actively participate in PCD signal transduction. Overall resistance to ROS attack is mediated by a combined survival factors such as antioxidant defense system and cellular redox buffering capacity. However, in due course of their low levels, the yeast elicits an essential final response to oxidative stress, initiation of apoptosis.

As observed in other osmo- and halo-tolerant yeasts, this altruistic mechanism tends to clear off old, unhealthy cells, which enhances the chance of survival of healthier and younger clones in stressed cells (20, 21), thereby favorably explaining the affected cell growth and percent survival of *Z. bisporus* under different concentrations of NaCl (stress stimulator) for specified stress exposure intervals in a previous study (6). In the present study, as expected from serial dilution assay, the salt induced osmotic stress reduced

and ultimately inhibited cell growth at highest observed concentration of 1.0M NaCl. Hence, the pattern of salt inhibition was exponential at this sub-lethal concentration in case of *Z. bisporus*. These results are in agreement with Silva-Graca and Lucas (4) for *Candida versatillis,* Sharma and Sharma (6) for *Z. bisporus* and Pribylova et al., (22) for *Z. rouxii*, where it was evident that cell survival reaches to near optimum values (as in pre-stress state) at the end of stress phase allowing growth restoration due to action of stress responsive factors.

The exponential phased cells in 60 minutes salt stress showed alterations and clustering of organelles. This finding explains the phenomenon of salinity induced PCD, previously reported in other halotolerant yeasts (23). Importantly, in TEM micrographs, nuclear condensation, which is one of the hallmark features of apoptosis, was visible soon after the onset of salt stress. Previous studies have suggested that primary salt induced ROS targets the unsaturated fatty acids leading to generation of wide variety of secondary reactive molecules such as autocatalytic lipid radicals and toxic lipid hydroperoxides (24, 25). Moreover, high levels of mitochondrial oxidative burst cause damage to membrane protein thiols via oxidation leading to decrease in membrane potential. These reactive protein and lipid carbonyls produced along the ROS cascade together with increased Na^+/K^+ ratio accumulate during the chronological aging before the appearance of apoptosis. Accordingly, mitochondria contain several pro-apoptotic molecules that directly cleave nuclear DNA and activate cytosolic proteins (24). These factors along with several other pathways cause fragmentation, cross linking, and unfolding of protein backbone and nucleic acids, which condition the cells to undergo apoptosis to efficiently remove irreparably damaged oxidized cell components such as mitochondria.

According to Watson (26), chemostat studies in *Saccharomyces cerevisiae* revealed that NaCl stimulates apoptosis largely due to increased requirement for energy generating substrates involved in the cell maintenance, thus leading to simultaneous decrease in cell growth yield. Our study compared stressed yeast cells with that of control, showing distorted surface morphology with irregular cell wall and plasma membrane. The blabbing and rupturing of cell wall was more pronounced in cultures with higher stress time, particularly at the lethal span of 60 and 90 minutes. It was evident from the TEM micrographs that stressed yeast cells showed significant changes in chromatin distribution, and lacked definite cell membrane with cytoplasmic condensation along the membrane, which might be crucial in explaining the mechanism of salt toxicity. Possibly, salt enters the cell by bounding to specific surface transporters.

The chromatin was either unrecognizable (Figure 2: B) or sequestered towards the outer layer of nuclear envelope (Figure 2: C). The disruption of outer layers and intense chromatin condensation adjacent to nuclear envelope in association with multiple nuclear fragmentations have also been previously reported as an important mechanism of salt mediated inhibition of microorganisms (27-29). These apoptotic phenotypes were rescued over prolonged stress spans by diverting the ATP usage in accumulation of anti-ROS metabolites rather than towards growth, which likely cause regression of ROS accumulation and cell death (6,9,10). Therefore, it confirms the postulated theory that salt induced ion toxicity prompted apoptosis as a component of adaptive response. This concludes that cell death might be a part of regulated adaptive process to ensure cell survival.

Altogether, salinity creates osmotic, ionic, and eventually ROS toxicity which are regulated mechanistically by haloadaptive responses such as compatible osmolyte production and accumulation (6), involvement of ion transporters across plasma membrane and cellular antioxidants like glutathione (9), modulation in cell wall properties, and apoptosis. Therefore, more of these research findings will be beneficial to food manufacturing industries since exhaustive information about food spoilage microbiota and their target foods, along with optimal growth conditions, can assist in choosing suitable natural preservatives, absolute processing techniques, and aids in improving safety issues, and controlling spoilage outbreaks. Additionally, the halophilic and halotolerant fungi are distinguished eukaryotic models for study of salt resistance owing to their characteristic growth rate in a wide range of pH, osmotic and ionic concentrations, including toxic drugs (29, 30).

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Disclosure statement:

No conflict of interest is declared

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Short Communication **Open Access**

Prevalence of intestinal helminthic infections among secondary school students in Edo State, Nigeria

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Abstract:

Background: Intestinal helminthic infections are generally common in children accounting for the largest disability adjusted life years (DALYs) of all the parasitic agents. In this study, we determined the prevalence of intestinal helminthic infections among secondary school students in a semi-urban community in Edo State, Nigeria.
Methodology: A descriptive cross sectional study of 489 students from four secondary schools in Esan West Local Government Area of Edo State, Nigeria was conducted between December 2018 and July 2019. The schools were selected by stratified random sampling and all eligible students in each school were enrolled. Stool samples were collected from each student into sterile universal bottle and direct wet mount as well as formol-ether concentrated samples were examined under compound light microscope at the Animal and Environmental Biology Laboratory of the University of Benin, Benin City, Nigeria. Structured questionnaire was administered to collect data on socio-demographic and potential risk factors for helminthic infection. Data were analysed with SPSS version 22.0 and associations between variables compared using Chi square or Fischer exact test, with $p<0.05$ as significant value.
Results: Of the total 489 students examined (261 males, 228 females), 6 had intestinal helminthic infection, giving an overall prevalence rate of 1.2%, with 0.7% (n=2) in males and 1.8% (n=4) in females ($p=0.4244$). Three helminthic parasites, *Ascaris lumbricoides, Trichuris trichiura* and *Ancylostoma duodenale* were identified. All the 6 subjects with helminthiasis had been de-wormed at interval of more than 1 year. Regular hand washing practices ($p<0.025$) and de-worming interval of 1 year or less ($p=0.000$) were factors significantly associated with low prevalence of helminthic infection while other risk factors were not significantly associated.
Conclusion: The study shows low prevalence of intestinal helminthic infections among secondary school students in Esan West LGA, Edo State, Nigeria. The high level of hand hygiene practices among the participants may account for the low prevalence, which emphasizes the effective role of hygiene practices in the control and elimination of intestinal parasitic infections. De-worming of students at regular interval (yearly) is recommended.

Keywords: Intestinal helminth, Prevalence, risk factors, hand hygiene, de-worming

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Prévalence des infections helminthiques intestinales chez les élèves du secondaire dans l'État d'Edo, au Nigéria

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Abstrait:

Contexte: les infections helminthiques intestinales sont généralement fréquentes chez les enfants représentant les

plus grandes années de vie corrigées de l'incapacité (Avci) de tous les agents parasitaires. Dans cette étude, nous avons déterminé la prévalence des infections helminthiques intestinales chez les élèves du secondaire dans une communauté semi-urbaine de L'État D'Edo, au Nigeria
Méthodologie: une étude transversale descriptive de 489 étudiants de quatre écoles secondaires dans la zone de Gouvernement Local D'Esan West de L'État D'Edo, au Nigeria, a été menée entre décembre 2018 et juillet 2019. Les écoles ont été sélectionnées par échantillonnage aléatoire stratifié et tous les élèves admissibles de chaque école ont été inscrits. Des échantillons de selles ont été prélevés chez chaque étudiant dans une bouteille universelle stérile et une monture humide directe ainsi que des échantillons concentrés de Formol-éther étaient examinés au microscope optique composé au Laboratoire de Biologie animale et environnementale de L'Université du Bénin, Benin City, Nigeria. Un questionnaire structuré a été administré pour recueillir des données sur les facteurs de risque sociodémographiques et potentiels d'infection helminthique. Les données étaient analysées avec la version SPSS 22.0 et les associations entre les variables comparées à L'aide du Chi carré ou du test Fischer exact, avec $p<0,05$ pris comme valeur significative.
Résultats: sur les 489 étudiants examinés (261 hommes, 228 femmes), 6 avaient une infection helminthique intestinale, ce qui donne un taux de prévalence global de 1,2%, avec 0,7% (n=2) chez les hommes et 1,8% (n=4) chez les femmes ($p=0,4244$). Trois parasites helminthiques, *Ascaris lumbricoides*, *Trichuris trichura* et *Ancylostoma duodenale* ont été identifiés. Tous les 6 sujets avec d'helminthiase avaient été vermifugés à intervalle de plus d'un an. Les pratiques régulières de lavage des mains ($p<0,025$) et l'intervalle vermifuge de 1 an ou moins ($p=0,000$) étaient des facteurs significativement associés à une faible prévalence de l'infection helminthique alors que d'autres facteurs de risque n'étaient pas significativement associés.
Conclusion: L'étude montre une faible prévalence des infections helminthiques intestinales chez les élèves du secondaire à Esan West LGA, dans L'État D'Edo, au Nigeria. Le niveau élevé de pratiques d'hygiène des mains chez les participants peut expliquer la faible prévalence, ce qui souligne le rôle efficace des pratiques d'hygiène dans le contrôle et l'élimination des infections parasitaires intestinales. Il est recommandé de déparasiter les élèves à intervalles réguliers (chaque année).

Mots-clés: helminthes intestinaux, prévalence, facteurs de risque, hygiène des mains, vermifuge

Introduction:

Gastro-intestinal helminthic infections are common parasitic infections globally and occur more frequently in children. They are caused by some species of nematodes, with over 2 billion people reportedly infected world-wide. They account for the highest number of infection burden in form of disability adjusted life years (DALYs) among parasitic disease causing agents (1). The role of environmental determinants, improved water supply, and impact of interventions such as increased availability of public and effective waste disposal system/facilities have been widely advocated (2,3). In reality, these suggestions have attracted relatively little or no attention especially in developing countries, which are endemic for these infections.

Meanwhile there are number of reasons to believe that the rate of intestinal helminth infection has decreased over the years. These include increased level of hygiene practice among individuals, proliferation of more private and clean public schools who often uphold good sanitary conditions (4), accessibility, availability, effectiveness of anti-helminthic drugs, and decreasing prevalence reports (5-10). With the burden of infection highest in children, efforts over the years have concentrated on primary school population. The present study therefore was aimed at determining the prevalence pattern in a population of secondary school students and highlighting the possible risk factors associated with acquisition of these infections.

Materials and methods:

Study area and setting

This study was carried out from December 2018 to July 2019 in four (4) post primary schools located in Esan West Local Government Area of Edo State, Nigeria (Fig. 1). They include three public and one private school (Ujoelen Grammar School, Eguare Secondary School, Ogwa Secondary School and Christ is the Answer Secondary School). The population of Esan West Local Government Area in the last 2006 National Population Commission report, stood at 77,483. The area is dominated by a tropical climate with high temperature, humidity and heavy rainfall. It is located on a relatively flat plateau referred to as the Esan plateau and it is approximately 466m above the sea level with two distinct characteristic seasons, wet season (between March to November) and dry season (November to February) (11). The water aquifer of the study area is very low and is put at approximately 150m, thus inhabitants depend more on water stored in wells and rain water for their water needs.

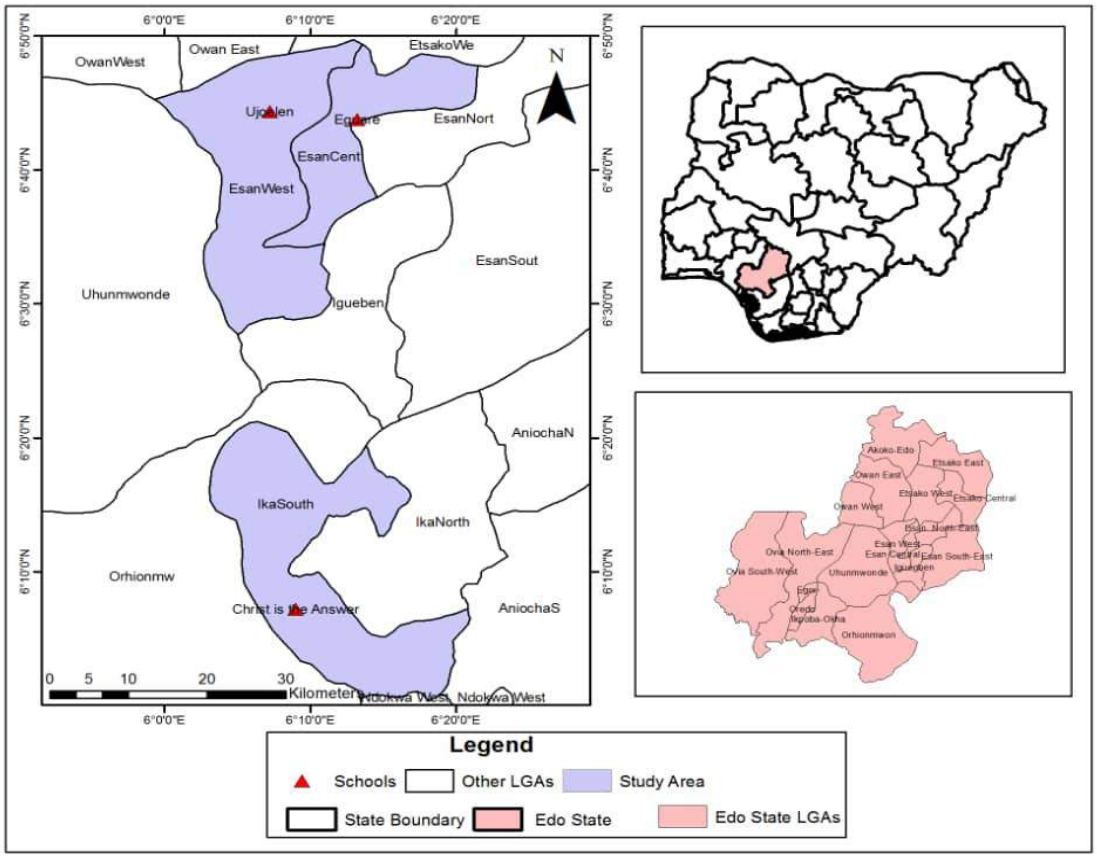

Fig 1: Esan West LGA showing the study location

Ethical clearance

Prior to the survey, ethical clearance was obtained from the Ministry of Education in Esan West Local Government Area Council with reference no EW/LGM BEE/001/9. Consent from the principals of the schools and representatives of the parents and teachers associations (PTA) were also obtained after due consultations.

All procedures contributing to the study complied with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975 as revised in 2008 and the author asserts that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

Subjects and sample size determination

The sample size for the study was determined using the formula of Charan and Biswas (12) for simple independent proportion with a mean prevalence of 25% from previous studies (13-15) in the study area. The calculated sample size was 288 but after adjusting for non-response rate, the sample size was increased to 489.

Sampling technique

The stratified random sampling technique was used first with random selection of four secondary schools in the local government area (LGA), and then enrolment of all students in each of the school. A total of 160 students were enrolled from Ujoelen Grammar School, 160 from Eguare, 85 from Ogwa and 84 from "Christ is the Answer" Secondary Schools.

Inclusion and exclusion criteria

The inclusion criteria were secondary school students who gave informed assent or whose parents/guardians gave informed consent. Subjects with gastrointestinal disorders manifesting as diarrhoea, dysentery, abdominal pain, bloating and nausea, and those who have received anti-parasitic drugs two weeks preceding the study were excluded.

Sample collection and examination

Each enrolled student was given a sterile specimen bottle for stool sample collection, which after collection was immediately transported to the Department of Animal and Environmental Biology Laboratory of the University of Benin, Benin City, Nigeria, for examination by direct wet mount preparation and Formol-ether concentration methods as previously described (16). This procedure was done over a period of 2 days in each school visited (17).

Data collection by questionnaire

Structured questionnaires were administered to all student participants to obtain socio-demographic data such as occupation of parents/guardians, family size, toilet types, household water sources and other information necessary to access potential risk factors.

Statistical analysis

Data were analysed with SPSS version 22.0, and association between variables were compared using Chi square (X^2) or Fischer exact test, with significant association set at $p<0.05$.

Results:

A total of 489 students (age range 10-17 years) from 4 randomly selected secondary schools in Esan West LGA of Edo State were examined for intestinal helminth infections, with 261 (53%) males and 228 (46.6%) females. A total of 160 students (74 males, 86 females) were from Ujoelen Grammar School; 160 (71 males, 89 females) from Eguare Secondary School; 85 (51 males, 34 females) from Ogwa Secondary School; and 84 (65 males, 19 females) from "Christ is the Answer" Secondary School (Table 1).

Table 1: Distribution of subjects by gender and schools in Esan West LGA, Edo State, Nigeria

School/ Gender	Male (%)	Female (%)	Total (%)
UGS	74 (46.3)	86 (53.7)	160
ESS	71 (44.4)	89 (55.6)	160
OSS	51 (60)	34 (40)	85
CSS	65 (77.4)	19 (22.6)	84
Total	261 (53.4)	228 (46.6)	489 (100)

UGS=Ujoelen Grammar School; ESS=Eguare Secondary School; OSS=Ogwa Secondary School; CSS=Christ is the Answer Secondary School

Of the total 489 students, 6 had intestinal helminthic infection, giving an overall prevalence rate of 1.2%. The prevalence of 0.7% (n=2) in the males was not significantly different from 1.8% (n=4) in the females (p=0.4244) and there was also no significant difference in the distribution of intestinal helminthiasis among the different age groups (p>0.05) (Table 2).

Of the 6 students with infection, 3 were from Ujoelen Secondary School, 2 from Ogwa Secondary School, and 1 from "Christ is the Answer" Secondary School. None of the students from Eguare Secondary School had intestinal helminthiasis. In total, 3 helminthic parasites, *Ascaris lumbricoides, Trichuris trichura* and *Ancylostoma duodenale* were identified with frequency of 0.8%, 0.2%, and 0.2% respectively (Table 3).

Table 3: Parasite types identified among secondary school students in Esan West LGA, Edo State, Nigeria

Parasite types	Male (%) (n=261)	Female (%) (n=228)	Total (%) (n=489)
A. lumbricoides	2 (0.8)	2 (0.9)	4 (0.8)
T. trichura	-	1 (0.4)	1 (0.2)
An. duodenale	-	1 (0.4)	1 (0.2)
Total	2 (0.8)	4 (1.8)	6 (1.2)

A=Ascaris; *T=Trichuris*; *An=Ancylostoma*

Table 2: Prevalence of helminthic infections among secondary school students with respect to age group and gender in Esan West LGA, Edo State, Nigeria

Age group (years)	Male		Female		Total	
	No examined	No infected (%)	No examined	No infected (%)	No examined	No infected (%)
10 – 12	60	–	51	2 (3.9)	111	2 (1.8)
13 – 15	117	1 (0.9)	101	1 (1.0)	218	2 (0.9)
16 – 17	84	1 (1.2)	76	1 (1.3)	160	2 (1.3)
Total	261	2 (0.7)	228	4 (1. 8)	489	6 (1.2)

Table 4: Factors associated with helminthic infections among secondary school students in Esan West LGA, Nigeria

Factors	Subjects		*p* value
	No examined (%) (n=489)	No infected (%) (n=6)	
Father's occupation			
Farmer	87 (17.8)	1 (1.2)	
Teacher	44 (9.0)	–	0.454
Artisan	111 (22.7)	3 (2.7)	
Trader	247 (50.5)	2 (0.8)	
Mother's occupation			
Farmer	49 (10.0)	–	
Trader	271 (55.4)	4 (1.5)	0.682
Artisan	93 (19.0)	2 (2.2)	
Teacher	76 (15.5)	–	
Type of toilet system			
Water closet	182 (37.2)	3 (1.7)	
Pit latrine	169 (34.6)	2 (1.2)	1.000
Open defecation	101 (20.7)	1 (1.0)	
Stream/river	37 (7.8)	–	
Sources of water			
Bore hole	16 (3.3)	–	
Well/Rain	384 (78.5)	6 (1.6)	1.000
Stream	38 (7.8)	–	
River	51 (10.4)	–	
Hand washing			
Yes (regularly)	151 (30.9)	1 (0.7)	
Sometimes	336 (68.7)	4 (1.2)	0.025*
No	2 (0.4)	1 (50.0)	
Onychophagy			
Yes	329 (67.3)	4 (1.2)	1.000
No	160 (32.7)	2 (1.3)	
Frequency of bathing			
Once daily	198 (40.5)	2 (1.0)	0.291
Twice daily	271 (55.4)	3 (1.1)	
Others	20 (4.1)	1 (5.0)	
Chewing money/items			
No	401 (82.0)	6 (1.5)	0.535
Yes	88 (18.0)		
Sanitary materials after passing faeces			
Tissue paper	119 (24.3)	1 (0.8)	
Water	100 (20.5)	–	0.109
Leaves	70 (14.3)	2 (2.9)	
Paper	151 (30.9)	1 (0.7)	
None	49 (10.0)	2 94.1)	
Family size			
<5	118 (24.1)	3 (2.5)	0.429
5-7	140 (28.6)	1 (0.7)	
>7	231 (47.2)	2 (0.9)	
Duration of residence in study area			
> 5 years	214 (43.8)	1 (0.5)	
5 years	124 (25.4)	3 (2.4)	0.141
2 years	72 (14.7)	2 (2.8)	
1 year	79 (16.2)	–	
Habit of washing fruits before eating			
Yes	366 (74.9)	4 (1.1)	1.000
No	123 (25.2)	2 (1.6)	
Consumption of raw aquatic food			
Yes	256 (52.4)	3 (1.2)	1.000
No	233 (47.7)	3 (1.3)	
De-worming interval			
3 months	311 (63.6)	–	0.000*
6 months	87 (17.8)	–	
12 months	91 (18.6)	6 (6.6)	

* Statistically significant

Table 4 shows the risk factors associated with prevalence of helminthic infections in the study population. Hand-washing practices and de-worming interval were the two factors associated with prevalence of helminthic infection. Most subjects in the study usually (n=151) or sometimes (n=336) perform handwashing while only few (n=2) do not perform hand-washing at all. Subjects who do not perform handwashing had significantly higher prevalence of helminthiasis (50%) than those who usually (1.2%) or sometimes (0.7%) perform handwashing (p<0.025). The prevalence of helminthic infection in subjects who were de-wormed at 3 months, 6 months and more than

1 year interval are 0% (0/311), 0% (0/87) and 6.6% (6/91) respectively, which showed significantly higher prevalence of helminthiasis in subjects with de-worming interval of more than 1 year (p=0.000). There was no significant association between prevalence of helminthiasis and other assessed risk factors (p>0.05).

Discussion:

This study reports a low prevalence rate (1.2%) of intestinal helminthic infection among secondary school students in Ekpoma, Edo State, Nigeria. Although, there have been many studies of helminthiasis among primary school children and other population groups such as pregnant women and students of tertiary institution in the same community (14,15,18), ours is the first survey among secondary school students in this environment. This rate is lower compared to those of Kamalu and colleagues (19) who reported 43% prevalence rate in a mixture of urban and rural high school students in Imo State, Nigeria. The rate is also lower than the previous study of Edogiawerie et al., (15) with 18.7% rate among primary school pupils. High prevalence rates have also been reported on different population groups in other studies from same community and elsewhere such as 33.3% (13), 24.5% (14), 12.5% (18) and 35.9% (21).

Meanwhile, a low prevalence (9.5%) of helminthiasis was reported among children attending a tertiary hospital in Benin City, the state capital which is about 80km from the study area (22), and therefore a much lower prevalence is likely in older population since evidence supports decrease in prevalence with increasing age (8,23,24). As there are recent reports of decreasing trend in the prevalence of intestinal helminthic infection across the country (8-10,25) compared to previous reports (6,7,24), the low prevalence rate reported in our study supports this trend. In addition, Salawu and Ughele (25) previously observed a decreasing pattern of helminth infection with increasing age because behavioural changes relating to hygiene and contact with soil improves compared to younger population whose behaviour may encourage frequent contact with helminth eggs in the soil (26). From our observation, more students reportedly practice good personal hygiene and regular hand washing.

Three helminthic parasites were identified in our study which is similar to previous report from a study on primary school children (25), and also to those of Ogbaini-Emovon (21) and Foghi and Nzeako (27), except for the presence of *Strongyloides stercoralis* and *Enterobius vermicularis* in their studies but absent in ours. Similarly, Isibor and colleagues (14) reported three helminth parasites including *Ascaris*, hookworm and *Trichuris*, with an additional protozoan, *Entamoeba histolytica*. However, our findings differs from those of Edogiawerie et al., (15) who identified seven helminthic parasites, and Omorondion et al., (18), who identified five helminths including *Taenia solium*, and two protozoan parasites in pregnant women and students of higher institution in the study area.

Between gender, females were more infected than males (though this was not significant) which corroborated earlier reports (14,15,28) but contrasted the report of Nmor and Oguanya (13,27). However, this gender observation should be taken with caution as the numbers of positive cases were too few for useful statistical inference. Nevertheless, infectivity has been said to be multifactorial and may not necessarily be influenced by gender, but other factors especially external or environmental may increase exposure of either gender to parasitic infection depending on level of contamination of the environment, personal hygiene and other infection prevention practices. It is also believed that age play an important role in the prevalence and infection pattern of intestinal helminths (29,30), and the age group 10-12 years were more infected in our study than other age groups. However, the number of positive cases in this study is too low to infer that age-group is a key factor in the prevalence and infection pattern of intestinal helminths.

Although most other factors investigated in our study did not influence the prevalence of helminthiasis significantly, hand washing practice was one factor that significantly influenced the prevalence. The prevalence of helminthiasis was high (50%) among those who do not perform hand washing at all, and low among those who sometimes (1.2%) and who regularly (0.7%) perform hand washing. Hand hygiene practices have been reported to hugely impact transmission of intestinal parasitic agents as eggs of parasites can attach to fingers or trapped in nails after defaecation or contact with contaminated soil (22). This in turn completes the transmission cycle enabling the maintenance of such parasites in the environment through continued infection of hosts who engage in poor hand hygiene practices. Our study agrees with that reported by Salawu and Ughele (25).

In conclusion, this study reports low prevalence of intestinal helminthiasis among secondary students in Esan West LGA, Edo State, Nigeria. The high level of hygiene practices among the student participants may be responsible for this, and highlights the importance of handwashing in the control and elimination of intestinal parasitic infections. Together with sustained yearly de-worming of students, successful control and prevention of intestinal helminthiasis can be achieved in this locality.

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Conflict of interest:

No conflict of interest is declared

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Corrigendum

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

[1*]Zatout, A., [2]Djibaoui, R., [2]Kassah-Laouar, A., and [3]Benbrahim, C.

Corrigendum to: Afr. J. Clin. Exper. Microbiol. 2020; 21 (1): 21-29. https://dx.doi.org/10.4314/ajcem.v21i1.3

There was an error made by the authors in the published article where 'oxacillin' instead of 'cefoxitin' was used in many places to report methicillin resistance in coagulase negative staphylococci. 'Oxacillin' should be corrected as 'cefoxitin' throughout the article.

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

[1*]Zatout, A., [2]Djibaoui, R., [2]Kassah-Laouar, A., et [3]Benbrahim, C.

Rectificatif à: Afr. J. Clin. Exper. Microbiol. 2020; 21 (1): 21-29. https://dx.doi.org/10.4314/ajcem.v21i1.3

Il y avait une erreur commise par les auteurs dans l'article publié où «oxacilline» au lieu de «céfoxitine» était utilisée dans de nombreux endroits pour signaler la résistance à la méthicilline dans les staphylocoques à coagulase négative. «Oxacilline» doit être corrigé en «céfoxitine» tout au long de l'article.

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1. Nsanze, H. Recommendation for management of gonorrhoea and genital ulcers in Africa. Afr J Sex Transm Dis. 1984; 1:5-7

2. Odugbemi, T. O., and Arko, R. J. Differentiation of *Kingella denitrificans* and *Neisseria gonorrhoeae* by growth on a semi solid medium and sensitivity to amylase J Clin Microbiol. 1983; 17: 389-391

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

For books:

4. Arya, O. P., Osoba, A. O., and Bennett, P. Tropical Venereology, Churchill Livingstone, Edinburgh, 1980 OR when referring to a chapter in a book and where the names of authors are also given, the reference should be as follows:

5. Easmon, C. S. F. Host-Parasite relationship in experimental staphylococcal infections. In: Macdonald, A., and Smith, G. (eds). The Staphylococci. University Press, Aberdeen 1981: 63-72

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