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Review Article **Open Access**

Prevalence of sulfate reducing bacteria in oral cavity: a narrative review

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Abstract:

Sulphate is used as terminal electron acceptor for the respiration of sulphate reducing bacteria (SRB) forming a specialized group of microbes. SRB have been known to cause microbiologically induced corrosion by forming metallic sulfides and oxides in the process of their dissimilatory respiration. Since oral cavity provides a conducive environment for corrosion, presence of SRB in oral cavity and their corrosive potential needs to be assessed. This article provides a narrative review of the available literature with the primary objective of evaluating the presence of SRB in oral cavity of patients.

Keywords: SRB; corrosion, oral cavity, patients

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Prévalence des bactéries sulfato-réductrices dans la cavité buccale: bilan narratif

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Abstrait:

Le sulfate est utilisé comme accepteur d'électrons terminal pour la respiration des bactéries réductrices de sulfate (SRB) formant un groupe spécialisé de microbes. On sait que le SRB provoque une corrosion induite par la microbiologie en formant des sulfures et des oxydes métalliques au cours du processus de respiration dissimilatoire. Étant donné que la cavité buccale crée un environnement propice à la corrosion, la présence de SRB dans la cavité buccale et leur potentiel corrosif doivent être évalués. Cet article fournit une revue narrative de la littérature disponible avec l'objectif principal d'évaluer la présence de SRB dans la cavité buccale des patients

Mots-clés: SRB; corrosion, cavité buccale, patients

Introduction:

Microorganisms play an important part in sulphur transformations. Sulphur which occurs in nature as pyrite, gypsum or sulphate is either taken up as a nutrient for metabolic energy such as sulphide oxidation by chemolithotropic sulphur bacteria and dissimilatory sulphate reduction by sulphate-reducing bacteria (SRB) or for producing sulphur containing amino acids or enzymes. SRB have been known to cause microbiologically induced corrosion by forming metallic sulfides and oxides in the process of their dissimilation (1, 2, 3). In accordance with this, the role of SRB in causing metal corrosion is of important concern in oral cavity where a lot of metallic components are used.

Assessing the prevalence of SRB in oral cavity is of utmost importance with reference to the same. This article provides a narrative review of the available literature with the primary objective of evaluating the presence of SRB in oral cavity of patients.

Materials and methods:

We searched PubMed and Embase databases. The search covered the period 1980 to March 2018. The main terminologies used for search were; "sulfate reducing bacteria and oral or dental or periodontal or saliva" AND "presence or prevalence of SRB and oral or dental or periodontal or saliva". We identified 123 articles in PubMed and 26 in Embase with these key words. Of these, 78 articles were selected after eliminating duplications. These 78 articles were screened and 68 articles were eliminated based on relevancy. The remaining 10 articles were further scrutinized and the article by Costinar et al., (4) was eliminated since it was related to dogs' oral cavity. The remaining 9 articles were included in the review (Fig 1).

Results:

Description of studies

Nine articles were finally selected for inclusion in the review. There were two major population groups studied, one involved healthy oral cavity and the other periodontally compromised. One article by Paster et al., (7) included samples from refractory periodontitis, acute necrotizing ulcerative gingivitis (ANUG) and HIV patients in addition to healthy and periodontitis patients. The sample size varied from 5 to 118. The details of the study representing the sample size, sampling sites and percentage of prevalence are given in Table 1.

Sampling sites and methods

The sampling sites were dorsum of tongue both anterior and posterior, palate, buccal mucosa, vestibular mucosa, supra and subgingival plaque for healthy patients. For periodontitis cases, the samples were taken from the deepest pocket. In the studies by Heggendorn et al., (5, 6), sampling was done on saliva in one study and from tooth fragment in another study. The samples were placed in reduced medium to facilitate the survival and growth of anaerobic bacteria in all of the studies with Postgate medium E being the most being the most common choice

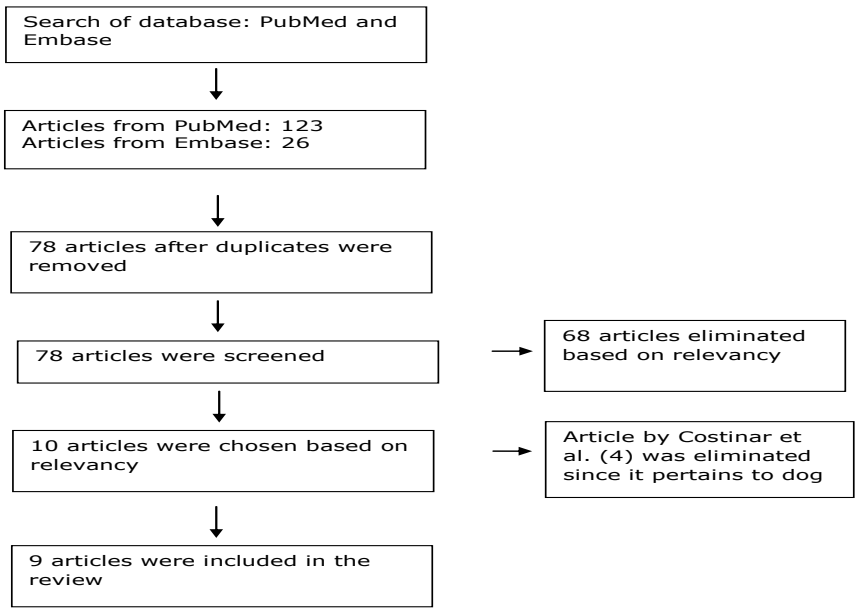

Fig 1: Flowchart for search and selection process

Table 1: List of articles selected for review

Study	Sample size	Groups	Sampling sites	Prevalence rate
Paster et al., 2001	31	Healthy (5) Periodontitis (9) Refractory periodontitis (11) ANUG(4) HIV (2)	Subgingival plaque from four most diseased or deepest site	Percentage not given but positive in periodontitis, refractory periodontitis and ANUG cases
Heggendorn et al., 2013	118	Healthy and periodontitis with other subgroups of gastritis, diabetes	Saliva	29.66%
Heggendorn et al., 2014	8	Healthy and periodontitis	Tooth fragment ,healthy tooth extracted tooth for orthodontic purpose	37.5%
Langendijk et al., 1999	41	Healthy and periodontitis	Pooled sample from posterior anterior palate, vestibular mucosa, dorsum of tongue, supragingival area, around the opening of parotid duct, gingival sulci deep pockets	Site wise: 10% in mucosa of healthy and periodontitis, 22% for tongue and supragingival, 86% for periodontal pockets
Langendijk et al., 2001	168	Periodontal SRB compared with other spirochetes	Subgingival plaque	SRB and *Porphyromonas* co-existed in 32% cases
van der Hoeven et al., 1995	43	Periodontitis	Subgingival plaque	58% had SRB
Langendijk et al., 2001	83	Periodontitis	Deepest area of the pocket	49% patients had SRB
Willis et al., 1995	12	Healthy	Posterior and anterior tongue, mid buccal mucosa, vestibular mucosa, supra and subgingival plaque	83%
Vianna et al., 2008	102	Healthy and periodontitis	Subgingival plaque	41.2% in periodontitis patients, insignificant presence in healthy

Description of outcome

Of the nine studies, seven gave the percentage prevalence of SRB in healthy and periodontitis patients, while the study by Paster et al., (7) reported the prevalence of SRB in association with periodontitis, refractory periodontitis, ANUG and HIV patients. The study by Langendijk et al., (8) assessed the co existence of SRB and *Porphyromonas gingivalis* in periodontitis patients.

***Saliva**:* One study did the sampling on saliva (5). Of 118 saliva samples collected, 35 were positive for the presence of SRB. Three positive samples were randomly chosen to identify the species of SRB by PCR and sequencing. The three selected samples were identified as *Desulfovibrio fairfieldensis*, *Desulfovibrio desulfuricans* and *Raoultella ornithinolytica*.

***Pooled sample**:* Two of the studies (9, 10) did a pooled sampling from posterior and anterior tongue, mid buccal mucosa, vestibular mucosa, supra and subgingival plaques. The study by Langendijk et al., (9) reported site-wise prevalence of 10% in mucosa of healthy and periodontitis, 22% for tongue and supragingival, and 86% for periodontal pockets.

***Subgingival**:* The study by Langendijk et al., (8) reported a prevalence percentage of 32% along with co-existence with *Porphyromonas gingivalis*. Van der Hoeven et al., (11) reported 58% for subgingival plaque. Vianna et al., (12) reported a prevalence of 41.2% in periodontitis patients while insignificant presence in healthy persons.

***Deep pockets**:* Langendijk (13) reported a prevalence of 49% for deep pockets.

Discussion

In this narrative review, we included nine articles describing the presence of SRB in oral cavity of healthy and periodontitis individuals. Almost all the articles have reported positively on the existence of SRB in oral cavity. Though the presence is more in periodontitis patients, healthy individuals also had presence of SRB. This has an important relevance since SRB has been associated with biological corrosion (1,2,3).

SRB are anaerobic microorganisms that are widespread in anoxic habitats, where they use sulphate as a terminal electron acceptor for the degradation of organic compounds, resulting in the production of sulphide (10). The sulphate ion acts as an oxidizing agent for the dissimilation of organic matter in sulphate reduction process like oxygen in conventional respiration. Under anaerobic conditions, a metal surface acts as the anode in an electrochemical reaction and is oxidized, yielding Me^{2+} ions. Bacteria produce S^{2-} ions, which enter into a reaction with Me^{2+} ions, resulting in the formation of metal sulfide. In the cathode area, H^{+} ions are produced and they react with hydroxyl groups. As a result, oxygen from sulfates is consumed for oxidizing metals which leads to the formation of metal oxides thereby corroding metals.

It has been documented that SRBs are capable of corroding metals like stainless steel and titanium intraorally (14, 15). There are very few literatures assessing the prevalence of SRB in oral cavity. More studies with larger sample size and randomized controlled trials (RCTs) are needed to have a clear understanding of the prevalence of SRB in oral cavity whether in healthy or periodontitis patients. With SRB being associated with microbiologically induced corrosion, it becomes even more relevant due to the use of metallic implants and other metallic materials in the oral cavity.

Conclusion

SRB is present in the oral cavity of both healthy and periodontitis patients but more commonly in periodontitis patients compared to healthy persons. The prevalence rates varied with studies. With the current knowledge on the prevalence of SRB in oral cavity, more quality studies, preferably RCTs with large sample size are needed in the future to evaluate the corrosive potentials of SRB on the metallic materials used intraorally.

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Original Article **Open Access**

Histopathological studies on kidney and liver of albino rat infected with toxigenic *Aspergillus flavus* after treatment with isolated *Lactobacillus* species from *Kunu*

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Abstract

Background: Aflatoxin is a metabolic product of *Aspergillus flavus* that causes several injuries to vital organs in the body.
Methods: The liver and kidney tissue of healthy rats challenged with toxigenic *A. flavus* after treatment with *Lactobacillus plantarum* and *Lactobacillus delbrueckii* were examined.
Results: The weight of the liver (3.61 g) and kidney (11.33 g) of infected rats with toxigenic mould were significantly reduced ($P<0.05$) when compared to the group treated with *Lactobacillus* spp.; BD+AP+LP, BD+AP+LD and BD+ AP+LPD. The rats fed basal diet and *Lactobacillus* spp. have a normal histological structure. Necrotic lesions, thickening of the glomerular basement membrane and collapse of the glomerulus were observed in the liver and kidney of rats induced with *A. flavus*. The rats infected with *Lactobacillus* spp. regained their strength and activity after treatment but showed mild necrosis in the liver and thickening of glomerular basement in the kidney.
Conclusions: The use of *Lactobacillus* species suppressed the growth and eliminated the potential risk of toxigenic *A. flavus* in the infected rats. This showed that *Lactobacillus* spp. possess some therapeutic properties due to their ability to secret secondary metabolites. The bioactive compounds can be exploited and used in food products to inhibit the growth of food borne pathogens.

Keywords: Aflatoxins, Lactic Acid Bacteria, Bio-control, Fermented beverage.

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Études histopathologiques sur les reins et le foie de rats albinos infectés par *Aspergillus flavus* après un traitement avec des espèces isolées de *Lactobacillus* de Kunu

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Abstrait

Contexte: L'aflatoxine est un produit métabolique d'*Aspergillus flavus* qui provoque plusieurs lésions des organes vitaux du corps
Méthodes: Les tissus hépatique et rénal de rats sains mis au défi par *A. flavus* toxigénique après un traitement par *Lactobacillus plantarum* et *Lactobacillus delbrueckii* ont été examinés.
Résultats: Le poids du foie (3,61 g) et du rein (11,33 g) des souris infectées atteintes de moisissure toxigénique était significativement réduit (P <0,05) par rapport au groupe traité par *Lactobacillus* spp. BD + AP + LP, BD + AP + LD et BD + AP + LPD. Les rats nourris avec un régime alimentaire de base et *Lactobacillus* spp. avoir une structure histologique normale. Des lésions nécrotiques, un épaississement de la membrane basale glomérulaire et un collapsus du glomérule ont été observés dans le foie et les reins de rats induits par *A. flavus*. Les souris infectées par *Lactobacillus* spp. ont retrouvé leur force et leur activité après le traitement, mais ont présenté une légère nécrose du foie et un épaississement du socle glomérulaire dans le rein
Conclusions: L'utilisation d'espèces de *Lactobacillus* a inhibé la croissance et éliminé le risque potentiel de toxine *A. flavus* toxigenic chez les souris ingérées. Cela a montré que *Lactobacillus* spp. possèdent certaines propriétés thérapeutiques en raison de leur capacité à sécréter des métabolites secondaires. Les composés bioactifs peuvent être exploités et utilisés dans des produits alimentaires pour inhiber la croissance d'agents pathogènes d'origine alimentaire

Mots-clés: Aflatoxines, Bactéries Lactiques, Bio-contrôle, Boisson fermentée

Introduction

Mycotoxins are secondary metabolites produced by some fungi. The fungal toxins; aflatoxins, fumonisins, ochratoxins, patulin, trichothecenes and zearalenone cause suppression of immune system, acute and chronic hepatocellular injury in animal or human (1). Nowadays, there is public concern about the type of mycotoxin produced by moulds in food. Aflatoxin is an important mycotoxin, commonly produced by *Aspergillus flavus* and *Aspergillus parasiticus* in food, unlike *Aspergillus nomius*, *Aspergillus bombycis*, *Aspergillus pseudotamari* and *Aspergillus ochraceoroseus* that are aflatoxigenic but less encountered in food. Biosynthesis of fungal toxin is highly influenced by fungal species, humidity, temperature, inadequate drying of the crops and type of foods (2). Food crops may come in contact with any of the phytopathogenic fungi on the field, during harvesting, post-harvest, storage or when processing and thus, adversely affect the quality of food products (3).

Consumption of food containing aflatoxigenic fungi or fungal toxins cause injuries in animals by decreasing their productivity due to chronic damage to their vital tissues and organs (4), Aflatoxin B1 produced by fungi is metabolized in the liver by the cellular cytochrome p450 microsomal enzymes to form an intermediate called aflatoxin B1-8, 9-epoxide, which thus, reacts with macromolecules such as lipid and DNA. The consequence of the reaction leads to disruption of transcription, lipid peroxidation, cellular impairment and abnormal cell proliferation (5). The acute intoxication of aflatoxin has devastating effects on the body. Hepato-cellular carcinoma, a liver cancer is also a primary disease associated with aflatoxin intake (6, 7).

However, the application of non-pathogenic microorganisms and their metabolites to prevent fungal infection will minimize public health hazards. Lactic Acid Bacteria (LAB) inhibit the growth of mould by secreting antimicrobial compounds, which bind with fungal toxins to eliminate their pathogenicity (8). The present study was therefore undertaken to reveal the protective role of LAB against the pathogenicity of aflatoxigenic *A. flavus* on the liver and kidney of induced albino rats.

Materials and Methods

Source of Toxigenic *Aspergillus flavus*

The studied toxigenic *A. flavus* had been screened for aflatoxins B1, B2, G1 and G2 in the previous studies of Jeff-Agboola (9). The fungus was sub-cultured

into Petri dishes with Potato Dextrose Agar (PDA, Oxoid, Hampshire, UK) and incubated at 28±1 ^{o}C for 7 days. After fungal full sporulation, their spores were harvested into sterile peptone water (0.2%) and filtered using sterile cotton filter to avoid the presence of conidia or mycelial debris (10). The fungal spore in peptone water was serially diluted and adjusted to the dilution 10^6 *A. flavus* spores/ml as the final concentrations.

Source of Lactic Acid Bacteria

Lactobacillus plantarum and *L. delbrueckii* were isolated from "Kunu", a traditionally fermented beverage. These *Lactobacill*i have been reported to have pronounced inhibitory effect against toxigenic fungus in our previous study (11).

Experimental Design

The male and female Wistar albino rats for the experiment were obtained from the Department of Animal Production and Health, The Federal University of Technology, Akure. The rats were 12-16 weeks old and weighing between 140 to 147 g. The animals were kept in a cage for 7 days to acclimatize them to the environmental conditions at 25±2 $^{\circ}$C, 12 h light-darkness cycle with adequate access to feed and water *ad libitum*.

Five rats were randomly assigned into each group and labeled as follows; BD: rats fed with basal diet; BD+AF: rats fed basal diet and injected with toxigenic fungus; BD+LP: rats fed basal diet and injected with *L. plantarum*; BD+LD: rats fed basal diet and injected with *L. delbrueckii*; BD+AF+LP: rats fed basal diet, injected with toxigenic fungi and *L. plantarum*; BD+AF+LD: rats fed basal diet, injected with toxigenic fungi and *L. delbrueckii*; and BD+AF+LPD: rats fed basal diet, aflatoxigenic fungi, *L. plantarum* and *L. delbrueckii*.

In this study, the experiment was performed in accordance with the institutional ethics and international standard of animal welfare described by National Research Council (12). The experiment was conducted using a completely randomized design.

Healthy rats challenge with toxigenic *A. flavus* and treatment with LAB

Five hundred microliter (500 µl) of the infectivity dose of the test fungi (10^6 spores/ml) was orogastrically administered into the rats, and after signs of infection were observed, 500 µl of suspension containing LAB (10^8 cfu/ml) was administered into the animals.

Histopathological Examination

All animals were sacrificed by cervical decapitation. Livers and kidneys were dissected out, washed with ice-cold saline and weighed using a digital scale (KERRO BL 200001, MxRady Lab Solutions Pvt. Ltd., Delhi, India). Thereafter, samples of liver and kidney tissues of each animal were excised and processed according to the methods of Drury et al. (13). Briefly, the tissue specimens were fixed with 10% neutral buffered formalin solution, dehydrated in alcohol and embedded in paraffin wax. Sections were cut at 5 µm thickness and stained with hematoxylin and eosin (H&E, Thermo Shandon, USA).

Statistical Analysis

Data obtained were analyzed by one-way analysis of variance (ANOVA). Means were compared by Duncan's New Multiple Range Test and considered statistically significant when $P<0.05$, using Statistical Package for Social Sciences (SPSS) software version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

The rats fed basal diet (BD) have liver and kidney weight of 6.0 g and 16.7 g respectively. The weight of liver (3.61 g) and kidney (11.33 g) in infected rats with toxigenic mould were significantly different ($P<0.05$) when compared to treated group of rats with LAB; BD+AF+LP, BD+AF +LD, BD+AF +LDP (Table 1). The rats fed basal diet and *Lactobacillus* spp. have a normal histological structure without visible lesions in their liver and kidney. Histopathological analysis of the infected albino rats with toxigenic fungus shows generalized ballooning, lesions, necrosis of hepatocytes

and congested central vein in the liver (Plate 1b). There were histological changes (Plate 3b) in the kidney of rats injected with aflatoxigenic *A*. *flavus*. The treated group of rats with LAB after infection caused by *A. flavus* showed mild necrosis of hepatocytes, less thickening of glomerular basement membrane and no gross change in their hepatic structures (Plates 2 and 4).

Table 1: Weight (g) of liver and kidney of experimental rats

Group of rats	Liver	Kidney
BD	6.00^{d} ±0.30	16.67^{d} ±0.41
BD+AF	3.61^{a} ±0.21	11.33^{a} ±0.50
BD+LP	5.60^{c} ±0.15	15.33^{c} ±0.35
BD+LD	5.60^{c} ±0.22	15.67^{c} ±0.51
BD+ AF+ LP	4.67^{b} ±0.15	13.67^{b} ±0.42
BD +AF +LD	4.67^{b} ±0.23	13.67^{b} ±0.36
BD +AF +LPD	4.67^{b} ±0.31	13.67^{b} ±0.43

Values are mean±sd of triplicates (n=3). Value with the same alphabet along column are not significantly different (P<0.05). The rats were fed;
BD=Basal diet
BD+AF= Basal diet and toxigenic *A. flavus*
BD+LP= Basal diet and *Lactobacillus plantarum*
BD+LD= Basal diet and *Lactobacillus delbrueckii*
BD+AF+LP = Basal diet, *A. flavus* and *Lactobacillus plantarum*
BD+AF+LD = Basal diet, *A. flavus* and *Lactobacillus delbrueckii*
BD+AF +LPD= Basal diet, *A. flavus*, *Lactobacillus plantarum and Lactobacillus delbrueckii*

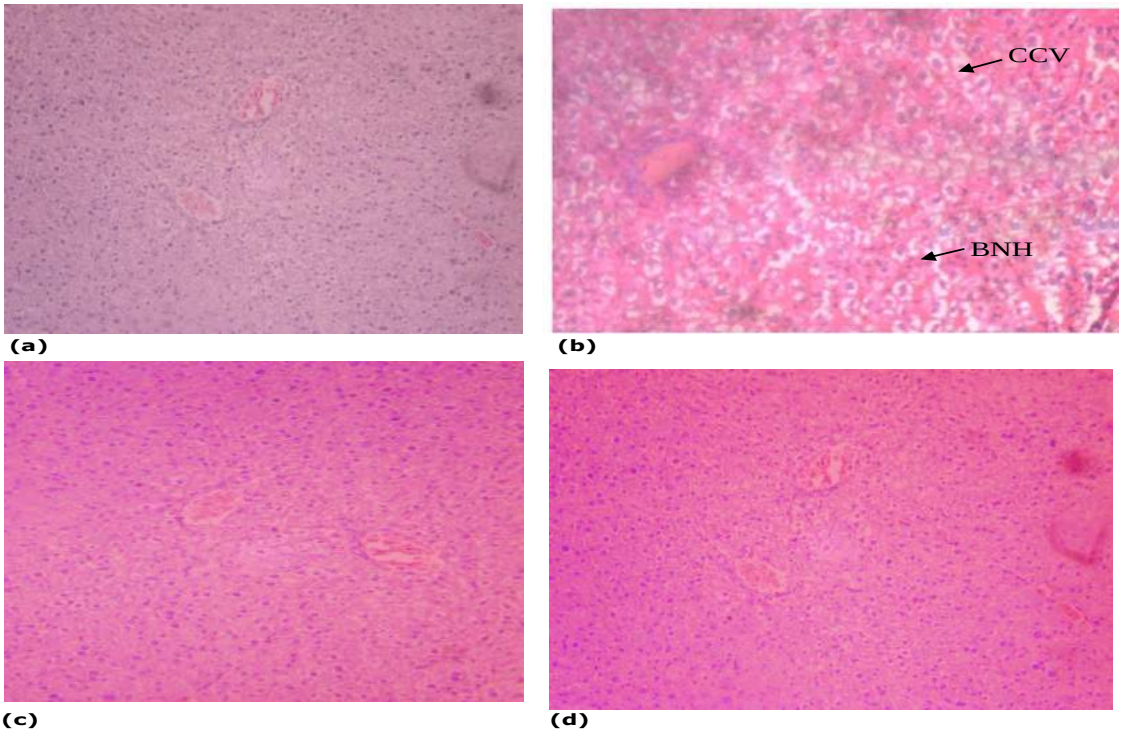

Plate 1: Photomicrograph of liver of albino rat fed (a) Basal diet (BD), (b) Basal diet with toxigenic *A. flavus* (BD+AF), (c) Basal diet with *L. plantarum* BD+LP and (d) Basal diet with *L. delbrueckii* (BD+LD). CCV = Congested central vein and BNH = Ballooning and necrosis hepatocytes.

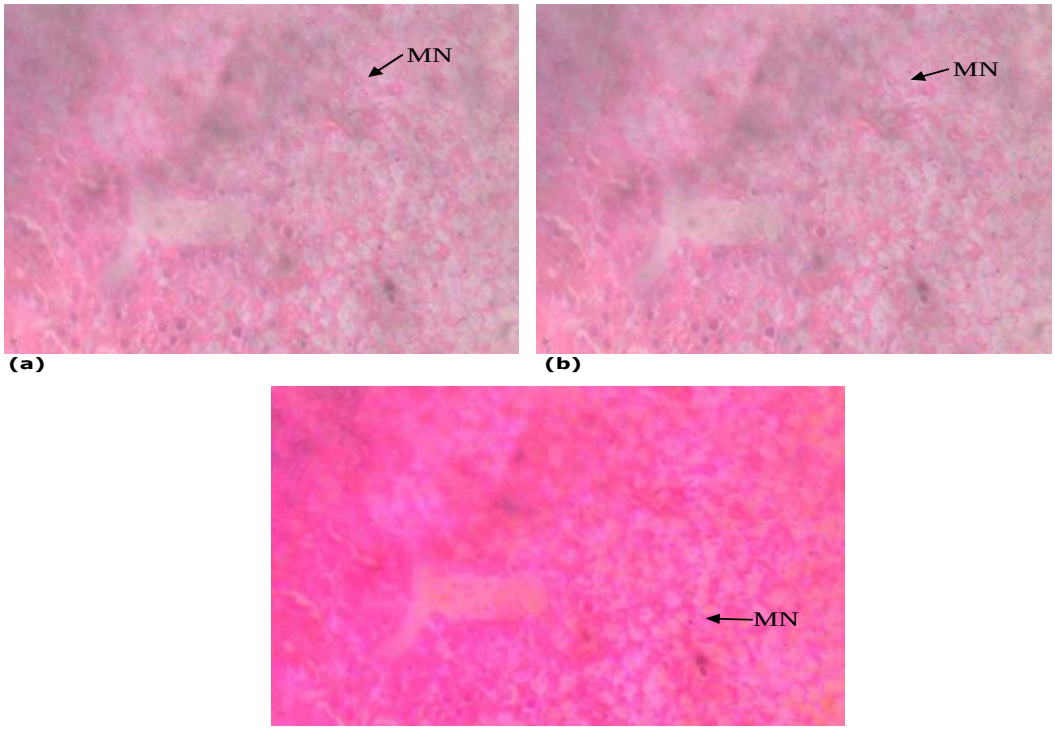

Plate 2: Photomicrograph of treated liver of albino rat fed (a) Basal diet, toxigenic *A. flavus* with *Lactobacillus plantarum* (BD+AF+LP), (b) Basal diet, toxigenic *A. flavus* with *Lactobacillus delbrueckii* (BD+AF+LD) and (c) Basal diet, toxigenic *A. flavus*, *Lactobacillus plantarum* with *Lactobacillus delbrueckii* (BD+ AF +LPD). MN: mild necrosis.

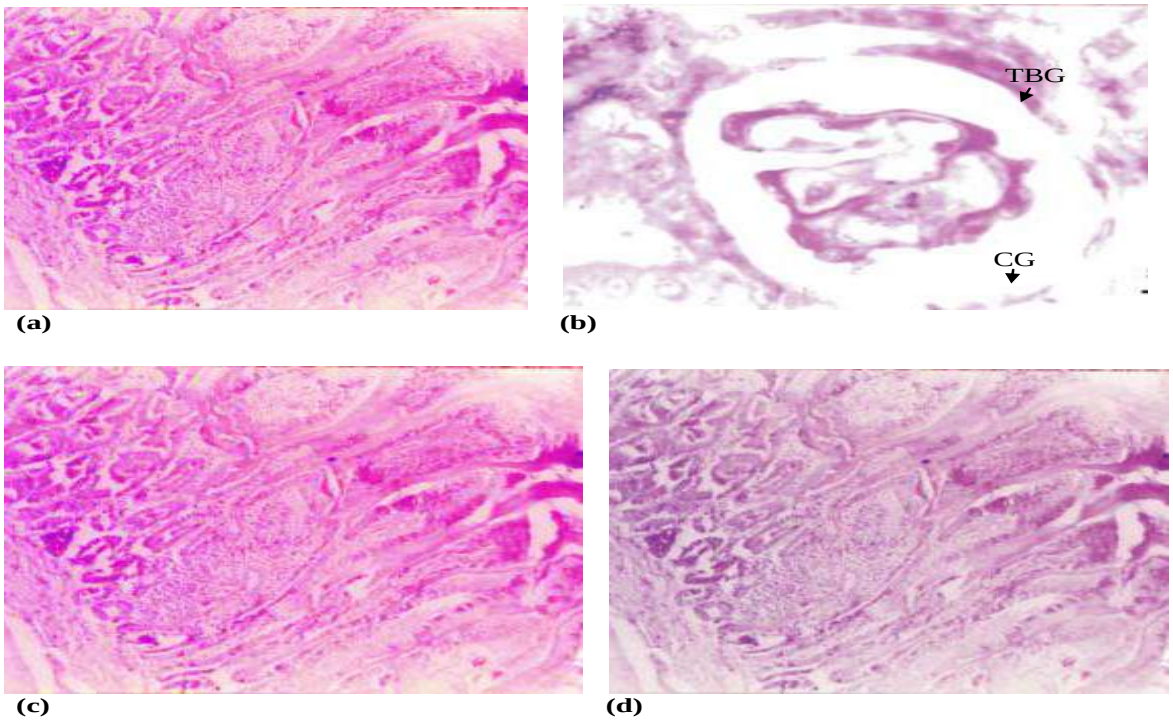

Plate 3: Photomicrograph of kidney of rat fed (a) Basal diet (BD), (b) Basal diet with toxigenic *A. flavus* (BD+AF), (c) Basal diet with *L. plantarum* BD+LP and (d) Basal diet with *L. delbrueckii* (BD+LD), TBG = thickened glomerular basement and CG = Collapse of glomerulus

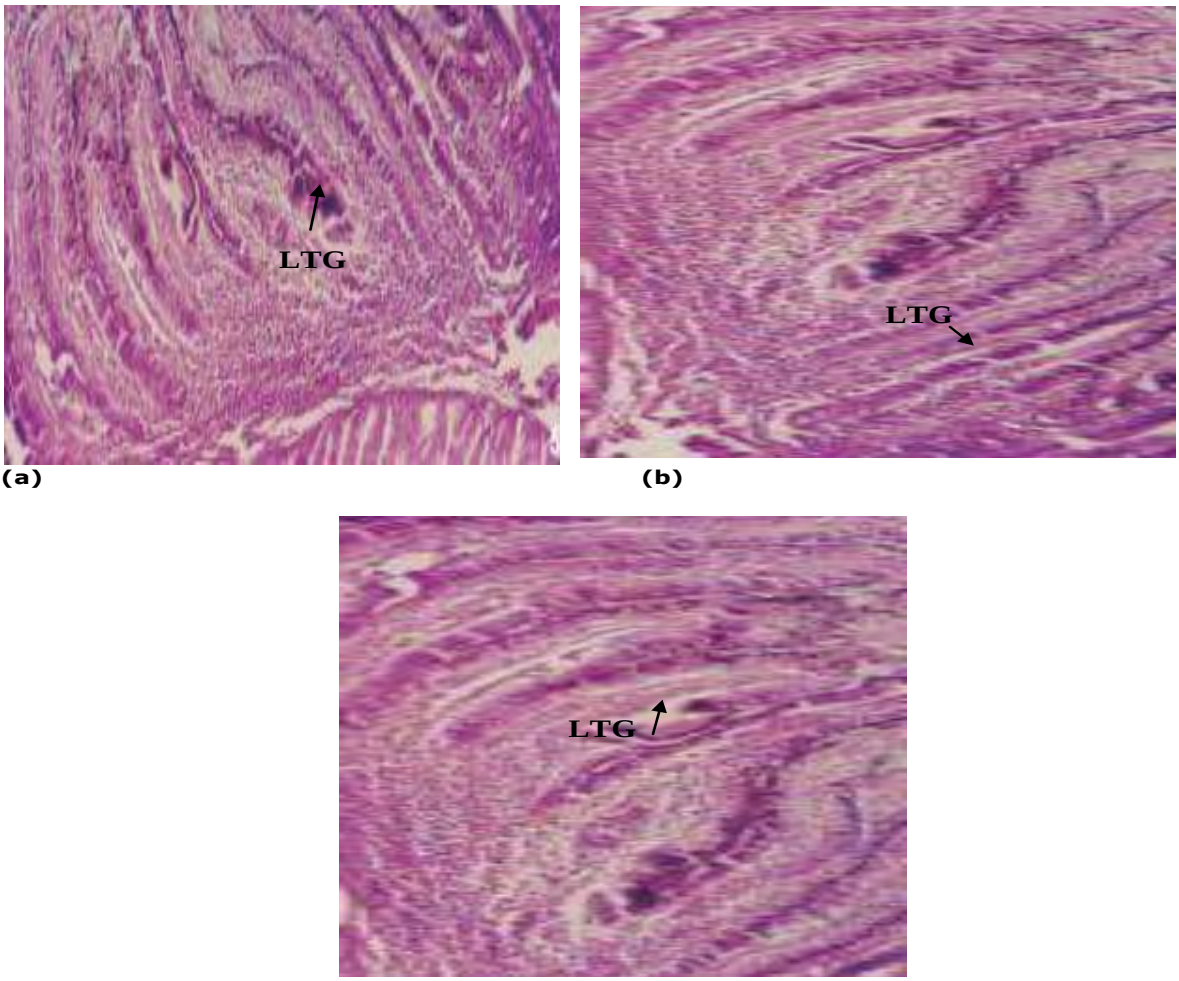

Plate 4: Photomicrograph of treated kidney of rat fed (a) Basal diet, toxigenic *A. flavus* with *Lactobacillus plantarum* (BD+AF+LP), (b) Basal diet, toxigenic *A. flavus* with *Lactobacillus delbrueckii* (BD+AF+LD) and (c) Basal diet, toxigenic *A. flavus*, *Lactobacillus plantarum* with *Lactobacillus delbrueckii* (BD+ AF +LPD). LTG = less thickened of glomerular basement membrane.

Discussion

Aflatoxigenic fungi, notably, *Aspergillus* spp. produced a secondary metabolite during hyphal growth in tissues, which causes inflammation or infections. The protective effect of LAB against the toxigenic activity of *A. flavus* on the liver and kidney of the infected rats was investigated. In an earlier study of Olonisakin et al. (11), the body weight of infected rats with toxigenic *A. flavus* was reduced to 120.23 g, while those treated with LP, LD and LPD after infection maintained their body weight as 150.43 g, 148.60 g and 155.84 g respectively. The reduction of feed intake and loss of bodyweight in infected rats conformed to the finding of Abdel-Wahhab *et al*. (14) who indicated those signs as adverse effects
and toxicity in rats injected with fungal toxin. The reduction in feed intake observed in tested rats has been reported to lead to protein catabolism, thereby causing impaired glomerular filtration and other kidney injuries (15).

The treated groups with LAB recovered with no symptoms of infection. A mixture of *Lactobacillus* species has been found to reduce fungal growth as well as aflatoxin production by *Aspergillus flavus* subsp. *parasiticus* (16). Hence, *Lactobacillus* spp. are probiotics, non-

pathogenic microorganisms with some therapeutic metabolites, which are essential for potential health benefits like maintaining normal intestinal microflora, modulating the immune system, detoxifying colonic contents, lowering serum cholesterol levels and promoting lactose tolerance in intestine (17).

The colour of the liver in infected rats with aflatoxigenic fungus was slightly pale and grey. This agreed with Yener *et al.* (18) who reported slightly pale, enlarged and grayish mottled in the liver of aflatoxin-induced rats, which indicated significant damage to the vital organs of the albino rats. The histopathological changes observed were in accordance with the studies of Yener *et al.* (18) and Gelderblom *et al.* (19) who stated that induced AFB_1 resulted to predominant lesions, extensive hydropic degeneration, necrosis, dysplastic and swollen hepatocytes. In the study of El-Nekeety *et al.* (20), they revealed that liver injury such as necrosis began to occur when the stored glutathione is almost exhausted due to their important role in the detoxification of toxic metabolites produced by fungi. Aflatoxigenic fungi generate Reactive Oxygen Species (ROS) and consequently caused lipid peroxidation, which lead to hepatotoxicity (21). Abdel-Wahhab *et al*. (22) and El-Mahalaway (23) had reported necrosis of tubules, degeneration of granular with cloudy swelling in the epithelial cell of proximal tubules and enlarged pale vacuolated cytoplasm. Hence, aflatoxigenic fungi are known to have a deleterious effect on immune system, cellular component, tissue and organs.

The bio-protective potential of LAB against the toxigenic fungus can be attributed to the production of antifungal compounds such as benzoic acid, methyl hydantoin, mevalonolactone, short-chain fatty acids, proteineous compounds and secretion of de-conjugated bile acids from bile salt synthesized by the host (24, 25). The detoxification of aflatoxins and elimination of their symptoms by LAB is a promising task toward an immuno-stimulatory property.

Conclusively, the protective effect of *Lactobacillus* spp. on the organs of rats induced with aflatoxigenic fungi can be attributed to some bioactive metabolites. The presence of natural antimicrobial products in *Lactobacillus* spp. can be used for competitive exclusion of pathogens, which will be a biological approach to eliminate food borne pathogens as well as decontaminating fungal toxin in foods.

Conflict of Interest

None

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Original Article **Open Access**

First report of enteropathogenic and enteroinvasive *Escherichia coli* with multiple antibiotic resistance indices from African catfish (*Clarias glariepinus*) in Nigeria

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Abstract

Background: There are increasing reports of food safety issues associated with intensive production of fish which increase the chances of disease outbreaks from stressful growth conditions accompanying mass production and presence of bacterial pathogens.
Methodology: Two hundred gastrointestinal tract (GIT) samples from two hundred African Cat Fish (*Clarias glariepinus*) were assessed for the presence of enteric *Escherichia coli* species including *E. coli* 0157, Enteropathogenic *E. coli* (EPEC) and Enteroinvasive *E. coli* (EIEC) which are traditionally associated with infantile gastroenteritis. The antibiotic resistance profile and Multiple Antibiotic Resistance Index (MARI) for these isolates were determined. The serogrouping of the *E. coli* isolates was done using *E. coli* agglutinating sera (Oxoid) and *E. coli* 0157 latex reagent (Oxoid). Antibiotic susceptibility was determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.
Results: A total of 35 (17.5%) *E. coli* isolates were recovered from the fish intestines among which 9 (25.7%) were EPEC and 2 (5.7%) were EIEC. No *E. coli* 0157 strain was recovered. Thirty-three (94.0%) isolates had a MARI greater than 0.2. Antibiotic resistance to cefoxitin and amoxicillin-clavulanic acid were 77.1% and 74.3% respectively. All isolates were susceptible to meropenem and amikacin but all EPEC and EIEC isolates were AmpC (resistance to all penicillins, cephalosporins and beta lactamase inhibitors) positive.
Conclusion: The isolation of EPEC and EIEC which can cause fatal gastroenteritis coupled with high MARI among isolates in this study represents a public health concern. Strict monitoring of administration of antibiotics in aquaculture is recommended.

Keywords: EPEC; EIEC; Multiple antibiotic resistance**;** Aquaculture

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Premier signalement d'*Escherichia coli* entéropathogène et entéro-invasif avec plusieurs indices de résistance aux antibiotiques chez le poisson-chat africain (*Clarias glariepinus*) au Nigéria

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Abstrait

Contexte: On signale de plus en plus de problèmes de sécurité sanitaire des aliments associés à une production intensive de poisson, qui augmentent les risques d'épidémies dues à des conditions de croissance stressantes accompagnant une production de masse et la présence d'agents pathogènes bactériens
Méthodologie: Deux cents échantillons du tractus gastro-intestinal (GIT) de deux cents poissons chats africains (*Clarias glariepinus*) ont été évalués pour la présence d'espèces entériques d'*Escherichia coli* comprenant *E. coli* 0157, *E. coli* entéropathogène (EPEC) et *E. coli* Enteroinvasive (EIEC). qui sont traditionnellement associées à la gastro-entérite infantile. Le profil de résistance aux antibiotiques et l'indice de résistance multiple aux antibiotiques (MARI) de ces isolats ont été déterminés. La sérogroupe des isolats de *E. coli* a été réalisée à l'aide de sérums agglutinants de *E. coli* (Oxoid) et du réactif latex *E. coli* 0157 (Oxoid). La sensibilité aux antibiotiques a été déterminée conformément aux directives du Clinical and Laboratory Standard Institute (CLSI).
Résultats: Au total, 35 isolats d'*E. coli* (17,5%) ont été retrouvés dans l'intestin des poissons, dont 9 (25,7%) étaient des EPEC et 2 (5,7%) des EIEC. *E. coli* 0157 n'a pas été retrouvé. Trente-trois (94,0%) des isolats avaient un IRS supérieur à 0,2. La résistance aux antibiotiques de la céfoxitine et de l'amoxicilline-acide clavulanique était respectivement de 77,1% et 74,3%. Tous les isolats étaient sensibles au méropénème et à l'amikacine, mais tous les isolats d'EPEC et EIEC étaient positifs pour AmpC (résistance à toutes les pénicillines, céphalosporines et inhibiteurs de la bêta-lactamase).
Conclusion: l'isolement des EPEC et des EIEC pouvant provoquer une gastro-entérite fatale, associé à un IAR élevé parmi les isolats de cette étude, constitue un problème de santé publique. Une surveillance stricte de l'administration d'antibiotiques en aquaculture est recommandée.

Mots-clés: EPEC; EIEC; Résistance multiple aux antibiotiques; Aquaculture

Introduction

Aquaculture is currently one of the fastest growing food production sectors with fish contributing about 60% of the world supply of protein (1). Fish and fish products are usually highly nutritious and safe, however there are increasing reports of food safety and environmental issues associated with intensive production of fish which increases the chances of disease outbreaks (2, 3). The common causes of such disease outbreaks in aquaculture include stressful growth conditions associated with mass production and presence of bacterial pathogens (4, 5). This has led to huge dependence on antibiotics in the management of bacterial infection in aquaculture which has in turn resulted in emergence of antibiotic resistance among micro-organisms isolated from fish. *Escherichia coli* is regarded as a commensal organism found in the GIT of humans and warm-blooded animals where they usually co-exist in a mutually beneficial relationship with the host organism, contributing to metabolic processes (6). In some instances, *E. coli* may cause opportunistic infections and other strains are considered to be truly pathogenic (7). *E. coli* serves as the most preferred indicator organism to test food and environmental samples for faecal contamination (8).

In developing countries like Nigeria, the artificial aquaculture of fish (especially the African Catfish) is popular, and no regulatory body exists to monitor and regulate the practice. In such settings, aquaculture practices are an issue of public health concern, being an important source of environmental pollution and possible contributor to the problem of antibiotic resistance. The aim of this study was to determine the prevalence of *E. coli* strains (EPEC, EIEC and *E. coli* O157) in the GIT of African Catfish (ACF) and the antimicrobial resistance profile of these strains.

Materials and methods

Sample collection:

Between October and December of 2016, intestinal contents of 200 ACF (one per ACF) were collected into sterile polythene bags during fish evisceration from the Jos Main Fish Market on alternate days between 7: 00a.m. to 12 noon. The GIT contents were placed on ice and transported to the diagnostic laboratory within sixty minutes of collection (9).

Isolation and identification:

In the diagnostic laboratory, the intestinal contents of each ACF were obtained aseptically using a sterile scalpel blade to dissect the intestine after which sterile cotton swabs were used to collect the intestinal contents; these were placed in peptone water and incubated overnight in aerobic conditions. The pre-enriched samples were then inoculated on Eosin Methylene Blue (EMB) Agar and MacConkey Agar, and incubated for 18- 24 hours at 37^{o}C (10).

Biochemical identification:

Single colonies from each sample were identified biochemically as *E. coli* using standard procedures including the Gram staining, morphology observation under microscope, Indole-Methyl Red-Voges Proskauer-Citrate (IMViC), lysine decarboxylase tests and triple sugar ion reactions. The *E. coli* isolates were seeded on nutrient agar for further processing.

Sero-grouping of isolates:

Further characterization of *E. coli* isolates was done using the *E. coli* agglutinating sera and *E. coli* 0157 latex agglutination assay according to the manufacturer's instructions (Oxoid, Basingstoke, UK).

Antimicrobial susceptibility testing:

The antimicrobial susceptibility test for each identified *E. coli* isolate was performed using the modified Kirby-Bauer disk diffusion method (11). Isolates were inoculated into peptone broth and incubated at 35-37^{o}C for 16 -18 hours in ambient air. The isolated *E. coli* were seeded onto the surface of freshly prepared, dry surfaced Mueller Hinton agar using sterile swabs after standardization of the inoculum. Using sterile forceps, the antimicrobial discs were placed on the agar plates and incubated at 35 -37^{o}C for 16 -18 hours in ambient air. The zone of inhibition was measured using a standard meter rule and results interpreted using the CLSI breakpoints (11).

All isolates were tested for sensitivity to the following antibiotics: amoxycillin (30µg), gentamicin (10µg), amikacin (30µg), sulfamethoxazole trimethoprim (30µg), ciprofloxacin (5µg), cefuroxime (30µg), cefoxitin (30µg), ceftriaxone (30µg), ceftazidime (30µg), amoxicillin-clavulanic acid (30µg), piperacillin tazobactam (30µg), and meropenem (10µg).

Screening for AmpC production:

The isolates were screened for AmpC beta lactamase production by testing their susceptibility to cefoxitin (30µg) using Kirby Bauer disk diffusion method as described by Tanushree and colleagues (12). The inhibition zone sizes were interpreted according to the CLSI guidelines (11). All the isolates with an inhibition zone diameter of less than 18 mm were presumed positive for AmpC β-lactamases production.

Extended spectrum β-lactamase detection:

This was carried out by the double disk synergy test (DDST). All isolates with reduced susceptibilities or resistance to an extended-spectrum cephalosporin namely ceftriaxone or ceftazidime were subjected to DDST to detect the presence of ESBL enzyme as described by CLSI (11). Mueller Hinton agar plates were inoculated with a 0.5 McFarland standard inoculum of *E. coli*. Control strains: *E. coli* ATCC 35218 served as positive control while *E. coli* ATCC 25922 served as negative control.

Multiple Antibiotic Resistance Index (MARI) determination:

The MARI of each isolate was determined using the formula first described by Krupperman (13). The MARI when applied to a single isolate is defined as a/b where; 'a' represents the number of antibacterial agents to which the isolate was resistant to, and 'b' represents the number of antibacterial agents to which the isolate was exposed to.

Results:

A total of 35 out of 200 ACF (*C. glariepinus*) GIT samples were positive for *E. coli*, giving a prevalence rate of 17.5 %. Serogrouping of the isolates revealed a total of 31.4% (11/35) of specimens were either Enteropathogenic *E. coli* (EPEC) or Enteroinvasive *E. coli* (EIEC). Among these, 14.3% were identified as EPEC with agglutinating sera for serotypes 026, 055, 0111, 0119, 0126; 11.4% for serotypes 086, 0114, 0125, 0127, 0128 and another 5.7% were identified as EIEC with agglutinating sera for serotypes 044, 0112, 0124 and 0142. None of the *E. coli* isolates was identified as *E. coli* 0157 using the Oxoid *E. coli* 0157 latex agglutination assay (Table 1).

The antimicrobial susceptibility testing of all isolates revealed high level resistance to cefoxitin (77.1%) and amoxicillin-clavulanic acid (74.3%), with other susceptibility patterns as shown in Table 2. A combined resistance to cefoxitin and amoxicillin-clavulanic acid is a phenotypic marker for *ampC* genes which usually confer resistance on the organism to all penicillins and cephalosporins including the extended spectrum beta lactamases (ESBLs) and beta lactamase inhibitors**.**

None of the isolates showed the characteristic dumbbell shape description for a positive ESBL phenotype on DDST**.**

Table 1: Serogroups of *E. coli* isolated from ACF sold within Jos, Nigeria

N = 35 Serogroups (Serotypes)	Frequency (%)
E. coli 0157	0 (0.0)
EPEC I (026, 055, 0111, 0119, 0126)	5 (14.3)
EPEC II (086, 0114, 0125, 0127, 0128)	4 (11.4)
EIEC (044, 0112, 0124, 0142)	2 (5.7)
Total	11 (31.4)

Key: EPEC = Enteropathogenic *E. coli*, EIEC = Enteroinvasive *E. coli*

All the 11 (100%) EPEC and EIEC isolates presumptively carried the *ampC* gene. The multiple antibiotic resistance indices showed that more than 90% of the isolates had a MARI greater than 0.2 (Table 3).

Table 3: Multiple Antibiotic Resistance Indices of *E. coli* Isolates from ACF sold within Jos, Nigeria

MARI	Frequency (%)
0.00 - 0.10	0 (0.0)
0.10 - 0.20	2 (5.7)
0.21 - 0.30	8 (22.9)
0.31 - 0.40	10 (28.6)
0.41 - 0.50	8 (22.9)
0.51 - 0.60	5 (14.3)
0.61 - 0.70	2 (5.7)
Total	35 (100.0)

Table 2: Antimicrobial susceptibility profile of *E. coli* isolated from ACF in Jos, Nigeria

Antibiotic	Susceptible (%)	Intermediate (%)	Resistant (%)
amoxycillin (30µg)	13 (37.1)	4 (11.4)	18 (51.4)
gentamicin (10µg)	31 (88.6)	0 (0.0)	4 (11.4)
amikacin (30µg)	35(100.0)	0 (0.0)	0 (0.0)
Sulfamethoxazole/trimethoprim (30µg)	19 (54.2)	1 (2.9)	15 (42.9)
ciprofloxacin (5µg)	5 (14.3)	23(65.7)	7 (20.0)
cefuroxime (30µg)	33 (94.2)	1 (2.9)	1 (2.9)
cefoxitin (30µg)	1 (2.9)	7(20.0)	27 (77.1)
ceftriaxone (30µg)	32 (91.4)	2 (5.7)	1 (2.9)
ceftazidime (30µg)	32 (91.4)	2 (5.7)	1 (2.9)
Amoxicillin/clavulanic acid (30µg)	0 (0.0)	9 (25.7)	26 (74.3)
Piperacillin/tazobactam (30µg)	28 (80.0)	6 (17.1)	1 (2.9)
meropenem (10µg)	35(100.0)	0 (0.0)	0 (0.0)

Discussion

Escherichia coli are regarded as commensal microflora in several living organisms including humans, animals and the African Catfish (14, 15). The presence of *E. coli* is also utilised as an indicator organism to monitor for faecal contamination of foods. In this study of ACF (*C. glariepinus*) sold at fish markets in Jos, Nigeria, the overall prevalence of *E. coli* was 17.5%. This prevalence is lower than what has been observed from studies on *E. coli* in ACF and pond water from various regions within and outside Nigeria (16-19).

Amande and Nwaka observed a 42% prevalence of *E. coli* in ACF harvested from ponds in Uyo, South-South Nigeria (16). Danba and co-workers obtained a prevalence of 54.27% in Kano, North-West, Nigeria (14). Studies in Ekiti, South-West Nigeria recorded 25.8% (17). Egbebi and colleagues recorded 24% prevalence also in Ondo, South-West Nigeria (18). However, a higher prevalence of 72.7 % from freshwater fish was observed in China (19). Nonetheless, Grema and colleagues in an analysis of bacterial flora of catfish obtained from different fish markets in Maiduguri, North-East Nigeria recorded a lower prevalence of 9% *E. coli* (20). In the West African country of Ghana, Takyi and colleagues documented a prevalence of 0% and 14.3% of *E. coli* in catfish obtained from two different fish farms (21).

The lower prevalence of *E. coli* in African catfish in this study compared to others, could have been influenced by the quality of the water source for aquaculture which would vary in the different studies, although, it can be argued that since the samples in this study came from the fish market, it would also serve as representative of various water sources. However, it may also signify that fish farmers in our study area pay closer attention to hygiene and their source of water for aquaculture. Fish obtained directly from ponds are also likely to have a higher load of microorganisms resulting from poor management, poor sanitary conditions in the farms and substandard hygiene practices associated with many artificial ponds especially in developing

countries (14). These practices provide favourable conditions for bacteria reproduction and development (14).

However, observations in this study revealed that fish marketers kept the fish for sale in large basins containing clean water without adding fish feeds. They also changed the water regularly perhaps to reduce bacteria growth and enhance sales. This practice could have lowered the chance of isolating *E. coli* from the fish. The average temperature of Jos is between 13^{o}C and 22^{o}C and can drop as low as 5^{o}C in the months of December and January (22). High temperature affects the population dynamics of *E. coli* and favours bacterial growth with peaks observed in the summer months (23). Hence, the lower temperatures that prevails in Jos could have contributed to the lower prevalence of *E. coli* obtained in this study. On the other hand, lower prevalence of *E. coli* from other studies in relation to this study (20, 21) could be because the fish samples were obtained from regulated markets and probably an indication of better management practices that prevail in the farms.

The occurrence of EPEC in fresh fish as revealed by this study emphasises that fresh fish could be a potential source of human infection, thus making this an issue of public health concern. The spread of such infectious agent to humans could occur not only by consumption of raw or undercooked fish, but also by environmental spread during handling or contact with contaminated surfaces, disposal of waste water from ponds, local transportation of the fish from farms to retail market in addition to gross mishandling and other human activities.

It is important to state that the *E. coli* serogroups found in this study have been identified in humans to cause severe infections including fatal cases of infantile gastroenteritis (24). They have also been isolated from animals (dogs, rabbits, monkeys, sheep, birds) and food items such as vegetables and other food products from animal source such as raw milk or cheese (24, 25). Barbosa and others recorded an overall prevalence of 43% EPEC serogroup from water and fresh fish in Brazil (9). The disparity in their observation in comparison with this study could be attributed to differences in the prevailing strains of *E. coli* colonising the humans and those found in the different environments. Similarities of many human and animal EPEC based on clonal relationship and virulent properties in other studies suggest interspecies transmission (9). The incidence of EIEC in this study was 5.7%. Reports of this pathotype in the environment or in food are rare. However, Barbosa et al., reported a similar EIEC incidence of 5% from fresh fish and water in Brazil (9).

Furthermore, ACF serves as a major source of protein in most developing countries like Nigeria. The identification of EPEC and EIEC in ACF could have major consequences. Particularly, EPEC and EIEC transmission via the food chain would affect nutrition, increase infection rates; increase hospital visits, stretch medical care resources thereby increasing poverty and might create a vicious circle of malnutrition, disease and poverty. With lack of safe food practices available, fish handlers in the markets were seen handling fish and equipment without proper hygienic practices. The lack of biosecurity and tight hygienic controls or policies within the fish market could have also contributed to the introduction of some of the pathogenic *E. coli* that were isolated.

It has been observed that some pathogenic and potentially pathogenic microorganism including *E. coli*, *Staphylococcus* and some anaerobes survive when uncooked and precooked fish foods were stored at freezing point (26). With the advent of grilled fish at bars, restaurants and eateries and the demand for fresh catfish in many places within Jos and other parts of the world, there is the danger of the transfer of these pathogens to both human and animals through anthropogenic activities. To the best of our knowledge this is the first

report of EIEC and EPEC from ACF in Nigeria.

Isolates in this study were tested against several classes of drugs including penicillin, cephalosporins, aminoglycoside, fluoroquinolones and carbapenem. Similar to our findings, Hleba et al., did not detect any *E. coli* resistant to meropenem and ceftriaxone from fresh water fish but found *E. coli* resistant to ampicillin and chloramphenicol (27). Also, Ryu et al., isolated 179 *E. coli* from commercial fish and sea food which were resistant to ampicillin (12 isolates) and to chloramphenicol (21 isolates). However, these authors found resistant strains to ceftriaxone in 3 isolates of *E. coli* (28). Lower sensitivity rates were observed with trimethoprim sulfamethoxazole and ciprofloxacin. Although, in an analysis of a large number of *E. coli* strains isolated from seawater samples collected from three beaches in Brazil, there were no strains resistant to ampicillin, cephalothin, gentamicin, tetracycline, sulfamethoxazole trimethoprim, chloramphenicol or ciprofloxacin (29).

The *E. coli* isolates in our study showed very little or no susceptibility to cefoxitin (2.9%) and amoxicillin-clavulanic acid (0%). This is an interesting finding, especially in the absence of ESBL production in the identified isolates. If indeed all the EPEC and EIEC *E. coli* isolates were harbouring Amp C type β-lactamase resistance, then cross transmission of these strains to humans could have catastrophic outcomes especially as there is little or no therapeutic options available. Isolates carrying *ampC* gene are usually resistant to all penicillins and cephalosporins including the extended spectrum and beta lactamase inhibitors. We were unable to confirm presence of *ampC* gene in the isolates due to lack of facility for genetic study. Further antibiotic resistance genomic studies are required to correctly identify what resistance genes were present in the isolates. However, the resistance to amoxicillin-clavulanic acid in this study is in agreement with the investigation of Adedeji et al., who reported 100% resistance to amoxicillin-clavulanic acid among bacterial isolates including *E. coli* from ACF (17).

The MARI of the isolates in this study ranged from 0.17 - 0.66. When the use of antibacterial agents in an aquaculture is seldom or low (low risk exposure), the MARI value is usually below or equal to 0.2. MARI value greater than 0.2 implies high level exposure to antibiotics (30). In this study, 94.3% of isolates had MARI value greater than 0.2, indicating high level exposure of fish in Jos to antibiotics. Varying MARI values have also been reported for different bacterial isolates from ACF (31). This corroborates other findings that there is high level exposure to antibacterial agents in ACF sold within Jos metropolis and other parts of Nigeria.

The high incidence of resistance among the isolates implies that practices such as use of sub-therapeutic doses of antibacterial agents and drug administration through feed medication which exposes both infected and uninfected fish population to antibacterial agents, are high in aquaculture in this environment. These practices enhance selection pressure and transfer of resistant genes among the fish population. This is a major risk to public health due to the resulting development of acquired antimicrobial resistance in fish pathogens and other aquatic bacteria. Bacteria in fish can act as reservoirs of resistance genes, from which such genes can disseminate to even commensal human pathogens (28, 29).

Conclusion

The occurrence of *E. coli* in ACF sold within the Jos metropolis is of public health and infection control significance. The pathotypes (EIEC and EPEC) identified are traditionally associated with gastroenteritis. The high rates of MARI in *E. coli* isolates from ACF also suggest overuse of antimicrobials in aquaculture practice in Jos. The aquaculture industry is

experiencing massive growth in many regions of the world and is of great importance for food and health. However, efforts are needed to prevent the widespread, intensive and unregulated use of antimicrobial agents in this area of animal food production, especially in developing countries such as Nigeria. International cooperation from organizations such as WHO and FAO is needed to support and assist developing countries in educating farmers, capacity building and implementation of preventive measures in animal husbandry and aquaculture.

The use of contaminated water sources for aquaculture should be prevented through adequate treatment. Hygienic practices should also be encouraged among fish farm workers and fish handlers in markets to reduce the risk of contamination during handling. It is important that governmental agencies set up hazard analysis and critical control point systems to monitor quality of foods available to the community at all times.

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Original Article **Open Access**

An immunoinformatic approach to design a novel vaccine against the human respiratory syncytial virus (hRSV) by targeting M2-1 protein

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Abstract:

Background: Human respiratory syncytial virus (hRSV) is the leading cause of upper and lower respiratory infection in infants, adults and immunocompromised persons. The matrix protein, M2-1 of hRSV is a cofactor of viral RNA polymerase that plays a crucial role during replication. This programmed study was designed to scrutinize potential immunogens from the M2-1 protein characterized from four different continents.
Methods: Sequence data obtained from NCBI databases were analysed by using a series of web and software based bioinformatics tools to find out the best epitope against hRSV.
Results: The phylogenetic data revealed a homogenized clustering of M2-1 protein for the African, European, and Asian clades while proteins from North American collections found to have a significant evolutionary detachment compared to three other clusters. Using various web-based bioinformatics tools, the study identified four common B-cell epitopes present in all the M2-1 proteins from four different clusters with higher antigenicity and conservancy. Among the 17 M2-1 protein investigated for T-cell epitopes, "VLQNLDVGL" peptide from A2 super-type, and "QSACVAMSK" and "CLNGRRCHY" from A3 super-type showed the highest antigenicity at >0.80 conservancy cut-off value. After evaluation of all antigenic properties, only "CLNGRRCHY" peptide qualified as a potential vaccine candidate against hRSV. Molecular docking revealed strong and stable binding of the epitope to major histocompatibility complexes (MHC) molecules in terms of hydrogen bonding.
Conclusion: The designed epitope could be used as a possible vaccine candidate against hRSV.

Keywords: hRSV; M2-1 protein; phylogenetic cluster; BCL and CTL epitopes; molecular docking

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Une approche immuno-informatique pour concevoir un nouveau vaccin contre le virus respiratoire syncytial (VRSH) humain en ciblant la protéine M2-1

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Abstrait

Contexte: Le virus respiratoire syncytial (VRSH) humain est la principale cause d'infection des voies respiratoires supérieures et inférieures chez les nourrissons, les adultes et les personnes immunodéprimées. La protéine matricielle M2-1 du hRSV est un cofacteur de l'ARN polymérase virale qui joue un rôle crucial lors de la réplication. Cette étude programmée a été conçue pour examiner les immunogènes potentiels de la
Méthodes: Les données de séquence obtenues des bases de données NCBI ont été analysées à l'aide d'une série d'outils bioinformatiques basés sur le Web et sur les logiciels, afin de déterminer le meilleur épitope contre le hRSV.
Résultats: Les données phylogénétiques ont révélé un regroupement homogénéisé de la protéine M2-1 pour les clades africain, européen et asiatique, tandis que les protéines des collections nord-américaines se sont révélées avoir un important détachement évolutif par rapport à trois autres groupes. À l'aide de divers outils bioinformatiques basés sur le Web, l'étude a identifié quatre épitopes de cellules B communs présents dans toutes les protéines M2-1 de quatre groupes différents présentant une antigénicité et une conservation plus élevées. Parmi les 17 protéines M2-1 étudiées pour les épitopes de lymphocytes T, le peptide «VLQNLDVGL» de super type A2, et «QSACVAMSK» et «CLNGRRCHY» de super type A3 ont montré la plus grande antigénicité à une valeur de coupure> 0,80. Après évaluation de toutes les propriétés antigéniques, seul le peptide «CLNGRRCHY» a été qualifié de candidat vaccin potentiel contre le hRSV. L'amarrage moléculaire a révélé une liaison forte et stable de l'épitope aux molécules du complexe majeur d'histocompatibilité (MHC) en termes de liaison hydrogène.
Conclusion: l'épitope conçu pourrait être utilisé comme vaccin potentiel contre le hRSV.

Mots-clés: hRSV; Protéine M2-1; groupe phylogénétique; Épitopes BCL et CTL; amarrage molèculaire

Introduction

Bronchiolitis is a severe respiratory tract infection caused by *Paramyxoviridae* family virus, human respiratory syncytial virus (hRSV). It is the leading cause of mortality in children under two years of age (1). It has also been reported to infect elderly and immunocompromised people, especially pregnant women and transplant patients (2, 3). Currently, there is no candidate vaccine or drug available to prevent hRSV infection in the young and adults. Although, some of the drugs showed promising preclinical trial results, their long-term application remained challenging due to problem of post-clinical trial development, adverse effects including allergenicity, and short-term efficacy in humans (4, 5). For instance, treatment with ribavirin is not only expensive but the drug also cause aerosol related side-effects (6).

M2-1 protein is an essential co-factor of hRSV viral RNA polymerase complex that plays a crucial function in transcription processivity, acting as an anti-termination factor. The M2-1 protein binds to RNA dependent RNA polymerase (RdRp) and also interacts with other components of viral RNA polymerase by its core domain at the N-terminus (7, 8). Inhibition of M2-1 protein activity caused a significant reduction of viral RNA transcription and viral assembly (7).

In-silico epitope-based drug design can provide rapid, reliable, inexpensive, and safe vaccine against targeted antigen using immunoinformatics platforms. Epitopes are usually 9–mer peptides from viral proteins that can generate potent antigenicity when directed against specific antigen(s) (9). In the post-genomic era, epitope-based computer-aided vaccine has proven to be successful against most of the pathogenic viruses including human viral pathogens such as Influenza, Chikungunya, Rota, Zika, Ebola, MARS-CoV, etc., and fish pathogens such as Edwardsiella, Flavobacterium, and shrimp

white spot virus (WSSV) (9–17). In humans particularly, proper definition and differentiation of major histocompatibility complex molecules (MHC class I and II), human leucocyte antigen (HLA), and readily available enhanced databases, make *in-silico* approach widely acceptable for vaccine design against viral and bacterial pathogens (17). The aim of the present study therefore was to discover potent B-cell and T-cell epitope(s) from the M2-1 matrix protein that can bind strongly to human MHC molecules and subsequently inhibit RSV replication in the host cell.

Materials and methods:

Retrieval of M2-1 protein sequences from the database

M2-1 protein sequences were downloaded in FASTA format from the National Center for Biotechnology Information (NCBI) protein data bases (https://www.ncbi.nlm.nih.gov/protein). M2-1 protein was selected for antigenicity screening and downstream bioinformatics analysis because of its role and association during respiratory infection (5,18,19). In the present study, we collected information on 17 M2-1 protein sequences from four different continents namely Asia, Africa, Europe, and North America for further immunoinformatics.

Although information about the epidemiology of hRSV infections is sparse in the literature, several outbreaks have been reported from these four continents (20, 21). Sequences having same isolation history and date were excluded in the present study.

Phylogenetic clustering

Extracted protein sequences were aligned using muscle alignment program in MEGA 7.0. Muscle alignment has been reported to be a highly efficient tool for protein sequence alignment and phylogenetic analysis (22). The phylogenetic tree was constructed by the neighbour-joining method in MEGA 7.0 using Kimura-J model where branches in the tree separated by 1000 bootstrap replicate (22). The evolutionary divergence in tree was calculated as p-distance for both transition and trans-version in default parameters.

Sorting antigenic protein

To determine the antigenic protein of M2-1 that can elicit necessary immune response, we used VaxiJen (v2.0) online based server (http://ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) for the classification of 17 sequences based on immunity score. This server can predict antigenicity of any protein sequence with 90% accuracy, and therefore, widely used for *in-silico* based drug design (23). The highest antigenic M2-1 sequences were selected for further studies.

Prediction of B-cell linear (BCL) epitopes

Immune Epitope Database (IDEB) (http://tools.iedb.org/main/) has different antigenicity analysis tools based on the protein sequences. Among the approaches, Kolaskar and Tongaonkar 1990 method has been widely used for B-cell linear epitope prediction due to its accuracy (>75%) (9). The tool can also efficiently stratify epitopes from the large group of protein sequences based on antigenic scores (17).

Helper T-lymphocytes (HTL) epitopes prediction

HTLs epitopes from the M2-1 protein sequences from four different clusters were screened by utilizing IEDB database for class II epitope prediction tool (http://tools.iedb.org/mhcii/) (24). In the webpage of IEDB, various prediction methods are available, but we chose the recommended option for the finding of best epitopes, and followed the process described earlier (25). The human was selected as the targeted species and recommended 27 MHC alleles were screened with >99% population coverage (26).

The selected MHC II alleles were; (i) HLA DRB1/01:01; (ii) HLA DRB1/03:01; (iii) HLA DRB1/04:01; (iv) HLA DRB1/04:05; (v) HLA DRB1/07:01; (vi) HLA DRB1/08:02; (vii) HLA DRB1/09:01;

(viii) HLA DRB1/11:01; (ix) HLA DRB1/12:01; (x) HLA DRB1/13:02; (xi) HLA DRB1/15:01; (xii) HLA DRB3/01:01; (xiii) HLA DRB3/02:02; (xiv) HLA DRB4/01:01; (xv) HLA DRB5/01:01; (xvi) HLA DQA1/05:01/DQB1/02:01; (xvii) HLA DQA1/05:01/DQB1/03:01; (xviii) HLA DQA1/03:01/DQB1/03:02; (xix) HLA DQA1/04:01/DQB1/04:02; (xx) HLA DQA1/01:01/DQB1/05:01; (xxi) HLA DQA1/01:02/DQB1/06:02; (xxii) HLA DPA1/02:01/DPB1/01:01; (xxiii) HLA DPA1/01:03/DPB1/02:01; (xxiv) HLA DPA1/01/DPB1/04:01; (xxv) HLA DPA1/03:01/DPB1/04:02; (xxvi) HLA DPA1/02:01/DPB1/05:01 and (xxvii) HLA DPA1/02:01/DPB1/14:01. During HTL epitope screening, allelic population coverage for four different clusters were taken into consideration so as to find more epitopes for wider coverage (>1 clusters).

Cytotoxic T lymphocyte epitopes prediction and conservancy analysis

Cytotoxic T-cells play a crucial role in generating MHC class I cellular response. They usually performed various functions including destruction of damaged, unresponsive, infected and cancerous cells by recognizing presented epitopes by MHC molecules on the cell surface. The CTL epitopes that can bind MHC class I molecule were predicted from CTLPred (http://crdd.osdd.net/raghava/ctlpred/) (27). The MHC binding ability of the predicted epitopes was measured by implementation of the artificial neural network (ANN) and stabilized matrix method implementation (SMM) at sensitivity cut-off value of 0.80. The 9-mer peptide (length) was selected because of its high binding ability (>75%) to MHC class I and class II molecules (28). The super-type A2, A3, and A7 were selected as a subtype to cover approximately 90% of the population around the globe (29). The C-terminal cleavage weight and tap transport efficiency cut-off value were set at 0.15 and 0.05, respectively.

The prediction threshold score was set at 0.80. The predicted binding scores of the CTL peptides against major human MHC molecules were calculated from the T-epitope designer portal http://www.bioinformation.net/script/hla_search.cgi. As conserved epitopes can provide border protection, therefore, we aimed to identify peptides with the higher conservancy. The conservancy values of the CTL epitopes were calculated from the IEDB conservancy analysis tool using NCBI protein reference data set NCBI (http://tools.iedb.org/ncbi_seq_browser/) (26).

Allergenicity of the predicted epitopes

The selection of non-allergen epitopes was one of the prime aims of this study. The allergenicity of the final epitopes those from four different clusters, having higher antigenicity scores and MHC binding affinity were evaluated using AllerTOP (v2.0) server at http://www.pharmfac.net/allertop/. This server transforms auto-cross covariance (ACC) into equal length vectors. Then the server classifies the protein to either known allergen or non-allergen based on the k-nearest neighbouring score after comparing 4420 allergenic and non-allergenic proteins from the same and different species (25).

Structure prediction and molecular docking of the peptide to MHC molecules

PEP-FOLD3 is a denovo peptide prediction server (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/) from corresponding amino acid sequences. PEP-FOLD3 can generate 3D models of peptide-based on greedy strategy by using information from the input epitope sequences (30). Molecular docking is the widely used tool to measure the binding affinity of any ligand to receptor molecules (31). Molecular docking of predicted of the predicted peptide to human leucocyte antigens, HLA-A0201 (PDB code 1HLA) and HLA-B*3508 (PDB code 1ZHL) were performed in CABS-DOCK server (http://biocomp.chem.uw.edu.pl/CABSdock) with default parameters. CABS-dock provides efficient and flexible docking with high accuracies (>80%) without predefined localization of the binding sites

(32, 33). Visualization tool PyMol (v2.0.5) was used to extract the 3D structure of docked protein-peptide complexes. The PyMol graphics system is capable of providing excellent visualization platform for analysing the efficacy of computer-aided drugs (34).

Population coverage

Population coverage is usually used to check whether the predicted final epitope and its HLA alleles can cover a significant percent of the world population or not. The IEDB population coverage tool (http://tools.immuneepitope.org/tools/population/iedb) was used to calculate the cumulative percent of world wide population coverage for the final predicted epitope for both MHC classes, as described previously (35).

Results:

Phylogeny and antigenicity of M2-1 protein sequences from four different clusters

Analysis of 17 M2-1 hRSV proteins from four different geographic locations revealed uniform clustering of sequences from Asian, African, and European continents. However, North American (USA) cluster had significant evolutionary divergence and distributed erratically in the phylogenetic tree (Figure 1).

Multiple alignments using muscle found a highly conserved region in M2-1 protein for all four clusters in position 7-26 and 82-101. The protein sequence Q5MKM1.1 from North American (USA) cluster had the highest evolutionary detachment value of 0.579 with other M2-1 sequences, followed by Q84132.1 (detachment value 0.164). African M2-1 protein cluster was found to be phylogenetically more detached from the other three groups. Preliminary screening of antigenicity for 17 M2-1 protein revealed Q84132.1 as the highest antigenic protein with the value of 0.5063, followed by Q5MKM1.1 (0.4787), APW78912.1 (0.4647, European cluster), APW78659.1 (0.4647, European cluster) respectively (Table 1).

Table 1: VaxiJen score of RSV M2-1 protein from four different clusters

Accession number	VaxiJen Score	Cluster
NP_056864.1	0.4	North American Cluster (NAC)
Q84132.1	0.5063	NAC
Q5MKM1.1	0.4787	NAC
AIY60641.1	0.4134	NAC
ASV49500.1	0.4087	NAC
APW78912.1	0.4647	European Cluster (EC)
APW78692.1	0.4615	EC
APW78681.1	0.4627	EC
APW78659.1	0.4647	EC
APW78868.1	0.4615	EC
AGN92849.1	0.4189	Asian Cluster (AC)
AGN92838.1	0.4189	AC
AOD40569.1	0.4164	African Cluster (AFC)
AOD41194.1	0.4172	AFC
AOD41183.1	0.4172	AFC
AOD41018.1	0.4172	AFC
AOD40803.1	0.4172	AFC

BCL, HTL, and CTL epitopes prediction

The present study found 23 B-cell linear epitopes from various sequence positions of 17 M2-1 proteins from four different clusters (Table 2). Among them,

Table 2: Antigenic properties of 23 linear B-cell epitopes of RSV M2-1 protein from four different clusters

Rank	Sequence	Start position	Score	Cluster
1	YFEWPPHALLVRQNFMLNKI	27	0.88	NAC, EC
2	VYNTVISYIESNRKNNKQTI	127	0.86	NAC, EC, AC
3	KNNKQTIHLLKRLPADVLKK	140	0.85	NAC, EC
4	SYIGSINNITKQSACVAMSK	82	0.84	NAC, EC, AC, AFC
5	CKFEIRGHCLNGRRCHYSHN	7	0.84	NAC, EC, AC, AFC
6	SACVAMSKLLIEINSDDIKK	94	0.83	NAC, AC
7	LIEINSDDIKKLRDNEEPNS	103	0.83	NAC, EC, AC, AFC
8	KTIKNTLDIHKSIIISNPKE	159	0.82	NAC, EC, AC, AFC
9	NGRRCHYSHNYFEWPPHALL	17	0.81	NAC, EC
10	PHALLVRQNFMLNKILKSMD	32	0.88	NAC, AC, AFC
11	RNPCKYEIRGHCLNGKKCHF	4	0.84	NAC
12	VISYIDSNKRNPKQTIHLLK	131	0.82	NAC
13	LGSVNNITKQSACVAMSKLL	84	0.8	NAC
14	KYSHKYWEWPLKTLMLRQNY	21	0.86	NAC
15	IACGSLITVLQNLDVGLVIQ	93	0.82	NAC
16	FDAPQRTAEYALGTIGVLKS	62	0.82	NAC
17	NTDAMSDVSGFDAPQRTAEY	52	0.82	NAC
18	GVLCNLIQSVISIEEKINSS	153	0.82	NAC
19	ESNRKNNKQTIHLLKRLPAD	130	0.84	AFC
20	PNSPKVRVYNTVISYIESNR	114	0.84	AFC
21	LLKRLPADVLKKTIKNTLDI	142	0.81	AFC
22	VRVYNTVISYIESNRKNNKQ	125	0.84	AFC
23	NRKNNKQTIHLLKRLPADVL	138	0.83	AC

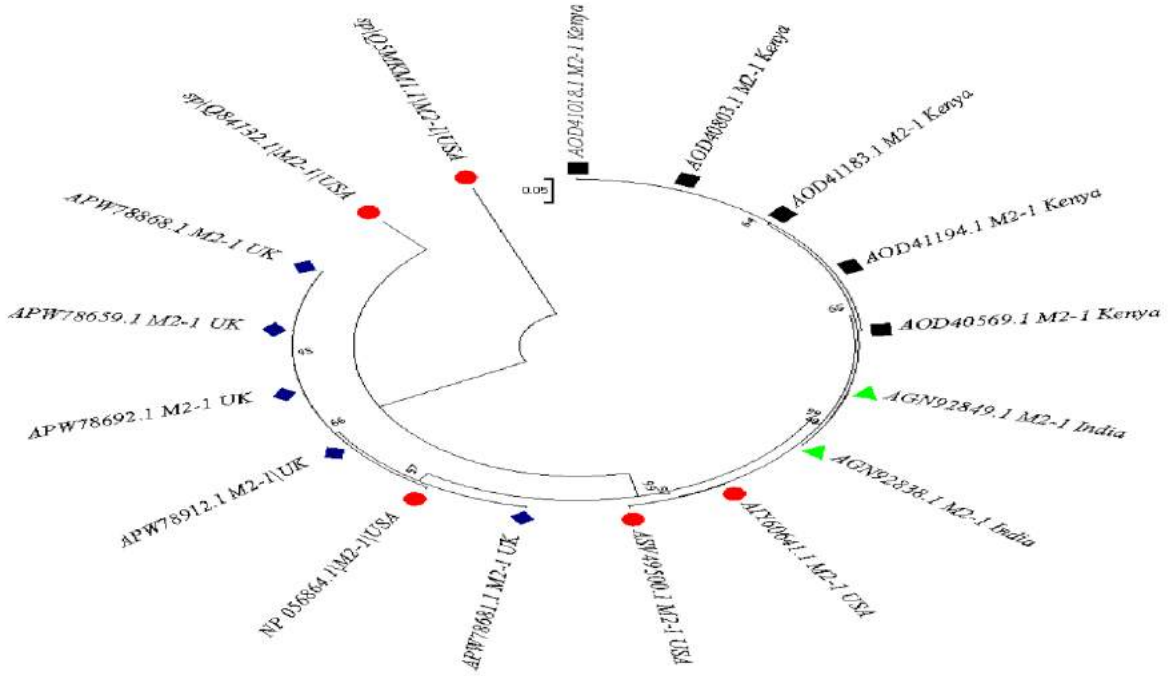

Figure 1: Phylogenetic clustering of 17 HRSV M2-1 Proteins from four different continents. African, Asian, North America, and European clusters are symbolized in black, green, red, and blue colours. The percentages of the replicate in trees in which the associated taxa clustered together by the bootstrap test (1000 replicates) are shown next to the branches. The tree was constructed using neighbour-joining method in mega7.

four were common in all four clusters (AC, AFC, EC, and NAC). Out of the four, epitope "SYIGSINNITKQSACVAMSK" and "CKFEIRGHCLNGRRCHYSHN" had higher antigenicity value of 0.84 compared to other two in the clusters. Among these two, the threshold of antigenicity for "CKFEIRGHCLNGRRCHYSHN" (1.039, only one residue had a score of below 1) was noticeably higher compared to "SYIGSINNITKQSACVAMSK" (1.028, seven residues had a score of below 1).

We found ten potential HTL epitopes candidate after the screening of a diverse set of alleles (Table 3). However, the majority of the epitopes were from HLA-DRB1*01:01 allele and some them had sequence similarity with BCL linear epitopes. CTL peptides are the potential vaccine candidates for the control of diseases. After screening of three main super type class, we found one common epitope "LLVRQNFML" in A2 with higher antigenicity (0.7122) and conservancy (1.03).

Table 3: HTL epitopes among matrix protein of hRSV

ID	Epitope	Allele	Method
74635	YLEKESIYY	HLA-DRB1*01:01	Consensus
28122	IPYSGLLLV	HLA-DRB3*01:01	Consensus
95757	RFAIKPME	HLA-DRB1*11:01	Consensus
121876	SRSALLAQM	HLA-DRB4*01:01	Consensus
122004	VRNKCLNGRR	HLA-DRB1*01:01	Consensus
144866	AITNAKII	HLA-DPA1*02:01/ DPB1*01:01	Consensus
542935	ILVKQISTPKGPS	HLA-DPA1*02:01	Consensus
546171	VNILVKQISTPKGPS	HLA-DRB4*01:01	Consensus
546172	VNILVKQISTPKGPSL	HLA-DRB5*01:01	Consensus
546173	VNILVKQISTPKGPSLR	HLA-DRB1*01:01	Consensus

Two epitopes "CLNGRRCHY" and "QSACVAMSK" from A3 super type were common in 15 out of 17 M2-1 proteins from four different clusters with antigenicity and conservancy score of 1.2599, 1.4418 and 0.8527, 1.3972, respectively (Table 4)

Table 4: Antigenic properties of major T-cell epitopes of RSV M2-1 protein from four different clusters at 0.80 conservancy

Accession number	T-cell antigen	Super type	VaxiJen Score	Conservancy (<-E)
NP056864.1	LLVRQNFML	A2 super type	0.7122	1.0329
	CLNGRRCHY	A3 super type	1.2599	0.8527
	QSACVAMSK		1.4418	1.3972
	ISYIESNRK		0.6024	1.278
	KQTIHLLKR		0.4361	0.8701
	KSIIISNPK		0.5318	1.1139
Q84132.1	LLVRQNFML	A2 super type	0.7122	1.0329
	SMDRSNDTL		0.428	0.8539
	QSACVAMSK	A3 super type	1.4418	1.4542
	KQTIHLLKR		0.4361	0.8558
Q5MKM1.1	LMLRQNYML	A2 super type	0.6751	1.1108
	VLQNLDVGL		2.1681	0.9847
	KTLMLRQNY	A3 super type	1.025	0.9069
	ALGTIGVLK		0.8243	1.3255
	RQIIHILKR		0.5421	0.8231
	LPVGVLCNL	B7 super type	0.4009	0.8792
AIY60641.1	LLVRQNFML	A2 super type	0.7122	1.03366
	QSACVAMSK	A3 super type	1.4418	1.4532
	ISYIESNRK		0.6024	1.278
	KQTIHLLKR		0.4361	0.8701
ASV49500.1	LLVRQNFML	A2 super type	0.7122	1.0366
	QSACVAMSK	A3 super type	1.4418	1.4532
	ISYIESNRK		0.6024	1.278
	KQTIHLLKR		0.4361	0.8701
APW78912.1	LLVRQNFML	A2 super type	0.7122	1.0329
	CLNGRRCHY	A3 super type	1.2599	0.8527
	QSACVAMSK		1.4418	1.3972
	ISYIESNRK		0.6024	1.282
	KQTIHLLKR		0.4361	0.8657
	KSITISNPK		0.8562	1.2084
APW78692.1	LLVRQNFML	A2 super type	0.7122	1.0329
	CLNGRRCHY	A3 super type	1.2599	0.8527
	QSACVAMSK		1.4418	1.3972
	ISYIESNRK		0.6024	1.282
	KQTIHLLKR		0.4361	0.8657
	KSITISNPK		0.8562	1.2091
APW78681.1	LLVRQNFML	A2 super type	0.7122	1.0329

	QSACVAMSK	A3 super type	1.4418	1.3972
	ISYIESNRK		0.6024	1.282
	KQTIHLLKR		0.4361	0.8652
	KSITISNPK		0.8562	1.2091
APW78659.1	LLVRQNFML	A2 super type	0.7122	1.0329
	CLNGRRCHY	A3 super type	1.2599	0.8527
	QSACVAMSK		1.4418	1.3972
	ISYIESNRK		0.6024	1.282
	KQTIHLLKR		0.4361	0.8652
	KSITISNPK		0.8562	1.2084
APW78868.1	LLVRQNFML	A2 super type	0.7122	1.0329
	CLNGRRCHY	A3 super type	1.2599	0.8527
	QSACVAMSK		1.4418	1.3972
	ISYIESNRK		0.6024	1.282
	KQTIHLLKR		0.4361	0.8657
	KSITISNPK		0.8562	1.2091
AGN92849.1	LLVRQNFML	A2 super type	0.7122	1.0366
	QSACVAMSK	A3 super type	1.4418	1.4532
	ISYIESNRK		0.6024	1.278
	KQTIHLLKR		0.4361	0.8701
AGN92838.1	LLVRQNFML	A2 super type	0.7122	1.0366
	QSACVAMSK	A3 super type	1.4418	1.4532
	ISYIESNRK		0.6024	1.278
	KQTIHLLKR		0.4361	0.8701
AOD40569.1	LLVRQNFML	A2 super type	0.7122	1.0366
	QSACVAMSK	A3 super type	1.4418	1.4532
	ISYIESNRK		0.6024	1.278
	KQTIHLLKR		0.4361	0.8701
AOD41194.1	LLVRQNFML	A2 super type	0.7122	1.0366
	QSACVAMSK	A3 super type	1.4418	1.4532
	ISYIESNRK		0.6024	1.278
	KQTIHLLKR		0.4361	0.8701
AOD41183.1	LLVRQNFML	A2 super type	0.7122	1.0366
	QSACVAMSK	A3 super type	1.4418	1.4532
	ISYIESNRK		0.6024	1.278
	KQTIHLLKR		0.4361	0.8701
AOD41018.1	LLVRQNFML	A2 super type	0.7122	1.0366
	QSACVAMSK	A3 super type	1.4418	1.4532
	ISYIESNRK		0.6024	1.278
	KQTIHLLKR		0.4361	0.8701
AOD40803.1	LLVRQNFML	A2 super type	0.7122	1.0366
	QSACVAMSK	A3 super type	1.4418	1.4532
	ISYIESNRK		0.6024	1.278
	KQTIHLLKR		0.4361	0.8701

Epitope "LLVRQNFML" failed to produce a satisfactory binding score with MHC molecule during scoring from the T-epitope designer website while positive scores recorded for "CLNGRRCHY" and "QSACVAMSK" epitopes against major HLA types.

Allergenicity of the predicted epitopes

The predicted peptide should be non-allergen in order to be considered as the safe vaccine. Among the final two selected epitopes, "QSACVAMSK" classified as "probable allergen" by the AllerTop database while "CLNGRRCHY" categorized as "probable non-allergen". Therefore, epitope "QSACVAMSK" was excluded for further studies.

Molecular docking of the peptide to MHC molecules

The structure of the predicted CTL epitope, "CLNGRRCHY" in PEP-FOLD3 with residues is shown in Figure 2. Molecular docking image of "CLNGRRCHY" to human MHC class I antigen, HLA-A2 (PDB code 1HLA) and MHC class II antigen, HLA-DRB1*04:01 (PDB code 5NIG) are presented in Figure 3. The designed peptide produced strong and stable binding with both MHC class I and II regarding hydrogen bonds. In the first case (peptide to 1HLA), the designed epitope formed seven hydrogen bonds, six (CYS, ARG, TYR, HIS) of which had a bond resolution of more than 3 °A. While in second interaction (peptide to 1ZHL), peptide-protein interaction aided by six hydrogen bonds, four (CYS, GLY, ARG) of which were more robust and stable (>3 °A).

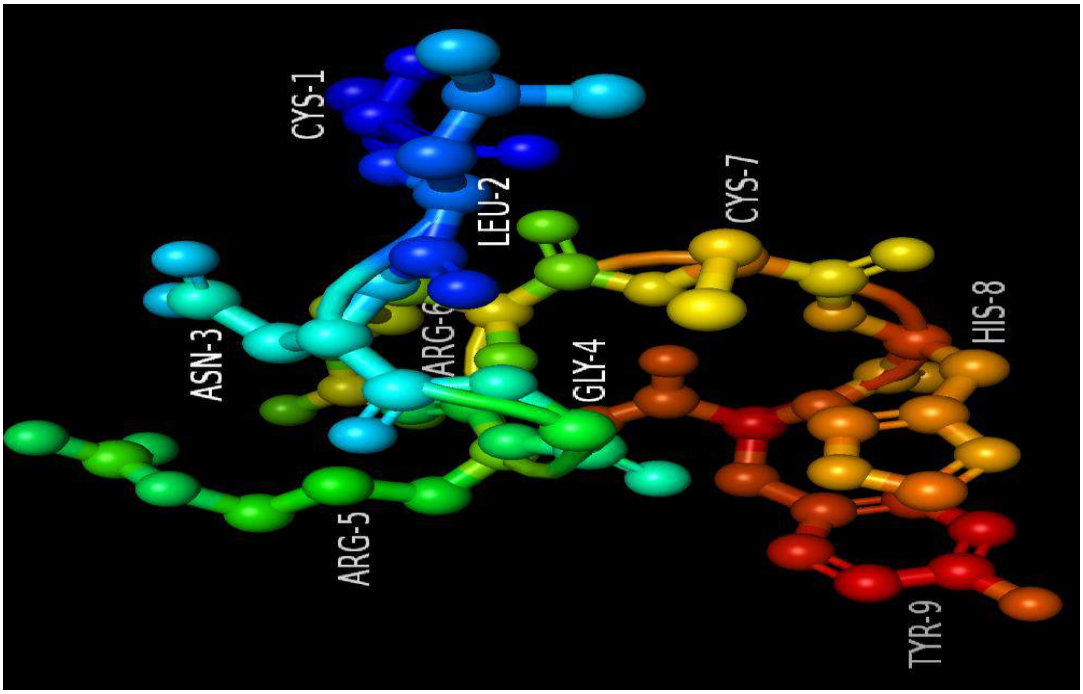

Figure 2: Modelled 3D structure of predicted "CLNGRRCHY" epitope using pep-fold3

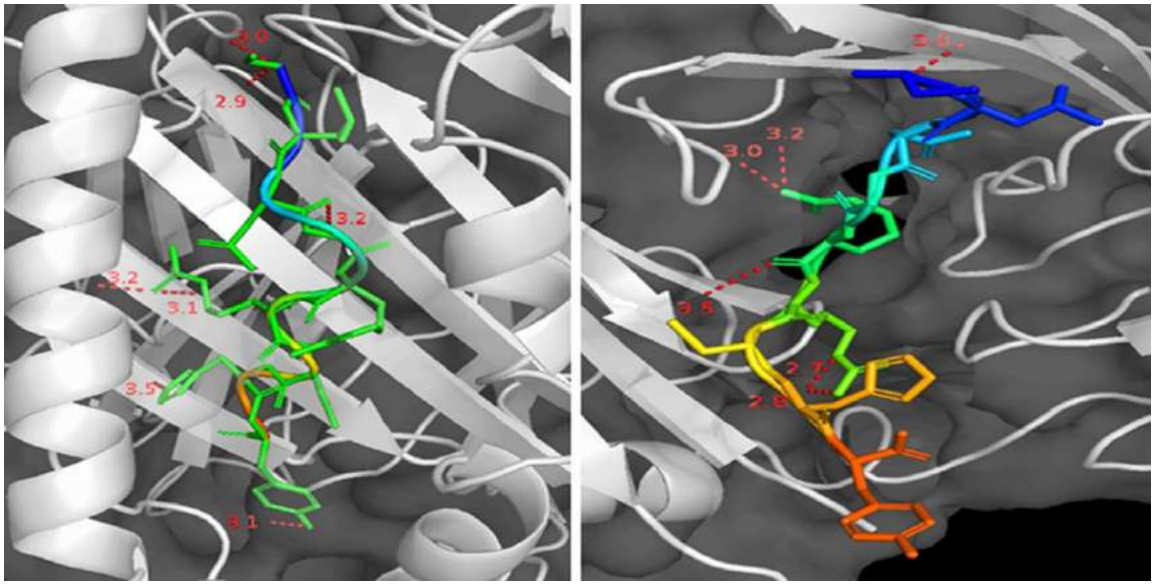

Figure 3: Molecular docking of the predicted epitope to HLA-A*0201, PDB-1HLA (left) and HLA-B*3508, PDB-1ZHL (right). The epitope "CLNGRRCHY" bound perfectly to the binding groove of MHC molecules in terms of strong hydrogen bonds (>3 °A).

Binding properties and population coverage of the predicted epitope

The binding properties of "CLNGRRCHY" revealed the percentile rank of epitope ranged from 0.4-0.75 in consensus method (ANN and SMM) and IC50 ranged from 135.87-553.88 for both ANN and SMM (Table 5). The deigned epitope covered 98.84% of world population with average hit of 2.57 and PC90 value of 1.04 by taking both class I and class II in counts where all major alleles (HLA-A*02:01, HLA-A*02:02, HLA-A*02:03, HLA-A*02:06, HLA-A*68:02, HLA-B*07:02, HLA-B*08:01, HLA-C*01:02, HLA-C*02:02, HLA-C*05:01) were taken into consideration (Figure 4).

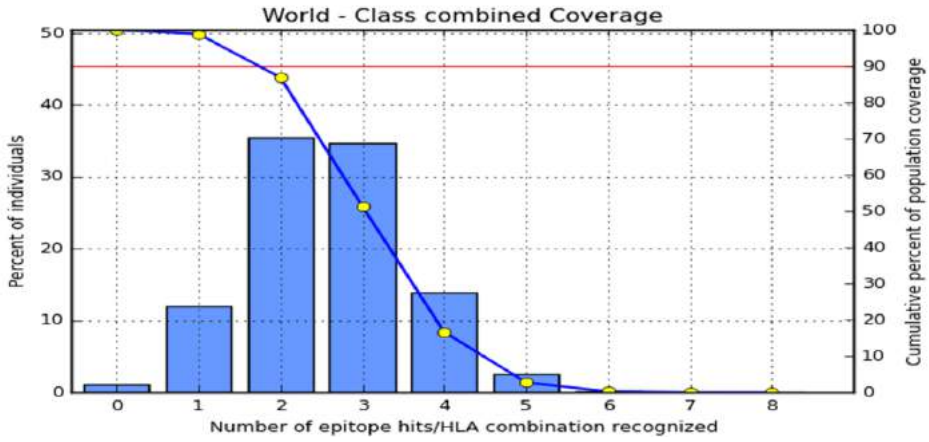

Figure 4: The world population coverage of predicted epitope based on MHC Restriction data. The cumulative coverage was 98.84% based on recommended ten HLA class. Individual bar represents number of hits for the corresponding HLA class generated by the predicted epitope

Discussion

In the present study, we aimed to identify the key antigenic epitope(s) with all necessary properties that can eventually bind strongly to MHC molecules to generate immunogenicity against hRSV. Several studies have been conducted to proffer solution to this, however no vaccine is yet to be licensed for hRSV (36). Transcription anti-termination factor M2-1 has been reported to be involved in viral replication and inactivation of M2-1 protein triggered inhibition of hRSV *in-vivo* (5). Hence, we selected this protein for *in-silico* drug design study.

The phylogenetic and evolutionary data suggested that the M2-1 protein from North America had more detachment and clustered everywhere. Therefore, we assumed that North America (NA) could be a potential source of hRSV transmission around the globe. However, these clusters had significantly higher bootstrap value (99%) and that means taxa's are well-supported by the reported data. We found more antigenicity in the M2-1 protein from the NA cluster, a well-studied sequence that can be a candidate vaccine. The four common BCL epitopes in 17 M2-1 protein sequences was one of the major findings in this study. A vaccine from one of these epitopes hereafter can inhibit hRSV from anywhere in the world. The sequence similarity of HTL and CTL epitopes with the BCL epitopes revealed more accuracies and wider coverage of the predicted peptides.

B-lymphocytes and hyper T-lymphocytes (HTLs) are the key players of adaptive immune response. They are the primary factors of the immune response mediated by activation of B-cells, cytotoxic T-cells and macrophages (9, 25). CTL peptides play a central role in governing adaptive immune response against broad range of infections (17). We comprehensively screened BCL, HTL, and CTL epitopes from M2-1 protein with consistency and flow strictly maintained. The two selected BCL epitopes were separately evaluated by a wide range of antigenic properties where "CKFEIRGHCLNGRRCHYSHN" showed its superiority over other.

Finally, among four CTL epitopes, only "CLNGRRCHY" qualified for all the necessary properties to be used as a candidate vaccine. Other three were excluded due to allergenicity, low affinity, and coverage. The conservancy values of selected BCL and CTL epitopes were more than 80%, a standard value to generate broad-spectrum antigenicity, and well-desired criteria for any predicted epitopes (17,37). A potential binder (epitope) should have three characteristics in order to generate strong immunogenicity, (i) an IC50 value of less than 500nM, (ii) percentile rank of below 1.5%, and (iii) population coverage of more than 80% (35,38). We found satisfactory IC50 value and percentile rank in our predicted epitope and population coverage of more than 98%, therefore, could be a potential immunogen against hRSV.

In molecular docking, epitope "CLNGRRCHY" formed strong and stable binding with MHC molecules. Usually, four hydrogen bonds are required to consider peptide as a strong binder of MHC molecules (16). Here we found seven (HLA-A2) and six hydrogen bonds (HLA-B*3508) in our protein-peptide dock for where nine formed a very strong and steady bond (> 3 °A) with both classes of HLA molecules. In both cases, arginine (R), cysteine (C), histidine (H), and tyrosine (Y) play a key role in protein binding. Arginine is strong protein binders which stabilize protein-protein interaction from aggregation, especially during the process of folding (39). These four amino acids have been used for the induction of protein binding ability of peptides in many vaccine preparation against broad range of diseases including cancer, pneumonia, and malaria (40–43). The introduction of cysteine zipper in the candidate vaccine leads to formation of inter-promoter disulfide rings that enabled stable coiled-coil trimers, generating satisfactory immunogenicity against hRSV (44).

Recent advances in bioinformatics have led to the rapid design of epitope-based vaccine against many human

pathogens. This method saves significant amount of time compared to previous lengthier vaccine design and long-lasting clinical trials. After analysing all the parameters, we hope that our designed epitope has all the necessary criteria as a future candidate vaccine that could produce protective neutralizing antibodies and cell-mediated immune responses to hRSV. Further synthesis and *in-vivo* laboratory trials are required to determine the exact potency of the designed epitope before commercial release.

Conflict of interest

The authors declare no conflict of interest

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Original Article **Open Access**

Phenotypic methods versus PCR-RFLP for the identification of dermatophyte species isolated from patients with dermatophytosis in Egypt

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Abstract:

Background: Dermatophytes are major causative agents of cutaneous fungal infections worldwide. Identification of dermatophyte species is based on macroscopic and microscopic morphology on different culture media. Molecular methods such as PCR-RFLP are rapid, reliable and precise identification methods. This local study aimed to identify the spectrum of dermatophyte species among the studied patients population using different phenotypic and genotypic methods.
Materials and methods: Hair, skin and nail specimens were collected from 135 patients with clinically suspected cases of dermatophytosis. All specimens were subjected to microscopic examination using KOH and culture on SDA and dermasel agar. Phenotypic identification was done by colony and microscopic morphology, and subculture on malt, PDA, lactrimel and urea agar plates. Molecular identification was done by PCR-RFLP using *Mva*I.
Results: Out of 135 patients included in the study, 78 (57.8%) were positive by culture for dermatophytes. Five different species were identified, the most commonly isolated species was *M. canis* (51.3%) followed by *T. violaceum* (42.3%). PCR-RFLP correctly identified the isolated dermatophyte species, producing unique restriction patterns.
Conclusion: Dermatophytosis is common in Egypt where humid hot climate and animal contact play important role in the spread of these fungi. The use of PCR-RFLP directly on clinical specimens rather than its use in the identification of dermatophytes from culture media is recommended.

Key words: Dermatophytosis, Dermasel agar, SDA, Sporulation media, PCR-RFLP

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Méthodes phénotypiques versus PCR-RFLP pour l'identification d'espèces de dermatophytes isolées chez des patients atteints de dermatophytose en Égypte

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Abstrait:

Contexte: Les dermatophytes sont les principaux agents responsables d'infections fongiques cutanées dans le monde. L'identification des espèces de dermatophytes est basée sur la morphologie macroscopique et microscopique sur différents milieux de culture. Les méthodes moléculaires telles que la PCR-RFLP sont des méthodes d'identification rapides, fiables et précises. Cette étude locale visait à identifier le spectre d'espèces de dermatophytes parmi la population de patients étudiée en utilisant différentes méthodes phénotypiques et génotypiques
Matériels et méthodes: Des échantillons de cheveux, de peau et d'ongles ont été recueillis chez 135 patients présentant des cas suspects de dermatophytose. Tous les échantillons ont été soumis à un examen microscopique utilisant du KOH et à une culture sur du SDA et de la gélose Dermasel. L'identification phénotypique a été réalisée selon la morphologie des colonies et microscopique, et la sous-culture sur des plaques de malt, de PDA, de lactrimel et d'urée. L'identification moléculaire a été réalisée par PCR-RFLP en utilisant *MvaI*
Résultats: Sur 135 patients inclus dans l'étude, 78 (57,8%) étaient positifs en culture pour les dermatophytes. Cinq espèces différentes ont été identifiées. L'espèce la plus communément isolée était M. canis (51,3%), suivie de T. violaceum (42,3%). La PCR-RFLP a correctement identifié les espèces de dermatophytes isolées, en produisant des profils de restriction uniques
Conclusion: la dermatophytose est courante en Égypte, où le climat chaud et humide et le contact avec les animaux jouent un rôle important dans la propagation de ces champignons. L'utilisation de PCR-RFLP directement sur des échantillons cliniques plutôt que son utilisation pour l'identification de dermatophytes à partir de milieux de culture est recommandée.

Mots-clés: Dermatophytose, gélose Dermasel, SDA, milieu de sporulation, PCR-RFLP

Introduction:

Dermatophytosis is a mycotic infection of skin, hair and nails caused by a unique group of fungi called dermatophytes, which have the ability to infect keratinous tissue. Dermatophytes include 3 important genera; Microsporum, Epidermophyton and Trichophyton (1). Diagnosis and identification of dermatophytes in the laboratory are based on conventional methods such as direct microscopic examination using KOH and culture-based identification. KOH mount is rapid, cost-effective and gives a presumptive diagnosis. However it cannot differentiate between different genera and species, and may give false positive or false negative results. Culture is regarded as the gold standard method in identifying dermatophytes but many species are slow growing. Culture on sporulation media is essential to stimulate conidiation, which facilitates species identification (1).

Precise species level identification of dermatophytes is mandatory to know the source of infection whether zoophilic, anthropophilic or geophilic, and to apply proper treatment and control measures (2). Identification of dermatophyte species by traditional methods relies on colony morphology and microscopic features of the colonies on different culture media and on biochemical and physiological tests. However, phenotypic identification is time consuming, requires experienced personnel due to identification overlap between species (3, 4).

Genotypic identification using different molecular techniques have been developed and include methods such as nested-PCR, PCR-RFLP, PCR-EIA, Real-time PCR, RAPD-PCR and microarray technology. These methods provide attractive alternative means of identifying fungi including dermatophytes (5). The present study was conducted to determine the prevalence of dermatophyte infections in Egypt, identify different dermatophyte species among the studied patients, and compare the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis with culture-based identification.

Materials and methods:

The study was approved by the Research and Ethical Committee of

Medical Microbiology and Immunology Department, Faculty of Medicine, Cairo University. Informed consent was obtained from all patients included in the study.

Demography and clinical history:

Detailed history obtained from eligible patients included age, sex, occupation, contact with animals, and use of systemic or local antifungal drug in the previous month. Patients who had received systemic or local antifungal therapy in the previous month were excluded from the study

Specimen collection:

A total of 115 hair, 11 skin and 9 nail specimens were collected from 135 patients with clinically suspected dermatophyte infection, referred to the laboratory of the Cairo Dermatological Hospital "El-Houd El-Marsoud" during the period January and December 2015.

Microscopy and culture of specimen:

All specimens were subjected to direct microscopic examination using KOH mount, and cultured on Sabouraud dextrose agar (SDA) (Oxoid, UK) and Dermasel agar supplemented with cycloheximide (0.4 g/L) and chloramphenicol (0.05 g/L) (Oxoid, UK). Cultures were incubated at 25 to 30°C and examined weekly for up to 4 weeks (6). Culture on SDA was used as the gold standard method for the diagnosis of dermatophytosis in the study.

Species identification of dermatophytes using phenotypic methods

Fungal growth on culture media was identified by; (i) macroscopic morphology including rate of growth, color of the surface and reverse of the colony, texture of the surface and topography (6); (ii) microscopic morphology using Lactophenol Cotton Blue stain of the Scotch tape preparation and examination under a bright field light microscope for the presence of septate hyphae, chlamydoconidia, microconidia, and/or macroconidia (7); (iii) subculture on 3 sporulation media; Malt agar (Oxoid, UK), Potato dextrose agar (Oxoid, UK) and Lactrimel agar with subcultures incubated at 25 to 30°C and examined weekly for up to 4 weeks by observing colony and microscopic morphology, and (iv) culture on urea agar to differentiate between *Trichophyton* species (6). Dermatophyte isolates were stored in saline at -70 °C for further processing by PCR-RFLP.

Species identification of dermatophytes using PCR-RFLP:

PCR-RFLP was used as a method for identification of dermatophyte species utilizing *Mva*I restriction enzyme patterns of PCR-amplified ITS1, 5.8S and ITS2 regions of the ribosomal DNA which revealed unique restriction patterns (1).

DNA extraction

DNA extraction was performed for all dermatophytes isolates using i-genomic BYD DNA Mini Kit (Intron Biotechnology, Korea), according to manufacturer's instructions.

Polymerase chain reaction assay

Amplification of internal transcribed spacer 1 (ITS1)-5.8S-ITS2 rDNA regions using the ITS1 (forward, 5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (reverse, 5'-TCC TCC GCT TAT TGA TAT GC-3') primer pairs was done according to previously described protocol (4). The thermal cycler (Biometra T 3000) conditions were as follows; 35 cycles of amplification (denaturation at 95°C for 1 min, annealing at 55°C for 1 min. and extension at 72°C for 2 min) ending with a final extension step at 72°C for 10 min. Amplified PCR products were run on 2% agarose gel electrophoresis and visualized by UV transilluminator (BiometraTi).

RFLP analysis

The PCR products were digested with the restriction endonuclease enzyme *Mva*I (Thermo scientific, USA), according to the manufacturer's directions. Restriction fragments were separated by 2% agarose gel electrophoresis and photographed.

Statistical analysis

Data were statistically described in

terms of frequencies (number of cases) and percentages and compared using Chi-square (χ^2) test. Exact test was used instead when the expected frequency is less than 5, *P values* less than 0.05 was considered statistically significant. All statistical calculations were done using computer program SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) release 15 for Microsoft Windows.

Results:

Age and gender distribution of patients:

The age of the patients ranged between 1 and 42 years, with a mean of 8.71 ± 6.236 years. The highest prevalence was observed in patients who were less than 5 years old (43.7%) and more males (63%) than females were affected.

Diagnosis by clinical presentations:

Out of the 135 patients; 115 (85.2%) were suspected clinically as tinea capitis, 11 (8.1%) as tinea corporis, and 9 (6.7%) as tinea unguium. Distribution of dermatophyte species in relation to clinical presentations is shown in Table 1

Table 1: Distribution of dermatophyte species in relation to clinical presentations:

Dermatophyte species	Tinea capitis (%)	Tinea corporis (%)	Tinea unguium (%)
M. canis (n =40)	36 (50.7)	4 (80)	0
T. violaceum (n=33)	33 (46.5)	0	0
T. mentagrophytes (n=1)	1 (1.4)	0	2
T. verrucosum (n=1)	1 (1.4)	0	0
E. floccusum (n=1)	0	1 (20)	0
Total (n=78)	71 (100)	5 (100)	2 (100)

Diagnosis by direct microscopic examination

Ninety five out of 135 (70.4%) specimens were positive on KOH mount, out of which 67 were positive on culture. Comparison between direct KOH examination and culture results is illustrated in Table 2, with a statistically significant difference between direct KOH examination and culture results (P < 0.001). The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of direct KOH examination compared to culture as the gold standard method in diagnosis of dermatophytes were 85.9%, 50.9%, 70.5%, 72.5%, 71.1% respectively.

Table 2: Comparison between direct KOH examination and culture results:

Culture	KOH			*P value*
	Positive	Negative	Total	< 0.001
Positive	67	11	78	
Negative	28	29	57	
Total	95	40	135	

Diagnosis by culture results:

Out of 135 patients that were included in the study, 78 (57.8%) were positive by culture for dermatophytes, while 57 (42.2%) were negative. Of 78 culture positive cases, 74 (94.9%) isolates grew on dermasel agar, 52 (66.7%) isolates grew on SDA agar and 48 isolates grew on both dermasel agar and SDA. There was a statistically significant difference between culture results on dermasel agar and SDA (P < 0.001) (Table 3).

Table 3: Comparison between culture results on dermasel agar and SDA

Culture on dermasel agar	Culture on SDA			*P value*
	Positive	Negative	Total	< 0.001
Positive	48	26	74	
Negative	4	57	61	
Total	52	83	135	

Faster rate of growth was observed with dermasel agar; 15 isolates grew in the 1st week on dermasel agar and in the 2nd week on SDA; one isolate grew in the 2nd week on dermasel agar and in the 3rd week on SDA. There was a statistically significant difference between rate of growth on both media (P = 0.002).

History of animal contact:

Out of 78 culture positive cases, 24 (30.8%) had positive history of animal contact (13 patients had history of contact with birds, 7 patients with cats, and 4 patients with dogs).

Species of dermatophytes:

Out of 78 dermatophyte isolates, five different species were identified by the rate of growth, colony morphology, microscopic examination, urease test and subculture on malt agar, potato dextrose agar, and lactrimel agar, the most commonly isolated species was *M. canis* (51.3%) followed by *T. violaceum* (42.3%) (Figure 1)

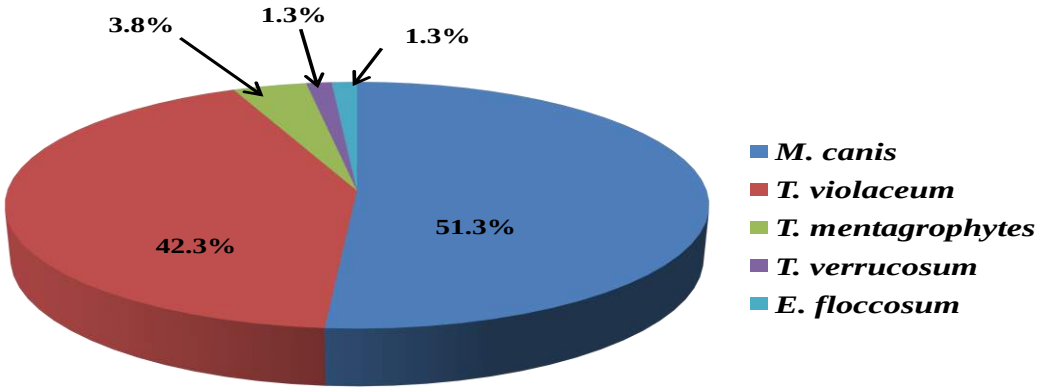

Figure 1: Distribution of dermatophyte species in 78 patients with dermatophytosis

Microsporium canis isolates were rapid growers, growing during the 1st week or early 2nd week. *T. violaceum* isolates were slower with growth appearing late in the 1st week or 2nd week. *T. mentagrophytes* and *E. floccosum* isolates also grew during the 2nd week, while *T. verrucosum* isolate was very slow-growing, and grew after 2 weeks of incubation**.** Growth on sporulation media showed that macroconidia was enhanced in all *M. canis* isolates, microconidia of *T. mentagrophytes* and macroconidia of *E. floccusum* isolates (Figure 2), while these media failed to stimulate sporulation of all *T. violaceum* isolates and macroconidia of *T. mentagrophytes*.

One isolate could not be identified due to failure of production of macro and microconidia but was suspected to be *T. verrucosum* based on its colony morphology.

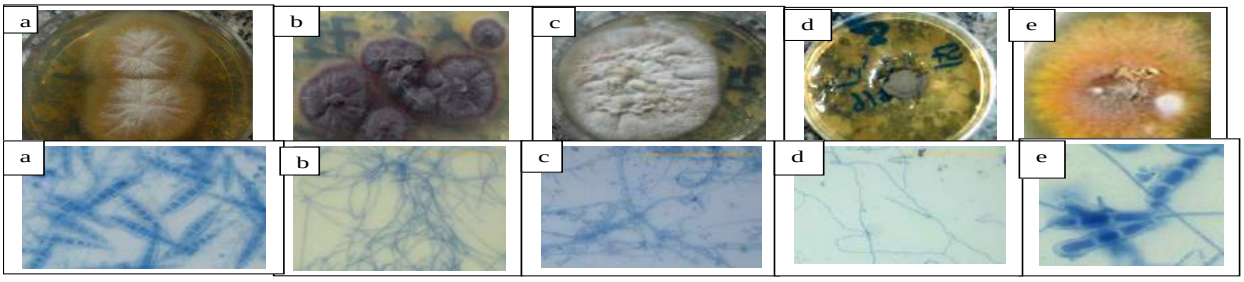

Figure 2: Colony morphology and microscopic morphology of different dermatophyte species on PDA; (a) *M. canis*, (b) *T. violaceum*, (c) *T. mentagrophytes*, (d) *T. verrucosum*, (e) *E. floccosum*

Molecular identification using PCR-RFLP:

PCR-based identification using ITS1/ITS4 primer set performed on the 78 dermatophyte isolates resulted in amplified products of approximately 690 bp in *T. violaceum* and *T. mentagrophytes* and 740 bp for the *M. canis* and *E. floccosum*. The one dermatophyte isolate which could not be identified phenotypically gave amplified product of approximately 690 bp before digestion with *Mva*I and the RFLP analysis resulted in four bands typical of *T. verrucosum*. *Mva*I digestion of the amplified products from each of the five isolated species revealed unique restriction patterns (Figure 3, 4).

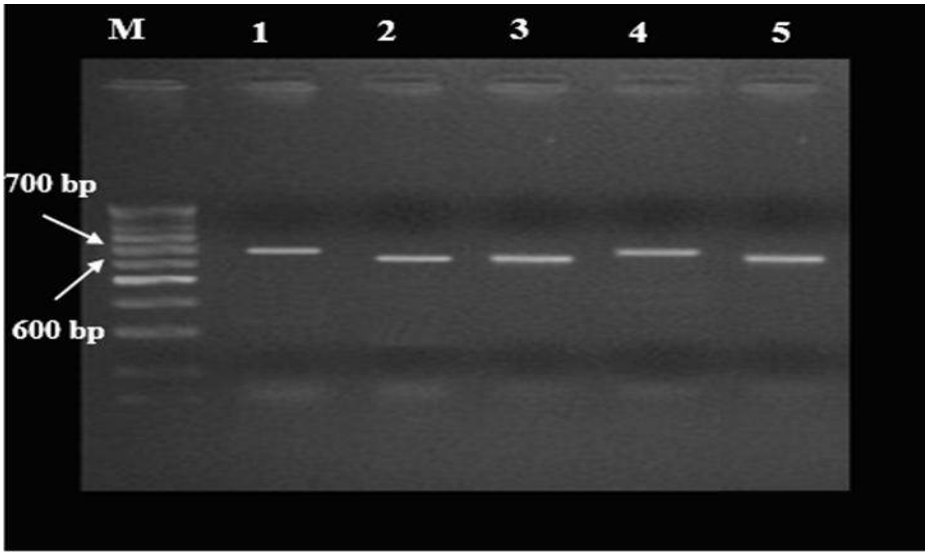

Figure 3: PCR products before digestion with *Mva*I:
Lanes: Lane M - 100 bp molecular weight marker; Lane 1 - *M. canis;* Lane 2 - *T. violaceum;* Lane 3 - *T. mentagrophytes;* Lane 4 - *E. floccosum;* Lane 5 - *T. verrucosum*

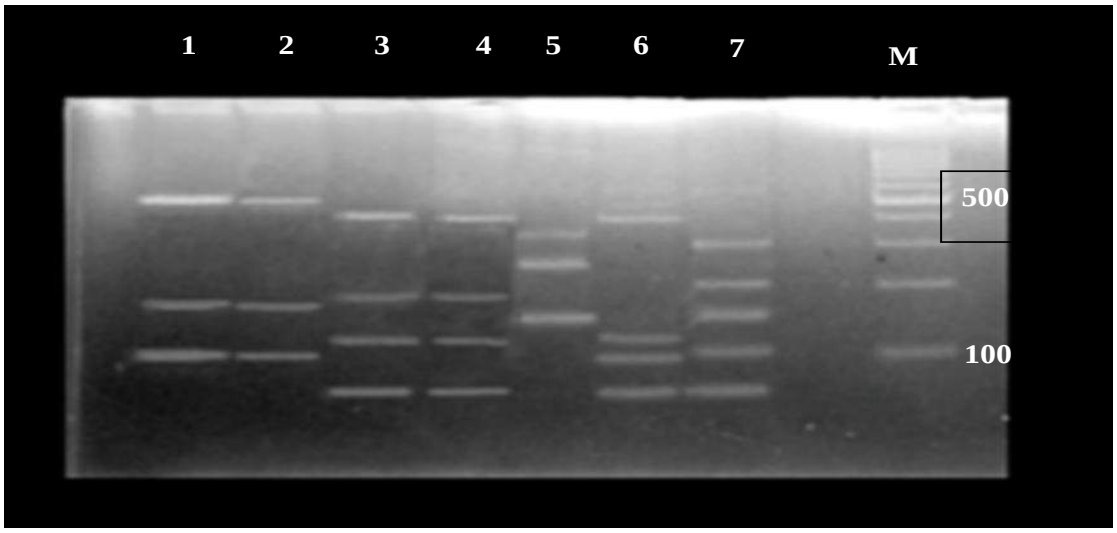

Figure 4: PCR products after digestion with *Mva*I:
Lanes: Lane M - 100 bp molecular weight marker; Lanes 1 and 2 - *M. canis*; Lanes 3 and 4 - *T. violaceum*; Lane 5 - *E. floccosum*; Lane 6 - *T. verrucosum*; Lane 7 - *T. mentagrophytes*

Discussion:

Dermatophytosis is a major health problem in Egypt. Rapid and precise identification of dermatophytes is essential in order to apply appropriate preventive measures and to direct empirical antifungal therapy (8). The current study was designed to determine the prevalence of dermatophyte infections, identify different dermatophyte species in a group of 135 patients with clinically suspected dermatophyte infections and to compare the PCR-RFLP analysis and culture-based identification methods. The prevalence of dermatophytosis reported in our study was 57.8%. Five different dermatophytes species were identified, *M canis* was the most commonly isolated species (51.3%) followed by *T. violaceum* (42.3%). The high prevalence of *M. canis* isolated from our study could be attributed to low socioeconomic standard and low hygienic measures of patients and close contact with pets (8).

Two earlier studies from Egypt have reported higher isolation rates for dermatophytosis of 81.5% and 61.9%, *M canis* being the most commonly isolated species in the first study (52.7%) and *T. violaceum* (37.3%) in the second study (8, 9). The variation in dermatophytes species isolated in different studies may be explained by the change in climate, environment, geography, lifestyles and occupation (10).

In the present study, the most common clinical presentation was tinea capitis (85.2%) followed by tinea corporis (8.1%) and tinea unguium (6.7%). *M. canis* was the most commonly isolated species in both tinea capitis and corporis, while *T. mentagrophytes* was the only species isolated in patients with tinea unguium. Previous studies in Egypt reported that tinea capitis was the most common presentation with rates of 44.5% and 35%, and *M. canis* and *T. violaceum* were the predominant causative agents (8, 9). Tinea capitis was the most common clinical presentation in the Middle East, with the predominance of *M. canis* in Saudi Arabia and Kuwait. *T. violaceum* was the most commonly isolated species in 83% of cases in the West Bank of Palestine, 39% in Iraq and 64% in Libya (11-15).

In Africa, dermatophytoses are common but are often undetected. The most prevalent clinical form is tinea capitis with *T. soudanense* as the main causative agent in Senegal and Nigeria (16, 17). In Ethiopia, a high incidence of tinea capitis mainly caused by *T. violaceum* has been reported (18). In India, tinea corporis was the main clinical presentation and in Japan, tinea pedis was the main clinical form, followed by tinea unguium, *T. rubrum* being the most frequently isolated causal species in both studies (19, 20). In Europe, tinea capitis represents about 1% of dermatophytosis. The predominance of anthropophilic dermatophytes such as *T. rubrum, M. audouinii, T. violaceum* and *T. tonsurans* has been reported (21).

In our study, tinea capitis occurred more commonly in males than females, and 93% occurred in children below 10 years of age. Male predominance could be explained by the short hairline, increased outdoor physical activities and sweating that favours growth of dermatophytes, and that also increased the chance of exposure to infection in males than females (9, 22). Children less than 10 years of age are most susceptible population to tinea capitis due to deficiency in protective fungistatic fatty acids in their scalp as the secretion of sebum starts at puberty, but other predisposing factors reported in literature include close contact with animals and soil, sharing of personal items, overcrowding, poor hygiene measures and low socioeconomic level (9, 22).

With respect to the history of animal contact in the present study, 30.8% of cases had a positive history of animal contact including birds, cats and dogs. The isolated dermatophytes among these patients were *M. canis* followed by *T. violaceum*. This finding agreed to a large extent with that of another study that reported 46% of cases with positive history of animal contact, and the predominance of *M. canis,* which made the

authors to conclude that animals play an important role in the transmission of zoophilic dermatophytes, and that they may be the source of anthropophilic dermatophyte infections in human (9).

Microscopic examination using KOH was not adequate for the diagnosis of dermatophytosis in our study. Although the sensitivity (85.9%) was high, the specificity (50.8%) was low. Another investigator however reported high sensitivity and specificity on direct examination of 88.2% and 76% respectively, which might have been attributed to the combined use of 10% KOH and 40% dimethylsulphoxide (DMSO) in that study (8).

Culture is regarded as the gold standard method for primary isolation of dermatophytes. In the present study, 49.6% of specimens were positive by both KOH and culture. KOH could not detect 8.1% of culture-positive cases, but detected 20.7% of culture-negative cases, while 21.5% of cases were negative with both KOH and culture. Our results are in consonance with other similar studies (23, 24). False negative cases have been attributed to lack of experienced personnel to correctly identify the dermatophytes while false positive cases to early unreported intake of antifungal drugs. In our study, dermasel agar was a useful selective medium for primary isolation of dermatophytes from clinical specimens with 94.9% of specimens growing on this medium compared to 66.7% on SDA agar. This agrees with another study (25) but other investigators have reported no significant difference between the isolation rate on both dermasel and SDA (8).

As many species of dermatophytes do not sporulate on SDA medium, it is important to stimulate conidiation for easier identification of species by the use of sporulation media (26). In our study, subculture on sporulation media (Malt agar, PDA and Lactrimel agar) led to enhancement of macroconidia of all *M. canis* and *E. floccosum* isolates as well as microconidia of *T. mentagrophytes* but the macroconidia of *T. mentagrophytes* were not stimulated. The media also failed to stimulate sporulation of all *T. violaceum* and *T. verrucosum* isolates. There was no significant difference between these media regarding sporulation and pigmentation. One study however reported that Lactrimel agar improved macroconidia production after 15 days and was the most useful medium to induce phenotypic characters in daily practice (27).

Identification of dermatophyte species by standard phenotypic methods is time consuming and requires experienced personnel (3, 8) necessitating the use of molecular methods several of which have been widely used for identification of dermatophytes. PCR-RFLP is a reliable, easy, simple and precise method for dermatophyte species identification (1). PCR-RFLP assay used in our study enabled the identification of five dermatophyte species and one isolate that phenotypic methods failed to detect was identified as *T. verrucosum* by PCR-RFLP. Several studies have used the PCR-RFLP method for the identification of dermatophyte species with the same single restriction enzyme (*Mva*I) which we employed in our study. Some investigators have reported that closely related species such as *M. canis, M. ferrugineum, T. rubrum* and *T. soudanense* gave similar restriction patterns with this enzyme (1, 28). Another investigator who performed PCR-RFLP using *Hae*III, *Dde*I and *Mva*I restriction enzymes reported that the use of *Dde*I and *Mva*I yielded unique band profile, however, *Hae*III produced similar band profiles and may therefore not be suitable for the identification of dermatophyte species (29).

Conclusion:

Dermatophytosis is common in our environment where hot humid climate and contact with animals play important role in the spread of these fungi. Tinea capitis was the most common clinical presentation in our study, especially in children below 10 years with male preponderance, and *M. canis* the most

commonly isolated dermatophyte species. PCR-RFLP correctly identified the isolated dermatophyte species producing unique restriction patterns.

Further studies performed over a longer period of time and covering larger population are recommended to enable better understanding of the epidemiology of dermatophytosis in Egypt. It is also recommended that PCR-RFLP analysis be applied directly on clinical specimens rather than on culture media for identification of dermatophytes.

Conflict of interest:

The authors declared no conflict of interest.

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Original Article **Open Access**

Micronutrient deficiencies among pregnant women with *Plasmodium falciparum* infection in Owo, Ondo State, Nigeria

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Abstract

Background: Two important barriers to a successful pregnancy outcome are maternal under nutrition and malaria. This study was conducted to determine some micronutrient deficiencies among pregnant women infected with *Plasmodium falciparum* in Owo, Ondo State, Nigeria
Material and methods: Two hundred and fifty four participants aged 18 to 42 years consisting of 154 pregnant women attending antenatal clinic of the Federal Medical Center, Owo, and 100 apparently healthy non-pregnant women as controls were randomly enrolled in this study. Blood specimen was collected and analyzed for the detection of *P. falciparum* using 10% Giemsa staining technique while micronutrients (calcium, copper, iron and zinc) were analyzed using Atomic Absorption Spectrophotometer (AAS).
Results: Out of 154 pregnant women studied, 91 (59.1%) had micronutrient deficiency (MND) while 5 out of 100 (5.0%) non-pregnant control had micronutrient deficiency ($p < 0.0001$). Forty three (27.9%) of the 154 pregnant women and 3 (3.0%) of 100 non-pregnant control had *P. falciparum* infection ($p < 0.0001$). Forty three of the 91 (47.3%) pregnant women and 3 of the 5 (60%) non-pregnant women with MND had *P. falciparum* infection ($p = 0.6681$). All 43 pregnant women with MND but none of the 63 pregnant women without MND had *P. falciparum* infection ($p < 0.0001$). Similarly, all 3 non-pregnant women with *P. falciparum* infection had MND but none of the 95 non-pregnant women without MND had *P. falciparum* infection ($p < 0.0001$). Multiple micronutrient deficiencies of iron and calcium (65.3%), iron and zinc (16.1%) and iron and copper (18.6%) were observed among pregnant women but none among non-pregnant women. Factors significantly associated with *P. falciparum* infection among pregnant women with MND were age group 23-27 years ($p = 0.0109$), first trimester gestational age ($p = 0.0234$), primiparity ($p = 0.0303$) and wet season ($p < 0.0173$). There was no significant association between anaemia and prevalence of *P. falciparum* infection in pregnant women with MND ($p = 0.1327$) but pregnant women with iron deficiency were more likely to be infected with *P. falciparum* than those with other micronutrient deficiencies ($p = 0.0013$)
Conclusion: This study reported a higher prevalence rate of 27.9% for *P. falciparum* infection in pregnant women compared to 3% in non-pregnant women population, but a much higher rate of 47.3% among pregnant women with micronutrient deficiencies.

Key words: Micronutrient deficiencies, *Plasmodium falciparum*, pregnant women, Owo

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Carences en micronutriments chez les femmes enceintes atteintes d'une infection à Plasmodium falciparum à Owo, dans l'État d'Ondo, au Nigéria

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Abstrait

Contexte: La sous-nutrition maternelle et le paludisme sont deux obstacles importants à la réussite de la grossesse. Cette étude avait pour objectif de déterminer certaines carences en micronutriments chez les femmes enceintes infectées par *Plasmodium falciparum* à Owo, dans l'État d'Ondo, au Nigéria
Matériel et méthodes: Deux cent cinquante-quatre participants âgés de 18 à 42 ans, dont 154 femmes enceintes fréquentant la clinique prénatale du Centre médical fédéral d'Owo et 100 femmes apparemment non enceintes et en bonne santé comme témoins ont été inscrits au hasard dans cette étude. Des échantillons de sang ont été prélevés et analysés pour la détection de P. falciparum en utilisant une technique de coloration à 10% de Giemsa, tandis que les micronutriments (calcium, cuivre, fer et zinc) ont été analysés à l'aide d'un spectrophotomètre d'absorption atomique (AAS)
Résultats: Sur 154 femmes enceintes étudiées, 91 (59,1%) avaient un déficit en micronutriments (MND), tandis que 5 sur 100 (5,0%) des témoins non enceintes avaient un déficit en micronutriments (p <0,0001). Quarante-trois (27,9%) des 154 femmes enceintes et 3 (3,0%) des 100 témoins non enceintes avaient une infection à *P. falciparum* (p <0,0001). Quarante-trois des 91 (47,3%) femmes enceintes et 3 des 5 (60%) femmes non enceintes atteintes de MND avaient une infection à *P. falciparum* (p = 0,6681). Les 43 femmes enceintes atteintes de MND, mais aucune des 63 femmes enceintes sans MND n'a été infectée par *P. falciparum* (p <0,0001). De même, les 3 femmes non enceintes atteintes de MND, mais aucune des 95 femmes non enceintes sans MND n'a été infectée par *P. falciparum* (p <0,0001). Des carences multiples en micronutriments en fer et en calcium (65,3%), en fer et en zinc (16,1%) et en fer et en cuivre (18,6%) ont été observées chez les femmes enceintes mais aucune chez les femmes non enceintes. Les facteurs significativement associés à l'infection à *P. falciparum* chez les femmes enceintes atteintes de MND étaient les groupes d'âge 23-27 ans (p = 0,0109), l'âge gestationnel au premier trimestre (p = 0,0234), primiparité (p = 0,0303) et saison des pluies (p <0,0173). Il n'y avait pas d'association significative entre l'anémie et la prévalence de l'infection à P. falciparum chez les femmes enceintes atteintes de MND (p = 0,1327), mais les femmes enceintes présentant une carence en fer étaient plus susceptibles d'être infectées par *P. falciparum* que celles présentant d'autres carences en micronutriments (p = 0,0013)
Conclusion: Cette étude a révélé un taux de prévalence plus élevé de 27,9% pour l'infection à *P. falciparum* chez les femmes enceintes, contre 3% chez les femmes non enceintes, mais un taux beaucoup plus élevé de 47,3% chez les femmes enceintes présentant des carences en micronutriments

Mots-clés: carences en micronutriments, *Plasmodium falciparum*, femmes enceintes, Owo

Introduction:

Malaria has been reported to be one of the three killer communicable diseases in Africa (1). An estimated 35,000 children in the Southern region of Nigeria die from malaria infection annually (2). Malaria is one of the main causes of maternal and child mortality with severe consequences on the population, social and economic development of countries ravaged by this disease (3, 4). It is estimated that each year over 125 million pregnant women living in low-and-middle-income countries are at risk of infection with *P. falciparum* and *P. vivax* (5). It has long been observed that populations residing in malaria endemic areas generally live under conditions that lead to poor nutritional status. Children and pregnant women are the worse hit by

malaria and also affected by poor nutrition (6).

Pregnancy is a period in the life cycle of a woman when additional nutrients are needed to meet the metabolic and psychological demands as well as the increased requirements of the growing fetus and placenta (7). Many anatomic, biochemical and physiologic changes occur during pregnancy to maintain a healthy environment for the growing fetus (8). Maternal nutrition does not only impact the mother in terms of morbidity but also on the growth and development of the fetus (9). The quality of nutrition in the first 1000 days beginning from the mother's pregnancy through her child's second birthday is a critical window when a child's brain and body develop rapidly and good nutrition is essential to lay the foundation for a healthy and productive future (10). Under nutrition is an underlying cause of death and it leaves millions more with lifelong physical and mental impairments (11).

Poor quality diet due to inadequate intake of animal source foods particularly in developing countries has been implicated as the major cause of micronutrient deficiencies. In addition, women who avoid meat and/or milk in developed countries have been reported to have higher risk of micronutrient depletion during pregnancy and lactation (12). Several micronutrient deficiencies have been established to contribute to abnormal prenatal development and/or pregnancy outcome (12). The common micronutrients are vitamins A, B, C, D and E, calcium, zinc, copper, iron, and magnesium (4, 13).

Deficiencies in maternal micro nutrient status may be a product of poor quality diets, high fertility rates, repeated pregnancies, short inter pregnancy intervals and increased physiological needs. All of these factors may be aggravated by inadequate healthcare systems with poor capacity, poverty and inequities, and socio-cultural factors such as early marriage, adolescent pregnancies and traditional dietary practices (14, 15, 16, 17, 18). The two important barriers to a successful pregnancy outcome are maternal undernutrition which contributes an estimated 800,000 neonatal mortality annually and malaria, estimated to cause about 900,000 low birth weight deliveries and over 100,000 infant deaths yearly (19, 20, 21). Foetal exposure to under nutrition is associated with congenital anomalies, intrauterine growth restriction, low birth weight, stunting in childhood, shorter adult height, lower educational attainment, and reduced economic activity (22).

Adequate intakes of micronutrients are needed for the development of efficient immune system (23). There is a growing body of reports linking micronutrient deficiencies and malaria caused by *P. falciparum* in developing countries where certain micronutrient deficiencies may be predisposing factors (24, 25, 26, 27). There is paucity of data on micronutrient deficiencies among pregnant women *with P. falciparum* infection in Owo, Ondo State. Against this background, this study was conducted to determine the micronutrient deficiencies among *P. falciparum* infected pregnant women in Owo, Ondo State.

Materials and methods:

Study area

This study was carried out at the Federal Medical Center, Owo, Ondo State. The hospital is a tertiary health institution with a referral status to Primary Health Care Centers, Maternity and Antenatal Clinics around Owo town.

Study population

This study was conducted between January and June, 2018 at the Federal Medical Center, Owo, Ondo State. Two hundred and fifty four participants consisting of 154 pregnant women attending antenatal clinics at the Federal Medical Center, and 100 apparently healthy, age matched non pregnant women as controls were enrolled in this study by simple random sampling technique. The participants' age ranged from 18-42 years.

Participants who refused informed

consent, pregnant women on treatment plan for trace elements (supplements) and those on antimalarial agents were excluded from the study. A well structured questionnaire was administered to collect demographic information from participants whereas informed consent was sought from participants before specimen collection. Information obtained through the questionnaire includes age, marital status, gestational age and parity amongst others. The Ethics and Research Committee of the Federal Medical Center, Owo, Ondo State approved the protocol for this study.

Collection of blood specimen

About 10 ml of venous blood was collected from each participant, 5 ml of which was dispensed into ethylene diamine tetra acetic acid (EDTA) container and properly mixed. The remaining 5 ml was dispensed into a plain tube and allowed to clot for the serum to separate.

Processing of specimen

Thick and thin blood films from each blood specimen were made, allowed to air-dry and stained in 10% Giemsa stain solution for 30 min. The stained smears were rinsed in buffer solution and allowed to air-dry. The stained thick films were examined under bright field light microscope for estimation of malaria parasite density while the thin films were examined for species of *Plasmodium*. A total of 200 fields per film were examined (28).

Haemoglobin concentration was determined using the automated analyzer-Sysmex Kx-21 (Sysmex Cooperation, Kobe, Japan). According to the WHO cutoff, anaemia was determined as haemoglobin concentration less than 11 g/dl for pregnant women and less than 12 g/dl for non-pregnant women (29, 30).

The micronutrients assayed for were calcium, copper, iron and zinc using Atomic Absorption Spectrophotometer (Buck Scientific 210 VGP, East Norwalk, CT). Briefly, for each element, the AAS was auto-zeroed using the flame from the lowest to the highest calibration. The corresponding absorbance was obtained and the graph of absorbance against concentration of the trace elements present being displayed in parts per million (ppm) (31).

Statistical analysis

The frequency data were analyzed by Chi-square test while the potential risk factors were analyzed using odd ratios. The statistical software used in the data analyses was INSTAT® (GraphPad Software Inc, La Jolla, CA, USA).

Results:

The micronutrients assayed in this study were calcium, copper, iron and zinc. Out of 154 pregnant women studied, 91 (59.1%) had micronutrient (iron) deficiency while 5 out of 100 (5.0%) non-pregnant control had micronutrient (iron) deficiency (OR 27.44, $p < 0.0001$). This trend was similar for copper, calcium and zinc (Table 1) which showed that pregnancy status was significantly associated with reduction in all micronutrients assayed with iron having the greatest reduction.

Table 1: Micronutrient deficiency status of pregnant and non-pregnant women population

MND status	Pregnant (%) n=154	Non-pregnant (%) n=100	Total (%) n=254	OR	95% CI	*P* value
Iron	91 (59.1)	5 (5)	96 (37.8)	27.44	10.559-71.332	< 0.0001
Copper	38 (24.7)	2 (2)	40 (15.7)	16.052	3.775-68.052	< 0.0001
Zinc	29 (18.8)	0 (0)	29 (11.4)	47.247	2.850-783.34	< 0.0001
Calcium	24 (15.6)	1 (1)	25 (9.8)	18.277	2.430-137.49	< 0.0001

MND = Micronutrient Deficiency

Table 2: Risk factors associated with MND and *P. falciparum* infection among studied population

Risk factors	No of pregnant women with MND (%)	No infected with *P. falciparum* (%)	OR	95% CI	*P* value
1. Age group					
18-22	26	9 (32.1)			0.0109
23-27	25	18 (72.0)			
28-32	15	8 (53.3)			
33-37	20	6 (30.0)			
38-42	5	2 (40.0)			
2. Marital status					
Single	19	7 (36.8)			0.5933
Married	62	31 (50.0)			
Widowed	10	5 (50.0)			
3. Gestational age					
1st trimester	28	19 (67.9)			0.0234
2nd trimester	38	16 (42.1)			
3rd trimester	25	8 (32.0|)			
4. Parity					
Primiparous	35	22 (62.9)	2.821	1.177-6.757	0.0303
Multiparous	56	21 (37.5)			
5. Seasonal variation					
Dry season	33	10 (30.3)	0.3294	0.1331-0.8185	0.0173
Wet season	58	33 (56.9)			
6. Pregnancy status in relation to MND and *P. falciparum* infection					
Pregnancy status	No of women with MND	No infected with *P. falciparum*	OR	95% CI	*P* value
Pregnant	91	43 (47.3)	0.5972	0.09519-3.747	0.6681
Not pregnant	5	3 (60.0)			

MND = Micronutrient Deficiency

Forty three (27.9%) of the 154 pregnant women and 3 (3.0%) of 100 non-pregnant control had *P. falciparum* infection (OR 12.526, $p < 0.0001$) indicating that pregnancy is a significant risk factor for acquisition of *P. falciparum* infection (Table 2). Forty three of the 91 (47.3%) pregnant women and 3 of the 5 (60%) non-pregnant women with MND had *P. falciparum* infection, indicating that MND is an independent risk factor for *P. falciparum* infection in both pregnant and non-pregnant subjects (OR 0.5972, $p = 0.6681$) (Table 2).

MND as a significant risk factor for *P. falciparum* infection among the studied population is further highlighted by the fact that all 43 pregnant women with MND but none of the 63 pregnant women without MND had *P. falciparum* infection (OR 113.9110, $p < 0.0001$). Similarly, all 3 non-pregnant women with *P. falciparum* infection had MND but none of the 95 non-pregnant women without MND had *P. falciparum* infection (OR 267.40, $p < 0.0001$) (Table 3).

Table 3: MND and *P. falciparum* infections in pregnant and non-pregnant women population

(a)

MND status	No with pregnancy	No with *P. falciparum* infection	OR	95% CI	P value
MND positive	91	43	113.9110	6.835-1898.3	< 0.0001
MND negative	63	0			

(b)

MND status	No without pregnancy	No with *P. falciparum* infection	OR	95% CI	P value
MND positive	3	2	267.40	10.694-6686.3	< 0.0001
MND negative	0	95			

MND = Micronutrient Deficiency

Multiple micronutrient deficiencies of iron and calcium (65.3%), iron and zinc (16.1%) and iron and copper (18.6%) were observed among pregnant women (Table 4) but no multiple micronutrient deficiency was observed among non-pregnant women.

Table 4: Micronutrient deficiency combinations in pregnant women

Micronutrient deficiency	Frequency
Iron + calcium	77 (65.3)
Iron + zinc	19 (16.1)
Iron + copper	22 (18.6)
Total	118

Age significantly affected the prevalence of *P. falciparum* infection among pregnant women with MND (p = 0.0109) with the 23-27 years age group having the highest prevalence (46.2%). Marital status did not affect the prevalence of *P. falciparum* infection among pregnant women with MND (p = 0.5933). Gestational age affected significantly the prevalence of *P. falciparum* infection in pregnant women with MND (p = 0.0234) with those in the 1st trimester having the highest prevalence of *P. falciparum* infection (67.9%). Primiparous women with MND were significantly more proned to *P. falciparum* infection (p = 0.0303), with a 1 to 6-fold risk of acquisition.

The prevalence of *P. falciparum* infection among pregnant women with MND was higher in rainy season (56.9%) than in dry season (30.3%) (p < 0.0173) (Table 2). There was no significant association between anaemia and prevalence of *P. falciparum* infection in pregnant women with MND (p = 0.1327) (Table 5). The three cases of *P. falciparum* infection in non-pregnant women with MND were also seen in those without anaemia. Pregnant women with iron deficiency are more likely to be infected with *P. falciparum* than those with other micronutrient deficiencies (p = 0.0013) (Table 6).

Table 5: Relationship between anaemia and prevalence of *P. falciparum* infection in pregnant women with MND

Pregnant women	No with MND	No infected with *P. falciparum*	OR	95% CI	P value
Anaemia	78	34 (43.6)	0.3434	0.0974-1.211	0.1327
No anaemia	13	9 (69.2)			

MND=Micronutrient Deficiency

Table 6: Comparison of micronutrient deficiencies among pregnant women infected with *P. falciparum*

Micronutrient	No with MND	No infected with *P. falciparum* (%)	P value
Iron	46	43 (93.5)	0.00013
Copper	20	12 (60.0)	
Zinc	13	9 (69.2)	
Calcium	12	6 (50.0)	

MND=Micronutrient deficiency

Discussion:

Pregnant women are often deficient in several nutrients particularly micronutrients, which can negatively impact on their well being as well as the health, growth and development of their fetus and infants (32). Micronutrient deficiencies in pregnancy have been associated with adverse health outcomes such as maternal anaemia, maternal and perinatal mortality, low birth weight, pre-term birth, intra-uterine growth retardation, altered immune response and cognitive deficits in the newborn (20, 33-37). Malaria has been associated with malnutrition and micronutrient deficiencies and certain micronutrient deficiencies may predispose to malaria especially among pregnant women (4). To the best of our knowledge, this is the first study to assess some micronutrient deficiencies among pregnant women with *P. falciparum* infection in Owo, Ondo State of Nigeria.

An overall prevalence of 27.9% for *P. falciparum* infection was observed among pregnant women population

studied but 47.3% among pregnant women with MND. The prevalence of 27.9% in this study is similar to 24% rate reported in Ouagadougou, Burkina Faso among pregnant women and 26.7% reported in Yaqshid district, Somalia also among pregnant women (38, 39). Pregnancy has been reported to reduce immunity as a result of general immunosuppression sustained by elevated level of serum cortisol which allows fetal allograft retention but renders the women susceptible to various infectious diseases (40). This may partly explain the reason for the high prevalence of *P. falciparum* in pregnancy in our study compared to the low rate of 3% among non-pregnant age matched control.

In this study, pregnancy was a significant risk factor for micronutrients deficiency for all the micronutrients assayed ($p < 0.0001$), with a 10 to 71-fold increased risk for pregnant women to be micronutrient deficient. Requirement for some micronutrients such as calcium, copper, iron and zinc are known to increase during pregnancy due to greater needs of the mother to increase her body tissue reserves, and to meet the high metabolic demands and development of the placenta and fetus (41, 42).

Micronutrients play an important role during pregnancy and reduction may cause physical abnormalities and diseases which increase the risk of adverse pregnancy outcomes (43). The levels of the four micronutrients assayed in our study were significantly reduced among the pregnant women, with highest reduction in the level of iron. Iron deficiency in pregnancy may cause anaemia which can lead to chronic fetal hypoxia, premature birth and perinatal mortality (44, 45). Pregnant women should therefore be encouraged particularly to consume iron-rich diet in order to forestall these adverse pregnancy outcomes.

There were cases of multiple micronutrient deficiencies observed among pregnant women in this study. In addition, pregnancy caused more reduction of the combination of iron and calcium levels (50%) when compared with other micronutrient combinations. Individually, deficiencies of any of these micronutrients have been associated with adverse outcomes such as preterm delivery, miscarriage, premature rupture of membranes, foetal nervous system damage, gestational hypertension, worsening of post-partum haemorrhage, maternal and fetal ossification disorders, and anomalies in DNA replication and transcription (46, 47, 48, 49, 50). Multiple micronutrients deficiency is expectedly associated with severe complications in pregnancy, childbirth and fetal development (51, 52). Adequate micronutrient supplementation in pregnancy is advocated to break the vicious circle that undernutrition can engender from pregnancy to the newborn through childhood, adolescence, and pregnancy in the next generation (53).

In this study, age was significantly associated with prevalence of *P. falciparum* infection among pregnant women with MND ($p = 0.0109$) with those in the 23-27 years age group having the highest prevalence (72.0%). The nutritional status of Nigerians has over the last few years been poor particularly with recent economic recession in the country. The National Demographic Health Survey 2013 reports indicated that nutritional status among Nigerian women aged 15-49 years has only minimally improved over a 10 year period, with prevalence of undernutrition of 15% in 2003 reduced to 11% in 2013 (54). This may partly explain our findings in this age group. Marital status was not associated with the prevalence of *P. falciparum* infection among pregnant women with MND ($p = 0.5933$) though the three cases of *P. falciparum* infection in the non-pregnant women were observed only in married subjects.

It has been reported that malaria in early pregnancy increase the risk of adverse pregnancy outcomes after organogenesis and placentation (55). In this study, pregnant women with MND in the first trimester had the highest prevalence of malaria (67.9%) when

compared to other trimesters which imply that majority of the women in our study are at high risk of adverse pregnancy outcome.

Our study revealed that primiparous women with MND had a higher risk (1 to 6-fold increased risk) of acquiring *P. falciparum* infection than multiparous women. This observation may be ascribed to inexperience in the act of pregnancy coupled with the fact that primiparous women have the propensity for not attending antenatal clinic early enough during the period they should have received health talks bothering on nutrition and health status during pregnancy.

P. falciparum infection among pregnant women occurred more frequently in rainy season with high prevalence rate of 46.6% compared to 16.7% during the dry season. Rainy season provides ecological alterations favouring the breeding of the mosquito vector which enhance transmission of malaria. Other possible factors for this include inadequate waste disposal facilities, poor drainage system and poor standards of living (56).

Contrary to the World Health Organization (WHO) report that 25% of the world population has anaemia with highest prevalence among preschool children and pregnant women (57), quite surprisingly, we did not find any association between anaemia and prevalence of *P. falciparum* in pregnant women with MND in our study (p = 0.1327). Also, the three non-pregnant women with MND who had *P. falciparum* infection did not have anaemia. The reason for this finding is not clear.

Iron deficiency and malaria have reportedly been described as significant co morbidities in most developing countries, and both maladies disproportionately affect pregnant women and children (58). In our study, pregnant women with iron deficiency were more likely to be infected with *P. falciparum* than those with other micronutrient deficiencies (p = 0.0013). Iron is essential for the survival of malaria parasite as an essential cofactor for the DNA replication enzyme, ribonucleotide reductase, required to fuel the rapid intra-erythrocytic proliferation of the parasite (59) and for pyrimidine and heme biosynthesis (60). This may explain the synergy of iron deficiency in pregnancy and *P. falciparum* infection in our study.

Conclusion:

Our study reported a higher prevalence rate of 27.9% for *P. falciparum* infection in pregnant women compared to 3% in non-pregnant women population, and a much higher rate of 47.3% among pregnant women with micronutrient deficiencies. Emphasis on health education, adequate intake of well balanced diets consisting of the appropriate micronutrients in the right proportion and intake of nutritional supplements are advocated in pregnancy.

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Original Article **Open Access**

Invitro, acidic, non-proteinaceous antifungal activities of lactic acid bacteria isolated from salad vegetables against human pathogenic *Candida albicans*

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Abstract:

Background: The antagonistic abilities of lactic acid bacteria (LAB) against clinical isolates of *Candida albicans* are not quite widely reported and such are even scarce in Nigeria. This study therefore investigated inhibitory potentials of LAB isolated from locally grown cabbage, cucumber and lettuce against four (4) clinical isolates of *C. albicans*.
Methods: The cell free supernatants (CFS) generated from LAB culture filtrate was evaluated for anti-candida activity using agar well diffusion method, and the CFS-LAB pH was measured and neutralized using standard methods. The proteinaceous inhibitory metabolites were assayed for using sodium dodecylsulphate polyacrilamide gel electrophoresis (SDS-PAGE) technique. The LAB strains used were previously isolated and identified by 16S rRNA partial sequencing and their data submitted to GenBank for accessioning.
Results: The CFS of six (6) LAB strains showed varying degrees of anti-candida activity. *Pediococcus pentosaceus* BTA 51 from cucumber showed the widest inhibition zone of 14 mm while at neutral pH, it was 12 mm diameter. *Weissella confusa* BTA 20, BTA 40 isolated from cabbage and lettuce produced 10 mm and 12 mm zones of inhibition at acidic and neutral pH respectively. *Lactobacillus plantarum* BTA 07 from lettuce showed inhibition zone of 12 mm while *L. fermentum* BTA 47 and BTA 62 from cucumber showed zones of 14 mm each in acidic pH only. The SDS-PAGE did not detect any proteinaceous substances.
Conclusion: In conclusion, LAB isolated from cabbage, cucumber and lettuce produced organic acids, non proteinaceous metabolites at neutral pH, exhibiting *invitro* inhibitory abilities against clinical isolates of *C. albicans*.

Keywords: *Invitro*, Lactic acid bacteria, 16S rRNA, antifungal, SDS-PAGE, salad vegetables

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Invitro, l'acide, non-protéiques des activités antifongiques des bactéries lactiques isolées à partir de la salade de légumes contre les pathogènes de l'homme *Candida albicans*

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Abstrait

Contexte: Les capacités antagonistes des bactéries lactiques (LAB) contre les isolats cliniques de *Candida albicans* ne sont pas très largement rapportées et sont même rares au Nigeria. Cette étude a donc examiné les potentiels inhibiteurs de LAB isolés de chou, de concombre et de laitue cultivés localement contre quatre (4) isolats cliniques de *C. albicans*.
Méthode: Les supernatants sans cellule (CFS) produits du filterate de culture de LAB ont été évalués pour l'activité d'anticandida suivre la méthode de diffusion de puits d'agar et aussi bien que le CSF-LAB pH a été mesuré et neutralisé suivre des méthodes standard.. On a analysé les métabolites inhibiteurs protéineux pour l'usage de la technique de l'électrophorèse de gel de polyacrylamide de dodecyl sulfate de sodium (SDS-PAGE). Les tensions de LAB utilisées ont été précédemment isolées et identifié par l'ordonnancement partiel du rRNA 16S et leurs données a soumis à GenBank pour accessioning.
Résultat: Le CFS de six (6) LAB tend des divers niveaux montrés d'activité d'anticandida. Le pentosaceus de pédiocoque BTA 51 de concombre a montré la zone d'inhibition la plus large de 14 millimètres tandis qu'à pH neutre, il était diamètre de 12 millimètres. Le confusaBTA 20, BTA 40 de Weissella a isolé dans le chou et la laitue a produit des zones de 10 millimètres et de 12 millimètres d'inhibition au pH acide et neutre respectivement, le lactobacille BTA plantarum 07 de laitue a montré la zone d'inhibition de 12 millimètres tandis que le fermentum de L. BTA 47 et BTA 62 de concombre montrait des zones de 14 millimètres chaque dans le pH acide seulement. Le SDS-PAGE n'a détecté aucune substance protéineuse
Conclusion: En conclusion, LAB isolé de chou, concombre et laitue a produit des acides organiques, des métabolites non protéiques à pH neutre, présentant des capacités inhibitrices invitro vis-à-vis des isolats cliniques de *C. albicans*

Introduction:

Lactic acid bacteria (LAB) found in different niches produce organic acids such as lactic, acetic, and other metabolites such as bacteriocins and hydrogen peroxide that have been shown to have antimicrobial activities (1, 2). Lactic acid produced in cell free supernatant by LAB and in co-culture has been shown to demonstrate anti-candidal activity. In a previous study, co-culture of LAB with *C. albicans* was reported to have led to loss of metabolic activity and eventually killing of the candida organism (2). In another study, four species of LAB belonging to *Lactobacillus* spp. and *Streptococcus thermophilus* showed varied inhibition against the fungal pathogen (3).

The protective effect of the LAB against infection with *C. albicans* in immunosuppressed Balb/c mice was demonstrated (4), while the anti-*Candida albicans* effects of probiotic LAB, *L. rhamnosus* GR-1 and *L. reuteri* RC-14 was speculated to have been due mainly to lactic acid produced by LAB at low pH (5). Human LAB isolate, *L. fermentum* Ess-1 was shown to interfere with the growth of the pathogen in an agar-overlay and cell free *Lactobacillus* culture filtrate (LCF) (6). Coman et al. (7) also reported a study in which LAB, *L. rhamnosus* IMC 501 and *L. paracasei* in a liquid co-culture with *C. albicans* resulted in spectacular inhibition of the candida pathogen.

The abilities of vaginal LAB, *L. acidophilus*, *L. jensenii*, *L. crispatus*, *L. gasseri*, *L. johnsonii*, *L. vaginalis*, *L. agilis*, *L. ruminus* and *L. salivarius* to auto and co-aggregate with *C. albicans* were demonstrated by Gill et al. (8) with all the LAB able to auto and co-aggregate at varied degrees. *Lactobacillus crispatus* exhibited the highest degree of co-aggregation, while *L. jensenii* and *L. acidophilus* produced highest amounts of lactic acid and H_2O_2 respectively.

In some other studies, proteinaceous, acidic, and anti-inflammatory activities of LAB against *C. albicans* have been demonstrated. For instance, Shekh and Roy (9) characterized biochemically an anti-candida protein (ACP) produced by *E. faecalis* while some probiotic LAB were demonstrated to suppress expression of inflammatory gene associated with *C. albicans* infection (10). More recently, *L. plantarum* HS, *L. curvatus* HH, *P. acidilactici* HC and *P. pentosaceus* HM all isolated from honey were shown to exhibit antifungal activities against pathogenic *Candida* spp. in both agar well diffusion and soft agar overlay assays (11).

There is paucity of reports on LAB from salad vegetables against clinical isolates of *C. albicans.* This study was therefore designed to investigate the *invitro* activities and possible inhibitory metabolites of LAB isolated from cabbage, cucumber and lettuce against *C. albicans* isolated from clinical cases of vulvo-vaginal candidiasis (VVC).

Materials and Methods:

Sources of *C. albicans* and LAB

Four isolates of *C. albicans* from women with vulvo-vaginitis were supplied by the culture bank of Department of Medical Microbiology, College of Medicine, University of Lagos, Nigeria. The LAB isolates were obtained from the culture Bank of the Molecular Biology and Biotechnology Department of the Nigerian Institute of Medical Research (NIMR), Nigeria. These LAB isolates have been previously recovered from cabbage, cucumber and lettuce grown in Nigeria, identified by partial sequencing of their 16S rRNA gene and submitted to GenBank (NCBI) and European Nucleotide Archive (ENA) (Table 1).

Microbiology

The *C. albicans* were sub-cultured from the stock onto Sabouraud Dextrose Broth (SDB) and incubated at 37^{o}C for 24 hours. Another inoculum was taken into Sabouraud Dextrose Agar (SDA) and incubated at same atmospheres. Single colonies were Gram stained and every oval to round shaped cell was tested for the growth on germ tube as follows; 3 drops of fresh pooled human serum were put into a tube and with the aid of a sterile wooding applicator stick, a yeast colony was transferred into the serum and incubated for 3hrs at 37^{o}C. A suspension of the culture was dropped on a clean microscope slide and cover slip placed on it. Using a high power objective, the presence of germ tube was confirmed.

The LAB isolates were sub-cultured in de Man Rogosa sharpe (MRS) agar to ascertain their purity. The typical LAB colonies with Gram positive reaction, and negative catalase, oxidase and spore reactions were used for anti-candida assay.

Centrifugation of MRS broth culture for bioassay test

All the LAB isolates were grown microaerophilically at 37^{o}C in sterile MRS broth for 24 hours. The control which comprised of sterile MRS broth was incubated and treated the same way as the culture. The cultures and controls were subjected to cold centrifugation (4^{o}C) at 10000g for 10min (Eppendorf, 5702 R). The supernatants were separated and filtered through a membrane (Millipore, 0.22µm) and the filtrates (cell free supernatant, CFS) used for anti-candida assay (12).

Acidity of cell free supernatants

This was measured by the use of pH meter (Thermo Electron Corporation, USA) after calibration at room temperature. The calibration was done using Thermo buffers of different pH and electrode storage solution.

Anti-candida assay

One hundred microliter (100µl) of the CFS was introduced into well bored (using sterile cork borer, 6mm diameter) on Mueller Hinton agar which has been seeded with 0.5 McFarland standard equivalent to 10^{8} colony forming unit per milliliter (CFU/ml) of *C. albicans* from

cultures on SDA. This was incubated in air at 37^{o}C for 24 hours after which the zones of inhibition were measured in millimeter (mm).

The pH of MRS broth before inoculation with LAB, and the CFS of the broth culture after centrifugation/filtration were taken. The CFS was neutralized (pH 7.0) by 1N NaOH. The neutralized CFS was used for another round of antagonistic assay as stated above (13).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on neutralized supernatants which showed inhibition, to rule out the presence of anti-candida protein (ACP). The CFS was treated with an equal volume of ice cold 20% trichloroacetic acid (TCA), incubated at 4^{o}C for 30 minutes and then centrifuged for 20 minutes at 13,500 rpm. The resulting pellets were washed with acetone, re-suspended in 50µl SDS loading buffer, then boiled for 5 mins and centrifuged for 1 min at 10,000 rpm, before being placed on ice.

The electrophoresis was done according to system of Laemmli (14) using 12% (w/v) separating gel and 5 % (w/v) stacking gel in SDS-PAGE Model (BIORAD, UK). The low molecular weight standard (Fermenters SM 0661 protein ladder) was loaded alongside. The apparatus was connected with constant electric current (30mA) till the bromophenol blue (BPB) reached the bottom of the plate (15), after which the gels were put into a container with staining solution containing 0.25% Coomassie brilliant blue R-250 dissolved in methanol with acetic acid and water (5:1:5). The destaining was done in a mixture of methanol, acetic acid and water (2:3:35, v/v/v) in a shaking water bath at room temperature until the bands became visible above the background.

Results:

The LAB produced metabolites inhibitory to test pathogen, *C. albicans* in *invitro* assays with *Pediococcus pentosaceus* BTA51 isolated from cucumber showing the widest zone of inhibition of 14mm diameter but with decreased (12mm) zone when neutralized CFS of the LAB was used. However, the CFS of 2 other LAB, *Weissella confusa* BTA20 from cabbage and *Weissella confusa* BTA40 from lettuce showed inhibition zones of 10mm and 12mm respectively in both acidic and neutralized CFS. Conversely, only acidic CFS of 3 other LAB showed anti-candida activities (Table 1). The result of SDS-PAGE showed no indication of any proteinaceous metabolites produced by the LAB and in the control.

Table1: Anti-candida activities of LAB strains from salad vegetables

LAB strain	Vegetable source	Accession number	Zone of inhibition (mm)	
			Acidic	Neutralized
P. pentosaceus BTA 51	Cucumber	MF580038	14	12
W. confusa BTA 20	Cabbage	MF580007	10	10
W. confusa BTA 40	Lettuce	MF580027	12	12
L. plantarum BTA 07	Lettuce	MF579994	12	-
L. fermentum BTA 47	Cucumber	MF580034	14	-
L. fermentum BTA 62	Cucumber	MF580049	14	-

- = No inhibition, mm = millimeter

Discussion:

The anti-candida investigations done in this study employed acidic and neutralized CFS of the LAB. This was to determine by elimination method, the inhibitory metabolites whether organic acids as reported by Adeniyi and Iveren (16) in Nigeria or others. While *L. fermentum* in the study of Adeniyi and Iveren (16) did not inhibit *C. albicans*, the *L. fermentum* BTA47 and *L. fermentum* BTA62 used in this study both exhibited the widest inhibition and this was demonstrated to be due to the effect of low pH. The pH of their CFS was 4.7 (unpublished). This was in tandem with the work of Manzoor et al (17). The yeast tested in the study of Adeniyi and Iveren (16) was standard strain instead of clinical isolates used in the present study. It is also worth noting that LAB isolates in our study have clearly identified by 16S rRNA partial sequencing and data submitted to GenBank.

Elsewhere, the CFS of human isolate of *L. fermentum* Ess-1 exhibiting some probiotic potentials was found to inhibit vulvo-vaginal *Candida albicans* (VVC), although non-acidic inhibition was demonstrated, proteinaceous metabolites were not excluded (18). To the best of our knowledge, this is the first study in Nigeria to demonstrate non-acidic, non-proteinaceous inhibition of clinical isolates of *C. albicans* by LAB from salad vegetables. The activities of *P. pentosaceus* BTA51, *W. confusa* BTA20 and *W. confusa* BTA40 were not due to proteinaceous metabolites as SDS-PAGE did not indicate presence of any protein. While the last 2 LAB species produced solely non-acidic anti-candida metabolites, the acidic CFS of *Pediococcus pentosaceus* BTA51 seemed to have a marginal anti-candida effect.

The SDS-PAGE was used in this study such that the size and number of any proteinaceous substances produced can be determined otherwise a protease such as trypsin or pepsin would have sufficed to rule out the effect of proteinaceous substances against the fungus. In studies by Shekh and Roy (9), Graham *et al* (19) and Ishijima *et al* (20), anti-candida activities of proteinaceous substances such as bacteriocins were reported while Lade *et al* (21) on the other hand, demonstrated lack of bacteriocin activities produced by *L. lactis* and *L. plantarum* against *C. albicans*.

Although, the mechanisms of inhibition of *C. albicans* by LAB have been reported to be poorly understood, various authors have demonstrated biofilm inhibition, anti aggregation, co aggregation, nanoparticle enhancement, suppression of *C. albicans* induced factors, anti-adhesion, anti-candida adjunct and many others (8-10, 22-25).

In conclusion, the acidic and non proteinaceous metabolites produced by LAB isolated from cabbage, cucumber and lettuce grown in Nigeria exhibited *invitro* inhibitory abilities against clinical strains of *C. albicans* while *W. confusa* BTA20 and *W. confusa* BTA40 in particular produced non-proteinaceous anti-candida factor at neutral pH.

Authors' contributions:

BAA and TAB conceptualized and designed the study; TAB performed field and laboratory work; TAB and SIS prepared the manuscript draft; TAB, BAA and SIS reviewed the final draft. All authors approved the final manuscript.

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Original Article **Open Access**

Prevalence of Hepatitis C virus and HIV among adults presenting for health screening in Lagos

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Abstract:

Background: Hepatitis C virus is an important cause of chronic liver disease with 71 million people worldwide living with chronic HCV infection as at 2015. Nigeria has the second largest burden of HIV in Africa, with 3.2 million Nigerians living with HIV/AIDS in 2016. Most people infected with the hepatitis C virus are asymptomatic and often unaware of their infection. This study was conducted to determine the prevalence of and risk factors for HCV and HIV infections among apparently healthy individuals in Lagos.
Materials and methods: This was a retrospective study in which the prevalence of hepatitis C virus antibodies and HIV was determined among apparently healthy individuals who presented for health screening at a private laboratory facility in Lagos from May 2014 to June 2016. The health records of those who met the inclusion criteria were systematically reviewed to extract demographic and clinical information including age, sex, history of blood transfusion, alcohol use, surgery and results of serological tests for antibodies to hepatitis C virus and HIV.
Results: Out of 1379 apparently healthy individuals included in the study, 60.1% were male. The prevalence rate of hepatitis C virus (HCV antibody) and HIV were 0.7% and 2% respectively. Hepatitis C virus infection was not significantly associated with history of blood transfusion, alcohol use, prior surgery and age. HIV infection was significantly associated with only prior history of surgery (OR 4.72, p=0.02).
Conclusion: In this study of apparently healthy Nigerians, the prevalence rates of HCV and HIV were low, no documented HCV-HIV co-infection and surgery was a risk factor for HIV infection. Screening of healthy individuals for HIV and HCV infection is very important to determine those who are infected and need to access treatment.

Keywords:

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Prévalence du virus de l'hépatite C et du VIH chez les adultes se présentant pour un dépistage médical à Lagos

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Abstrait:

Contexte: Le virus de l'hépatite C est une cause importante de maladie hépatique chronique avec 71 millions de personnes dans le monde vivant avec une infection chronique au VHC en 2015. Le Nigéria est le deuxième fardeau du VIH en Afrique, avec 3,2 millions de Nigérians vivant avec le VIH/sida en 2016. La plupart des personnes infectées par le virus de l'hépatite C sont asymptomatiques et ignorent souvent leur infection. Cette étude visait à déterminer la prévalence et les facteurs de risque d'infection par le VHC et le VIH chez des individus apparemment en bonne santé à Lagos

Matériels et méthodes: Il s'agit d'une étude rétrospective dans laquelle la prévalence des anticorps anti-virus de l'hépatite C et du VIH a été déterminée chez des individus apparemment en bonne santé qui se sont présentés pour un dépistage médical dans un laboratoire privé à Lagos de mai 2014 à juin 2016. Les dossiers médicaux de ces personnes les personnes répondant aux critères d'inclusion ont été systématiquement examinées pour extraire des informations démographiques et cliniques, notamment l'âge, le sexe, les antécédents de transfusion sanguine, la consommation d'alcool, la chirurgie et les résultats de tests sérologiques pour la recherche d'anticorps anti-virus de l'hépatite C et du VIH

Résultats: Sur les 1 379 individus apparemment en bonne santé inclus dans l'étude, 60,1% étaient des hommes. Le taux de prévalence du virus de l'hépatite C (anticorps anti-VHC) et du VIH était respectivement de 0,7% et 2%. L'infection par le virus de l'hépatite C n'était pas associée de manière significative aux antécédents de transfusion sanguine, à la consommation d'alcool, à une intervention chirurgicale antérieure et à l'âge. L'infection par le VIH était associée de manière significative aux antécédents d'opération (OR = 4,72, p = 0,02).

Conclusion: Dans cette étude portant sur des Nigérians apparemment en bonne santé, les taux de prévalence du VHC et du VIH étaient faibles. Aucune co-infection documentée VHC-VIH et la chirurgie ne constituaient un facteur de risque d'infection par le VIH. Le dépistage de l'infection par le VIH et le VHC chez les personnes en bonne santé est très important pour déterminer les personnes infectées qui ont besoin d'un traitement

Mots clés:

Introduction:

Hepatitis C virus is a hepatotropic virus well known to lead to chronic liver disease such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. In 2015, 71 million people were estimated to be living with chronic HCV infection and the global prevalence of HCV was 1% (1). Viral hepatitis was said to be responsible for 1.3 million deaths in 2015. Worldwide, 2.3 million persons living with HIV were also infected with hepatitis C virus. The burden of hepatitis constitutes a global public threat which made the World health assembly in 2016 to approve a global strategy for the elimination of viral hepatitis by the year 2030 (3). Therefore if people living with hepatitis b and hepatitis c infection are not diagnosed and treated, the number of deaths will continue to rise (1).

In 2016, the prevalence rate of HIV infection in Nigeria was 2.9% which translates into 3.2 million people living with HIV/AIDS. There were 220,000 new HIV infections and an estimated 160,000 adult and children died of AIDS (2).

The prevalence of hepatitis C in Nigeria from a population based screening is 0.9% (4). In Nigeria, its prevalence is higher among populations at high risk such as patients who are positive for the human immuno deficiency virus with 2.3% (5), mentally ill individuals 12.6% (6), intravenous drug users 7.7% (7) and patients with sickle cell anaemia 5% (8).

Most people with chronic HCV infections are asymptomatic and those infected most often have no knowledge of their infection. Testing for HCV is poor as shown in a 2015 study by the World health organisation that reported that only 20% of those living with HCV infection knew their status (1). In Nigeria only 34% of people living with HIV infection know their status (2). Therefore testing for both HIV and HCV is very important to know the burden of infection and know those who need to access care and treatment.

One of the strategies for the elimination of viral hepatitis is testing and treatment. Most of the studies on

hepatitis C in Nigeria have tested few individuals and subgroups of the population. This study was conducted to determine the prevalence and risk factors of HCV and HIV among apparently healthy individuals in Lagos from data collected from a health screening programme.

Materials and methods:

Study design

This was a retrospective study to determine the prevalence of hepatitis C virus antibodies and HIV among apparently healthy individuals who presented for health screening from May 2014 to June 2016 at a private medical diagnostic centre in Lagos. The health records of those who met the inclusion criteria were systematically reviewed to extract the demographic and clinical information including age, sex, history of blood transfusion, alcohol use, surgery and results of serum liver enzymes and serological tests for antibodies to hepatitis C virus and HIV. The detail of the identity of the subjects was kept confidential with all identifiers removed except for the demographic and clinical information mentioned above.

The centre is a leading medical diagnostic centre in Lagos state. It has a health assessment clinic with different health check-up plans consisting of a range of laboratory and radiological tests and clinical examination to detect lifestyle diseases early (when chances of treatment and cure are better) or prevent their occurrence.

The eligibility criteria included individuals aged 18 years and above who registered for health check-up plans within the study period that included screening tests for Hepatitis C virus antibody or HIV.

Laboratory procedures

Antibody to Hepatitis C virus was tested on samples collected from the clients using the Roche Elecsys HCV Antibody (Roche Diagnostics Ltd, Mannheim, Germany) assay. This is an electrochemiluminescence immunoassay "ECLIA" performed on Cobas e 411 immunoassay analyser. Antibodies to Human immunodeficiency virus were tested for using the Alere Determine HIV 1/2 test kit (Alere, USA). The tests were done according to manufacturer's instructions. Ethical approval was obtained from the Lagos University Teaching Hospital Health Research and ethics committee.

Data analysis

Data was analysed using SPSS version 20. Discrete variables were analysed using the Chi-square test while Student's t–test was used to evaluate differences between means. Odds ratio (OR) was used as a measure of the strength of association and p value <0.05 was be considered statistically significant.

Results:

Sociodemographic characteristics of study participants

Out of 1379 apparently healthy individuals included in the study, over half of them were male (829, 60.1%). Of the 1379 participants, 1356 individuals were tested for hepatitis C antibody, 1280 individuals tested for HIV. The mean age was 47.1 ± 12 years and 795 (57.6%) of them were in the age group 31 – 50 years. There was no significant difference between the mean ages of male (47.5 ± 11.7 years) and females (46.6 ± 12.4 years). Majority (1190, 87.4%) of the participants were married, while 8.8% were single. Most of the participants had no history of blood transfusion (1318, 95.6%) or prior history of surgery (987, 71.6%), see Table 1.

Table 1: Socio-demographic and clinical characteristics of the study participants

Variables	Frequency (n = 1379)	Percentage (%)
Gender		
Female	550	39.9
Male	829	60.1
Age		
<=30	91	6.6
31-50	795	57.6
>50	493	35.8
Blood Transfusion		
Yes	61	4.4
No	1318	95.6
Surgery		
Yes	987	71.6
No	392	28.4
Alcohol		
Yes	582	42.2
No	796	57.8
Marital status		
Married	1190	87.4
Single	120	8.8
Widow	40	2.9
Separated	8	0.6
Divorced	3	0.2

Prevalence of hepatitis C virus

The prevalence of hepatitis C virus (HCV antibody) was 0.7% (n=9, 95% CI 0.3 – 1.1%). The prevalence of hepatitis C virus in females was 0.4% and 0.9% in males but the difference was not statistically significant. The mean age of participants who tested positive for HCV antibody was 42.6 ± 12years. The prevalence of HCV antibody was highest in the age group 30 years or younger (1.1%) but the difference was not statistically significant (p = 0.68).

Risk factors for hepatitis C virus infection

In the bivariate analysis, 0.7% of the married participants tested positive for HCV antibody but there was no statistical significance between hepatitis C virus infection and marital status (p = 1). Hepatitis C virus infection was not significantly associated with history of blood transfusion, alcohol use, prior surgery and age (p >0.05) (Table 2).

Table 2: Risk factors associated with hepatitis C virus infection

Variable	Hep C positive N (%)	Hep C negative N (%)	P value
Gender			
Female	2 (0.4)	538 (99.6)	0.33
Male	7 (0.9)	809 (99.1)	
Age-group			
<=30	1 (1.1)	88 (98.9)	0.68
31 -50	4 (0.5)	782 (99.5)	
>50	4 (0.8)	477 (99.2)	
Transfusion			
Yes	0	60 (100)	1
No	9 (0.7)	1287 (99.3)	
Alcohol			
Yes	1 (0.2)	575 (99.8)	0.09
No	8 (1.0)	771 (99)	
Surgery			
Yes	2 (0.5)	383 (99.5)	0.68
No	7 (0.7)	964 (99.3)	
Marital status			
Married	8 (0.7)	1160 (99.3)	1.0
Single	1 (0.8)	118 (99.2)	
Widow/Divorced/Separated	0	51 (100)	

Table 3: Risk factors associated with HIV infection

Characteristic	HIV positive N (%)	HIV negative N (%)	OR (CI)	P value
Gender				
Female	6 (1.2)	500(98.8)	1	0.11
Male	19 (2.5)	755 (97.5)	2.09 (0.83 –5.29)	
Age group				
<30	3 (3.4)	84 (96.6)	3.1(0.73 – 13.22)	0.13
31-50	17 (2.3)	737 (97.7)	2.0 (0.73 – 5.46)	0.17
>50	5 (1.1)	434 (98.9)	1	
Transfusion				
Yes	1 (1.8)	54 (98.2)	1	0.94
No	24 (2)	1201 (98)	1.08 (0.14 – 8.13)	
Alcohol				
Yes	13 (2.4)	537 (97.6)	1.45 (0.65 – 3.19)	0.36
No	12 (1.7)	717 (98.3)	1	
Surgery				
Yes	2 (0.6)	365 (99.4)	1	0.02
No	23 (2.5)	890 (97.5)	4.72 (1.11 – 20.11)	
Marital Status				
Married	21 (1.9)	1080 (98.1)	1.13 (0.26 – 4.87)	0.80
Single	2 (1.7)	116 (98.3)	1	
Widow/Divorced/Separated	2 (4.3)	43 (95.7)	2.7 (0.37 – 19.75)	0.31

OR = Odd Ratio; CI = Confidence Interval

Prevalence of HIV

In this study, the prevalence of HIV was 2% (n = 25, 95% CI 1.3 – 2.7%). The prevalence of HIV in females was 1.2% and 2.5% in males. The difference was not statistically significant. The mean age of participants who tested positive for HIV was 43.4 ± 12.4years The prevalence of HIV was highest in the age group 30 years or younger (3.4%) but the difference was not statistically significant (p = 0.24). None of the participants was co-infected with both HCV and HIV.

Risk factors for HIV infection

In the bivariate analysis, HIV infection was significantly associated with prior history of surgery. Prior history of surgery increased the risk of HIV infection by 5 times (OR 4.72, 95% CI 1.11–20.11, *p*=0.02). HIV infection was not significantly associated with history of blood transfusion, alcohol use, age and marital status (See Table 3).

Discussion:

This study represents the results of health screening for hepatitis C virus infection and HIV in asymptomatic Nigerians. The prevalence of HCV in this study was low at 0.7%. This is similar to findings of 0.4% among University under-graduates in Ogbomoso (9), a population based screening in Lagos 0.9% (4), and among first time blood donors in Abeokuta 1.5% (10). It is also similar to findings in studies from other African countries such as Eritrea 0.7%, Somalia 0.9% and Sudan 1% (11 – 12). However, the prevalence of HCV antibody in this study is lower than that reported among healthy blood donors in Port Harcourt 5%, Calabar 10%, a rural population in Cameroon 6.3% and the WHO estimated African regional HCV prevalence of 5.3% (13 – 16). The differences in prevalence across the studies may be due to differences in demographics, socioeconomic status, risk behaviour, and the method of testing employed. The low prevalence in this study may also be due to the fact that the subjects live in Lagos which is an urban area, because the prevalence of HCV has been reported to be higher in rural versus urban areas (17).

The prevalence of HIV infection in this study was found to be 2%, which is similar to studies from Burkina Faso with 1.8%, and Tanzania 2.2% (18, 19). It is lower than the Nigerian national prevalence of 2.9% (2), other studies from Nigeria such as 3.1% from Osogbo (20) and 6.2% from Abeokuta (10), and studies from other African countries such as Ethiopia 3.8%, and Cameroon 4.1% (21–22). The prevalence of 2% is higher than the reported rates of 0.3% in Eritrea and 1.2% in Mauritania among blood donors (11, 23). Differences in demographic factors, socioeconomic status, level of education and exposure

to risk factors may account for these differences.

Many studies have demonstrated co-infection with the hepatitis C virus and HIV. However, no co-infection was demonstrated in this study, and this is similar to other studies from Benin-city and Ethiopia (24 – 25). In this study, older age was not found to be a risk factor for hepatitis C virus infection as it has been previously described. Those who were 30 years or younger had the highest prevalence of HCV antibody but age was not statistically significant. Males had a higher rate of HCV infection in this study but gender was not a significant risk factor. Unsafe blood supply has contributed to HCV transmission in developing countries but in this study previous history of blood transfusion was not significantly associated with risk of HCV infection. Marital status, previous history of surgery, and alcohol use, were not significantly associated with the risks of HCV infection. In as many as one third of subjects with HCV, a risk factor could not be identified for their infection (26 – 28).

In this study, those with no previous history of surgery were 5 times more likely to be infected with HIV. No history of previous history of surgery was significantly associated with the risk of HIV infection. This is surprising because surgical procedures which are invasive offer opportunity for transmission of HIV through poor infection control practices such as use of contaminated surgical instruments, equipment and injections. It may also be due to a cofounding factor. The reason for this is not clear. Transfusion of infected blood and blood products contribute to transmission of HIV infection and these products are routinely screened for HIV. However, prior history of blood transfusion was not significantly associated with risk of HIV infection in this study.

The highest rate of HIV was found in subjects younger than 30 years, as is reported in other studies however age was not significantly associated with the risk of HIV infection. Noteworthy is our finding that the highest rates of HIV occurred in those who had been previously married (divorced or separated or widowed) even though this was not statistically significant. Men were 2 times more likely to be HIV infected in this study but gender was not significantly associated with HIV infection in this study. Some studies have shown that women have significantly higher HIV prevalence rates than men across countries and age groups (29).

In conclusion, this study among apparently healthy Nigerians shows that prevalence rates of HCV and HIV were lower than earlier documented and there was no documented HCV-HIV co-infection. Previous history of surgery was the only significant risk factor for HIV infection. Screening of healthy individuals for HIV and HCV infection is very important to determine those who are infected and therefore need to access treatment.

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Original Article **Open Access**

Prevalence and pattern of infectious dermatoses referrals to clinical microbiologists in a tertiary hospital in Southern Nigeria

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Abstract:

Background: Infectious dermatoses are rife in low resource tropical countries. The consultative roles of clinical microbiologists in these countries are not portrayed in the literature. Specifically, their role in the management of infectious dermatoses has not been emphasized. The objective of this study is to determine the prevalence and pattern of infectious dermatoses referrals to an out-patient infectious disease clinic run by clinical microbiologists.
Method: A retrospective analysis of all outpatient referrals to the clinical microbiologists in a tertiary hospital in southern Nigeria from October 2016 to September 2018 was conducted. The infectious dermatoses referrals were further analysed for patient demographics and frequency of clinical diagnosis.
Results: A total of 545 consults were received, 82 (15.0%) of which were outpatient referrals. Infectious dermatoses accounted for 67 (81.7%) of the outpatient referrals. The male to female ratio was 0.9:1 and mean age was 39.7 ± 17.9 years. Paediatric referrals accounted for 10.4%. The commonest referral diagnoses were tinea corporis/capitis in 28 (41.8%), onchodermatitis in 15 (22.4%) and onychomycosis in 10 (14.9%). Leprosy was suspected in 5 cases (7.5%), lymphatic filariasis in 5 cases (7.5%), and pityriasis in 2 cases (3.0%). Referral diagnosis in 27 cases (38.8%) was a skin-related neglected tropical disease. Microbiological testing confirmed clinical suspicion in 31.3% of cases.
Conclusion: Infectious dermatoses referrals constitute a substantial proportion of referrals to clinical microbiologists in the study location. Clinical microbiologists must be adept in the diagnosis of mycotic and parasitic dermatoses. The trending global health interest in integrated management of skin NTDs should be exploited to advocate for more sensitive diagnostic testing for infectious dermatoses.

Keywords: Clinical, Microbiology, Infectious dermatoses, Consultation, Nigeria

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Prévalence et caractéristiques des dermatoses infectieuses adressées à des microbiologistes cliniques dans un hôpital tertiaire du sud du Nigéria

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Abstrait:

Contexte: Les dermatoses infectieuses sévissent dans les pays tropicaux aux ressources limitées. Les rôles consultatifs des microbiologistes cliniques dans ces pays ne sont pas décrits dans la littérature. Plus précisément, leur rôle dans la gestion des dermatoses infectieuses n'a pas été souligné. L'objectif de cette étude est de déterminer la prévalence et les caractéristiques des dermatoses infectieuses dirigées vers une clinique de traitement des maladies infectieuses ambulatoire gérée par des microbiologistes cliniques
Méthode: Une analyse rétrospective de toutes les consultations externes vers les microbiologistes cliniques dans un hôpital tertiaire dans le sud du Nigéria d'octobre 2016 à septembre 2018 a été réalisée. Les références de dermatoses infectieuses ont ensuite été analysées pour déterminer les données démographiques des patients et la fréquence des
Résultats: Un total de 545 consultations ont été reçues, dont 82 (15,0%) étaient des consultations externes. Les dermatoses infectieuses représentaient 67 (81,7%) des consultations externes. Le ratio hommes/femmes était de 0,9: 1 et l'âge moyen était de 39,7 ± 17,9 ans. Les références pédiatriques représentaient 10,4%. Les diagnostics de référence les plus fréquents étaient les suivants: tinea corporis/capitis dans 28 (41,8%), onchodermite dans 15 (22,4%) et onychomycose dans 10 (14,9%). La lèpre était suspectée dans 5 cas (7,5%), la filariose lymphatique dans 5 cas (7,5%) et le pityriasis dans 2 cas (3,0%). Le diagnostic de référence dans 27 cas (38,8%) était une maladie tropicale négligée liée à la peau. Les tests microbiologiques ont confirmé la suspicion clinique dans 31,3% des cas
Conclusion: Les références de dermatoses infectieuses constituent une proportion substantielle des références aux microbiologistes cliniques sur le lieu de l'étude. Les microbiologistes cliniques doivent être compétents dans le diagnostic des dermatoses mycotiques et parasitaires. L'intérêt actuel pour la santé mondiale dans la gestion intégrée des maladies tropicales négligées de la peau devrait être exploité pour plaider en faveur de tests de diagnostic plus sensibles des dermatoses infectieuses.

Mots-clés: Clinique, Microbiologie, Dermatoses infectieuses, Consultation, Nigéria

Introduction:

Promoted by poverty and the typically tropical climate, infectious dermatoses are rife in resource limited settings (1-3). A number of these dermatoses are cutaneous manifestations of neglected tropical diseases (NTDs), communicable diseases which occur among one billion of the most impoverished populations in the developing world (4). For many infectious dermatoses, particularly the skin-related NTDs, clinical signs are of variable specificity for making diagnoses: the manifestations of leprosy overlap with other skin diseases; chronic skin ulcers may be Buruli ulcer, cutaneous leishmaniasis or yaws; the lower limb swelling typical of lymphatic filariasis may also arise in podoconiosis, tuberculous lymphadenitis or even systemic diseases like heart failure (4). In some cases such as the dermatophytoses, clinical diagnosis is straightforward but laboratory identification is necessary to direct treatment and institute prophylactic measures (5). These reasons place a premium on the clinical microbiology laboratory in the management of infectious dermatoses

Clinical microbiologists possess an in-depth knowledge of infectious disease aetiology which positions them to provide clinical services extending beyond the laboratory bench (6). The extent to which they become involved in direct patient management is often dictated by other specialists who demand their services. Thus, their contributions are often not as visible as surgical or medical specialties (7). In western societies, such clinical roles have been highlighted in scholarly

reviews and audits for the enlightenment of the medical community (6-9). The literature from sub-Saharan Africa is, however, devoid of studies reporting the consultative activities of clinical microbiologists.

At the University of Benin Teaching Hospital in Southern Nigeria, there has been a perceived demand for the skills of clinical microbiologists in sampling and diagnosis of infectious dermatoses. The aim of this study is to document the prevalence and pattern of infectious dermatoses referrals to clinical microbiologists at a tertiary hospital in Nigeria.

Materials and methods:

Study setting

This study was conducted at the University of Benin Teaching Hospital in Edo state, southern Nigeria. The hospital, which serves as a referral centre for surrounding states such as Ondo and Delta, is a 700 bed tertiary facility with a busy outpatients' department. The Medical Microbiology department houses the microbiology laboratory and offers clinical microbiology services provided by consultant clinical microbiologists sub serving administrative, consultative, teaching and research functions. Consultative functions include running of outpatient infectious disease diagnostic clinics.

Study design

This was a two-year retrospective review of referrals sent to the clinical microbiologists from October 2016 to September 2018. All out-patient infectious dermatoses referrals were retrieved and analysed for patient demographics. The distribution of dermatoses based on clinical suspicion was determined. Consultations involved brief history-taking; specimen collection; specimen processing and; microbiological testing including microscopy and culture. Sampling techniques employed were skin scraping for tinea and scabies, nail scraping for onychomycosis, skin snips for onchocerciasis, slit skin smears for leprosy and collection of night blood specimen for lymphatic filariasis.

Data analysis

Data were collated using Microsoft Excel and analysed. Descriptive statistics were calculated for key demographics and referral diagnoses. Frequencies were presented using tables and chart

Ethical statement

As this study involved a retrospective analysis of records, ethical review and approval was not required.

Results:

A total of 545 consults were received during the two-year review period; 463 ward-based consults and 82 referrals to the infectious disease clinic. Infectious dermatoses accounted for 67 (81.7%) of the outpatient referrals. The dermatology clinic was the source of referral in 60 cases (n= 67, 89.6%) while the general practice clinic accounted for the other referrals. Other outpatient referrals were ten (12.1%) sexually transmitted infections (STIs), three (3.7%) chronic respiratory tract infections and two (2.4%) chronic urinary tract infections.

Patient profile

Sixty-seven patients with infectious dermatoses were seen: 32 males (47.8%) and 35 females (52.2%). The male to female ratio was 0.9:1. Mean age was 39.7 ± 17.9 years with a range of five to 83 years. Seven patients (10.4%) were below 18 years of age**.**

Referral diagnosis

Fig 1 shows the relative distribution of infectious dermatoses suspected in the referrals received. As a single entity, the classical NTDs (onchodermatitis, lymphatic filariasis, leprosy and scabies) accounted for 27 (38.8%) referrals. The commonest referral diagnosis in patients under 18

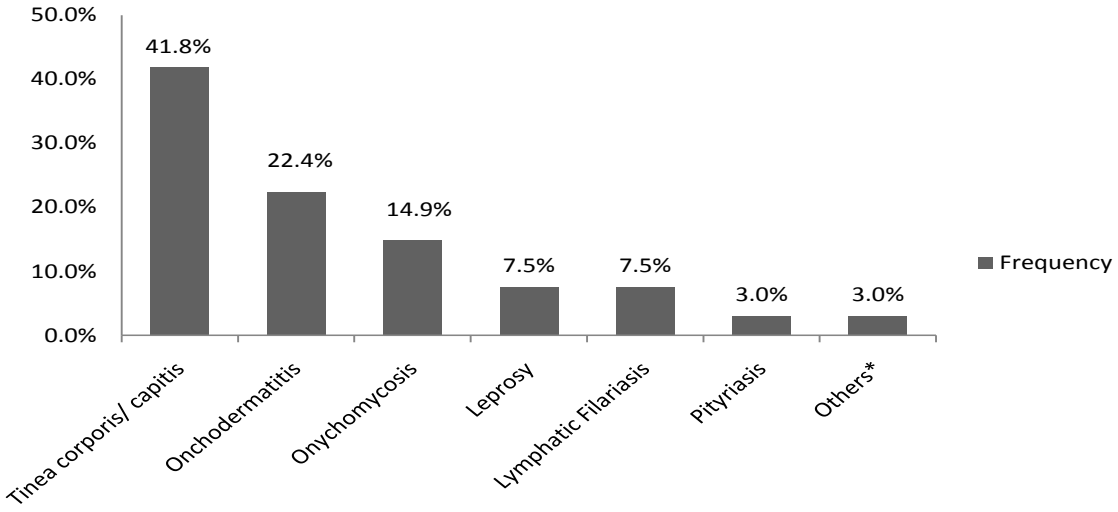

Fig 1: Frequency of infectious dermatoses according to clinical suspicion
*Others = Scabies, Mycobacterial cutaneous ulcer

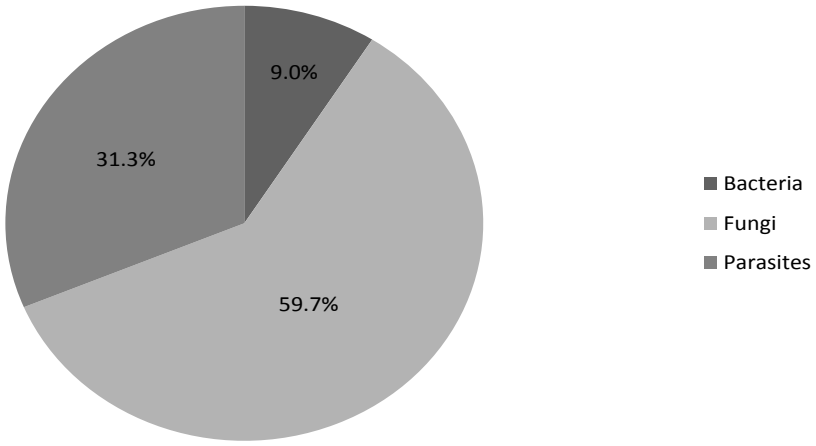

Figure 2: Frequency of infectious dermatoses referral diagnosis according to causative agents

years was tinea in six (n = 7, 85.7%). Distribution of referral diagnosis based on causative agent is depicted in Figure 2.

Microbiological analysis

Microbiological testing which involved microscopy in all cases and culture for suspected fungal infections yielded a diagnosis in 31.3% of cases. Table 1 shows the categories of suspected dermatoses and the number with microbiological confirmation. Among confirmed cases of onychomycosis, there was one tinea unguim (microscopic evidence of dermatophyte infection only), one *Candida* onychomycosis caused by *Candida krusei* and two non dermatophyte mould (NDM) onychomycosis caused by *Aspergillus niger*.

Table 1: Frequency of microbiological confirmation in suspected infectious dermatoses

Dermatosis	Number of consultations	Number with microbiological confirmation (%)
Tinea capitis/ corporis	28	7 (25.0)
Onychomycosis	10	4 (40.0)
Onchodermatitis	15	3 (20.0)
Lymphatic filariasis	5	0 (0.0)
Leprosy	5	4 (80.0)
Pityriasis	2	2 (100.0)
Scabies	1	1(100.0)
Mycobacterial skin ulcer	1	0 (0.0)
Total	67	21 (31.3)

Discussion:

A key responsibility of clinical microbiologists in Nigeria is the running of STI and/ or infectious disease clinics but literature pertaining to the provision of this service is sparse. In this two-year retrospective analysis, infectious dermatoses cases constituted the bulk of out patient referrals to clinical microbiologists in the study location. The results also show the range of infectious dermatoses with which clinical microbiologists need to be adept within a single tertiary hospital.

The actual number of infectious dermatoses cases was low considering the fact that skin infections are the second most common group of dermatoses seen in Nigerian dermatology practice (10). This connotes a reliance on clinical acumen over laboratory diagnosis. The extent to which this is justified varies for individual conditions. For example, the lack of clinical microbiology consultation in the diagnosis of classical scabies is probably justified because the paucity of adult mites on an individual, usually ten to fifteen, makes sampling and microscopic identification insensitive (11). Tinea caused by dermatophytes, on the other hand, are relatively easier to diagnose in the laboratory. Otrofanowei *et al* noted that 80.8% of diagnoses of dermatophytosis in a tertiary hospital were made on clinical grounds without laboratory confirmation (12). This scenario is not unique to low resource settings; according to the European Onychomycosis Observatory, most general physicians and about 60.0% of dermatologists do not sample cases of suspected dermatophytosis (13).

This study revealed microbiological confirmation of the referral diagnoses in approximately three out of ten cases. This is slightly higher than the 28.0% of true positive microbiology test results for skin specimens reported by Xia *et al* in a United States based study (14). However it should be noted that this figure was based on bacterial and fungal cultures since parasitic diseases were not considered. While it is tempting to equate the frequency of microbiological confirmation with the accuracy of clinical judgment, one must consider variations in sensitivity of the different tests which may

have resulted in some false negative results. For example, microscopic examination of skin snips, although highly specific, is inadequate for detecting pre-patent and light *Onchocerca volvulus* infections (15). Sensitivity depends on a number of factors including the number of snips examined, the number of fertile female worms harboured, the distribution of microfilariae in the skin, the snip incubation medium and duration and host immune response (16). PCR and diethylcarbamazine patch test are more sensitive alternatives especially where prevalence is low (17).

Given their knowledge of quality assurance and specimen management, it is reasonable to expect that clinical microbiologists will strive to eliminate pre-analytical variables which could affect test accuracy. The importance of appropriate and adequate sampling of infectious dermatoses lesions can not be overemphasized as sampling errors can account for a large number of negative tests. In dermatophytosis, for example, such errors include taking specimens from the clearing of tinea lesions instead of the edge; not targeting the junction between healthy and affected tissue in onychomycosis; not collecting adequate amount of specimen; and failing to ensure that patients have abstained from antifungal agents in the weeks prior to sampling (5).

The referrals to clinical microbiologists were predominantly mycoses and parasitic dermatoses. Bacterial skin infections other than leprosy and viral diseases like warts were conspicuously absent. Pyodermas (a terminology used to describe all superficial bacterial skin infections associated with production of pus including impetigo, ecthyma and furunculosis) are common skin infections in children both in community surveys and dermatology clinics within and outside Nigeria (18, 19) The absence of referrals is probably because the diagnosis of these infections is straightforward, the causative agents being usually *Staphylococcus aureus* and *Streptococcus pyogenes* which are likely to respond well to antibiotics. Since these infections are commoner in children, it may be the reason why few paediatric referrals were seen in this study. A few studies have reported methicillin resistance rate of 7.4% to 13.0% in *S.aureus* isolates from pyoderma in Asia (20, 21). Similar studies are needed in Nigeria to guide institutional antibiotic policy formulation for treatment of pyodermas.

Fungal dermatoses, especially tinea capitis/corporis were the most common referrals seen. The clinicomycologic correlation in tinea of 25.0% was lower than 48.6% to 66.0% reported from Indian studies (22-24). Thus clinical overdiagnosis of tinea may be the case in the study location. Clinicomycologic correlation for onychomycosis, at 40.0%, was higher than for tinea. Efuntoye *et al* reported a similar rate of 41.4% in south-western Nigeria but Gupta et al reported 59.2% in India (25, 26). Differentials of onychomycosis which include psoriasis, lichen planus and traumatic onchodystrophy could account for negative cases (5). Moreover, *Candida* and NDM were encountered among patients with microbiologically confirmed onychomycosis. These fungi may not respond to antifungals commonly prescribed for the treatment of onychomycosis, further buttressing the need for laboratory diagnosis.

With respect to the parasitic dermatoses, onchodermatitis was the commonest referral, followed by lymphatic filariasis and only one case of scabies. This distribution varies significantly from the findings in a previous dermatology clinic-based study conducted by Airuauhi *et al* in the same location (27). In that study, scabies was the predominant parasitic dermatosis over the five-year study period (1993 to 1998). The paucity of scabies referrals in the index study cannot be attributed to reduced prevalence of the disease because sources have reported resurgence and increasing prevalence (28). A more plausible explanation is that the diagnosis of scabies is typically clinical. Some other

parasitic dermatoses reported by Airuahi *et al* such as myiasis, cutaneous larva migrans and pediculosis pubis were not encountered in this study. Interestingly, the report of Airuahi *et al* suggests that all diagnoses of scabies and other parasitic dermatoses were supported by laboratory evidence. Since their study was retrospective in nature, the practice of subjecting all clinically suspected cases to laboratory investigation is not likely to have been motivated by research. This implies that there has been a change in the way dermatologists utilize microbiology laboratory services in the study location. The reasons need to be identified and if necessary addressed.

Referral diagnosis of skin related NTDs (onchodermatitis, lymphatic filariasis, leprosy and scabies) was common and almost as prevalent as tinea. Majority of cases of NTDs are expected to be encountered in rural communities but skin-related NTDs are reportedly also seen in urban dermatology clinics (29). Hotez *et al* described Nigeria as 'ground zero' for NTDs because of the high prevalence of these diseases in the country (30). Specifically, Edo state where the study site is located is highly endemic for onchocerciasis (31). In the ensuing years since the London declaration on NTDs in 2012, giant strides have been made in the control of NTDs with chemotherapy reaching a billion people in 2014. However, the World Health Organisation (WHO) and partners have focused on mass drug administration (MDA) to the detriment of diagnostics (32).

More sensitive diagnostics are imperative in the era of successfully implemented NTD control programmes because microscopy-based tests are not sufficiently sensitive to detect residual infection. This may have accounted for the lack of microbiological confirmation in some of the cases of onchodermatitis and lymphatic filariasis seen in this location as MDA directed programmes have been implemented to control both diseases in surrounding areas (31).

An emerging concept in the control of NTDs is the integrated management of skin NTDs which is predicated on the knowledge that many of these diseases are associated with skin manifestations (4, 33). This trend can be leveraged to advocate for the provision of diagnostics for the skin NTDs and in extension other infectious dermatoses. Existing diagnostic tests including immunochromatographic tests like the Binax filarial antigen for lymphatic filariasis and the Dual Pathway syphilis test for yaws need to be made widely available in routine diagnostic laboratories via reliable and consistent supply chains. Nucleic acid amplification tests, increasingly available in point of care (POC) formats, should be deployed for diagnosis of skin NTDs and rolled out in a fashion akin to Gene X-pert TB. This will go a long way to improve diagnostic accuracy.

The findings of this study may not apply to other locations of practice in Nigeria. The number and variety of cases should not be interpreted as the magnitude and diversity of infectious dermatoses in the location but as those which various practitioners, mainly dermatologists, felt needed a clinical microbiologist's input. Further studies are required to assess the burden of infectious dermatoses seen in the dermatology clinic and place the demand for microbiological diagnosis in better context. The factors which determine the dermatologist's decision to consult a clinical microbiologist and the impact of such consultations on patient management need to be formally assessed.

To conclude, fungal and parasitic infectious dermatoses are commonly referred to clinical microbiologists who, therefore, need to be adept in diagnosing these conditions. Insensitive testing platforms may militate against effective diagnosis. The trending interest in integrated management of skin NTDs can be capitalized upon to advocate for more sensitive diagnostic platforms for infectious dermatoses. Further studies are needed to evaluate the demand for clinical microbiology services and their impact on the management of infectious dermatoses.

Author Contributions:

The author was responsible for the concept, data collation and analysis, and manuscript preparation.

Conflict of Interest:

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Short Communication **Open Access**

Non detection of *mecA* gene in methicillin resistant *Staphylococcus aureus* isolates from pigs

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Abstract:

Background: Methicillin resistant *Staphylococcus aureus* (MRSA) have become a global health problem causing infections in both humans and livestock, ranging from skin and soft tissue to life threatening blood stream infections. The *mecA* gene is known to confer resistance to MRSA isolates. This study investigated the carriage of *mecA* gene by MRSA isolates from pigs.
Methods: One hundred non duplicate staphylococcal isolates recovered from blood samples of pigs in Bariga district of Lagos State at the Molecular Biology and Biotechnology unit of the Nigerian Institute of Medical Research were used in the study. *S. aureus* was identified by cultural characteristics, and positive catalase, coagulase and deoxyribonuclease tests. Phenotypic methicillin resistance was determined by the modified Kirby Bauer disk diffusion method and *mec*A gene was detected by conventional polymerase chain reaction (PCR) assay.
Results: Twenty-five *S. aureus* were identified, of which 11 (44%) were MRSA by phenotypic method. All the isolates were *mecA* negative on PCR.
Conclusion: The MRSA phenotype observed in the pig isolates in this study appears not to be the classical *mec*A mediated resistance. There may be alternative mechanisms of resistance in MRSA isolates in pigs.
Key words: MRSA, phenotypic, *mec*A gene, PCR, pigs

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Non détection du gène *mecA* dans les isolats de porc provenant de *Staphylococcus aureus* résistant à la méthicilline

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Abstrait

Contexte: Le *Staphylococcus aureus* résistant à la méthicilline (SARM) est devenu un problème de santé mondial provoquant des infections chez l'homme et le bétail, allant de la peau et des tissus mous aux infections du flux sanguin constituant un danger de mort. On sait que le gène *mecA* confère une résistance aux isolats de SARM. Cette étude portait sur le portage du gène *mecA* par des isolats de SARM chez le porc.
Méthodes: Une centaine d'isolats de staphylocoques non dupliqués, récupérés à partir d'échantillons de sang de porcs dans le district de Bariga, dans l'État de Lagos, de l'unité de biologie moléculaire et de biotechnologie de l'Institut nigérian de recherche médicale ont été utilisés dans l'étude. *S. aureus* a été identifié par ses caractéristiques culturelles et par des tests positifs à la catalase, à la coagulase et à la désoxyribonucléase. La résistance phénotypique à la méthicilline a été déterminée par la méthode de diffusion sur disque de Kirby Bauer modifiée et le gène *mecA* a été détecté par un test classique de réaction en chaîne de la polymérase (PCR).
Résultats: 25 *S. aureus* ont été identifiés, dont 11 (44%) étaient des SARM par méthode phénotypique. Tous les isolats étaient *mecA* négatifs en PCR.
Conclusion: le phénotype de MRSA observé dans les isolats de porc dans cette étude ne semble pas être la résistance à médiation classique de *mecA*. Il peut exister d'autres mécanismes de résistance des isolats de SARM chez les porcs

Mots-clés: MRSA, phénotypique, gène *mecA*, PCR, porcs

Introduction:

Staphylococcus aureus is one of the most frequent bacterial pathogens encountered in humans where it causes variety of hospital and community associated infections ranging from mild skin and soft tissue infections, catheter-associated urinary tract infections, surgical site infections, osteomyelitis, to life threatening bacteraemia, septicemia and endocarditis (1). Infections by methicillin resistant *S. aureus* (MRSA) strains have become major public health problem worldwide (2). Since 2000, there have also been reports of zoonotic transmission of livestock-associated MRSA clonal complex 398 (LA-MRSA CC398) leading to increasing number of human infections in Denmark and other European countries with industrial pig production (3, 4, 5).

In Africa, there are only few reports of MRSA in pigs (6, 7, 8) and particularly in Nigeria, only two studies have reported MRSA in pigs (9, 10) with one of them reporting genetically confirmed MRSA strains in pigs that appeared to have originated from a typical community associated MRSA (CA-MRSA) clone in the human population (10).

The *mecA* gene is known to mediate resistance to methicillin and other β-lactamase resistant penicillins but some reports have shown absence of *mecA* gene in MRSA strains (11, 12). The aim of this study therefore is to investigate the genetic basis of MRSA isolates from pigs in Nigeria

Methods:

Study setting and culture isolation

A total of 100 staphylococci isolated at the Molecular Biology and Biotechnology division of the Nigerian Institute of Medical Research Lagos from non duplicate blood samples of pigs in Bariga district of Lagos, were employed in the study. The staphylococcal isolates were first subcultured on Brucella medium to confirm purity and secondarily isolated on Mannitol salt agar after incubating cultures at 37°C for 24 hours. *S. aureus* isolates were identified by characteristic golden yellow colouration, and positive catalase, tube coagulase and deoxyribonuclease (13, 14).

Detection of methicillin resistance by phenotypic test

Resistance to methicillin was performed on all confirmed *S. aureus*

isolates by the modified Kirby-Bauer technique using methicillin (5 µg) and oxacillin (1 µg) disks (Oxoid, UK) performed on Mueller Hinton (MH) agar plates containing 5% sodium chloride, which were incubated at 35°C for full 24 hours (15). The diameter of zone of growth inhibition around each disk was measured and interpreted using the Clinical and Laboratory Standards Institute (CLSI) guidelines (16).

Detection of *mecA* gene by PCR

PCR for amplification of *mecA* gene was performed on the 11 MRSA isolates identified by phenotypic method. DNA was extracted by subjecting overnight cultures of each isolate grown in Brain Heart Infusion (BHI) broth to centrifugation and processing according to the procedure of Arakere et al., (17). The isolated DNA was stored at -20°C until further use. PCR was performed as previously described (18) in a thermal cycler (Gradient Thermocycler, Biologix, China) with mecA1 forward primer 5-AAAATCGATGGTAAAGGTTGGC-3' and mecA2 reverse primer sequence 5'-AGTTCTGCAGTACCGGATTTTGC-3' (18).

The PCR conditions were as described by Oliveira et al., (19) and included an initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 60 seconds, 53°C for 30 seconds, and 72°C for 35 seconds with a final extension at 72°C for 10 minutes. The PCR products were electrophoresed on 2% agarose gel, visualized under UV transilluminator and the image captured with 16 MP Nikon Camera. Amplification of *mecA* gene was expected to produce a band pattern at 533 bp (18).

Results:

Twenty five of the 100 (25%) staphylococci were confirmed as *S. aureus* out of which 11 were resistant to both methicillin and oxacillin. The prevalence of MRSA in the pig population studied is 11% (11/100) but the rate among the *S. aureus* population from the pigs is 44% (11/25). PCR results indicated that all 11 MRSA isolates did not amplify for *mec*A gene with absence of the expected band at 533 bp (Fig 1).

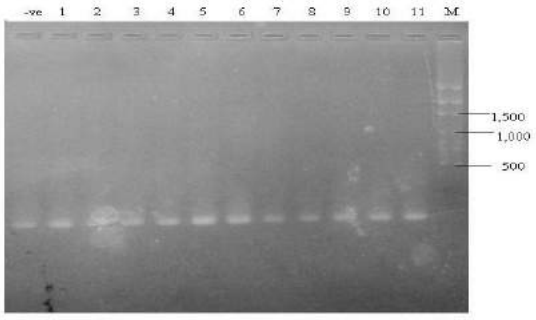

Fig 1: Gel electrophoresis of *mecA* gene

lane M = molecular weight marker; lane 1-11 = *mecA* negative; lane 1 - internal negative control

Discussion:

Although the prevalence of 11% for MRSA isolates among the 100 pigs in this study is lower than the 39% reported in the Netherlands among 540 pigs (20), the MRSA rate of 44% among the *S. aureus* population in the pigs is high. This is very worrisome as many studies including some from Nigeria have shown possible transmission of MRSA infections from pigs to human and vice versa (3-10). A high rate of MRSA in pigs therefore makes them a reservoir and potential source of MRSA transmission and outbreaks, which may constitute a public health challenge in the nearest future.

All the MRSA isolates in our study did not contain the *mecA* gene that is the classical gene responsible for methicillin resistance carried by a unique class of mobile genetic elements, staphylococcal cassette chromosome *mec* (SCC*mec*) (21) originally found in MRSA (22). Other *mec* genes such as *mecC* in *S. aureus* (23, 24) and *mecB* and *mecD* in *Macrococcus caseolyticus* (25, 26) have however been described to mediate methicillin resistance, with capacity for transfer of these genes from one species to another. We did not investigate other *mec* gene types in our study, which is one of the limitations.

Some researchers in Nigeria and elsewhere have also reported absence of *mec*A gene as well as the gene product, PBP2a or PBP2', in phenotypic MRSA

strains and have suggested the possibility of hyper-production of *β*-lactamase as a cause of this phenomenon (11, 12). We also did not test for this in our study.

Although these were obvious limitations in this study, the possibility of *S. aureus* exhibiting resistance to methicillin and other beta-lactamase resistant penicillins other than through *mecA*-mediated resistance should be considered, especially among livestock MRSA strains.

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