

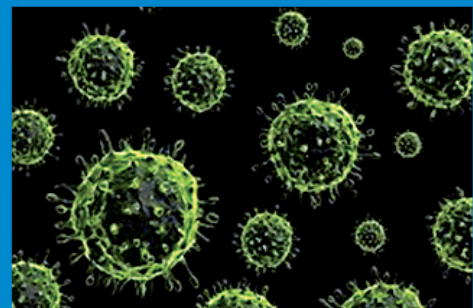


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Review

An Overview of Inhibitory Methods in MAPK Signaling Pathway for the Treatment of Covid-19 Infection

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Abstract

A new coronavirus called SARS-CoV-2 (severe acute respiratory Syndrome-Coronavirus-2) first detected in Wuhan, China, in December 2019, has spread to other regions of China and all over the world as of January, 2020. The coronavirus disease (COVID-19) is a global problem associated with infection caused by a newly discovered coronavirus and causes severe respiratory disorders. Accordingly, various studies have been conducted on it and different aspects of the disease have been identified. ACE2 (Angiotensin converting enzyme 2) is the main receptor for SARS-CoV-2. ACE2 protein allows the virus to re-enter a wide range of human cells and to manage and treatment the Covid-19, special attention should be paid to this feature. Angiotensin II has an inhibitory effect on the expression level of ACE2 through activation of two strategic proteins, ERK1 (extracellular signal-regulated kinase 1) and ERK2. Propagation of these viruses in permissive cells activates signaling pathways, which leads to activation of the MAPK (mitogen-activated protein kinase) superfamily. Accordingly, some studies have explored the molecular mechanisms of MAPK signal pathways involved with inhibitory treatment. Based on recent studies, this article summarizes the mechanisms of MAPK signal transduction pathways involved with inhibitory treatment. This adds great value to the research of molecular mechanisms of inhibitory treatment and also establishes an effective source for its clinical use.

Keywords: Infection, ACE2, MAPK pathway, Coronavirus, Inhibitory treatment

Резюме

През декември 2019 г. за първи път в Ухан, Китай беше открит нов коронавирус, наречен SARS-CoV-2 (тежък остър респираторен синдром Coronavirus-2), който се разпространи в други региони на Китай, както и по целия свят от януари 2020 г. Болестта COVID-19 е глобален проблем, свързан с инфекция, причинена от новооткрития коронавирус и която води до тежки респираторни нарушения. В резултат на провежданите изследвания се установяват различни аспекти на заболяването. ACE2 (ангиотензин конвертиращ ензим 2) е основният рецептор за SARS-CoV-2. ACE2 протеинът позволява на вируса да навлезе в широк спектър от човешки клетки. За да се управлява и лекува болестта, трябва да се обърне специално внимание на този отличителен белег. Ангиотензин II има инхибиращ ефект върху нивото на експресия на ACE2 чрез активиране на два стратегически протеина, извънклетъчна сигнално-регулирана киназа 1 (ERK1) и ERK2. Размножаването на вируса в пермисивни клетки активира сигналните пътища, което води до активиране на т.н. митоген-

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активирана протеин киназа (МАРК) от суперсемејството на МАР киназите. Ето зашто, молекуларните механизми на МАРК сигналните патишта, свързани с инхибиторното лечение са обект на интензивно изследване. Настоящата статия обобщава най-новите данни, публикувани относно механизмите на активиране на патищата на МАРК сигналната трансдукция, свързани с инхибиторното лечение. Настоящият обзор може да допринесе за изясняване на молекуларните механизми на инхибиторното лечение, а също така е и от полза за ефективната му клинична употреба.

Introduction

The coronaviruses' structure is well known and they are single-stranded, enveloped and positive sense RNA viruses. The length of their genome is approximately 30 kb (29903 nucleotides). Studies based on electron microscopy have shown that these viruses range from 60 to 140 nm in diameter with spike-like projections on their surface giving them a crown-like appearance, that is why they are called coronavirus (Richman *et al.*, 2016). The viral genome is packaged by proteins called nucleocapsid and this structure is surrounded by a bilayer lipid structure which consists of membrane, envelope, and spike proteins (Bagca and Avci, 2020) and contains structural proteins that include: spike (S), nucleocapsid (N), envelope (E), and membrane (M) (Siddell *et al.*, 2010). To infect the host cells, the S gene encodes the spike glycoprotein that binds to the human ACE2 receptor (Walls *et al.*, 2020). While the membrane and envelope proteins encoded by the E and M genes join the bilayer lipid envelope structure on the exterior surface of the virus, the nucleocapsid protein encoded by the N gene directly interacts with the viral genome (Zeng *et al.*, 2020). Based on genetic properties, the classification of coronaviruses is into four sub-families, alpha, beta, gamma, and delta. Two important species, SARS-CoV and MERS-CoV, belong to the subcategory beta-coronavirus and can cause severe respiratory diseases in humans (Hilgenfeld and Peiris, 2013).

Symptoms and pathogenesis

The systemic and respiratory disorders caused by COVID-19 infection include fever, cough, fatigue, sputum production, haemoptysis, acute cardiac injury, dyspnea lymphopenia, diarrhea, rhinorrhea, sneezing, sore throat, pneumonia, ground glass opacities, RNAemia and acute respiratory distress syndrome (Rothan and Byrareddy, 2020). Patients infected with COVID-19 show abnormal respiratory findings, higher leukocyte numbers, and increased levels of plasma pro-inflammatory cytokines. One COVID-19 case report showed a patient at 5 days of fever presenting with a cough, body temperature of 39.0°C and coarse breathing sounds in both lungs. The patient's sputum showed positive real-time polymerase chain reaction results that confirmed

COVID-19 infection (Lei *et al.*, 2020). Remarkably high blood levels of chemokines and cytokines were noted in patients with COVID-19 infection. Some patients in critical condition who were admitted to the ICU (intensive care unit) showed high levels of inflammatory cytokines, including IL2 and TNF α , which increased the severity of the disease (Huang *et al.*, 2020).

Entrance mechanism and function

ACE2 is a monocarboxy-peptidase well known for cleaving different peptides surrounded by the renin-angiotensin system and other substrates such as apelin. This enzyme is widely expressed in different organs, such as the gastrointestinal tract and the kidneys, with comparatively low level of expression in the lungs, but barely present in the circulation (Serfozo *et al.*, 2020). ACE2 protein was recognized as the receptor for SARS-CoV on the surface of human cells (Letko and Munster, 2020) and it is a key component of the renin-angiotensin system (RAS). This protein is produced and secreted by a widely different cells and tissues, including the heart, gastrointestinal tissues, brain, and the kidneys (Donoghue *et al.*, 2000; Diz *et al.*, 2008). Recent studies showed that ACE2 is also required for 2019-nCoV entry (Letko and Munster, 2020) and plays an important function in the onset of lung disorder (da Silveira *et al.*, 2010). SARS-CoV-2 genome sequencing has been performed, with its close resemblance to SARS suggesting that they originated from a similar source (Zhou *et al.*, 2020). The COVID-19 pathogen, like other family members, uses a spike protein S1 that enables the insertion of the pathogenic agent into the cell membrane by interacting with the host ACE2 receptor (Netland *et al.*, 2008; Wrapp *et al.*, 2020). An earlier study has demonstrated that the ACE2 protein binding affinity of the 2019-nCoV spike protein ectodomain is 10–20-fold higher than that of the SARS-CoV spike protein (Wrapp *et al.*, 2020).

MAPKinase, a family of serine-threonine protein kinases, consists of three major members: growth factor-regulated ERK1/2, p38 and stress-activated JNK, which play important functions in the regulation of certain cellular attributes (Severin *et al.*, 2010; Yasuda, 2015). Previous studies have

shown that MAPKs signaling pathway is activated in ALI (acute lung injury), and inhibiting MAPKs can effectively alleviate the disease (dos Santos *et al.*, 2018; Zhang *et al.*, 2018). Recent discoveries have indicated that inhibiting the MAPK/NF- κ B pathway can protect against pulmonary fibrosis by up-regulation of ACE2 (Meng *et al.*, 2014). NF- κ B can be activated by MAPKs, a vital role in regulating the activation, survival and differentiation, and regulates transcription factors of inflammation and in ALI, activated NF- κ B increases transcription of inflammatory factors and accelerates disease progression (Luo *et al.*, 2010; Xie *et al.*, 2012; Liu *et al.*, 2017). Several studies indicated that all three MAPK families have a vital role in the regulation of apoptosis (Zhang *et al.*, 2003). ERK is a survival mediator involved in the suppressive effects of growth factors in programmed cell death (Ballif and Blenis, 2001), also JNK and p38 MAPKs are usually involved in the inflammation and induction of apoptosis after exposure to various factors (Harpur and LoGrasso, 2001). MAPK cascades are vital signaling pathways that convert extracellular signals into cellular responses (Cohen, 2002). MAPK signaling transduction includes several members, including Raf/MEK/ERK, which cause many alterations in the cells, such as proliferation and differentiation, stimulus-specific changes in gene expression and induction of programmed cell death (apoptosis). The induction of this pathway occurs due to extracellular agents and pathogens such as RNA viruses. At first there was little information about this, but today the correlation between RNA viruses and this signal transduction pathway is well known. This vital control center of cellular responses operates by different mechanisms to protect the proliferation of several important human pathogenic RNA viruses, including Ebola, hepatitis C, influenza, and SARS corona viruses (Pleschka, 2008).

Management and treatment by MAP-Kinase pathway inhibitors

In March 2003, a new case of CoV was isolated from patients who showed atypical pneumonia. This was subsequently recognized as the causative factor of the respiratory disease now referred to as SARS. By studying the effects of the transiently expressed viral spike protein (S) of SARS-CoV, it was indicated that the S protein plays a necessary function in virus-stimulated COX-2 (cyclooxygenase-2) expression (Cai *et al.*, 2007). COX-2 is a prostaglandin synthetase enzyme involved in inflammatory processes (Wymann and Schneider, 2008) that is closely modulated by various factors

as cytokines (Funk and FitzGerald, 2007). The upstream calcium-dependent PKC α (protein kinase C type α) that regulates the downstream Raf/MEK/ERK pathway is induced by the S protein of SARS-CoV. It was shown that ERK is involved in S protein-induced activation of the COX-2 promoter and the production of COX-2 protein in HEK293T cells. This result helps to describe the vital role of SARS-CoV S protein in SARS pathogenesis (Liu *et al.*, 2007).

More information on the MAPK signaling pathway activated by coronavirus was obtained for MHV (mouse hepatitis virus). MHV infection of cultured cells resulted in activation of all members of this pathway such as Raf/MEK/ERK (Cai *et al.*, 2006) and inhibition of this signaling pathway by UO126, a highly selective inhibitor of both MEK1 and MEK2 through inhibition of the catalytic activity of the active enzyme, or knockdown of MEK and ERK by siRNAs remarkably destructed MHV progeny production (Battcock *et al.*, 2006; Wang *et al.*, 2018). Treatment by inhibitory properties of UO126 did not affect virus entry or cellular and viral mRNA synthesis. Nevertheless, production of viral genomic and sub-genomic RNAs was strongly suppressed by using UO126. These results clearly show that the MAPK signaling pathway is involved in RNA synthesis of MHV (Cai *et al.*, 2007). One of the main reasons for the virus to activate the MAPK pathway is that this up-regulation seems to be involved in the downregulation of IFN synthesis and thus suppresses the cellular innate immune response, which would be useful for viral proliferation (Battcock *et al.*, 2006). There are studies that show that RNA viruses induce MAPK signaling via viral surface structures, which then can activate Ras or PKCs, leading to activation of Raf protein (Ludwig *et al.*, 2004; James *et al.*, 2015).

It remains to be determined what specific interactions between the viral factor and host components trigger MAPK signaling. However, it can be predicted that the viral protein either recruits intracellular signaling factors or interacts with receptor kinases on the cell surface. Replication of the genome and transcription process seems to be modulated by the activity of the pathway. This demonstrates that MAPK cascade activation might be necessary for efficient virus proliferation and suggests that this signaling pathway can be considered an interesting cellular target for potent anti-viral approaches. For example, notable anti-viral action against both types A and B influenza viruses in cell culture (Ludwig *et al.*, 2004) and infected mice

(Ludwig *et al.*, 2003) could be shown for MEK inhibitors, such as U0126, PD098059 and others. Moreover, these compounds have little toxicity in both culture medium (Planz *et al.*, 2001) and mice (Sebolt-Leopold *et al.*, 1999).

Various promising MAPK pathway inhibitors, especially MEK protein, such as CI-1040 are also being tested in clinical trials as anti-cancer approaches to be used over longer periods of time, supporting the idea that these compounds might be beneficial in anti-viral inhibitory treatment (Cohen, 2002; Friday and Adjei, 2008). In addition, inhibitors that involve MAPK signaling agents are being tested in clinical trials in the treatment of HCV-caused HCC based on: this pathway is an important molecular mechanism involved in HCC consist of protection and proliferation. Thus, inhibition of the MAPK pathway would help to fight cancer cells and the causative agent, HCV. Furthermore, MAPK suppression blocks stimulation of the pathway by extracellular signals, such as TGF α and EGF (Pang and Poon, 2007). Furthermore, MEK inhibitors did not demonstrate any tendency to induce replication of resistant viruses (Ludwig *et al.*, 2004). According to a study by Cai *et al.* (2007), two main inhibitors of MEK1/2 mean U0126 and PD98059 suppressed MHV proliferation. In addition, the effects of the MEK1/2 inhibitors on virus replication closely correlated with their inhibitory effects on phosphorylation of the substrate ERK1/2, suggesting a function for the MAPK signaling pathway in MHV proliferation.

Furthermore, studies showed that the expression of Egr-1 gene following MHV infection meaningfully increases in DBT cells. Because Egr-1 gene is a downstream substrate of the MAPK pathway and based on induction by MHV, treating the cells was blocked when the MEK inhibitor U0126 was used. These results strongly support the theory that the components of MAPK pathway play an important function in MHV infection, probably through the induction of its downstream agents such as Egr-1 (Cai *et al.*, 2006). Manipulating cells with U0126 inhibitor, often remarkably suppressed the propagation of influenza A virus, Borna disease virus, Cocksackie-virus B3, and HCMV (Johnson *et al.*, 2001; Planz *et al.*, 2001; Pleschka *et al.*, 2001; Luo *et al.*, 2002). Thus, it appears that the MEK1/2 inhibitors have a broad effect on the propagation of viruses from various families, such as DNA viruses, retroviruses, positive-strand and negative-strand RNA-viruses (Cai *et al.*, 2006). Based on a study by Kennedy *et al.* (2006), the MAP kinases JNK,

p38, and ERK1/ERK2 are also activated by ET-1, microarray analysis of cardiac myocytes stimulated with ET-1 in the absence or presence of the ERK1/ERK2 inhibitor UO126 suggested that the majority of alterations in the level of gene expression in response to ET-1 are regulated by induction of the ERK1/ERK2 signaling pathway. According to a study by Gallagher *et al.* (2008), it was shown that treatment of myocytes with two inhibitors complex, such as PD-98059 and UO126, to prevent the MEK and ERK activation blocked both the ET-1 and ANG-II mediated inhibition of ACE2. The research results of Gallagher *et al.* (2008) show that activation of ERK1/ERK2 and the ERK-mediated regulation of gene transcription represent the molecular mechanism for the reduction of ACE2 by either agonist. The 5' upstream element of the ACE2 promoter contains a number of putative sites for the binding of transcriptional factors regulated by ANG II, including sites for c-jun, c-fos, AP-1 and AP-2 (activator protein) and nuclear factor- κ B.

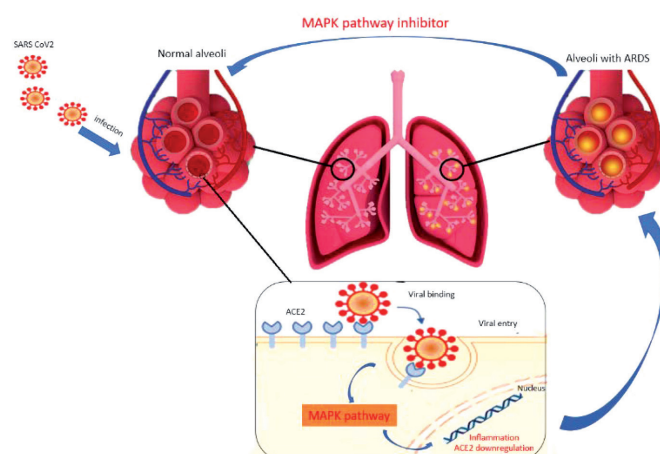


Fig. 1: ACE2 is a receptor for the virus to enter the cell. But because of its protective role, decreased expression through the MAPKinase path can be associated with ARDS.

Conclusion

The COVID-19 virus is a new virus linked to the same family of viruses as SARS. ACE2 is a protein that sits on the surface of various types of cells in the human organs, including the lung, heart, and intestinal surface, and inside the nose. Also ACE2 is a key goal in biochemical-metabolisms that modulates wound healing, blood pressure and inflammation. Amino acid sequence of the ACE2 forms a grooved pocket, allowing it to snag and chop up a destructive protein called angiotensin II, which drives up blood pressure and damages tissues. But angiotensin II is not the only thing that fits in ACE2's pocket. The structures of the coronavirus

and ACE2 protein are complementary so that this protein allows the virus to enter the cell, then infection begins by hijacking the cell's protein-making agents to make copies of itself.

An increasing number of studies indicating the importance of RNA virus-induced MAPK signaling cascade for viral propagation is emerging. Inhibition of MAPK pathways may provide novel anti-inflammatory therapies and this is connected to thorough exploration of virus and host interactions and provides new avenues in virus pathology. It can therefore be expected that our knowledge of RNA virus-induced MAPK signaling will steadily increase over time. The main effects of the ERK signaling pathway are cell proliferation, differentiation and development. Previous studies have shown that ERK signaling plays a key function in lung injuries and ERK1/2 pathway can up-regulate the inflammatory cytokines as IL-1 β and TNF- α during lung inflammation processes. Accordingly, pharmacologic inhibition of ERK molecule, as an inhibitory treatment, provides a promising new therapeutic strategy against lung inflammatory diseases, especially Covid-19. However, the properties of these mechanisms are not yet well understood and require further study; a better understanding of this issue will increase our understanding of the management of the disease. In confirmation of these results, the findings show that inhibition of MEK1/2 and ERK1/2 with specific siRNAs significantly suppressed MHV replication and knockdown of Egr-1 by siRNA inhibited MHV replication. Also, several studies showed that the MAPK p38 plays an important function in acute lung injury. The use of inhibitors such as UO126 and similar compounds can be effective in managing the disease. According to results, it is expected that inhibitory treatment methods are recognized as a beneficial way against Covid-19.

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Production of 2,3-butanediol from fructose by *Bacillus licheniformis* 24

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Abstract

Bulgarian isolate *Bacillus licheniformis* strain 24 possesses considerable advantages as a producer of 2,3-butanediol (2,3-BD) with potential for industrial implementation – the strain is non-pathogenic, has a broad substrate spectrum, cellulolytic activity, and ability to convert glucose, cellobiose, and mannose in 2,3-BD with high amounts, yield, and productivity.

The present study aims to investigate the ability of *B. licheniformis* 24 to produce 2,3-butanediol (2,3-BD) from fructose. In optimized media and process parameters, in batch fermentation from 200 g/L fructose at 33rd hour of fermentation was achieved 83.5 g/L 2,3-BD with yield and productivity of 0.46 g/g and 2.53 g/L/h, respectively. In fed-batch mode from a total of 370 g/L fructose, the obtained concentration of 2,3-BD after 72 hours of fermentation was 156.1 g/L with a yield of 0.42 g/g and productivity 2.17 g/L/h – one of the best results for 2,3-BD production from any substrate obtained to date. This indicates that *B. licheniformis* 24 would be extremely useful in the bioconversion of fructose-containing wastes or feedstocks for economical 2,3-BD production.

Keywords: 2,3-butanediol, fructose, *Bacillus licheniformis*

Резюме

Българският изолат *Bacillus licheniformis* 24 притежава редица качества, определящи го като изключително перспективен продуцент на 2,3-бутандиол (2,3-БД) в индустриален мащаб - той е непатогенен, притежава широк субстратен спектър, целулазна активност и способността да конвертира редица захари в 2,3-БД във високи концентрации, добив и продуктивност. Настоящото изследване цели да установи способността на *B. licheniformis* 24 да продуцира 2,3-БД от неизползван до момента субстрат – фруктоза. При периодичен процес в оптимизирани за щам хранителна среда и условия, от 200 г/л фруктоза, след 33 часа ферментация, бяха получени 83.5 г/л 2,3-БД с добив 0.46 г/г и продуктивност 2.53 г/л/ч. При периодичен процес с подхранване, при същите условия, от общо 370 г/л фруктоза, за 72 часа бяха продуцирани 156.1 г/л 2,3-БД с добив от 0.42 г/г и продуктивност 2.17 г/л/ч. Тези резултати са едни от най-добрите, получени до момента за 2,3-БД от какъвто и да е субстрат, което прави щам *B. licheniformis* 24 изключително перспективен като продуцент на 2,3-БД чрез биоконверсия на отпадъци и суровини, съдържащи фруктоза.

Introduction

2,3-Butanediol (2,3-BD) is a versatile platform chemical, indispensable in the manufacturing of drugs (antiseptics, cryoprotectants), cosmetics, food additives, fuels, antifreeze, resins, paints, solvents, lacquers, and as a precursor for the synthesis of thermoplastic polymers (Ji *et al.*, 2011; Hakizimana *et al.*, 2020; Tinôco *et al.*, 2021). It can be obtained by chemical and biochemical routes, however, the rising price of fossil fuels and the need for reduction of greenhouse gas emissions, fo-

cus the contemporary attention exclusively on the biorefinery concept. White biotechnologies comprise a search for microbial approaches to synthesize chemicals that in the past could only be produced chemically as 2,3-BD (Frazzetto, 2003). In the case of 2,3-BD production, the biological route provides environmental and economic advantages due to the use of renewable substrates, the selective production of 2,3-BD isomers, and the possibility for strains and processes improvement. However, although significant progress, including fermenta-

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tion and downstream processes intensification, to date the microbial production of 2,3-BD remains uncompetitive. As the main reasons are considered the relatively high costs of the used substrates, the pathogenic nature of the best producers such as *Klebsiella pneumoniae* (Petrov and Petrova, 2010), *K. oxytoca* (Cho *et al.*, 2015), *Enterobacter cloacae* (Priya and Lal, 2019), *Serratia marcescens* (Zhang *et al.*, 2010; 2016), and *Raoultella terrigena* (Ripoll *et al.*, 2021), as well as the huge losses of the product during its extraction and purification. Hereof, investigations are focused on finding new, non-pathogenic producers, capable of converting cheap and abundant carbon sources, such as industrial or agricultural wastes, into 2,3-BD with high titer, yield, and productivity, thus diminishing the importance of the inevitable losses.

Isolated from soil, strain *B. licheniformis* 24 was proved the best 2,3-BD producer from all non-pathogenic strains in our collection (Petrova *et al.*, 2020). This strain produces 2,3-BD in two stereoisomeric forms – the optically inactive meso-form and the optically active D-form. Also, the strain 24 possesses several advantages, defining it as very promising for implementation as a 2,3-BD producer on an industrial scale: (i) ability to convert in 2,3-BD a broad spectrum of carbon sources, including glucose, cellobiose, mannose, galactose, starch, inulin and glycerol (Petrova *et al.*, 2020); (ii) extremely high 2,3-BD tolerance, allowing it to withstand titers of 14% (w/v) 2,3-BD (Tsigoriyna *et al.*, 2021); (iii) being extremely aerobic, *B. licheniformis* 24 produces 2,3-BD instead of acetoin even at high aeration rates.

The present work aims to investigate the capabilities of *B. licheniformis* 24 to convert fructose into 2,3-BD. The obtained results would be of high importance to determine the abilities of the strain to convert fructose-containing industrial or agricultural wastes.

Materials and Methods

Strain and cultivation conditions

The strain *B. licheniformis* 24 was isolated from soil near the banks of the Yantra River in Veliko Tarnovo and was identified by 16S rDNA sequencing (NCBI GenBank accession number MK461938). It was stored frozen at -80°C in a 30% glycerol solution.

Batch and fed-batch processes were performed in 1L stirred bioreactor (Biostat® A Plus, Sartorius Stedim Biotech, Gottingen, Germany) additionally equipped with bumpers, air pump, and

rotameter in an attempt to ensure higher levels of oxygen supply. In both cases was used medium optimized for 2,3-BD production from glucose (Tsigoriyna *et al.*, 2021) supplemented with 200 g/L fructose instead of glucose. In fed-batch processes, fructose was added as an 87% (w/v) sterile water solution. The pH, temperature, and aeration rate were maintained at their optimal values of 6.23 (by addition of 6M NaOH or 5M HCl), 37.8°C, and 3.68 vvm, respectively, as they were already optimized for 2,3-BD production by *B. licheniformis* 24 (Tsigoriyna *et al.*, 2021). Inoculums were prepared with 100 mL media in 500 mL flasks on a rotary shaker at 37°C and 200 rpm. Inoculation of 10% was made when inoculums reached an optical density of 2.3 ($OD_{600}=2.3$).

Analytical methods

Fructose and soluble products were determined by HPLC analysis using YL Instruments 9300 HPLC System (YL Instrument Co., Ltd., Anyang, South Korea). The components were separated by an Aminex HPX-87H column (BioRad Laboratories, Hercules, CA, USA) as fructose, meso-2,3-butanediol, D-2,3-butanediol, glycerol, and ethanol were detected by RI detector (YL 9170 RI Detector), while acetoin and lactic acid – by UV detector (YL 9120 UV/Vis Detector) at 190 and 210 nm, respectively.

Results and Discussion

Batch fermentation of fructose

In the batch process from 200 g/L fructose, the maximum concentration of 2,3-BD was achieved after 33 hours of fermentation – 83.5 ± 1.5 g/L, when the fructose was completely depleted (Fig. 1a). It is remarkable that the so obtained fructose consumption rate of 5.52 g/L/h by *B. licheniformis* 24 significantly surpasses the glucose consumption rate of the strain of 3.98 g/L/h, received in batch fermentation in the same conditions and initial substrate concentration (Tsigoriyna *et al.*, 2021). Similarly, although the obtained titer 2,3-BD from fructose is less than that from glucose (83.5 vs 91.2 g/L), it was received by significantly higher productivity – 2.53 g/L/h, instead of 1.94 g/L/h from glucose. The 2,3-BD yield from fructose was 0.46 g/g, less than that from glucose – 0.488 g/g.

The main co-product of fructose fermentation is glycerol. At the moment when fructose was completely consumed, 37 g/L glycerol was formed and no other soluble metabolites were detected, except insignificant quantities of ethanol (about 0.1 g/L). Lactic acid appeared to be a temporary prod-

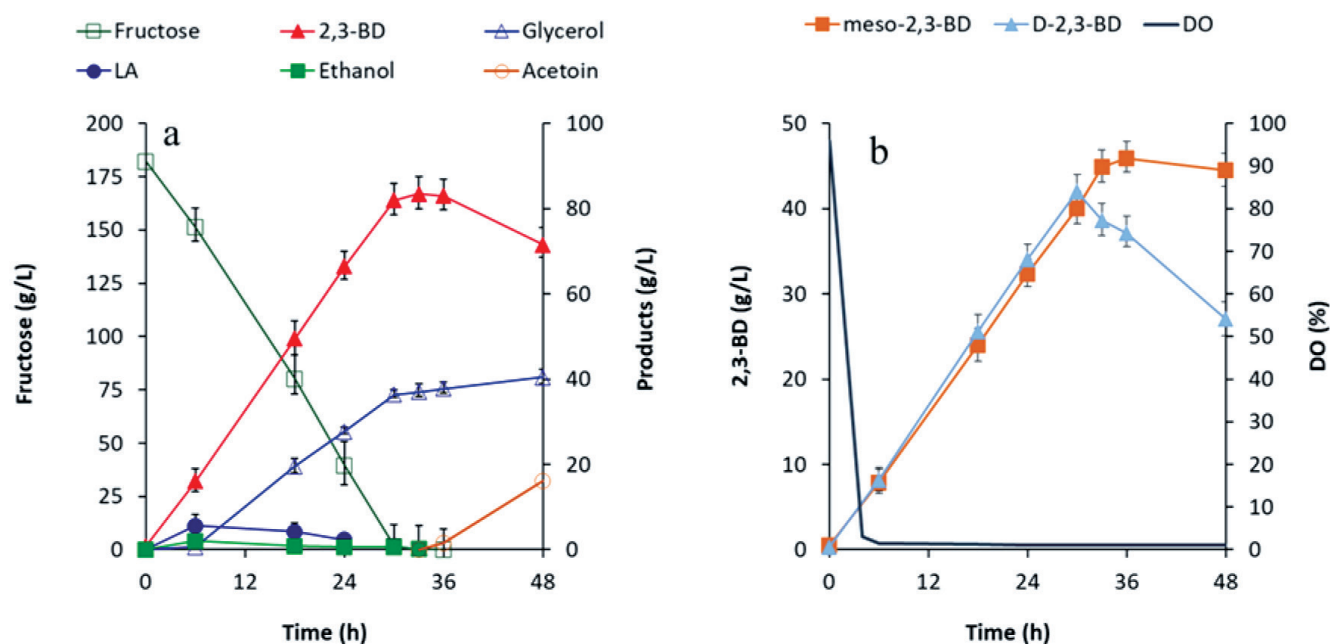


Fig. 1. Batch fermentation of 200 g/L fructose by *Bacillus licheniformis* 24: a) Time profiles of fructose consumption and product formation; b) Time profiles of meso- and D-2,3-BD accumulation and dissolved oxygen availability.

uct of fermentation, reaching its maximum titer of 5.67 g/L at the 6th hour of fermentation. Thereafter, its concentration decreases and completely disappear after the 24th hour. Similar is the time profile of ethanol accumulation in the broth. Ethanol titer reached its maximum of 2.05 g/L at the 6th hour and slowly decreased afterward (Fig. 1a).

After the 33rd hour, when the carbon source was no more available, 2,3-BD sharply was converted into acetoin. The quantification of the different 2,3-BD isomer concentrations shows that in acetoin is converted especially D-2,3-BD form, while the meso-form remains almost in the same concentration at least 15 hours after the complete substrate depletion (Fig. 1b). In this period acetoin concentration in the broth increased from 0 to 16.1 g/L, and dissolved oxygen concentration remained at its lowest level (1-2 %) which indicates that the biomass is still vital (Fig. 1b).

Although all these effects are already observed in glucose fermentation of *B. licheniformis* 24, two significant differences should be noted. In batch fermentations with high initial substrate concentrations (i.e. 200 g/L), substrate consumption and 2,3-BD production are quite faster when fructose is used. Likewise, fructose is the single substrate, from which strain 24 produces D-2,3-BD in higher concentrations than meso-2,3-BD (Fig. 1b).

Fed-batch fermentation of fructose

Usually, the fed-batch mode is performed in an attempt to achieve the aim product in the highest

possible concentration and productivity by eliminating substrate inhibition. For 2,3-BD production, fed-batch is performed regularly because of its relatively less toxic nature and necessity to be obtained in concentrations as high as the producer's tolerance allows.

The fed-batch fermentation of fructose by *B. licheniformis* 24 was provided in the same media and conditions as the batch fermentation, including the initial fructose concentration. During the process, additional fructose was added as sterile water solution when fructose concentration decreases to below 50 g/L. Thus, after 72 hours of fermentation, a total of 370 g/L fructose was consumed and 156.1 ± 3.6 g/L 2,3-BD was produced (Fig. 2a).

As soluble by-products were detected glycerol (43.1 g/L) and acetoin (16.7 g/L). Lactic acid and ethanol appeared to be only temporary products of fermentation and were not detected after the 48th hour. On the contrary, acetoin begins to accumulate in the broth only after 30 hours (Fig. 2a). At the 72nd hour, when 2,3-BD maximal concentration was reached, it was produced in stereoisomer ratio meso-2,3-BD/D-2,3-BD of 1.00/1.35. The detailed kinetics of stereoisomers accumulation reveals that until the 24th hour, both isomers were produced approximately in equal quantities, but thereafter, the produced D-2,3-BD was significantly higher bringing the ratio of 1.00/1.51 (meso-form/D-form) at the 60th hour. After that, between 60th and 72nd hour, when acetoin accumulation began, although

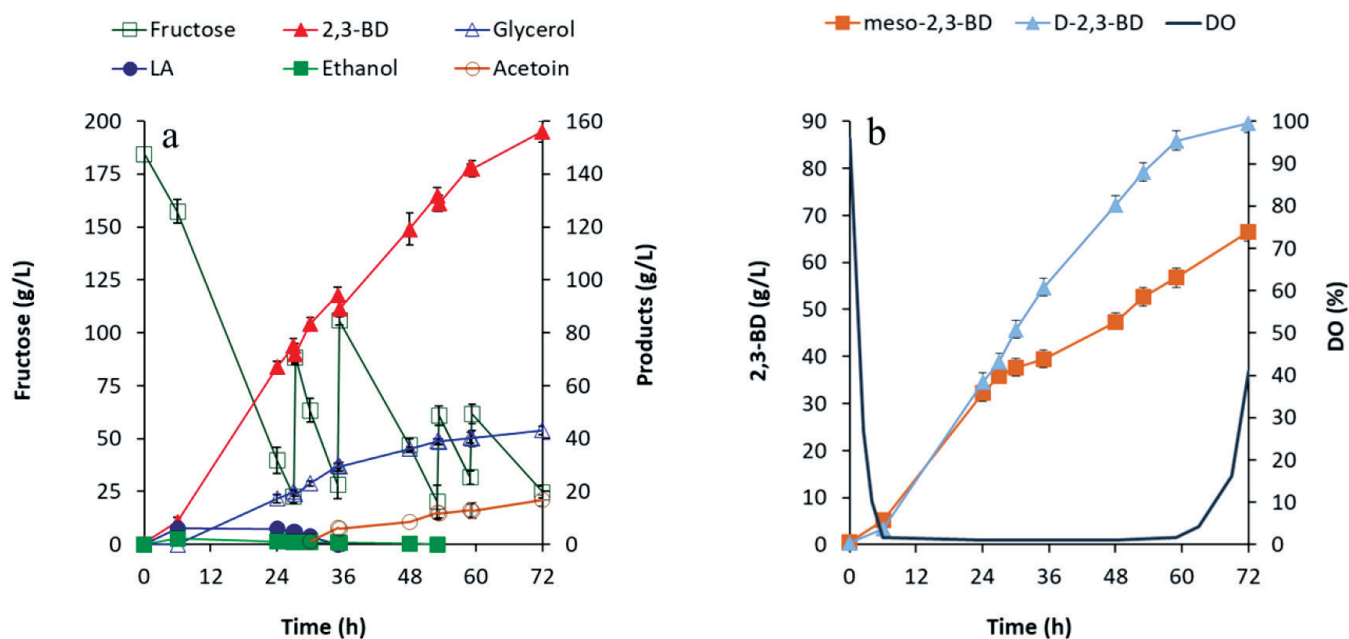


Fig. 2. Fed-batch fermentation of fructose by *Bacillus licheniformis* 24: a) Time profiles of fructose consumption and product formation; b) Time profiles of meso- and D-2,3-BD accumulation and dissolved oxygen availability.

the concentrations of both isomers continue to increase, the ratio returns to 1.00/1.35 (Fig. 2b). This indicates, that when acetoin is produced, obviously this is mainly at the expense of the D-2,3-BD. The level of the dissolved oxygen in the broth dropped sharply from 95.8% to 1.7% during the first 6 hours of the fermentation and remained almost constant until the 60th hour. Then, it gradually increased from 1.7% to 40.9% at the 72nd hour, most probably due to the decrease of the number of living cells (Fig. 2b).

These results suggest, that when *B. licheniformis* 24 is used for 2,3-BD production, fructose appeared to be a better carbon source than glucose. Both the obtained maximum concentration and productivity of 2,3-BD from fructose were higher than those from glucose, with 12.5% (156.1 g/L vs 138.8 g/L), and with 87.1% (2.17 g/L/h vs 1.16 g/L/h), respectively. Likewise, the fructose consumption rate of 5.14 g/L/h was more than two-fold higher than the corresponding glucose consumption rate of 2.41 g/L/h (Tsigoriyna *et al.*, 2021). As glucose is considered to be the preferred carbon source, one possible explanation for this phenomenon lies in the ability of *B. licheniformis* 24 to produce exopolysaccharides (EPSs) (Petrova *et al.*, 2021). EPSs are produced in very high amounts and form a polysaccharide capsule, which leads to an increase in the viscosity of the culture broth. From glucose and fructose, strain 24 produces different EPSs as from glucose the produced amounts of EPS are nearly

double (Petrova *et al.*, 2021). As a result, when glucose is fermented, the culture broth is much more viscous and it was more difficult to maintain the proper aeration.

The other remarkable fact is that from fructose only, strain 24 produces more D-2,3-BD than meso-2,3-BD. By all other substrates used, the produced ratio meso-2,3-BD/D-2,3-BD is between 1.6/1.0 and 1.1/1.0 (Petrova *et al.*, 2020; Tsigoriyna *et al.*, 2021). It was observed that with a higher 2,3-BD concentration this ratio decreases, regardless of the substrate used. This dependence is also confirmed in fructose fermentation, except for the fact that D-2,3-BD is the predominantly produced form.

In conclusion, it should be noted, that the obtained concentration of 156.1 g/L 2,3-BD in fed-batch fermentation from fructose is the highest amount reached by any microorganism to date. Remarkably, this was achieved employing a non-pathogenic strain and from a substrate other than glucose. Although remaining below the record values obtained by the pathogenic producers of 2,3-BD such as *K. pneumoniae* (Ma *et al.*, 2009), *Enterobacter ludwigii* (Maina *et al.*, 2019), or *Serratia marcescens* (Zhang *et al.*, 2010) ranging between 3.5 and 4.2 g/L/h, the productivity of 2.17 g/L/h of *B. licheniformis* 24 is relatively high for a representative of the genus *Bacillus*.

Conclusions

The presented results revealed that *B. licheniformis* 24 is capable to produce 2,3-BD from fruc-

tose in extremely high concentrations with useful productivity and yield. Its non-pathogenic nature, exceptional resistance to 2,3-BD, and high fructose consumption rate determine the strain as very promising for bioconversion of fructose-containing substrates in industrial scale.

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Antimicrobial Sensitivity and Distribution of *Pseudomonas aeruginosa* in Cantonal Hospital of Zenica, Bosnia and Herzegovina

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Abstract

Pseudomonas aeruginosa (PA) is one of the most common nosocomial pathogens that occurs in the human population. In 2017, PA was recognised as one of the most life-threatening bacteria microorganisms. Infections caused by PA isolates are exceedingly difficult to treat due to intrinsic antibiotic resistance and an incredible capability to gain resistance to multiple groups of antimicrobial agents. The purpose of this study was to determine the incidence of PA isolates in inpatient and outpatient samples, to determine the incidence of PA isolates in different hospital wards and to analyse the sensitivity of PA isolates to the tested antimicrobial drugs. The study included a collection of 152 biological samples and 6 swabs of inhumane material inside the hospital obtained and processed in the Microbiological laboratory of the Cantonal Hospital in Zenica, Bosnia and Herzegovina (BiH), over a period of 3 months from October to December 2018. The samples which were positive for PA were 31.58% of hospital population, and 68.42% were outpatient population. The largest number was taken from the surgical ward (60.42%), while urine was the most frequent sample with 47.92%. PA showed susceptibility to amikacin 89.6%, imipenem 83.3%, piperacillin tazobactam 81.3%, ceftazidime 79.2%, cefepime 68.8%, gentamicin 62.5% and ciprofloxacin 60.4%. In most cases PA was isolated from outpatient samples. PA showed the highest susceptibility rate to amikacin, while it showed the greatest resistance to ciprofloxacin. The largest number of samples in the hospital environment originated from the surgical department.

Keywords: *Pseudomonas aeruginosa*, isolates, intrahospital infections, antimicrobial resistance

Резюме

Pseudomonas aeruginosa (PA) е един от най-често срещаните нозокомиални патогени в човешката популация. От 2017 г. PA е призната за една от най-застрашаващите живота бактерии. Инфекциите, причинени от изолати на PA, са изключително трудни за лечение поради присъщата антибиотична резистентност и невероятната способност за придобиване на резистентност към множество групи антимикробни агенти. Целта на това проучване е да се определи разпространението на PA изолатите в болнични и амбулаторни проби, да се установи тяхното разпространение в различни болнични отделения и да се анализира чувствителността на тези изолати към проучваните антимикробни лекарствени средства. Проучването включва събиране на 152 биологични проби и 6 тампона от нехуманен материал в болницата, обработени в Микробиологичната лаборатория на Кантоналната болница в Зеница, Босна и Херцеговина, за период от 3 месеца - от октомври до декември 2018 г. Положителните проби за PA от болничната популация са 31.58%, а от извънболничната популация - 68.42%. Най-много са изолатите от проби, взети от хирургичното отделение (60.42%), докато при пробите от урина се отчита 47.92% честотата. Чувствителност към изследваните антибиотици е

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както следва: амикацин - 89.6%, имипенем - 83.3%, пиперацилин тазобактам – 81.3%, цефтазидим – 79.2%, цефепим – 68.8%, гентамицин – 62.5% и ципрофлоксацин - 60.4%. В повечето случаи PA е изолиран от амбулаторни проби. PA показва най-висока степен на чувствителност към амикацин, а най-голяма резистентност - към ципрофлоксацин. Най-голям брой проби в болничната среда произхождат от хирургичното отделение.

Introduction

Pseudomonas aeruginosa (PA) is an opportunistic pathogen that exists within the body of living creatures as a part of the normal microbiota but might result in mild to extreme diseases if they reach sterile sites of the body. Among infections caused by Gram-negative rods, PA has a leading position, mainly in immunocompromised patients. Antimicrobial resistance has caused an extreme restriction in treatment alternatives for PA infections. PA is a pathogen presenting a large genome that may develop wide variety of factors related to antibiotic resistance involving almost all classes of antibiotics (Gale *et al.*, 2015; Gomila *et al.*, 2018).

In 2017, PA was recognised as one of the most life-threatening bacteria microorganisms and indexed as priority pathogen for Research and Development of new antibiotics by the World Health Organization (WHO) (World Health Organization, 2017). PA is liable for 10–15% of nosocomial infections global (Blanc *et al.*, 1998). The WHO study confirmed that the highest prevalence of nosocomial infections occurs in orthopaedic wards and intensive care units (ICUs), that is explained via an extensively higher quantity of invasive procedures and a higher frequency of antibiotic use in those wards. PA is one of the most common pathogens that causes intrahospital infections, especially in ICUs. Factors that help this pathogen to be dominant in this kind of sensitive department are mechanical ventilation, surgical interventions, and previous exposure to imipenem (Prasad *et al.*, 2009).

Infections caused by PA isolates are exceedingly difficult to treat due to intrinsic antibiotic resistance and an incredible capability to gain resistance to multiple groups of antimicrobial agents (Strateva and Yordanov, 2009). Such infections are associated with excessive mortality rates, which range from 18 to 61% (Kang *et al.*, 2003).

Carbapenems are regarded as a drug of final resort and are used to treat severe infections caused by multidrug-resistant PA. Though, growing use of carbapenems has increased the prevalence of carbapenem-non-susceptible (CnS) PA strains. Moreover, such strains are regularly resistant against other drugs which include β -lactams and quinolones (Codjoe and Donkor, 2017). According to surveillance from the USA and Europe, the prevalence

of CnSPA multiplied from 4% within the 1900s to 14–36% inside the 2000s. In China, the prevalence of CnSPA is >30% (Souli *et al.*, 2008; Shi *et al.*, 2019). Thus, CnSPA has induced many nosocomial outbreaks (Peña *et al.*, 2003; Zavascki *et al.*, 2005; Logan *et al.*, 2017).

Earlier studies display that irrelevant antimicrobial therapy and/or postponed introduction of effective antimicrobial therapy is related with a damaging outcome (Kang *et al.*, 2003; Codjoe and Donkor, 2017).

The purpose of this study was to determine the incidence of PA isolates in inpatient and outpatient samples in Cantonal hospital of Zenica (CHZ), Bosnia and Herzegovina, to determine the incidence of PA isolates in different hospital wards and to analyse the sensitivity of PA isolates to the tested antimicrobial drugs.

Materials and Methods

Collection of specimens

The study included a collection of 152 biological samples and 6 swabs of inhumane material inside the CHZ obtained and processed in the Microbiological laboratory. These specimens which included wound swabs, tracheostoma swabs, bronchoalveolar lavage fluid, urine, ear swabs and other swabs were collected over a period of 3 months. Another six samples of inhumane material were taken during the earlier period in the surgical, physical, paediatric department and from the hospital pharmacy and kitchenette of the hospital. These included incubator water, rivanol, aspirator, distilled water and tap water.

Processing of specimen and preliminary identification of isolates

Swab samples were inoculated onto Blood agar and MacConkey agar. The plates were incubated at 37°C for 24 – 48 hours. After incubation, isolated colonies undergo biochemistry tests to examine the biochemistry characteristics of the isolated strain. The morphology of the colonies on pure culture was used in the preliminary identification of PA isolates. Colony characteristics such as size, colour, shape, and consistency were observed, pigment production, the outline of the edges and haemolysis on blood agar. For biochemical testing, Kligler's Iron Agar Test, Indole Test, Simmons Citrate Agar,

Christensen Urease Test and Parisian Mannitol were used. PA was citrate positive. Nitrate reduction test and oxidase test were positive.

Oxidase test

The oxidase test is a test used to identify bacteria that can secrete the enzyme cytochrome oxidase. When a bacterium has cytochrome oxidase as an integral part of its respiratory chain, we say that the bacterium is oxidase positive. To be able to record this property, it is necessary to use a reagent that will change colour depending on the presence of enzymes. The filter paper is soaked in tetramethyl-p-phenylenediamine dihydrochloride reagent, and several colonies of the test culture are applied to it and wait 10-30 seconds for the finding to be interpreted. If the inoculated area of the paper changes colour to purple, the test is said to be positive. In the case of PA there is a change in colour to dark purple.

Antimicrobial susceptibility testing of PA isolates

The modified disc-diffusion method of Bauer and Kirby was used to determine susceptibility of PA isolates according to EUCAST (The European Committee on Antimicrobial Susceptibility Testing) standards to the following antibiotics: cefepime 30 µg, ceftazidime 10 µg, imipenem 10 µg, ciprofloxacin 5 µg, amikacin, gentamicin 10 µg, piperacillin-tazobactam 30/6 µg. Mueller-Hinton agar was used to examine the antimicrobial sensitivity of PA.

Statistical analysis

The results were processed using standard statistical methods using the SPSS Statistical Package for Social Sciences version 13.0 (Chicago, IL, USA). Kolmogorov-Smyrn test, or Shapiro-Wilk test, was used to assess the normality of the distribution of continuous variables. The results for independent continuous variables are expressed as median Me (X) and interquartile interval (Iq), and as absolute N and percentual values (%). The significance of the difference for independent continuous variables that did not follow the normal distribution

was used in the Mann-Whitney-U test. In the analysis of the differences between the observed groups for categorical variables, Hi-square, or Fisher's exact test, was used. The value of $p < 0.05$ was taken as statistically significant.

Results

All of PA isolates showed a positive oxidative test. Out of a total of 152 samples, 48 of them (31.58%) were inpatient, while the other 104 (68.42%) were outpatient samples (Table 1). Out of a total of 48 hospital patients in whom PA was isolated, 26 (54.17%) were male, while the remaining 22 (45.83%) were female. Out of a total of 104 outpatients in whom PA was isolated, 54 (51.92%) were male, while the remaining 50 (48.08%) were female. The mean age of a hospital examinees in whom PA were detected was 64 years (35.75-74.0), while in non-hospital examinees it was 57 years (28.0-71.0).

The frequency of the age group from 0 to 10 years inpatients was 6 (12.5%), the age group from 11 to 60 years the frequency was 14 (29.17%), while the age group over 60 years was 28 (58.33%). The frequency of the age group from 0 to 10 years in outpatients was 23 (22.12%), the age group from 11 to 60 years the frequency was 38 (36.54%), while the age group over 60 years was 43 (41.35%). A statistically significant difference was found in the frequency of occurrence of different age groups of inpatients and outpatients from whose samples PA was isolated ($p < 0.001$) (Table 1).

Out of a total of 48 hospital patients in whom PA was isolated, 29 (60.42%) were from the surgical ward, 13 (27.08%) were from the internal medicine ward, while the other 6 (12.5%) were from the paediatric ward. The difference in the distribution of PA isolates in relation to the hospital ward was statistically significant ($p < 0.001$) (Fig. 1).

The largest number of hospital samples in which PA was isolated was obtained from the surgical ward and its frequency in the age group of 11 to 60 years was 12 (25%), and in the age group over 60 years it was 17 (35.42%) (Fig. 2).

Table 1. Frequency of PA isolates in relation to sex, age and sample origin

	Inpatient	Outpatient
<i>P. aeruginosa</i> isolates, n (%)	48 (31.58)	104 (68.42)
Male, n (%)	26 (54.17)	54 (51.92)
Mean age	64 (35.75-74)	57 (28-71)
The age group from 0 to 10 y, n (%)	6 (12.5)	23 (22.12)
The age group from 11 to 60 y, n (%)	14 (29.17)	38 (36.54)
The age group over 60 y, n (%)	28 (58.3)	43 (41.35)

Results are shown as absolute numbers N and percentage values (%); p – probability

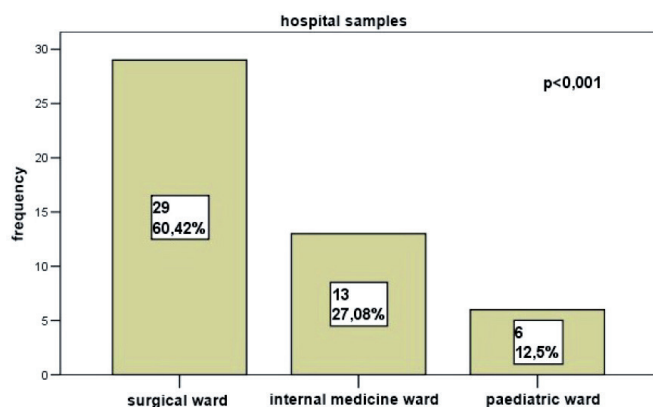


Fig. 1. Distribution of PA isolates in relation to the hospital ward. Results are shown as absolute numbers N and percentage values (%) p – probability

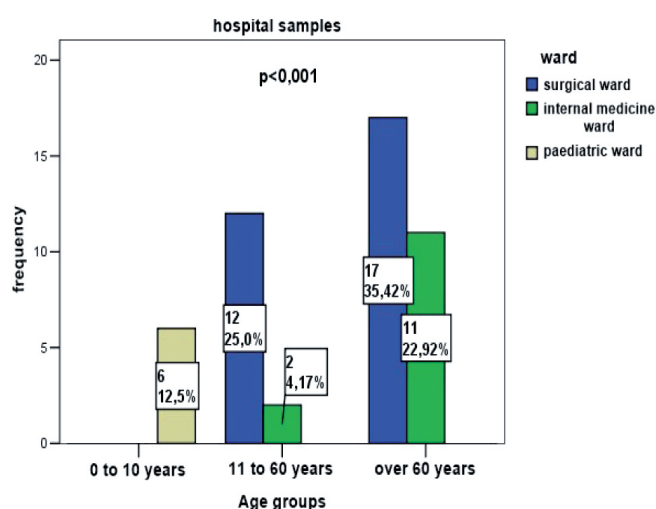


Fig. 2. Frequency of age groups in hospital patients in whom PA was isolated in relation to the hospital ward from which the samples were taken. Results are shown as absolute numbers N and percentage values (%). p – probability

The frequency of hospital samples in which PA was isolated from internal medicine wards in the group from 11 to 60 years was 2 (4.17%), and in the

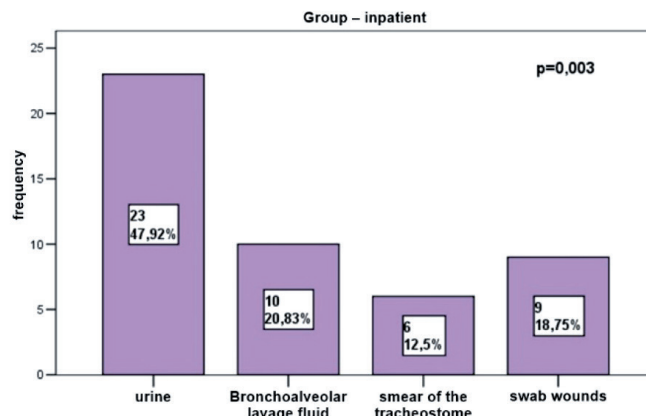


Fig. 3. Distribution of different types of PA samples in hospital patients

age group over 60 years 11 (22.92%). The difference in the representation of age groups in hospital samples in which PA was isolated in relation to the hospital ward from which the samples were taken was statistically significant ($p < 0.001$).

Out of a total of 48 hospital patients in whom PA was isolated, 23 (47.92%) had bacteria detected in urine, 10 (28.83%) in bronchoalveolar lavage fluid, and 6 (12.5%) from the smear of the tracheostoma and in the other 9 (18.75%) from the wound swab (Fig. 3). Out of a total of 104 outpatients in whom PA was isolated, 43 (41.35%) had bacteria detected in urine, 38 (36.54%) from wound swabs, and 15 (14.42%) from ear swabs, and in the other 8 (7.69%) from other swabs).

In our study, PA showed the highest antimicrobial sensitivity to the aminoglycoside drugs, amikacin with 89.6%, followed by the carbapenem drug, imipenem with 83.3%, piperacillin/tazobactam with 81.3% and ceftazidime, as a third-generation cephalosporin with 79.2% (Table 2). Susceptibility to cefepime as a fourth-generation cephalosporin was 68.8%, to gentamicin from the aminoglycoside group 62.5% and to ciprofloxacin,

Table 2. The sensitivity of PA isolates to the tested antimicrobial drugs

Antimicrobial drug n (%)	Hospital patients		Outpatients		Statistical significance ($p < 0.05$)
	Resistant n (%)	Sensitive n (%)	Resistant n (%)	Sensitive n (%)	
Imipenem	8 (16.7)	40 (83.3)	10 (9.6)	94 (90.4)	$p = 0.163$
Ceftazidime	10 (20.8)	38 (79.2)	15 (14.4)	89 (85.6)	$p = 0.233$
Cefepime	15 (31.3)	33 (68.8)	20 (19.2)	84 (80.0)	$p = 0.078$
Amikacin	5 (10.4)	43 (89.6)	9 (8.7)	95 (91.3)	$p = 0.469$
Gentamicin	18 (37.5)	30 (62.5)	43 (41.3)	61 (58.7)	$p = 0.395$
Ciprofloxacin	19 (39.6)	29 (60.4)	42 (40.4)	62 (59.6)	$p = 0.535$
Piperacillin+ Tazobactam	9 (18.8)	39 (81.3)	10 (9.6)	94 (90.4)	$p = 0.096$

a member of the fluoroquinolone group 60.4%. The highest resistance in our study was shown by ciprofloxacin with 39.6%, followed by gentamicin with 37.5% and cefepime with 31.3%.

Discussion

PA is in second place in the critical group of bacteria, which is an alarm to find new antibiotics for this pathogen (World Health Organization, 2017). This pathogen is the cause of many nosocomial infections, especially in ICUs, where in 2012, 23% of patients during hospitalization acquired an infection associated with nosocomial infections. The most common infections are: ventilator-associated pneumonia, skin infections, and bacteremia, all of which are difficult to cure because most patients are either immunocompromised or have a state of reduced immunity response (Hoang *et al.*, 2018).

In our study, PA is more prevalent in outpatient samples. In a study conducted in India, out of 229 samples 70% samples were of inpatient (Dash *et al.*, 2014). A study in Saudi Arabia had similar results in favor of inpatient samples (Al-Tawfiq, 2007). Since it was not possible to find out the diagnosis of patients in whom PA was isolated, the probability that these patients were hospitalized earlier, or that they were in the hospital environment in the previous weeks is high. Another possibility is that the patients became positive for PA when they went to their Health Centers where they underwent medical interventions (such as dressing wounds, placing urinary catheters). The third possibility is that there is PA in the waters consumed by the population, which causes various infections in people with weakened immunity.

In our study, the mean age of the hospital patients was 64 years. The age of outpatients did not differ significantly and was 57 years. A similar study was conducted in Serbia in 2015, where the mean value of inpatients was 65 years, while in outpatients it was 50 years (Milojković *et al.*, 2020).

When it comes to different age groups of hospital patients, the largest representation was the age group of patients older than 60 years (58.33%). The age group from 11 to 60 years had a representation of 29.17%, and the third age group up to 10 years had a representation of 12.5%. The results of a study conducted at a hospital in Iraq showed different results where the highest percentage of prevalence was in the age group of 11 to 60 years and amounted to 74.7%. The prevalence of PA in the age group over 60 years was 14.6%, and the age group up to 10 years had the lowest PA prevalence and it amounted to 10.7% (Hussein *et al.*,

2018). A study conducted in Turkey from January 2008 to July 2011 showed that 58.3% of PA infected are in the age group older than 60 years, corresponding with our study (Sonmezer *et al.*, 2016). Also, a study conducted by a hospital in Kathmandu, Nepal, showed that the percentage of PA prevalence was high in the age group over 60 years and it amounted to 31% (Shahid, 2013).

The age groups of outpatients are similar to the age groups of inpatients, except for the category from 0 to 10 years. The prevalence of PA within the age group from 11 to 60 years amounted to 36.54%, and in the age group of patients older than 60 years, the prevalence was 41.35%.

The different prevalence of PA within different age groups can be explained by the weaker immunity that has not yet been sufficiently developed in children and immunity that declines over time in the elderly who eventually become more susceptible to different types of infections (Simon *et al.*, 2015).

In our study, the largest number of samples in the group of hospital patients in whom PA was isolated was taken from the surgery department, while the second in frequency was internal medicine. The third most frequent was the department of pediatrics with a frequency of 12.05%. In a study conducted in Egypt between May 2009 and July 2011, the greatest number of inpatients positive to PA, 47.2%, was from the surgery department while there were no positive samples in the internal medicine and pediatric department (Mahmoud *et al.*, 2013). The results of a research conducted between December 2012 and June 2013 in Turkey corresponds with our survey, with the greatest number of PA inpatients within the surgical department (54.9%), then, the post-internal medicine department and pediatric department (6.7 %) (Nazli *et al.*, 2014).

In our research, the largest number of PA isolates among inpatients was from urine culture with 47.92%, followed by bronchoalveolar lavage, wound swabs, while the last was tracheostomy swab. In a study conducted in Nigeria, urine culture accounted for 51.1% of all samples, followed by swab wounds with a prevalence of 41.3% (Olayinka *et al.*, 2004). In the research in Serbia, urine culture accounted for 21% of the samples, while the wound swab had a higher percentage (Milojković *et al.*, 2020). In a research conducted in India, a high percentage of cases were derived from sputum, while urine culture accounted for 20.45% of the cases (Shaikh *et al.*, 2015).

PA is a dominant pathogen that is often isolated from bronchopulmonary specimens in ICUs, where patients are connected to mechanical ventilation (Vincent, 1995). This pathogen is a significant cause of nosocomial urinary tract infections (UTIs), especially those associated with the use of urinary catheters. About 40% of all nosocomial infections are UTIs, which are a common source of bacteraemia (Warren, 1997). PA plays a major role in skin and subcutaneous tissue infections, whether surgical wound infections or burnt skin infections, and this pathogen is often isolated from wound swabs (Masaadeh and Jaran, 2009).

In our research, PA showed the highest antimicrobial sensitivity to the drug from the group of aminoglycosides, amikacin with 89.6%, then to the drug from the group of carbapenems, imipenem with 83.3%, to piperacillin tazobactam with 81.3% and ceftazidime, as third-generation cephalosporins with 79.2%. According to a study conducted in USA in 2015, amikacin showed a sensitivity of 96.4%, piperacillin / tazobactam 82.5% and ceftazidime 85.8% (Sader *et al.*, 2017). Similar results of PA antimicrobial sensitivity have been reported in studies conducted in Canada and Malaysia (Fazlul *et al.*, 2011; Walkty *et al.*, 2017).

We have shown that the highest resistance was shown by ciprofloxacin with 39.6%, followed by gentamicin with 37.5% and cefepime with 31.3%. In a study in India, 40% of the samples showed resistance to gentamicin, while resistance to ciprofloxacin was 39% (Ramana and Chaudhury, 2012).

Ceftazidime had a resistance percentage of 20.8%, piperacillin tazobactam 18.8%, followed by imipenem with 16.7% resistant samples and finally amikacin, as the most effective antimicrobial drug, with a percentage of resistance of 10.4%.

When we talk about statistically significant differences between inpatient and outpatient PA-positive samples, all drugs except gentamicin and ciprofloxacin, showed greater resistance in the hospital setting compared to the outpatient, which is not statistically significant. Also, we presented that gentamicin and ciprofloxacin were even more resistant in an outpatient setting. In the outpatient setting gentamicin shows resistance of 41.3%, while in the inpatient setting it is 37.5%. In the outpatient setting ciprofloxacin shows resistance of 40.4%, while in the inpatient setting it is slightly lower and amounts to 39.6%. The reason for such data may be that these two antibiotics are used irrationally in everyday practice.

In order to reduce the resistance of bacteria to antibiotics, it is necessary to follow certain measures in prescribing antibiotics in everyday practice, from increasing the rational use of antibiotics, rotation and cyclic prescribing of antimicrobial drugs. It is also necessary to educate and raise the awareness of the entire population that antibiotics should not be used without a justifiable reason. Unfortunately, today, more than ever, doctors are under pressure from their patients to constantly prescribe antibiotics, guided by the slogan that if they do not receive an antibiotic, they are not provided with quality medical care. In the prevention of intrahospital infections, it is necessary for all health workers to participate and take different measures for the prevention of such infections, which include the implementation of sanitary and hygienic procedures during work, hand and skin hygiene, disinfection of medical devices and the environment as well as sterilization of equipment and supplies, monitoring of antibiotic consumption, formation of a reserve group of antibiotics and regular monitoring of resistance to the most common causes of nosocomial infections.

Conclusion

Considering the danger of increasing incidence of intrahospital infections with multidrug-resistant PA, it is necessary to implement measures to prevent such infections. That prevention includes the implementation of sanitary and hygienic procedures during the work, hand and skin hygiene, disinfection of medical devices and the environment and sterilization equipment and accessories. Also, it is necessary to monitor the consumption of antibiotics as well as regular monitoring of resistance to the most common causes of nosocomial infections. In every local environment it is necessary continuous monitoring of nosocomial infections, which might enable timely prevention and treatment.

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Synthesis of Biocompatible Carboxylic Acid-Functionalized Graphene Oxide as Stimulator of Bacterial Growth

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Abstract

Researchers have shown great interest in graphene and its potential applications in various fields, such as electronics, energy, materials and biomedical areas. The effects of graphene, graphene oxide (GO) and its derivatives on bacteria activities are still controversial. Thus, how graphene and its derivatives interact with microorganisms and the mechanisms of their interactions are important issues for nanotechnology which need proper exploration. In the present investigation, graphene oxide (GO) has been synthesized and functionalized by chemical method. The GO and COOH-functionalized GO have been characterized by Fourier transforms infrared spectroscopy; Raman spectroscopy and SEM and haemocompatibility study. The effect of GO and COOH-GO on bacterial growth has been observed. The FTIR, Raman and SEM data confirmed the successful functionalization of GO with carboxyl (COOH) group. Interestingly, functionalized graphene oxide can significantly stimulate bacterial growth for gram-positive and gram-negative bacteria, whereas as-made GO shows no effect. It can be concluded that carboxylic acid-functionalized GO may act as a new, positive regulator for the growth of bacterial cells.

Keywords: functionalized graphene oxide; biocompatibility; bacterial growth

Резюме

Интересът към графена и неговите потенциални приложения в различни области, като електроника, енергия, биомедицина и др. нарастват непрекъснато. Данните, относно ефектите на графена, графеновия оксид (GO) и неговите производни върху развитието на бактериите все още са противоречиви. В същото време, остават нерешени редица въпроси, които имат важно значение за нанотехнологиите, като напр. какъв е механизма на въздействие на графена и неговите производни върху микроорганизмите. В настоящото изследване графеновият оксид (GO) е синтезиран и функционализиран чрез химичен метод. Функционализираните GO и COOH-GO са охарактеризирани чрез инфрачервена спектроскопия с трансформации на Фурие, Раманова спектроскопия и SEM и изследване на хемосъвместимостта. Наблюдаван е ефектът на GO и COOH-GO върху бактериалния растеж. Данните от FTIR и Raman и SEM потвърждават успешната функционализация на GO с карбоксилна (COOH) група. Интересно е, че функционализираният графенов оксид може значително да стимулира бактериалния растеж на грам-положителни и грам-отрицателни бактерии, докато GO не показва ефект. Може да се заключи, че функционализираният с карбоксилна киселина GO може да действа като нов, положителен регулатор за растежа на бактериалните клетки.

Introduction

Recently, graphene and its derivatives have shown their prompt significance in the field of nanobiotechnology having potential applications in widespread fields. Among the graphene derivatives, graphene oxide (GO) has been considered as a potential material for nano-medicine as it is a water-soluble derivative containing abundant

functional groups and a large surface area. The abundant functional groups (epoxide, hydroxyl and carboxyl groups) in GO increase its solubility in water and form a stable colloidal suspension. These groups also provide various active sites for the process of functionalization and hybridization with other materials like metals, genes, drugs and

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proteins (Wang *et al.*, 2014).

A large number of literature reports related to biomedical research demonstrate the applications of graphene and its derivatives in various fields such as bio-imaging, biosensing, cancer therapy, drug and gene delivery, protein modulation as well as tissue engineering (Liu *et al.*, 2014; Zhou *et al.*, 2014; Pal 2015; Yang *et al.*, 2015). Numerous studies have concluded that graphene or GO usually impose adverse toxic effects on the cells or animals, such as genotoxicity, immunotoxicity, dose-dependent or size-dependent toxicities (Akhavan *et al.*, 2012). However, when these materials are modified or functionalized by proper methods/modifications, their toxic effects could be ameliorated significantly.

Recently, scientists have shown substantial interest in the effects of graphene derivatives on microorganisms. Various reports revealed that GO possesses antibacterial activity, thus it can be used to inactivate bacteria. The antibacterial activity of GO may be due to ROS generation, interaction between the sharp edges of GO sheets and the bacterial cell wall to disrupt cell integrity and wrapping bacteria with GO sheets to reduce their activities (Akhavan *et al.*, 2012). On the other hand, a few reports have demonstrated that GO may enhance bacterial activities (Wang *et al.*, 2013). The effects of graphene oxide alone on the bacterial cells have been explored (Tang *et al.*, 2013). The controversies in the reported results may be due to the differences in the experimental protocols (e.g. preparation of samples, sample size variations and their chemical states, as well as the culture procedure of bacteria cells). As the effects of GO on bacteria cells have been demonstrated, the studies related to the interactions of biocompatible, carboxylic acid functionalized GO with the microorganisms along with the underlying mechanisms have not been explored. Such interactions and effects are very important for nanotechnology as well as microbiology, and therefore need great attention.

Thus, in the present investigation, graphene oxide (GO) has been synthesized and functionalized chemically to form carboxylic acid (-COOH) functionalized graphene oxide. The GO and functionalized GO have been characterized and their interaction with gram positive bacteria (*B. subtilis*, *Staphylococcus aureus*) as well as gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) has been analysed to explore its potential applications in microbiology and nanotechnology.

Materials and Methods

Materials

Graphite and all other reagents were purchased from Sigma Aldrich. All the reagents were freshly prepared with ultrapure water.

Preparation of graphene oxide and carboxylic acid functionalized graphene oxide

Graphene oxide (GO) was produced from graphite using Hummer's method with slight modifications (Jin *et al.*, 2012). Synthesized graphene oxide (GO) was suspended in water in a concentration of 2 mg/ml to form GO aqueous suspension, and then the bath was sonicated for 1 h. The resultant 2 mg/ml GO suspension (100 ml) was mixed with NaOH (1.2 g, 30 mmol) and chloroacetic acid (Cl-CH₂-COOH) (1.0 g, 10.6 mmol), and then the bath was sonicated for 2 h. The -OH groups on the GO were converted to -COOH moieties. The resulting GO-COOH solution was neutralized with hydrochloric acid and purified by repeated rinsing and filtrations until the product was well dispersed in water.

Characterization of graphene oxide and carboxylic acid functionalized graphene oxide

The synthesized GO and COOH-GO were characterized using Raman spectroscopy (in Via Reflex Raman Spectrometer, Renishaw, U.K.), Fourier transform infrared (FTIR) spectroscopy (SHIMADZU IR prestige-21, Japan), and scanning electron microscopy (SEM) (JEOL JSM 6390LV, USA). FTIR spectra were recorded in Attenuated Total Reflectance mode at ambient temperature and pressure. SEM measurements were carried out at 10 kV.

Biocompatibility study

The haemolysis test was carried out to investigate the biocompatibility of the synthesized COOH-GO. Different concentrations of COOH-GO (20, 40, 60 and 100 µg/ml) were placed in test tubes containing N-saline (0.9% NaCl) and incubated at 37°C for 30 min for providing temperature stabilization. The anticoagulated and diluted blood (N-saline: blood; 8: 10) (0.2 ml) was then added to the test tube and incubated for 60 min after mixing properly. The optical density (OD) of the incubated solution was measured in an UV spectrometer at 545 nm. N-saline was used as a negative control and sodium carbonate (1%) as a positive control. The haemolysis percentage was calculated using the formula (Sharma *et al.*, 2010; Chakravarty *et al.*, 2016).

$$\text{Haemolysis(\%)} = \frac{\text{OD}_{(\text{Uncd sample})} - \text{OD}_{(\text{negative})}}{\text{OD}_{(\text{positive})} - \text{OD}_{(\text{negative})}} \times 100.$$

The accepted norm was that if the haemolysis percentage was less than 10, the test sample was considered to be haemocompatible, and if it was less than 5, the sample was deduced to be highly haemocompatible.

Bacterial culture

The microorganisms *P. aeruginosa*, *B. subtilis*, *E. coli* and *S. aureus* were used for the study. A fresh subculture was prepared for each microorganism individually. One loopful of individual microorganism was transferred to each autoclaved test tube containing media and incubated in a shaking incubator for 24 hours at 37°C. Further, the culture was again inoculated (1:100) into fresh medium and grown in an incubator at 37°C for 2-3 h until an optical density of 0.5 at 600 nm was obtained.

Bacterial viability assay

The required number of prepared media was transferred into each sterilized test tube. The different concentrations of graphene oxide and functionalized graphene oxide (20, 40, 60 and 100 µg/ml) were added into the media. When graphene oxide solution was added it formed a distinct lower layer, which is solubilised by shaking the test tube 1-2 minutes. After that, one loopful of microorganisms was added into all test tubes and incubated for 24 hours at 37°C. Finally, all test tubes were checked for turbidity.

The cultures were further diluted and seeded in 96-well plates. The medium containing the same number of nanomaterials but no bacterial cells was considered as the blank for each culture. The growth as well as the metabolic activities of bacteria were analyzed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Tang *et al.*, 2013). All readings were measured in triplicate.

Another method used to analyze the numbers of viable bacterial cells was the colony-forming unit (CFU) counting method. The gradient dilutions of each culture were plated on agar plates in triplicate. The plates were further incubated at 37°C for 24 h. The number of bacterial colonies formed was counted.

Cell viability assay of mammalian cells

Lymphocytes were collected from albino mice and a single cell suspension was prepared under sterile conditions. The suspension was filtered

through 100-micron filter and centrifuged at 3000 rpm for 10 min at 4°C. Lymphocytes were re-suspended in RPMI-1640 medium (Sharma *et al.*, 2016). The cells at a density of 1.0×10^4 cells per well were plated in a 96-well plate along with different concentrations of COOH-GO (20-200 µg/ml) and incubated at 37°C in a CO₂ incubator for 24 h. Ten microlitres (µl) of 3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 5 mg/ml in phosphate-buffered saline was further added to each well, 4 h before the completion of incubation. The plate was centrifuged, the supernatant was removed and 100 µl of dimethyl sulfoxide (DMSO) was further added to dissolve the formazan formed. The absorbance was read at 530 nm in a microplate reader (Pathak and Khandelwal, 2006).

Results and Discussion

Preparation and characterization of functionalized GO

Graphene oxide was prepared from graphite by following Hummer's method with few changes and further GO was functionalized chemically to form carboxylic acid functionalized graphene oxide. The GO and functionalized GO were characterized by Fourier transform infrared spectroscopy; Raman spectroscopy and SEM analysis. FTIR is the widely used technique to characterize the bonding and chemical bonding present in the material. In the FTIR spectra of GO (Fig. 1a), a broad absorption peak was observed at 3012 cm⁻¹. This absorption peak was due to the -OH stretching vibration of GO. The peak at 1,706 cm⁻¹ corresponds to carbonyl group (C=O stretching vibration), the peak at about 1,600 cm⁻¹ was due to the skeletal vibrations of the sp² carbon atoms. Stretching peaks at about 1,218 cm⁻¹ and 939 cm⁻¹ were due to the vibrations of COOH and C-O-C groups, respectively. From the FTIR spectra of GO-COOH, it was observed that the -OH stretching peak (3101 cm⁻¹) became sharper than the native GO (Fig. 1b).

This peak sharpening may be due to the introduction of carboxylic acid functional group and the nitrogen containing surface group (-NH). In addition, the characteristic bands at 1708, 1511 and 1088 cm⁻¹ correspond to C=O stretching vibration of carboxyl group, the O-H vibration in carboxylic acid and C-O vibration in epoxy group, respectively.

Another most reliable tool to confirm the formation of GO-COOH is Raman spectroscopy. Graphite has dominant Raman features at 1,620 cm⁻¹ (G band) and 1,375 cm⁻¹ (D band), as shown in Fig. 2. The G mode, present in most graphite-like materials, is related to the planar vibrations of

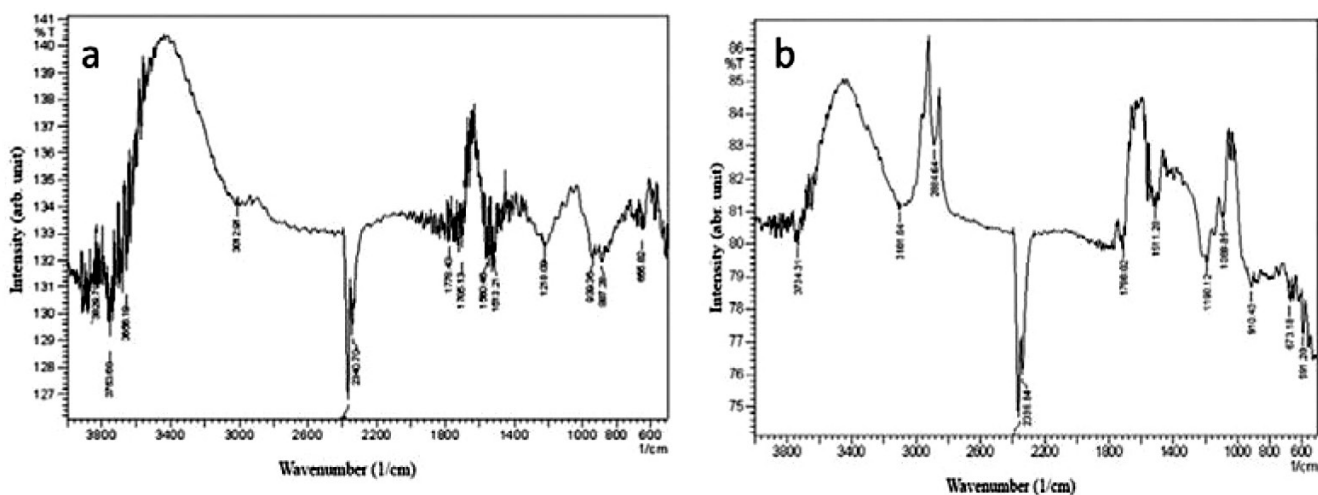


Fig. 1. FTIR plot of (a) Graphene oxide and (b) –COOH functionalized Graphene oxide.

carbon atoms. The D mode, present in all graphite-like carbon materials, corresponds to the structural defects. The increase in the intensity of D mode (probably becomes higher than the G mode) may be due to the structural disorder in the sp² pattern induced by oxygen containing groups on the carbon basal plane or at the edges. The changes between graphene oxide and COOH- functionalized graphene oxide can be observed by analysing the plot, which shows that the shifting of the intensity at 1,620 cm⁻¹ and 1,375 cm⁻¹ is sharper and wider in functionalized graphene oxide rather than graphene oxide. Hence, it proves that the structural changes occurred and the functionalization was done successfully.

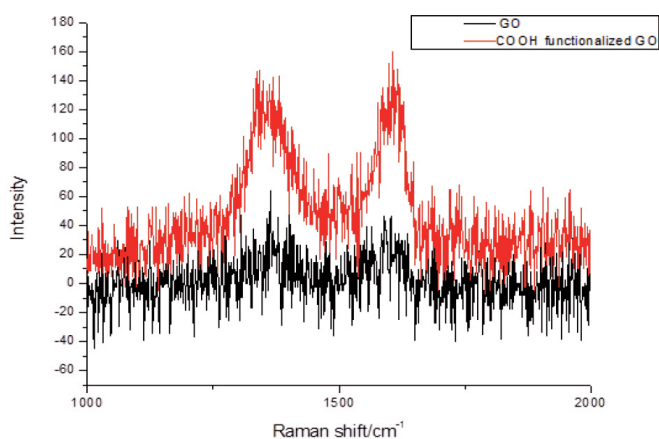


Fig. 2. Raman spectra of Graphene oxide and (-COOH) functionalized Graphene oxide.

The SEM images of GO and modified GO (1000x and 5000x) are presented in Fig. 3. From the SEM images it can be seen that GO has more or less smoothly arranged, brick by brick structure. It lacks any type of surface growth and fluffiness. However, in modified GO the arranged structure is totally disrupted. Moreover, some petal-like surface growth was observed at high-

er magnification. These changes in surface property and topology may be an indication that GO was successfully modified to its carboxylic form.

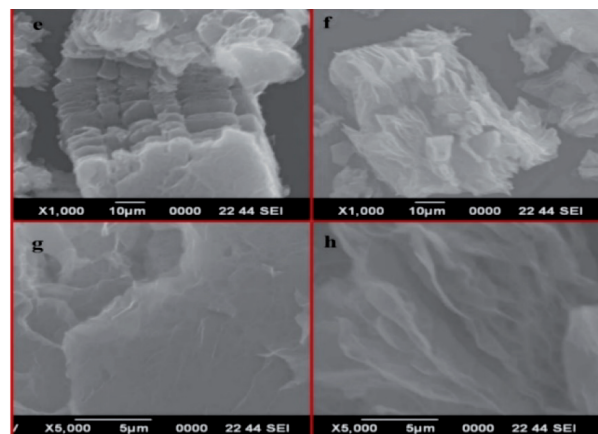


Fig. 3. The SEM micrographs of (e) GO (f) Functionalized GO at 1000x, 10 μm (g) GO (h) Functionalized GO at 5000x, 5 μm.

Biocompatibility of synthesized COOH-GO

Biocompatibility is the most important requirement for all materials used in biotechnology. A biomaterial should also not have any effect on the proteins adsorbed on the surface during the growth of microorganisms, nor should it percolate into any substance, which can induce toxic effects to the organisms. The biocompatibility of synthesized COOH-GO was observed by the haemolysis test. Different concentrations of COOH-GO (20, 40, 60 and 100 μg/ml) were used for the test. The result of the haemolysis test (Table 1) implies that carboxylic acid functionalized GO is biocompatible.

The effects of as-made GO and COOH-GO on the viability of gram-positive bacteria (*B. subtilis*, and *S. aureus*) as well as gram-negative bacteria (*E. coli* and *P. aeruginosa*) were analyzed using MTT assay. The results demonstrated that without functionalization, GO alone did not exert any effect on the cell viability of any of the bacteria (Fig. 4).

Table 1. Biocompatibility study of COOH-GO

S. no	Samples	Absorbance	% Haemolysis
1	NaCl (-ve control)	0.234	0%
2	Na ₂ CO ₃ (+ve control)	0.714	100%
3	20 µg/ml	0.238	0.83%
4	40 µg/ml	0.242	1.67%
5	60 µg/ml	0.246	2.5%
6	100 µg/ml	0.257	4.8%

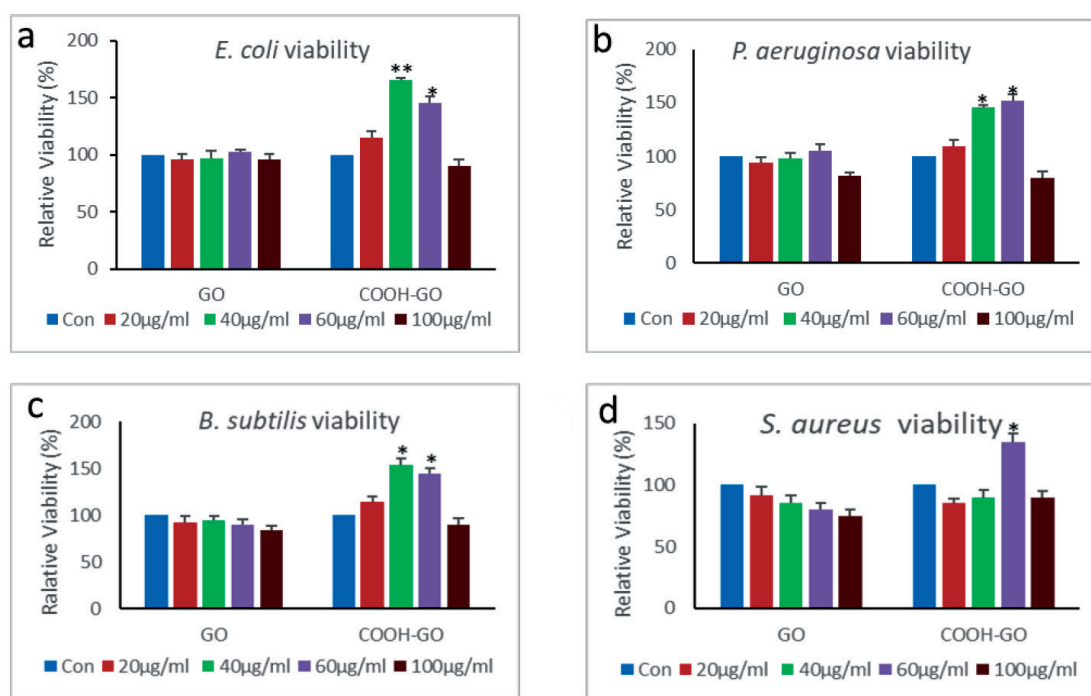


Fig. 4. Effects of COOH-GO on bacterial viability (a) *E. coli*, (b) *P. aeruginosa*, (c) *B. subtilis*, (d) *S. aureus*. The bacteria cells were grown in the medium with different concentrations (20 - 100 µg/ml) of GO and COOH-GO for 24 hrs. Their viabilities were analyzed using the MTT method. Error bars represent the standard deviations ($n \geq 3$). ** $P < 0.01$, * $P < 0.05$.

This result is almost consistent with the literature reports. Functionalized COOH-GO at the concentration of 20 µg/ml also did not have any effect on the viability of any of the bacterial cells. Interestingly, COOH-GO strongly increased *E. coli* viability to around 165% at the concentration of 40 µg/ml. The higher concentration (60 µg/ml) also showed a significant increase in the cell viability but to a lesser extent. In *P. aeruginosa* and *B. subtilis*, both concentrations of COOH-GO (20 and 40 µg/ml) showed significant cell growth. The highest concentration (100 µg/ml) did not affect the viability of cell in any of the bacterial species. In *S. aureus*, only the 60 µg/ml concentration enhanced cell viability in a significant manner.

The results of the CFU counting method also confirmed the growth stimulating effect of COOH-GO (Fig. 5). The growth of all the bacterial species

increased after treatment with COOH-GO. This result is similar to the results of the MTT assay. Such stimulation effect on bacterial growth which may be due to surface chemistry modification is fascinating. The interaction between bacterial cells and COOH-GO might be different from the interaction of cells with GO. Therefore, it is possible that COOH-GO with more carboxylic groups may provide a different biocompatible interface between bacteria and the nanomaterial, and thus affect bacterial growth. Its mechanism needs to be explored further.

Effect of functionalized GO on murine lymphocyte viability

Since carboxylic functionalized GO can stimulate the activity and growth of bacterial cells, its effect on the growth of eukaryotic cells was analyzed further. Murine lymphocytes were used in

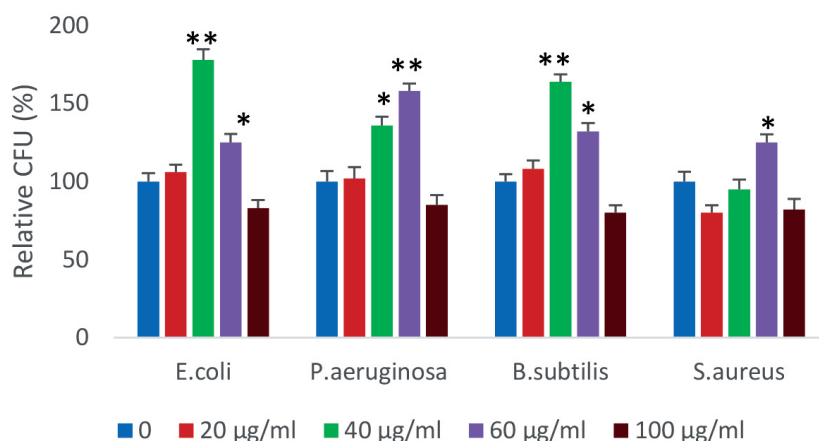


Fig. 5. Effects of COOH-GO on CFU counting of viable bacteria. CFU counting of viable bacteria in the cultures after being treated with indicated concentrations of COOH-GO at 37 C for 24 h. Error bars represent the standard deviations ($n \geq 3$). ** $P < 0.01$, * $P < 0.05$.

the study and COOH-GO concentrations similar to those used in the bacterial study were chosen. As shown in Fig. 6, murine lymphocytes, after being treated with COOH-GO (20-200 µg/ml) for 24 hrs, had only about 15% increase in cell growth with the lowest concentration, i.e. 20 µg/ml. A further increase in the concentration of COOH-GO (up to 200 µg/ml) could not enhance cell growth but exhibited cytotoxic effects towards lymphocytes. Higher concentrations (100 and 200 µg/ml) showed even more than 50% cell death.

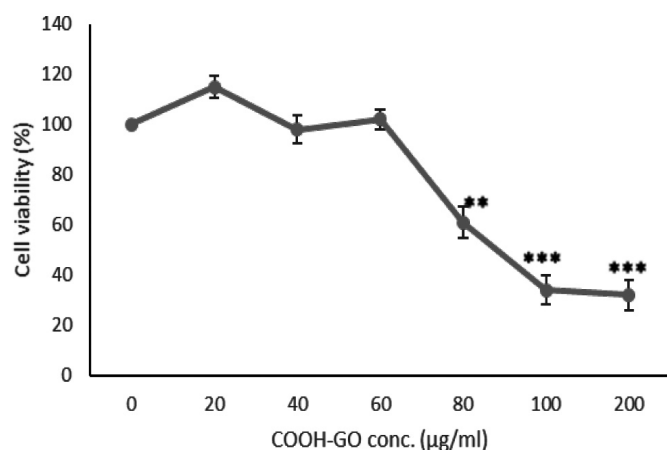


Fig. 6. Effects of COOH-GO on viability of murine Lymphocytes. Murine lymphocytes were treated with COOH-GO (20 - 200 µg/ml) for 24 h. Results are expressed as mean $\pm$ SD, $n=3$. *** $P < 0.001$, ** $P < 0.01$ when compared to control.

Conclusion

In summary, GO and COOH-GO were synthesized chemically and characterized using Raman spectroscopy, FTIR and SEM. A biocompatibility study of the synthesized samples was carried out and their effects on *P. aeruginosa*, *B. subtilis*, *E. coli* and *S. aureus* were carefully investigated. GO alone did not show any effect on the growth and

viabilities of bacteria, whereas COOH-GO showed a rather significant stimulating effect on the growth of bacterial cells (both Gram positive and Gram negative). Further investigation of the mechanism underlying the stimulation of the growth of bacteria and any type of toxicity towards bacteria is required in future. The present work has revealed that the interactions between nanomaterials (GO) and microorganisms (bacterial cells) could be closely related with the surface properties of nanomaterials. In the present investigation, specific carboxylation on the GO surface could generate different surface chemistry for interaction with bacteria. Thus, it can be concluded that carboxylic acid functionalized GO may act as a new, positive regulator for the growth of bacterial cells. This indicates the role of carboxylic acid functionalized GO as a promising nanomaterial in microbial engineering.

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Study of the Antimicrobial Action of Some Commercial Mouthwash (MW) Solutions against Biofilm Formation by Oral Bacterial Microflora

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Abstract

The aim of the study was to evaluate the *in vitro* antibacterial action of some commercial mouth washing solutions against biofilm formed due to oral microflora from saliva of healthy persons. In this study, four oral mouthwash (MWs) solutions, Listerine 100%, Oral B 100%, Chlorhexidine 0.12%, and Sensodyne 100%, were selected for evaluating the antibacterial activity against biofilm formation. About 10 ml of saliva sample was taken from 5 healthy donors in the morning before eating and brushing their teeth and collected in sterile containers. Half of the collected saliva was filter-sterilized and used for coating and biofilm formation on a 96 ELISA-well microplate, the other part was used to study the antimicrobial activity of MW solutions. Biofilm formation was evaluated with crystal violet methods. Crystal violet-based method was used to determine the amount of biofilm, and optical densities (OD) of the wells in the plate were measured at a wavelength of 600 nm using an ELISA microplate reader to determine the effect of mouthwash solutions. The results showed that the amount of biofilm formed by the samples treated with Listerine, Oral B, Chlorhexidine and Sensodyne was found to be 28.4%, 32.95%, 37.5%, 60.22%, respectively. All mouthwash solutions showed a highly significant reduction in the biofilm amount (P -value = 0.00). Accordingly, in this study, all the commercial MW solutions showed antibiofilm activity, but Oral B was found to be the most effective mouthwash solution against oral biofilm formation.

Keywords: biofilm, chlorhexidine, listerine, microflora, oral b, saliva

Резюме

Целта на изследването е да се оцени *in vitro* антибактериалното действие на някои търговски разтвори за изплакване на уста срещу биофилм, образуван от орална микрофлора в слюнката на здрави хора. За оценка на антибактериалната активност срещу образуването на биофилм са избрани четири разтвора за орално приложение на вода за уста (MWs), а именно Listerine 100%, Oral B 100%, Chlorhexidine 0.12% и Sensodyne 100%. Сутрин преди хранене и измиване на зъбите се взема около 1 мл слюнка от 5 здрави донора и се поставя в стерилни контейнери. Половината от събраната слюнка се стерилизира през филтър и се използва за покриване и образуване на биофилм върху 96 ямкова ELISA микроплака, а другата част се използва за изследване на антимикробната активност на MW разтворите. Образуването на биофилм се оценява чрез оцветяване с багрилото кристал виолет. За определяне на количеството биофилм се използва метод, базиран на промяната в цвета на същото багрило. Оптичната плътност (OD) на ямките в плаката се измерва при дължина на вълната 600 nm с помощта на ELISA четец за микроплаки, за да се определи антибактериалния ефект на разтворите за вода за уста. Резултатите показват, че количеството биофилм, образувано от пробите, третирани с Listerine, Oral B, Chlorhexidine и Sensodyne, е съответно 28.4%, 32.95%, 37.5% и 60.22%. Всички разтвори вода за уста показват статистически високо намаляване на количеството биофилм (P -стойност=0,00). В заключение, всички търговски разтвори MW демонстрират антибиофилмна активност, но Oral B е най-ефективният разтвор срещу образуването на орален биофилм.

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Introduction

To maintain further oral hygiene, there is a wide use of mouthwash solutions. Some compounds found in these products (natural and/or synthetic) have their actions on microorganisms through inhibiting the growth and blocking some enzymatic reactions of these microorganisms, thereby playing an essential role in the control of biofilm formation (Saad *et al.*, 2011). Biofilms are organized microbial cell communities which adhere to surfaces and show phenotypic heterogeneity (Ammons *et al.*, 2014). The microorganisms form a microecosystem, where there will be a release of by-products (e.g. acids) that may affect the surface integrity. These by-products can lead to accumulation into a biofilm, in addition to using them in metabolizing carbohydrates derived from the host's diet. If they are found on the teeth, biofilms can participate in tooth demineralization and therefore development of dental caries. Mouthwash solution products are usually divided into therapeutic or cosmetic. Therapeutic mouthwashes contain active ingredients that help mechanical control and maintain oral health. In many systematic reviews, the effectiveness of various formulations like Delmopinol (Del) (Addy *et al.*, 2007), Hexetidine (Afennich *et al.*, 2011), Povidone-iodide (Sahrman *et al.*, 2010), Chlorhexidine gluconate (CHX) (Gunsolley, 2006; Gunsolley, 2010; Van Leeuwen *et al.*, 2011, Essential oil (EO) (Gunsolley, 2006; Stoeken *et al.*, 2007), Cetylpyridinium chloride (CPC) (Haps *et al.*, 2008) and Hydrogen peroxide were reported (Hossainian *et al.*, 2011). Therapeutic mouthwash solutions function as anti-gingivitis and anti-plaque agents, causing prevention of the first steps of biofilm formation, i.e., bacterial adhesion. Fluoride mouthwashes act as cariostatic agents, leading to dental hard tissue remineralizations and increasing its acid resistance (O'Mullane *et al.*, 2016). Mouthwash solutions that contain fluorides are recommended as part of caries-prevention strategy for people at high risk of caries, e.g. people undergoing orthodontic treatments or those with hyposalivary functions (Pitts *et al.*, 2012). Fluoride mouthwash solutions contain different fluoride compounds, such as sodium fluorides, SnF, sodium monofluorophosphates and AmF (Rošin-Grget *et al.*, 2013). The aim of this study was to study the efficacy of some selected commercial mouthwash solutions (MWs) on polymicrobial biofilm formed from saliva of normal healthy donors and study the reduction of the amount of the biofilm under the treatment with these MWs.

Materials and Methods

Collection of saliva sample

Unstimulated saliva samples were collected from 5 donors by a spitting method, as they contain more bacteria than those obtained by drooling (Chiappin *et al.*, 2007) in sterile falcon tubes in the morning before eating and before brushing the teeth.

SHI broth media preparation

The SHI medium was composed of the following: proteose peptone (Difco) 10 g/L, trypticase peptone (Difco) 5.0 g/L, yeast extracts (Difco) 5.0 g/L, KCl 2.5 g/L, sucrose 5.0 g/L, hemin 5.0 mg/L, VitK 1 mg/L, Urea 0.06 g /L, arginine 0.174 g/L, mucin (type III, porcine, gastric, Sigma Chemical Co. St Louis, Mo) 2.5 g/L, sheep blood (Colorado serum company) 5% and N-acetylmuramic acid (NAM) 10 mg/L (Tian *et al.*, 2010).

Coating

About 10 mL of unstimulated saliva sample was collected from 5 healthy donors by spitting in sterile falcon tubes in the morning before eating and before brushing their teeth. About 5 mL of saliva sample was centrifuged at 2600 g for 3 minutes, after that the supernatant was transferred to new tubes and mixed with equal volumes of PBS (10x), then this mixture was centrifuged again at 14,000 rpm and 4°C for 10 minutes. For sterilizing the mixture, all the supernatant was filtered using 0.45 µm Millipore membrane filter. From this sterilized saliva, 100 µL was taken and transferred into the wells of a 96-well microplate and incubated at 37°C for 1 hour. At the end of incubation, the microplate was left under ultraviolet light (UV) for 1 hour to sterilize it and then stored under sterile conditions until use. The remaining amount of this aliquot was used to study the antimicrobial activity under different types of mouthwashes without sterilization with filter.

Treatment of biofilm formation

After sterilizing, the mixture of 5 mL portions was transferred from aliquot and used to investigate antimicrobial action with different types of mouthwash solutions as shown in Table 1.

For this reason, 0.5 mL was distributed to 5 tubes and labeled as Listerine100%, Oral B100%, Chlorhexidine 0.12, Sensodyne100%, and the fifth tube was used as a positive control (water mixed with aliquot), with 0.5 ml from each of (Listerine 100%, Oral B 100%, Chlorhexidine 0.12, Sensodyne 100%). The whole mixture inside the 5 tubes was slowly turned upside down and mixed for few minutes. After that, all the 5 tubes were centrifuged

Table 1. Type of mouthwash (MWs) solutions used in this study and their manufacturers

No	Mouthwash type	Contents	Manufacturer
1	Listerine	Alcohol, Sorbitol, poloxamer407, Bezoic Acid, Sodium Saccharin, Eucalyptol, Aroma, Methyl Salicylate, Thymol, menthol and Sodium Benzoate	Johnson and Johnson (U.S.A)
2	Oral B	Aqua, Glycin, Alcohol, Aroma, Methylparben, Poloxamer, Sodium floride, Cetylpyridium Chloride, Sodium Saccharin and Propylparaben	Procter & Gamble (Germany)
3	Chlorhexidine	% 0.12 Chlorhexidine digluconate	Gülşa Diş Ticaret LTD. ŞTI (Turkey)
4	Sensodyne	Aqua, Glycerine, Sorbitol, Potassium Nitrate, PEG-60 Hydrogenated Castor Oil, Poloxamer 407, Sodium Benzoate, Aroma, Disodium Phosphate, Methyparaben, Propylparaben, Sodium Phosphate, Sodium Fluoride, Sodium Saccharin, CI 42090 Containing 3% w/w Potassium Nitrate & 0.048% w/w Sodium Fluoride (217ppm fluoride)	GlaxoSmithKline, UK

at 10,000 rpm for 3 minutes at 4°C. The supernatant was removed, and the pellet of each of the 5 tubes was mixed with 1 ml of sterilized saliva sample and 1 mL of SHI broth. This mixture was used for testing biofilm formation, for this purpose 250 µL of this mixture was transferred and distributed to 96 ELISA microplate wells (flat type) previously coated with saliva (coating steps), while for the control negative well, only 250 µL SHI broth was transferred. For each of the positive and negative control and mouthwash solution, 8 wells were used. The plate was incubated at 37°C for 24 hours.

Identification of biofilm formation by crystal violet staining

At the end of the incubation period, the contents of the wells were discarded, and the wells were washed manually twice with PBS (pH 7.2) and left to dry. After that, each well was loaded with 50 µL of crystal violet (0.1%). The microplate was incubated at room temperature for 20 minutes, after that the crystal violet stain was discarded, the microplate was washed twice with PBS (pH 7.2), and then each well was loaded with (200) µl of ethanol (96%). In order to remove all the remnant crystal violet stain, the plate was left at room temperature under slow shaking. After that, the mixture of each well was transferred to a new microplate well and the biofilm formation was determined by reading the optical density (OD) of each well at 600 nm using ELISA plate reader.

Evaluation the anti-biofilm activity of mouthwash solutions

The anti-biofilm activity of the 4 mouthwash

types was measured depending on the OD value, and the % of the biofilm remaining after treatment with each type of mouthwash was calculated using the following formula: Percentage of biofilm remaining = (1-mean of treatment- mean of control/ mean of control×100%) (Fred, 2012).

Data analysis

The difference between variables was estimated by using the one-sample t-test. The SPSS V.23 program was used to analyze the current data and test the level of significance of $\alpha=0.05$. The remaining biofilm formation (%) after treatment was calculated using MS excel application (2010).

Results

A sample of morning saliva was collected from 5 healthy individuals using a spitting method. Biofilm formation from the saliva sample containing oral microflora bacteria is shown in Fig. 1.

The antibiofilm activity of the selected 4 types of commercial mouthwashes in Table 1 showed that the Oral B 100% was the most effective mouthwash solution against biofilm formation using optical density measurement to evaluate the antibiofilm activity of the mouthwash solution. The mean value of inhibition was (0.25±0.04) for Oral B 100%, followed by Listerin 100% (0.29±0.05) and Chlorhexid 0.12% (0.33±0.03), while the lowest activity was shown by Sensodyne solution (0.53+ 0.1) (Fig. 2).

Tables 2 and 3 and Fig. 2 in comparison with the result of positive and control showed different means of inhibition - 0.88 and 0.22, respectively. Statistically, all the selected mouth washing solutions showed antibiofilm activity (P-value= 0.00).

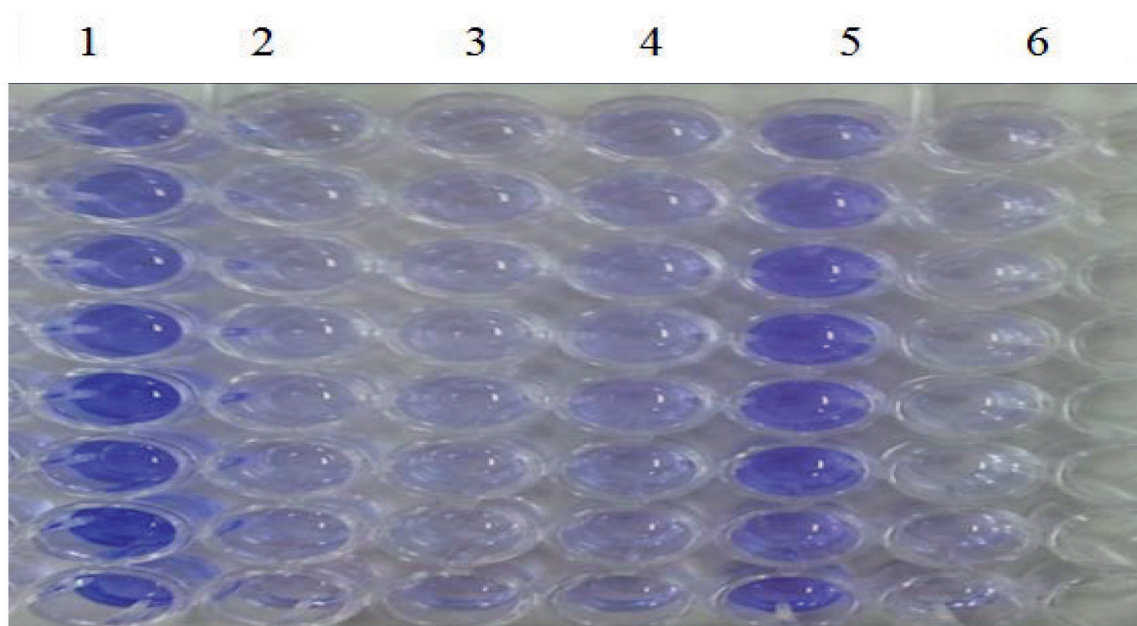


Fig. 1. Result of biofilm formation; column 1: Positive control (SHI broth+ saliva sample), columns: 2-5; Listerine 100%, Oral B 100%, Chlorhexidine 0.12%, Sensodyne 100% 1 and column 6: Negative control contains only sterile saliva.

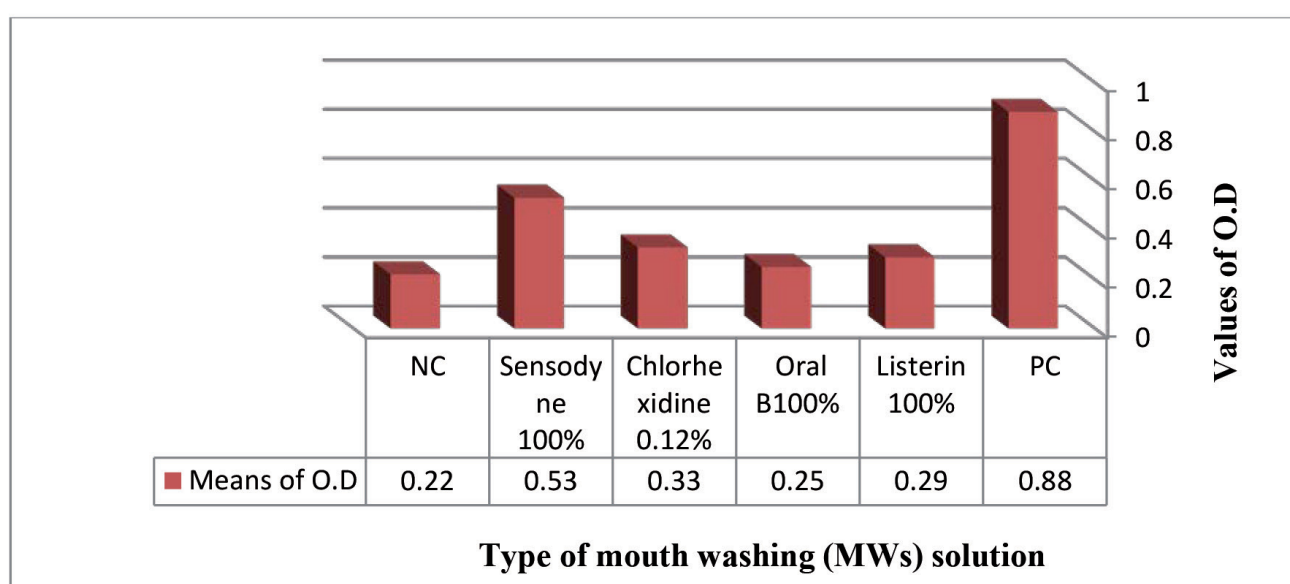


Fig. 2. Comparison between the antibiofilm activities of selected mouthwashes (MWs) compared with positive and negative controls by measuring the OD at 600 nm.

Table 2. Comparison between the antibiofilm activities of selected mouthwashes compared with positive and negative controls by measuring the O.D at 600 nm

Type of test	N	Mean	Std. deviation	Maximum	Minimum	T-test	*P-value
Positive control	8	0.88	0.29	1.35	0.51	8.579	0.00 (H.S)
Listerine	40	0.29	0.05	0.42	0.20	33.744	0.00 (H.S)
Oral B	40	0.25	0.04	0.34	0.19	40.163	0.00 (H.S)
Chlorhexidine	40	0.33	0.03	0.41	0.28	67.782	0.00 (H.S)
Sensodyne	40	0.53	0.10	0.69	0.29	31.806	0.00 (H.S)
Negative control	8	0.22	0.04	0.30	0.18	15.331	0.00 (H.S)

*; One-Sample Test; H.S; highly significant

Table 3. Biofilm remaining ratio (%) after treatment of saliva sample with four types of commercial mouth washing (MWs) solutions

Type of mouth washing solution	Mean of (PC)	Means of biofilm formation after treatments	% of biofilm remaining
Oral B (%100)	0.88	0.25	28.40
Listerine (% 100)	0.88	0.29	32.95
Chlorhexidine (% 0.12)	0.88	0.33	37.50
Sensodyne (100%)	0.88	0.53	60.22

*; One-Sample Test; H.S; highly significant

In this study, the biofilm remaining ratio (%) after treatment of the formed biofilm was calculated as shown in Fig. 3; the results revealed that the Oral B % 100 type was the most active (28.4%) in inhibiting biofilm formation, while the less active mouthwash solution was Sensodyne with (60.22 %) of biofilm remaining after treatment with this type.

Discussion

The careful treatment of oral biofilms, which are composed of complex microbial communities, is necessary for preventing onsets and progressions of oral diseases (Seneviratne *et al.*, 2011). In comparison with planktonic bacteria, the microbial communities which form biofilms are more resistant to external stresses such as toxic exposures or nutrient depletions, owing to the existence of bacterial extracellular polysaccharide (Stoodley *et al.*, 2002; Yu *et al.*, 2017). Controlling biofilms may be insufficient for several individuals, although the removal of biofilms is regarded essential to maintain oral

health (Van der *et al.*, 2005). Although tooth brushing is regarded as the first-line oral hygienic procedure, the biofilm-removal effect can be restricted among some patients or certain areas (Arweiler *et al.*, 2018). Hence, for achieving the best oral health status, tooth brushing must accompany using chemotherapeutic agents (Berger *et al.*, 2018). Among the antimicrobial agents, chlorhexidine gluconate (CHX) has been observed to reduce oral biofilms effectively, in addition to prevention and treatment of gingival inflammations (Arweiler *et al.*, 2006). The current study assessed the antibiofilm action of available and some most commonly used commercial MWs brands in the center of Baghdad city against polymicrobial biofilm from the saliva of normal healthy individuals, which is regarded as the primary cause of oral diseases. In our study, Listerine and Oral B were used at a 100% concentration, while Chlorhexidine was used at 0.12% concentration. As seen in the results, Oral B is the most effective commercial mouthwash solution

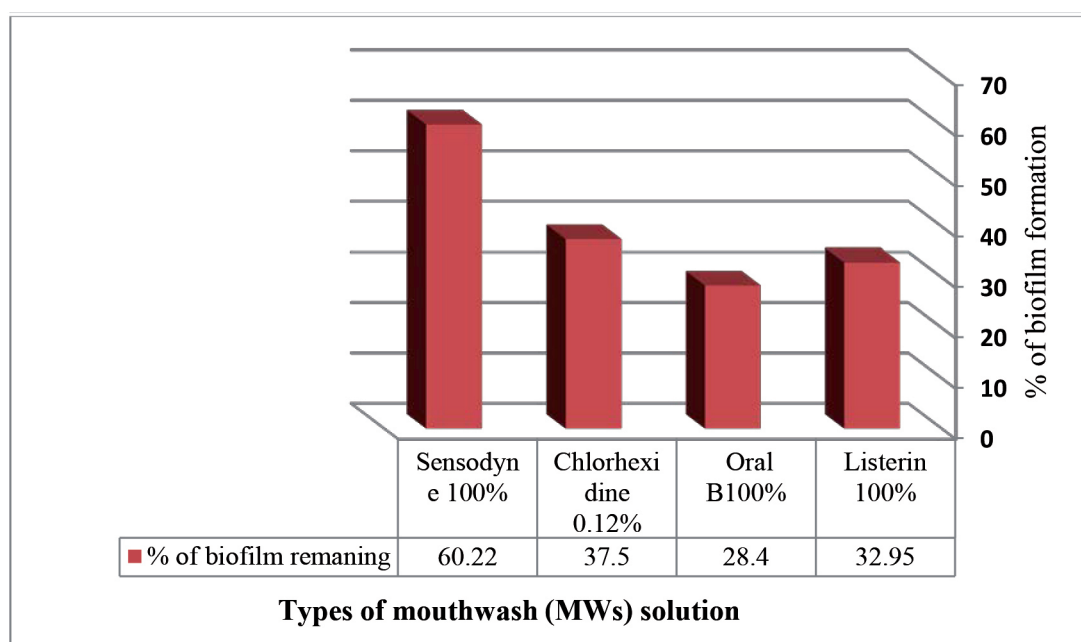


Fig. 3. Biofilm remaining ratio (%) after treatment of saliva sample with four types of commercial mouthwash (MWs) solutions

against oral biofilm formation. This is followed by Listerine. But it should be noted that the rate of use of Chlorhexidine is very low compared to Listerine and Oral B. Despite this, its biofilm inhibiting feature is not much different from Listerine and Oral B. Mouthwashes containing different antibiotics have been used to inhibit carcinogenic bacteria in plaque. Among them, Chlorhexidine (CHX) is an antimicrobial chemical which kills both gram-negative and gram-positive bacteria via attacking bacterial inner or cytoplasmic membranes. It was shown that CHX application is efficacious in inhibiting the growth of *S. mutans* and retarding acid production by carcinogenic bacteria (Kocak *et al.*, 2009). Antimicrobial essential oil mouth rinses such as Listerine can be accessed over the counter. Essential oil can kill microbes via cell wall disruption and inhibition of enzyme activities. It can prevent the aggregation of bacteria with gram-positive pioneer species and extracts endotoxin from gram-negative bacteria (Stoeken *et al.*, 2007). Additionally, it can lower bacterial loads, slow down plaque maturation and reduce plaque mass and pathogenicity (Ouhayoun, 2003). Other reports have shown that essential oil can reduce numbers of *S. mutans* in saliva (Fine *et al.*, 2000). Listerine can also inhibit the *S. mutans* biofilm formation *in vitro* (Baffone *et al.*, 2011), and may have a role in preventing the development of caries (Filoché *et al.*, 2005). Fluoride has well-known caries-preventative effects (Zhang *et al.*, 2004). A pilot study using combined 0.2% CHX gluconate and 0.055% sodium fluoride as mouth rinses showed that the mineral gain of bovine teeth for this CHX/fluoride mouth rinse was significantly higher than for both the CHX and placebo treatment groups. Similarly, fluoride combined with an essential oil mouth rinse may also have a better preventive effect on caries. The higher concentration of CHX has a direct bactericidal effect in penetrating the bacterial cell wall and causing cytoplasm precipitation, while lower concentrations, such as 0.012% CHX, are bacteriostatic (Hennessey, 1973). The main component of Listerine is an essential oil which was shown to be antiseptic. *In vivo* has found that essential oil can reduce levels of plaque and inhibit plaque formation (Moran *et al.*, 1997). Other research found that Listerine and CHX-containing mouth rinses showed equivalent antimicrobial activity when used on biofilm from human saliva (Pan *et al.*, 2010). Several clinical studies confirmed the plaque-inhibitory properties of Listerine. Twice daily application of Listerine mouth rinse suppressed *de novo* plaque forma-

tions following 4 days (Riep *et al.*, 1999) and after 21 days in the absence of mechanical tooth cleaning (Brex *et al.*, 1990; Riep *et al.*, 1999). The essential oil mouth rinse resulted in reduction of total recoverable *Streptococci* and *S. mutans* and other anaerobes in plaque, with corresponding reductions in saliva (Fine *et al.*, 2000; 2007). It has been well accepted that fluoride can prevent caries by inhibiting bacteria and inducing remineralization. In the present study, the concentration of biofilm treated with fluoride Listerine and fluoride of Sensodyne showed differences in the means of biofilm concentration 0.29 ± 0.05 , (0.53 ± 0.1) , respectively (Table 2, 3). The fluoride of Oral B 100% solution appeared to act better in preventing biofilm formation than the fluoride of Sensodyne 100%.

Conclusions

Oral B 100% mouthwash used in this study was the most effective in inhibiting the biofilm formation, with little differences in activity with Listerine 100%, although there are differences in the compositions, while the CHX 0.12% and Sensodyne were shown to be less effective compared with other the other mouthwash solutions.

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Antimicrobial and Cytotoxic Properties of Leaf and Stem Bark Extracts of *Faidherbia albida*

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Abstract

This study evaluated the antimicrobial, cytotoxic and phytochemical properties of *Faidherbia albida* leaf and stem bark extracts. The methanolic extract of the plant parts was obtained and screened for phytochemical properties according to standard techniques. The antibacterial activities of the plant extract were investigated against ten clinical isolates using the agar well diffusion method while its cytotoxic potentials were assessed in accordance with established procedures. Flavonoids, tannins, alkaloids, saponins, glycosides, triterpenes, xanthoproteins, and anthraquinones were present in the leaf and stem bark extracts, and the extracts exerted antibacterial activities against the isolates with zones of inhibition showing no statistical difference from that of the positive control ($p = 0.089$). The minimum inhibitory concentrations of stem bark and leaf extracts ranged from 0.781 to 6.25 mg/ml and 0.472 to 6.43 mg/ml, respectively, while their minimum bactericidal concentrations ranged from 0.391 to 1.562 mg/ml and 0.290 to 1.782 mg/ml, respectively. There was no statistical difference between the activities of stem bark and leaf extracts ($p > 0.106$). The cytotoxicity test against the human embryonic kidney (HEK) 293 cells showed that the leaf and stem bark extracts were toxic at 0.1059 and 0.1179 $\mu\text{g/ml}$, respectively. The relatively high therapeutic indexes (TI) of the plant extracts indicated their safety. This study revealed that the leaf and stem bark extracts of *F. albida* possess robust antibacterial and cytotoxic properties that could be exploited in the treatment of related diseases and curtail further spread of antimicrobial-resistant pathogens.

Keywords: Bacteria, cytotoxic, *Faidherbia albida*, medicinal plant, MBC, MIC, phytochemical, therapeutic index.

Резюме

Това проучване оценява антимикробните, цитотоксичните и фитохимичните свойства на екстрактите от листа и кора на стъбла от *Faidherbia albida*. Получен е метанолов екстракт от растителните части на *F. albida* и са изследвани фитохимичните му свойства съгласно стандартни техники. Проучени са антибактериалните активности на екстракта срещу десет клинични изолата чрез метода на дифузия в агар, а неговите цитотоксични свойства са оценявани в съответствие с установени в лабораторната практика процедури. Резултатите показват, че в екстрактите от листата и кората на стъблото присъстват флавоноиди, танини, алкалоиди, сапонини, гликозиди, тритерпени, ксантопротеини и антрахинони. Екстрактите проявяват антибактериално действие срещу изолатите със зони на инхибиране, показващи недостоверна статистическа разлика от тази на положителната контрола ($p = 0.089$). Минималните инхибиторни концентрации на екстракти от кора и листа варират съответно от 0.781 до 6.25 mg/ml и 0.472 до 6.43 mg/ml, докато минималните им бактерицидни концентрации варират от 0.391 до 1.562 mg/ml и 0.290 до 1.290 mg/ml съответно. Няма статистически достоверна разлика между активността на екстрактит от кората на стъблото и екстрактите от листа ($p > 0.106$). Тестът за цитотоксичност срещу клетките от човешки ембрионален бъбрек (HEK 293) показва, че екстрактите от листата и кората на стъблото са токсични съответно при 0.1059 и 0.1179 $\mu\text{g/ml}$. Относително високите терапевтични индекси (TI) на растителните екстракти по-

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казват тяхната безопасност. Следователно, това проучване установи, че екстрактите от листата и кората на стъблото на *F. albida* притежават силни антибактериални и цитотоксични свойства, които могат да бъдат използвани при лечението на свързани заболявания и да ограничат по-нататъшното разпространение на патогени, резистентни към антимикробни препарати.

Introduction

The use of medicinal plants is recognized globally for the treatment of diseases and disorders including bacterial infections. Bacteria, viruses, and fungi are the major cause of morbidity and mortality globally with bacteria contributing to the highest disease burden. Also, communicable diseases account for over 50% of the annual mortality in low-income countries (Ngemenya *et al.*, 2019). However, the biggest challenge facing the chemotherapy of bacterial infection is the development of antibiotic resistance to conventional drugs. This has led to the emergence of drug-resistant and pan-drug resistant phenotypes in bacterial species (Bello *et al.*, 2018). Science has been struggling to catch up with the emergence of new diseases; however, the process has been marred by the slow rate of new drug development. Sub-Saharan Africa has the highest incidence of disease outbreak across all continents necessitating thigh use of antibiotics, which inadvertently will lead to high levels of antimicrobial resistance (Essack *et al.*, 2016). The need to develop new drugs, therefore, becomes imperative in order to combat the global challenge of antimicrobial resistance.

Faidherbia is a monotypic genus. It contains only one species: *Faidherbia albida* (Del.) A. Chev. *F. albida* belongs to the *Acacieae* family and it is used ethnomedicinally for the treatment of respiratory, tooth, and eye infections (Wood, 2019). The bark and roots have been reportedly used for the treatment of digestive disorders, malaria, and fever. It is a leguminous plant native to Africa and the Middle East which was later introduced to Pakistan and India. The use of *F. albida* in traditional agro forestry systems is associated with the inverse phenological rhythm of the species and which is important because of the lack of competition between trees and crops during the rainy season, while the persistent foliage during the dry season is an essential source of fodder (Bello *et al.* 2015).

A number of indigenous plants in Nigeria have been studied and found to contain some tremendous therapeutic properties. Many of such plants have been successively screened for their phytochemical and biological constituents including their antibacterial activities; however, many more are yet to be documented for antibacterial activity; and where such activity is reported, toxicity and safety might

not have been reported (Dlana *et al.*, 2016). This study, therefore, evaluated the phytochemical and cytotoxic properties alongside the antibacterial activities of the stem bark and leaf extracts of *F. albida* against selected clinical pathogens in southwest Nigeria.

Materials and Methods

Collection of plant sample

Fresh samples of *F. albida* (leaf and stem bark) were collected from Ede in Osun State, Southwest Nigeria. The identification and authentication of the plant and plant parts were done at the Department of Botany (IFE Herbarium), Obafemi Awolowo University, Ile-Ife.

Preparation of the plant samples

The investigated parts of the plant were separately rinsed in tap water, air dried and pulverized into fine powder. A 200 g portion of the powdered sample of each of the plant parts was extracted separately with 80% methanol for 72 h. These were filtered and the filtrates concentrated *in vacuo* in order to obtain the crude methanol leaf and stem bark extracts (Akinpelu *et al.*, 2018).

Source of microorganisms

The bacteria investigated in this study were collected from National Collection of Industrial Bacteria (NCIB) and locally isolated organism (LIO). These include *Bacillus subtilis* (NCIB 3610), *Bacillus cereus* (NCIB 6349), *Bacillus stearothermophilus* (NCIB 8222), *Enterococcus faecalis* (NCIB 775), *Escherichia coli* (NCIB 86), *Klebsiella pneumoniae* (NCIB 418), *Pseudomonas aeruginosa* (NCIB 950), *Proteus vulgaris* (LIO), *Shigella* sp. (LIO), and *Staphylococcus aureus* (NCIB 8588). These organisms have been implicated in infectious diseases.

Preparation of bacterial cultures

The method of Jorgensen *et al.* (1990) was adopted for the preparation of the bacterial cultures. Each pure bacterial culture was prepared freshly on nutrient agar and incubated at 37°C for 24 h. The fresh cultures were then transferred into freshly prepared nutrient broth and standardized to 0.5 McFarland turbidity standards using the spectrophotometer to obtain the desired cell density of 1.5×10^8 cells/ml.

Antimicrobial activity of extracts of the plant's leaf and stem bark

Dimethyl sulfoxide (DMSO) was used to separately prepare the crude extracts which were screened for antibacterial activity. A 0.1 ml proportion of the test organism was aseptically and homogenously smeared on the surface of solidified nutrient agar medium inside the Petri plate and allowed to stand for 5 minutes. A cork borer of 6 mm diameter in size was used to create five wells on the agar medium and few drops of the working solution were introduced into each of the wells and labeled. This was allowed a pre-diffusion time of 30 min and then incubated at 37°C for 24 h. The diameters of the zones of complete inhibition were measured with a metre rule and recorded. Ampicillin and streptomycin were used as positive controls and all tests were performed in triplicates (Jorgensen *et al.*, 1990).

Determination of the Minimum Inhibitory Concentration

The minimum inhibitory concentrations (MICs) of the plant extracts that showed positive results in the disk diffusion assay by inhibiting the growth of the test organisms were determined using the microplate dilution method as described by Mishra *et al.* (2015). Using DMSO, serial dilutions of the plant extracts were prepared. The standardized inoculum of test organisms with turbidity adjusted to 0.5 McFarland standard to prepare 1×10^8 bacterial cell/ml. Then 150 μ l of different concentrations of plant extracts were separately introduced to each well of the 96-well microplate and 50 μ l proportion of the bacterial suspension added. Ampicillin and streptomycin were used as the positive controls while 10% DMSO and plant extracts lacking bacterial suspension were the negative controls. The incubation of the microtiter plates was at 37°C for 24 h. The lowest concentration of the plant extract that inhibited the visible growth of the test organism was taken as the MIC.

Determination of the Minimum Bactericidal Concentration (MBC)

The wells of the microtiter plate showing growth at the lowest concentration of the plant extract and those with no growth in the MIC assay were considered for the determination of MBC. A 100 μ l proportion of the bacterial cells from the selected wells were streaked on the surface of freshly-prepared nutrient agar medium and plates incubated at 37°C for 24 h. The lowest concentration that showed no growth was taken as the MBC of

the extract (Bello *et al.*, 2019).

Phytochemical screening of the aqueous extract

The extracts from the plant were screened for phytochemical constituents such as flavonoids, tannins, alkaloids, saponins, glycosides, triterpenes, xanthoproteins, and anthraquinones of as described in the methods of Harbans (1973), Trease and Evans (2002), and Sofowora (2006).

Cytotoxicity assay

The cytotoxicity of the plant extracts was tested on human embryonic kidney (HEK) 293 cells as reported by Zanariah *et al.* (2013). Exactly 100 L of HEK cell suspension (1×10^5 cells/ml) was added to the inner walls of a 96-well plate and 200 L of incomplete medium Dulbecco's modified eagle's medium (DMEM) to the outer wells. In order to allow cell attachment to the bottom of the plate, incubation was done at 37°C for 24 h. The plant extracts, which had been dissolved in DMSO, were serially diluted to obtain dilution factor of up to 10^8 . A 100L proportion of each concentration was introduced to the 96-well plate in triplicates. A reference plate to account for the colour of the plant extracts was also prepared. This plate comprised the plant extracts and medium without bacterial cells, and was incubated at 37°C for 72 h. A 50L proportion of XTT reagent (ROCHE) was introduced to the wells of the microtitre plate and incubated at 37°C for 3 h. After incubation, the plates were read using ELISA with the KC junior software. Actinomycin D and DMSO were used as the positive and negative controls, respectively, while 10% fetal bovine serum Dulbecco's Modified Eagle Medium (DMEM) Penicillin/Streptomycin was the complete medium.

Data analyses

Data were collated and statistically analyzed using MedCalc statistical software, version 17.2. A Chi-square test was used to correlate the antibacterial activities exerted by different concentrations of the stem bark and leaf extracts. The difference between the activities exerted by the leaf and stem bark extracts was considered significant $\alpha \leq 0.05$ (i.e. at 95% level of confidence).

Results and Discussion

Zone of inhibition

The antibacterial sensitivity of the test pathogens is shown in Table 1. The stem bark and leaf extracts demonstrated a high inhibitory effect against *P. vulgaris*, *B. cereus*, *P. aeruginosa* and *S. aureus* and competed favourably with the positive control.

The stem bark extract inhibited *P. vulgaris*

Table 1. Antibacterial activities of *F. albida* stem and leaf extracts against selected pathogens

Bacterial strains	Zone of Inhibition (mm)			
	Stem	Leaf	Streptomycin	Ampicillin
<i>B. cereus</i> (NCIB 6349)	23	22	22	20
<i>P. vulgaris</i> (NCIB 67)	29	20	20	17
<i>E. coli</i> (NCIB 86)	20	19	19	21
<i>Serratia marcesens</i> (NCIB 1377)	21	15	15	22
<i>B. subtilis</i> (NCIB 3610)	16	29	29	20
<i>P. aeruginosa</i> (NCIB 950)	23	20	20	15
<i>S. aureus</i> (NCIB 8588)	17	25	25	19
<i>Micrococcus luteus</i> (NCIB 167)	19	22	22	20
<i>Vibrio fluvialis</i> (LIO)	20	20	20	21
<i>K. pneumoniae</i> (NCIB 418)	16	29	29	20

(NCIB 67) with inhibition zone of 29 mm while the leaf extract equipotently inhibited *B. subtilis* (NCIB 3610) and *K. pneumoniae* (NCIB 418) with inhibition zone of 29 mm each, and *S. aureus* with 25 mm. Therefore, there was no significant difference in the antibacterial sensitivity test between the stem bark extract and the leaf extract (Table 1).

MIC and MBC of the stem bark and leaf extracts of *F. albida*

The MIC and MBC of the stem bark and leaf extracts of *F. albida* are presented in Table 2. The stem bark and leaf extracts inhibited *M. luteus* at MICs of 0.781 and 0.472 mg/ml, respectively, while the MBCs were 0.391 and 0.290 mg/ml, respectively. *E. coli*, *P. aeruginosa* and *K. pneumoniae*, however, required high extract concentrations of 6.25 mg/ml. For all the bacterial pathogens tested, the MIC and MBC of the stem bark extract were lower than the leaf extract except for *M. luteus* (NCIB

167). The difference, however, was not significant ($p=1.07$). This suggests that the stem bark extract had a better broad spectrum antibacterial activity than the leaf extract.

Cytotoxicity and Therapeutic Index (TI) of the stem bark and leaf extracts of *F. albida*

The result of cytotoxicity test against the HEK cells showed that the leaf and stem bark extracts were toxic at 0.1059 and 0.1179 $\mu\text{g/ml}$, respectively. The TIs of the leaf extract were 0.067, 0.38, 0.14, 0.49, and 0.89 for *S. aureus*, *E. coli*, *K. oxytoca*, *S. enterica*, and *S. sonnei*, respectively. The TIs of the stem extract were 0.057, 0.38, 0.17, 0.38, and 0.64 for *S. aureus*, *E. coli*, *K. oxytoca*, *S. enterica*, and *S. sonnei*, respectively (Table 3).

Phytochemical constituents of the stem bark and leaf extracts of *F. albida*

The phytochemical constituents of the stem bark and leaf extracts of *F. albida* are shown in

Table 2. MIC and MBC of *F. albida* stem bark and leaf extracts

Bacterial Strains	MIC (mg/ml)		MBC (mg/ml)	
	Stem	Leaf	Stem	Leaf
<i>B. cereus</i> (NCIB 6349)	3.125	3.590	1.562	1.762
<i>P. vulgaris</i> (NCIB 67)	3.125	3.145	0.781	0.871
<i>E. coli</i> (NCIB 86)	6.25	6.36	1.562	1.582
<i>S. marcesens</i> (NCIB 1377)	3.125	3.246	1.562	1.467
<i>B. subtilis</i> (NCIB 3610)	3.125	3.158	1.562	1.782
<i>P. aeruginosa</i> (NCIB 950)	6.25	6.34	1.562	1.642
<i>S. aureus</i> (NCIB 8588)	3.125	3.134	1.562	1.678
<i>M. luteus</i> (NCIB 167)	0.781	0.472	0.391	0.291
<i>V. fluvialis</i> (LIO)	1.562	1.633	0.391	0.290
<i>K. pneumoniae</i> (NCIB 418)	6.25	6.43	1.562	1.562

$p = 0.089$

Table 3. Cytotoxicity and therapeutic index (TI) of the leaf and stem bark extracts

Extracts	Cytotoxicity ($\mu\text{g/ml}$)	Therapeutic Index				
		<i>S. aureus</i>	<i>E. coli</i>	<i>K. oxytoca</i>	<i>S. enterica</i>	<i>S. sonnei</i>
Leaf	0.1059	0.067	0.38	0.14	0.49	0.89
Stem bark	0.1179	0.23	0.97	0.25	0.27	0.41

$p = 0.106$

Table 4. These showed that flavonoids, tannins, alkaloids, saponins, glycosides, triterpenes, xanthoproteins, and anthraquinones were present in both the stem bark and leaf extracts of plant. However, steroids were absent in both the stem bark and leaf extracts.

This study reflected the antibacterial and cytotoxicity potentials of *F. albida* leaf and stem bark extracts. The discovery of antibiotics resistance among bacterial pathogens necessitates the search for safe and effective broad-spectrum antibiotics from natural products. In spite of the medical advances in the chemotherapy of human infections, bacterial infections still remain one of the greatest challenges facing the medical sciences.

Table 4. Phytochemical constituents of the stem bark and leaf extracts of *F. albida*

Phytochemical	Stem bark	Leaf extract
Flavonoids	+	+
Tannins	+	+
Alkaloids	+	+
Saponins	+	+
Steroids	-	-
Glycosides	+	+
Triterpenes	+	+
Phlobatanins	+	-
Xanthoproteins	+	+
Anthraquinones	+	+

Key: (+) = Present; (-) = Absent

Both the stem bark and leaf extracts of *F. albida* inhibited all the bacterial pathogens tested with varying inhibition zones of diameter. No significant difference in the antibacterial sensitivity of the bacterial species occurred between the stem bark extract and the leaf extract, although the leaf elicited relatively better activity. This implies that any of the plant parts could be used for treatment of bacterial infections caused by the investigated organisms in this study.

Umar *et al.* (2017) reported that the combined stem bark extracts of *F. albida* and *Psidium guajava* elicited a better inhibition diameter (21 mm) against methicillin-resistant *S. aureus* than the indi-

vidual stem bark extracts (8-10 mm). This corroborates the finding in this study, where the leaf extract exhibited a better inhibitory activity (25 mm) than the stem bark extract (17 mm) against *S. aureus*. Therefore, for the treatment of *S. aureus*-mediated infections, such as diarrhea and urinary tract infections (UTIs), the leaf decoction of *F. albida* would be preferably recommended.

The lowest concentration of the plant extracts that completely inhibited the growth of microorganisms was taken as the MBC. The absence of turbidity in the recovery medium used in MIC test was taken as a proof for bactericidal activity of *F. albida* extracts; and was considered as the lowest concentration from the MIC range, which resulted in no growth in the recovery medium of the MBC test. Both the leaf and stem bark extracts of *F. albida* elicited potency against the tested isolates at different MIC and MBC, but the leaf extract was found to elicit higher inhibitory and bactericidal effects against *Micrococcus luteus* (NCIB 167). *M. luteus* was reported to be resistant to antimicrobial treatment with *Lactobacillus casei* and *Lactobacillus brevis* (Amuche *et al.*, 2019). Therefore, the use of *F. albida* extracts could serve as a cheap and effective alternative in combating *Micrococcus luteus*-mediated infections.

The leaf and stem bark extracts were toxic at concentrations of 0.1059 and 0.1179 $\mu\text{g/ml}$, respectively. TI compares the blood concentration at which a drug is either toxic or safe to the end user. When the TI is large, the drug is considered safe. Therefore, the leaf and stem bark extracts of *F. albida* are suitable for the treatment of *S. sonnei*, *S. enterica*, and *E. coli*-mediated infections.

The phytochemical analysis of *F. albida* revealed the presence of flavonoids, tannins, alkaloids, saponins, glycosides, triterpenes, xanthoproteins, and anthraquinones. Therefore, the broad spectrum antibacterial activity observed in this study could be attributed to the presence of these active ingredients. Tannins and alkaloids had been reported to possess excellent antibacterial activity (Umar *et al.*, 2017). Tannins elicit their antibacterial activity by binding to and precipitating proteins. Alkaloids act by affecting a wide range of cellular

molecular targets, such as biological membranes and nucleic acids (Roberts and Wink, 1998; Akiyama *et al.*, 2001).

Ohouko *et al.* (2020) reported that leaves, fruits and stem bark of *F. albida* revealed common chemicals groups, such as coumarins, leucoanthocyanins, mucilage, gallic tannins, and saponins. The authors reported only anthocyanins in the leaves and stem bark of the plant, and flavonoids found in the stem bark were absent in the leaves and fruits, which is contrary to the findings of the study. The study of Karoune *et al.* (2015) revealed the presence of phenolic acids and flavonoids in the leaves, fruits and stem bark of *F. albida*. Alkaloids were also reported to be present in the seeds of *F. albida* (Evans *et al.*, 1977).

This study revealed that *F. albida* leaf and stem bark extracts possess robust antibacterial and cytotoxic properties. The plant parts could serve as good candidates for antibacterial and drug discovery. The low MIC and MBC values of the plant extracts suggest strong antibacterial activity; while low therapeutic index recorded with the plant extract suggests possible toxic effects. More studies are required to assess the degree of toxicity of these plant parts.

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Possibility of Saccharide-Binding Protein to Interactions of *Acanthamoeba castellanii* and Several Bacteria

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Abstract

The ability of *Acanthamoeba castellanii* to ingest bacteria means suggests the presence of a mechanism of contact dependence. It has been confirmed that it is important to induce toxicity to target cells through lectin, a glycoprotein, with the strong pathogenicity of amoeba. In this study, it was analyzed the interaction between pathogenic bacteria and *A. castellanii* trophozoites and cysts using various concentrations of a monosaccharide called mannose. When the concentration of mannose was increased to 100 mM, the association of methicillin-resistant *Staphylococcus aureus* (MRSA) was reduced by more than 2 times. Moreover, it was confirmed that the number of MRSA reduced by about 80 times could invade *A. castellanii* trophozoites with 100 mM mannose. The association of *Enterococcus faecalis* with *A. castellanii* trophozoites was approximately 41% lower and the invasion of *E. faecalis* into *A. castellanii* trophozoites was reduced by about 2 to 60 times compared to the bacterial association, and *E. faecalis* never survived within the *A. castellanii* trophozoites. Interestingly, survival of *Pseudomonas aeruginosa* within *A. castellanii* trophozoites was reduced by 20 times but was significantly higher than that of other bacteria. The invasion or survival of *P. aeruginosa* was 0% in all results of *A. castellanii* cyst. The mechanism of contact dependence via monosaccharide-binding proteins other than mannose in *A. castellanii* is not only a way to inhibit *A. castellanii*, but also an analysis of the interaction.

Keywords: *Acanthamoeba castellanii*, saccharide, MRSA, *Enterococcus faecalis*, *Pseudomonas aeruginosa*

Резюме

Способността на *Acanthamoeba castellanii* да поглъща бактерии предполага наличието на механизъм на контактна зависимост. Установено е, че въглехидрат-базираната разпознавателна система - лектин (гликопротеин) е токсична за таргетните клетки и е от значение за силната патогенност на амебата. В това проучване се анализира взаимодействието между някои патогенни бактерии и трофозоити и кисти на *A. castellanii*, като се използват различни концентрации на монозахарида маноза. При повишаване концентрацията на манозата до 100 mM, свързването на метицилин-резистентния *Staphylococcus aureus* (MRSA) се намалява повече от 2 пъти. Установено е също така, че намаления с около 80 пъти брой на MRSA, може да инвазира трофозоитите на *A. castellanii* при същата концентрация на маноза. Свързването на *Enterococcus faecalis* с трофозоитите на *A. castellanii* е приблизително с 41% по-ниско, а инвазията на *E. faecalis* в трофозоитите на *A. castellanii* е намалена с около 2 до 60 пъти в сравнение с бактериалното свързване, като *E. faecalis* никога не оцелява вътре в трофозоитите на *A. castellanii*. Интересен е резултатът, че преживяемостта на *Pseudomonas aeruginosa* в трофозоитите на *A. castellanii* е намалена с 20 пъти, но е значително по-висока от тази на други бактерии. Инвазията или оцеляването на *P. aeruginosa* е 0% при всички получени резултати при изследване на кисти на *A. castellanii*. Механизмът на контактна зависимост посредством монозахарид-свързващи протеини, различни от маноза, в случая с *A. castellanii*, е не само начин за инхибиране на *A. castellanii*, но дава и възможност за анализ на взаимодействието.

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Introduction

Acanthamoeba castellanii is a single-celled protozoan distributed in natural environments such as soil and rivers (Khan, 2006; Ruqaiyyah and Khan, 2012; Fakae *et al.*, 2020). *A. castellanii* is also known as the causative agent of amoebic keratitis when swimming in lakes or rivers in humid areas, and wounds in the cornea of the eye (Schuster and Visvesvara, 2004; Jung *et al.*, 2008). The fact that *A. castellanii* can digest a variety of microorganisms in lakes and rivers means how important the interaction between bacteria and *A. castellanii* is (Alsam *et al.*, 2006). Interactions with microorganisms that can cause human infection can also have important implications. *Acanthamoeba* acts as a reservoir for several pathogenic bacteria, such as *Escherichia coli* K1 as an agent of meningitis, *Legionella pneumophila* as an agent of Legionnaire's disease, *Pseudomonas aeruginosa* as an agent of keratitis, etc. (Michel *et al.*, 1995; Cengiz *et al.*, 2000; Alsam *et al.*, 2006). There is a report of different degrees of ingestion of bacteria caused by the pathogenicity of amoeba. If so, it could be suggested that the pathogenicity of amoeba is related to the uptake of bacteria, that is, interaction with bacteria (Alsam *et al.*, 2006). The higher rates of bacterial invasion and survival for pathogenic amoeba than non-pathogenic amoeba means that pathogenic amoeba is slightly better in bacterial uptake than non-pathogenic amoeba. However, this report relates to the intake of bacteria in the nutrient type of amoeba, but there is no study on the cyst type of *A. castellanii* that survives in a poor environment.

The ability of *A. castellanii* to ingest bacteria means that there is a mechanism of contact dependence. According to Khan's report, it was confirmed that it is important to induce toxicity to target cells through lectin, a glycoprotein, among which mannose-binding protein (MBP) was associated with the strong pathogenicity of amoeba (Garate *et al.*, 2006; Fakae *et al.*, 2020).

In order to understand this process, it was suggested that the mannose-binding protein that the research team had previously studied would be an important factor in the interaction, so that the *A. castellanii* trophozoite and cyst were pretreated with mannose or saturated to determine how much pathogenic bacteria could enter the amoeba. Therefore, this study analyzed the interaction between bacteria that cause various infections in the human body and *A. castellanii*, which can ingest bacteria, using various concentrations of a monosaccharide called mannose. In addition, an analysis was con-

ducted on how many bacteria the *A. castellanii* cyst can interact with.

Materials and Methods

Culture of A. castellanii trophozoites and inducement of A. castellanii cyst

In this study, a T4 genotype isolated from keratitis patients (American Type Culture Collection, ATCC 50492) was used for *A. castellanii* trophozoite and cyst form based on previous studies, and *A. castellanii* was cultured with PYG medium [Proteospeptone 0.75% (w/v), yeast extract 0.75% (w/v) and glucose 1.5% (w/v)] in a T-75 culture flask at 37°C (Krishna-Prasad and Gupta, 1978). When 70% confluence was reached, it was desirable to change to a new medium. Most of cultured amoebial types were trophozoites and were used in polysaccharide experiments.

A. castellanii encystment method was performed to study the interaction of bacteria with cyst of *A. castellanii* (Jung *et al.*, 2007). Briefly, the above cultured amoeba trophozoites were transferred to a non-nutrient agar plate (prepared using 3% (w/v) purified agar, Oxoid limited, Seoul, Korea) and the plate was placed at 30°C for up to 96 h. Periodic cyst induction was observed under a bright microscope. Ten ml of PBS was added to the non-nutrient agar plate in which cysts were induced, and the agar surface was gently scraped using a cell scraper to determine the number of amoeba cysts, and it was observed whether the cysts were completely induced under a microscope.

Culture of pathogenic three bacteria

Gram-positive and gram-negative bacteria, such as MRSA, *E. faecalis* and *P. aeruginosa* with strong pathogenicity were used in this study. Bacterial cultivation was performed using tryptic soy agar (TSA, MB cell, Korea), which is an enrichment medium, rather than using bacterial selection medium, to evaluate the number of bacterial colonies. A single colony was subcultured on a TSA plate at 37°C and double confirmed by Gram staining procedure (Lim *et al.*, 1988). Single colonies obtained to cultivate correct colonies were diluted with 0.85% NaCl and a McFarland turbidity of 0.5, which can produce 0.5×10^3 to 1.5×10^6 colony forming units (cfu)/ml, for example (Song and Jung, 2017).

Association, invasion and survival of bacteria with A. castellanii trophozoites and cysts

The experimental procedures were referred by Jung *et al.* (2007). The following experimental

procedures used *A. castellanii* trophozoites, and mannose at a concentration of 10, 50 and 100 mM was used to pretreat the amoeba for 30 min, which were afterwards washed twice with PBS. Briefly, *A. castellanii* trophozoites were cultured in 24-well culture plates with PYG medium. *A. castellanii* was incubated with each bacterium (2×10^5 cfu/0.5 ml of PBS) and plates were incubated for 1 hour at room temperature (RT). Obtained single colonies were diluted with 0.85% NaCl as mentioned above by McFarland turbidity assay (Song and Jung, 2017). After incubation and triple washing with PBS, amoebae were dissolved by adding SDS (0.5% final concentration) to each well for 30 min and the number of bacteria was enumerated by plating on TSA agar plates (Schuster and Visvesvara, 2004). The percentage of bacterial association was calculated as follows: recovered bacteria (cfu)/total bacteria (cfu) $\times$ 100 = % bacteria associated with *A. castellanii*. For the invasion assay, after the bacteria were incubated with *A. castellanii* trophozoites, gentamicin antibiotics was added for 45 min (100 μ g/ml, final concentration) to remove extracellular bacteria. Bacterial plating was performed as mentioned above. The percentage of bacterial invasion was calculated as follows: recovered bacteria (cfu)/total bacteria (cfu) $\times$ 100 = percentage of intracellular bacteria. In order to determine how many bacteria survived, amoeba with invading bacteria were incubated at 37°C for 24 h. Thereafter, all bacteria in the supernatant were removed and the number of surviving bacteria was counted as in the above calculation method to evaluate the survival rate. For an experiment with amoeba cysts, cysts grown in a non-nutrient medium were transferred to a 24-well culture plate. The concentration of mannose treated in the cyst was performed the same as in the trophozoites as described above.

Statistical analysis

Student two-sample *t* test was performed using the mean and standard deviation values of the experimental results to determine statistical significance. The statistically significant difference was $P < 0.05$.

Results

Effect of mannose on A. castellanii trophozoites in the interaction with pathogenic MRSA

In order to determine the relationship of mannose by the contact-dependent pathway, the association of bacteria was analyzed by adding bacteria to *A. castellanii* treated with mannose. Several components present in bacteria (e. g., OmpA, LPS, etc.)

have been reported to be related to contact with *A. culbertsoni* (Jung *et al.*, 2007). As shown in Fig. 1, MRSA was found to have a nearly 160% association with *A. castellanii* trophozoites.

The association between *A. castellanii* trophozoites treated with 10 mM mannose and MRSA did not show much difference from that of untreated, but when the concentration of mannose was increased to 100 mM, the association of bacteria was reduced by more than 2 times. As a result of analyzing the degree of invasion of MRSA related to *A. castellanii* trophozoites to which mannose was already bound into *A. castellanii* trophozoites, it was confirmed that the number of MRSA reduced by about 80 times could invade *A. castellanii* trophozoites with 100 mM mannose (Fig. 1 B1 and B2). On the other hand, the survival of MRSA invading *A. castellanii* trophozoites was not significantly different from the concentration of mannose, and this fact showed that already invading MRSA could survive in *A. castellanii* trophozoites regardless of the contact-dependent pathway outside the amoeba (Fig. 1 C1 and C2).

Effect of mannose on A. castellanii trophozoites in the interaction with pathogenic E. faecalis

In fact, the selection medium of *E. faecalis* is bile esculin agar (BEA), but in this experiment, TSA medium was used to confirm the bacterial proliferation pattern, not to observe the complete growth or properties of bacteria (Fig. 2).

The association of *E. faecalis* with *A. castellanii* trophozoites was approximately 41% lower, and the overall pattern of interaction with mannose-treated *A. castellanii* trophozoites was similar to that of MRSA (Fig. 2). On the other hand, the invasion of *E. faecalis* to *A. castellanii* trophozoites was reduced by about 2 to 60 times compared to the bacterial association and *E. faecalis* never survived within the *A. castellanii* trophozoites.

Effect of mannose on A. castellanii trophozoites in the interaction with pathogenic P. aeruginosa

The results of *P. aeruginosa* showed very high values compared to the results of MRSA or *E. faecalis* above (Fig. 3).

In previous studies, it has been reported that *P. aeruginosa* interacts with *A. castellanii* very well, and this study also showed similar results. The association of *P. aeruginosa* to *A. castellanii* trophozoites was 3 times higher than MRSA and 5 times higher than *E. faecalis* (Fig. 3 A1 and A2). The invasion by *P. aeruginosa* of *A. castellanii* trophozoites was slightly higher than that of MRSA,

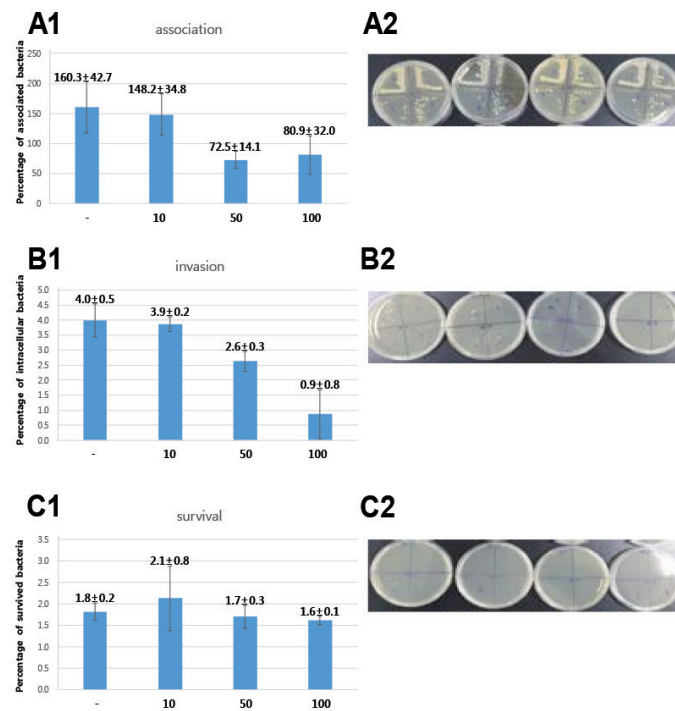


Fig. 1. Effect of mannose on the interactions of *A. castellanii* trophozoites with MRSA. Mannose of 10, 50 and 100 mM concentration was pre-treated to *A. castellanii* and then added to MRSA. A1, B1 and C1 indicated the percentages of bacterial association, invasion and survival, respectively. A2, B2 and C2 described the bacterial plates showing the bacterial cfu of A1, B1 and C1, respectively. This experiment was performed in triplicate, and data were described by mean ± standard deviation (SD) value. Asterisks indicated significant differences between groups.

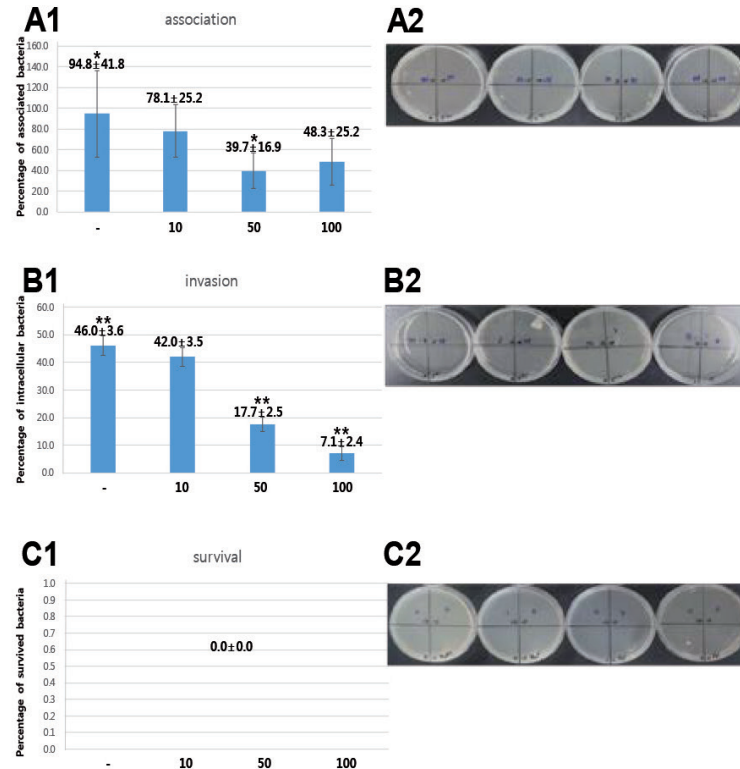


Fig. 2. Effect of mannose on the interactions of *A. castellanii* trophozoites with *E. faecalis*. Mannose of 10, 50 and 100 mM concentration was pre-treated to *A. castellanii* and then added to *E. faecalis*. A1, B1 and C1 indicated the percentages of bacterial association, invasion and survival, respectively. A2, B2 and C2 described the bacterial plates showing the bacterial cfu of A1, B1 and C1, respectively. This experiment was performed in triplicate wells with three times, and data were described by mean ± standard deviation (SD) value. Asterisks indicated significant differences between groups.

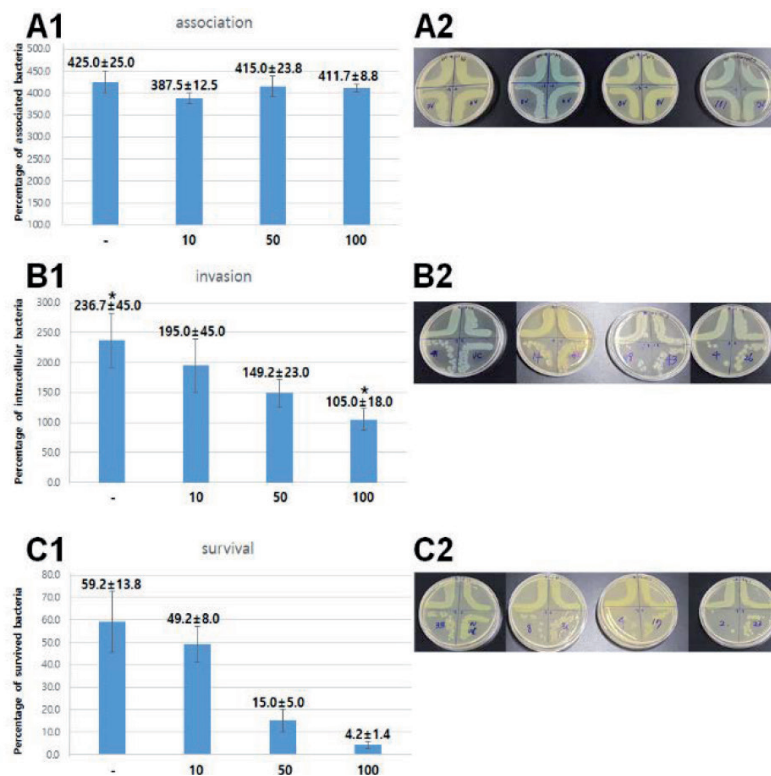


Fig. 3. Effect of mannose on the interactions of *A. castellanii* trophozoites with *P. aeruginosa*. Mannose of 10, 50 and 100 mM concentration was pre-treated to *A. castellanii* and then added to *P. aeruginosa*. A1, B1 and C1 indicated the percentages of bacterial association, invasion and survival, respectively. A2, B2 and C2 described the bacterial plates showing the bacterial cfu of A1, B1 and C1, respectively. This experiment was performed in triplicate wells with three times, and data were described by mean $\pm$ standard deviation (SD) value. Asterisks indicated significant differences between groups.

and the effect pattern of mannose treatment was similar. Interestingly, survival within *A. castellanii* trophozoites of *P. aeruginosa* decreased 20-fold when *P. aeruginosa* invaded *A. castellanii* trophozoites, but showed a much higher survival rate than other bacteria (Fig. 3 C1 and C2).

Effect of mannose on *A. castellanii* cysts in the interaction with pathogenic *P. aeruginosa*

The cyst and trophozoite of *A. castellanii* are very different morphologically. For example, cysts have a very thick double-membrane cell wall, but relatively less motility than vegetative ones. In this experiment, the association, invasion, and survival of *P. aeruginosa*, which showed higher values compared with MRSA and *E. faecalis* for *A. castellanii* cysts were analyzed, and the effect of mannose on *A. castellanii* cysts was analyzed (Fig. 4). The association between *P. aeruginosa* and *A. castellanii* cysts was about 2.75%. When treated with a maximum of 100 mM mannose, about 2.11% of *P. aeruginosa* became associated with *A. castellanii* cysts. Therefore, it was shown that the association of *P. aeruginosa* decreased by about 0.6% when treated with mannose. However, the invasion or survival of *P. aeruginosa* was 0% in all results (data

not shown). These results showed that *P. aeruginosa* cannot enter *A. castellanii* cysts.

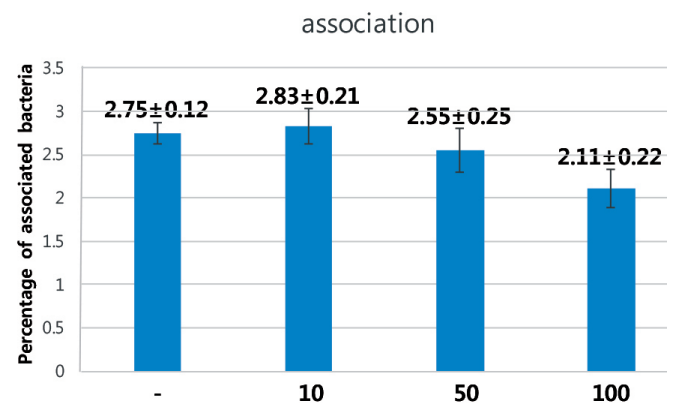


Fig. 4. Effect of mannose on the interactions of *A. castellanii* cysts with *P. aeruginosa*. Mannose of 10, 50 and 100 mM concentration was pre-treated to *A. castellanii* and then added to *P. aeruginosa*. A1, B1 and C1 indicated the percentages of bacterial association, invasion and survival, respectively. A2, B2 and C2 described the bacterial plates showing the bacterial cfu of A1, B1 and C1, respectively. This experiment was performed in triplicate wells with three times, and data were described by mean $\pm$ standard deviation (SD) value. Asterisks indicated significant differences between groups.

Discussion

According to a previous report, among the factors related to the association between *A. culbertsoni* and bacteria, the MBP in *A. culbertsoni* trophozoites was confirmed to play an important role in the pathogen interactions (Jung, 2020). Therefore, in this study, mannose was used to pretreat *A. castellanii* trophozoites among free-living amoebae, and the association, invasion, and survival of various pathogenic bacteria were analyzed. Also, how the cyst form of *A. castellanii* was related to bacteria was also analyzed. These pathogen-pathogen interactions and survival within the amoebae may suggest the possibility of multiple infections. However, there are currently no reports, and this is probably because most of the research still uses a single entity. Since *A. castellanii* can mainly move using the active pseudopodia, there will be many saccharide-binding proteins distributed in the pseudopodia. This fact means that if the proteins constituting the pseudopodia or various pathogenic factors are inhibited, the binding ability of *A. castellanii* will be poor. MBP, which was mainly distributed in the pseudopodia and expressed mainly in the direction of movement, was associated with contact-dependent cytotoxicity with the host cells (Garate *et al.*, 2004; Kim *et al.*, 2012). In this study of *A. castellanii* cysts, bacterial association, invasion, and survival rates were reduced under the assumption that trophozoites with many pseudopodia played an important role in contact-dependent binding. Among the three types of bacteria, such as MRSA, *E. faecalis*, *P. aeruginosa* with strong pathogenicity, *P. aeruginosa* showed the highest proportion of association, invasion, and survival to *A. castellanii* trophozoites and cysts. In addition, this study showed that the rates of bacterial association, invasion, and survival were also reduced depending on the concentration of mannose. *A. castellanii* causes amoebic keratitis, and understanding *A. castellanii* interactions with host cells can be helpful in understanding *A. castellanii* pathogenicity. The mechanism of contact dependence by monosaccharide-binding proteins other than mannose in *A. castellanii* is not only a way to inhibit *A. castellanii*, but also an analysis of the interaction between *A. castellanii* and bacteria as a host of pathogenic bacteria should be carried out further in the future.

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Isolation of Canine Parvovirus from Rectal Swabs of Suspected Dogs from Various Regions in Northern India

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Abstract

Canine parvovirus (CPV) is a highly contagious pathogen causing acute gastro-intestinal illness among the canine population. The study aimed to isolate canine parvovirus in a Madin-Darby Canine Kidney (MDCK) cell line from rectal swabs of dogs having signs of gastroenteritis. A total of 45 rectal swabs were collected from dogs exhibiting clinical signs of canine parvovirus infection from various regions of northern India, viz., Punjab, Assam, Delhi, Chandigarh and Jammu. The samples were processed and subjected to MDCK cell isolation, and isolates that exhibited cytopathic effects (CPE) like rounding of cells, clumping and detachment of cells were confirmed by PCR and nPCR. Out of 45 samples, 12 (26.66%) isolates showed CPE on MDCK. Further, it was observed that out of these 12 isolates, three isolates were from dogs which had history of vaccination for the disease.

Keywords: Canine parvovirus, MDCK cell line, isolation, Northern India, cytopathic effects.

Резюме

Кучешкият парвовирус (*Canine parvovirus*, CPV) е силно заразен патоген, причиняващ остро стомашно-чревно заболяване сред кучешката популация. Проучването има за цел да изолира кучешки парвовирус в клетъчна линия Madin-Darby Canine Kidney (MDCK) от ректални тампони, взети от кучета с признаци на гастроентерит. Събрани са общо 45 ректални тампона от кучета, показващи клинични признаци на кучешка парвовирусна инфекция от различни региони на Северна Индия, а именно Пенджаб, Асам, Делхи, Чандигарх и Джаму. Пробите са обработени и изолирани в MDCK клетъчна линия. Изолатите, които проявяват цитопатични ефекти (CPE) като закръгляване, струпване и отделяне на клетките са потвърдени чрез PCR и nPCR. От 45 проби ректални тампона на предполагаеми за носителство кучета, 12 изолата (26.66%) показват CPE върху клетки MDCK. От тези 12 изолата, три изолата са от кучета, които са с данни за ваксинация срещу болестта.

Introduction

Canine parvovirus (CPV) is the leading cause of highly contagious and a relatively common cause of acute, infectious gastro-intestinal illness, especially in young/unvaccinated dogs. Canine parvovirus is one of the smallest known viruses (~25nm in diameter) belonging to the genus *Protoparvovirus* of the family *Parvoviridae* (Appel, 1978; Berns, 1990). CPV is a non-enveloped icosahedral, linear, non-segmented, negative-sense, single-stranded DNA genome of 5,323 bases which encodes for three structural proteins (VP1, VP2 and VP3) and two non-structural proteins (NS1 and NS2). VP2 is the major structural protein of capsid and its key antigenic determinant. The earliest known CPV positive sera were reported in Greece in 1974. CPV

was first identified in the mid-1970s as an emerging pathogen (Appel, 1978; Appel *et al.*, 1979) and was referred to as CPV-2 to distinguish it from CPV-1 (Parrish and Carmichael, 1983). In India, CPV-2 was first reported from Madras by Balu and Thangaraj (1981) and it was first isolated in India by Ramadass and Khader (1982). A few amino-acid substitutions in the VP2 gene sequence can lead to mutations in CPV and are responsible for different antigenic variants of the virus (Phromnoi *et al.*, 2010; Kaur *et al.*, 2016). Presently, there are four known antigenic types of CPV circulating throughout the world, viz., CPV-2, CPV-2a, CPV-2b and CPV-2c. As the knowledge regarding the current antigenic types of CPV in northern India is still

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limited, more seroprevalence data and detection of ongoing mutations in the VP2 gene would be of great help in the early detection and identification of the possible new emerging CPV strains. Thus, the present study was designed to isolate CPV from the rectal swabs of suspected dogs.

Materials and Methods

Sample collection and preparation of virus inoculum

A total of 45 rectal swabs in two ml of phosphate buffer saline (pH=7.2) from dogs exhibiting clinical signs of parvoviral enteritis like gastroenteritis/hemorrhagic enteritis with pyrexia, vomiting, dehydration etc. were collected from New Delhi (n=11), Chandigarh (n=6), Jammu (n=3), Punjab (n=24) and Assam (n=1). The rectal swabs were squeezed around the walls of the collection tube and centrifuged at 3000 rpm for 15 minutes to collect supernatant and 0.1 ml of antibiotic and antimycotic 100x solution was added and kept for incubation at 37°C for 30 minutes. Then it was stored at -20°C till further use.

Virus isolation

Dulbecco's Modified Eagle's Medium (DMEM) growth medium (GM) with 10% fetal bovine serum (FBS) was used for virus isolation in Madin-Darby Canine Kidney (MDCK) cell line. After a complete monolayer of MDCK cells was obtained on 12 -well culture plate, 0.1 ml of prepared viral inoculum was added to each well and culture plates were kept for incubation at 37°C for 1.5 h for the adsorption of the virus onto cells in each well. Then after washing each well, 1ml DMEM maintenance media (MM) with 2% FBS was added and the culture plates were incubated at 37°C with 5% CO₂ for 3-7 days to observe CPE. The cell culture fluid was collected after three cycles of freezing and thawing from the wells that showed CPE.

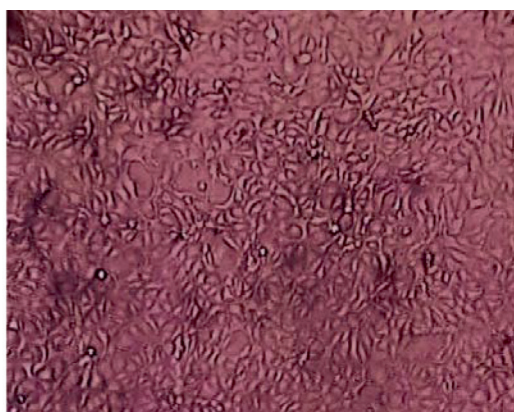


Fig. 1. Negative control having monolayer of MDCK cells at 10x magnification

Confirmation of isolates

DNA was extracted from the cell culture fluid showing CPE (Russell and Sambrook, 2001) and was subjected to PCR and NPCR. For PCR and NPCR, primers were used as per Mizak and Rzezutka (1999). For PCR, the reaction mixture was prepared by adding 15µl of the template DNA, 5.0 µl of 10x PCR buffer (with 15 mM MgCl₂), 1.0 µl of forward and reverse primer (20 pm/µl) each, 1.0 µl of dNTPs mix (10 mM), 0.2µl Taq DNA polymerase 5 units/µl and the final reaction mixture volume was made up to 50 µl using nuclease-free water. For further confirmation by NPCR, the reaction mixture was prepared by adding 5 µl of the PCR product (from above reaction), 2.5 µl of 10x PCR buffer (with 15 mM MgCl₂), 1.0 µl each of forward and reverse primer (20 pm/µl), 1.0 µl of dNTPs (10 mM), 0.2 µl Taq DNA polymerase (5 units/µl) and the final volume was made up to 25 µl by adding nuclease-free water.

Both PCR and NPCR reactions were set at thermocycling parameters with 35 cycles of denaturation at 94°C for 60s, annealing at 55°C for 60s, elongation at 72°C for 150s and a final elongation at 72°C for 10 min. Both PCR and NPCR products (10 µl each) were subjected to Agarose Gel Electrophoresis by using 1% agarose at the rate of 5 volts/cm with GeneRuler Ladder plus 100 bp (New England Biolabs, USA). The products on gel were visualized and documented using Gel documentation system (Syngene, USA).

Results and Discussion

Out of the 45 samples subjected to virus isolation, 12 (26.66%) samples exhibited cytopathic effects (CPE) in the MDCK cell line. The CPE observed were rounding of cells within 24 h (Fig. 1), clumping of cells within 48 h (Fig. 2), and detachment of cells within 72 h of inoculation (Fig. 3,

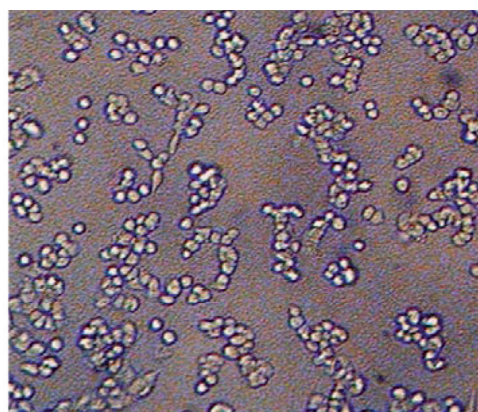


Fig. 2. The cytopathic effect: Rounding of MDCK cells by CPV at 20x magnification

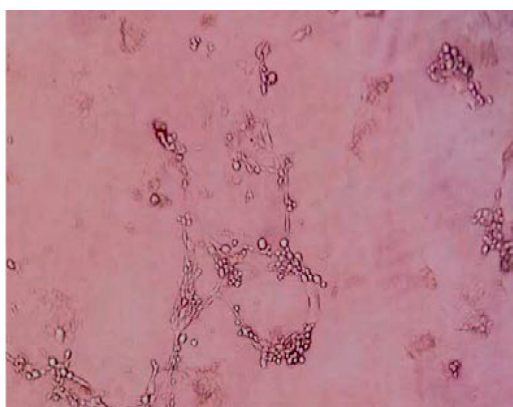


Fig. 3. The cytopathic effect: Clumping of MDCK cells by CPV at 10x magnification

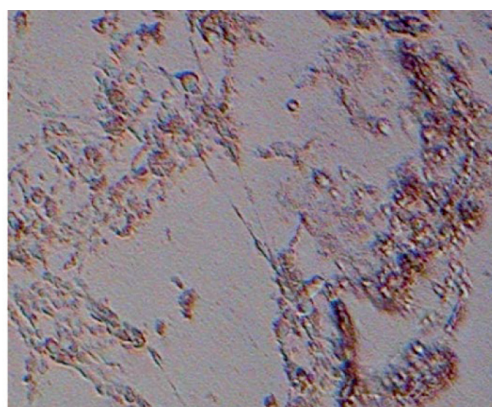


Fig. 4. The cytopathic effect: Detachment of MDCK cells by CPV at 20x magnification

Fig. 4). Out of the 12 samples that exhibited CPE in MDCK cell line, eleven samples (D4, P4, P5, P15, P16, C3, C6, J1, G28, G30, A35) showed CPE in the first passage and one sample (D5) showed CPE in the 2nd passage, whereas the remaining samples did not exhibit any CPE even after the 3rd passage (Table 1).

In PCR and NPCR, 1198bp product and 548bp product were amplified respectively, which confirmed the presence of CPV. Casewise analysis revealed that four CPE positive samples (D4, D8, P15 and P16) were from the dogs having haemorrhagic gastroenteritis; three CPE positive samples (P4, P5 and C6) were from vaccinated dogs, and

Table 1. CPV samples positive with cytopathic effects

S. No.	Sample No.	Age (Months)	Sex	Breed	Diarrhoea	CPE (I Passage)	CPE (II Passage)	Vaccination/Booster Status
1	D4	1.5	M	Mongrel	Hemorrhagic	Clumping and Detachment	...	✗
2	D8	2	F	Mongrel	Hemorrhagic	No CPE	Rounding and Clumping	✗
3	P4	4	M	Indian Bully	Non-Hemorrhagic	Rounding and Detachment	...	✓
4	P5	6	M	Pitbull	Non-Hemorrhagic	Rounding and Detachment	...	✓✓
5	P15	2.5	M	Mongrel	Hemorrhagic	Clumping and Detachment	...	✗
6	P16	1.5	F	GSD	Hemorrhagic	Clumping and Detachment	...	✗
7	C3	2.5	M	Labrador Retriever	Hemorrhagic	Clumping and Detachment	...	✗
8	C6	4	F	Cocker Spaniel	Non-Hemorrhagic	Rounding	...	✓✓
9	J1	4	F	Mongrel	Hemorrhagic	Clumping and Detachment	...	✗
10	G28	2	F	Mongrel	Non-Hemorrhagic	Rounding and Clumping	...	✗
11	G30	3	F	Labrador Retriever	Non-Hemorrhagic	Rounding and Clumping	...	✗
12	A35	2.5	M	Mongrel	Non-Hemorrhagic	Rounding and Clumping	...	✗

eight CPE positive samples (D4, D8, P15, P16, C3, G28, G30, A35) were from dogs below 3.5 months of age.

CPV causes a highly contagious form of acute gastro-intestinal illness often leading to high mortality among dogs all around the globe. CPV has been isolated from many regions all over the world and is known to continually evolve into its various antigenic variants due to high mutation rates, which has raised a concern for potential CPV enteritis outbreaks, hence exposing the health of dogs to risk. Thus, studying the ongoing status of CPV geographical prevalence of any new antigenic types is of key importance in order to predict and ensure the good health of dogs in future.

Parvovirus is a fastidious pathogen and difficult to isolate, since replication is hostcell dependent and takes place only in actively dividing S-phase of cells, where cellular DNA polymerase is synthesized abundantly (Tattersall, 1972; Berns, 1990). Isolation of CPV on cell lines was reported by various workers in the past. Canine parvovirus was first isolated in the year 1978 by Appel *et al.* (1979), where they used primary cultures of canine foetal lung, kidney as well as feline foetal lung and kidney, which had given consistent results. A-72 cell line was used by Sagazio *et al.* (1998) for isolation of CPV-2 using co-cultivation method. The CPE was observed for 3-4 days. Indirect fluorescent antibody test (IFAT) using a canine serum with antibodies to CPV-2 was used to confirm the virus.

In another study, Nandi *et al.* (2010) isolated CPV on MDCK cell line grown in DMEM containing 10% fetal calf serum and observed CPE up to 72 hours post infection. Further, out of five samples, only one showed cytopathic changes, including rounding of cells, granulation, aggregation of cells similar to the cytopathic changes observed in the present study. In another study, Kumar *et al.* (2010) subjected 25 samples for the isolation of CPV using MDCK cell line and reported that 9 samples developed CPE in the MDCK cell line.

Raj *et al.* (2010) observed that out of fifty-one samples passaged in CRFK cells, sixteen isolates were confirmed by PCR. Similarly, isolation was attempted from Puducherry, South India, in 2010 (Parthiban *et al.*, 2010), in which CPV was isolated from rectal swabs from suspected cases of dogs similar to the present study. Thus, isolation of CPV has been done by various researchers from India and across the world over and they had similar observations of the cytopathic effects produced by canine parvovirus in MDCK or CRFK cell line (Zhao

et al., 2013; Deka *et al.*, 2015; Kaur *et al.*, 2015; Wang *et al.*, 2016; Vieira *et al.*, 2017; Jaune *et al.*, 2019; Abdel-Rehman *et al.*, 2019; Chen *et al.*, 2019). Thus, from the study it could be concluded that isolation of CPV from rectal swabs of dogs was successful. Further, these isolates can be subjected to sequencing of VP2 in order to study variation.

Conclusions

Canine parvovirus could be isolated in MDCK cells from rectal swabs collected from clinical cases of dogs in the regions of Punjab, New Delhi, Chandigarh, Jammu and Assam. Twelve isolates were obtained which can be used to gain wider insights on the possible mutations and prevailing antigenic types of CPV in different geographical regions of India.

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Physico-Chemical and Bacteriological Screening of Household Water Supplies in Selected Communities in Edo State, Nigeria

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Abstract

Over 70% of the earth crust is filled with water but access to potable water remains a global challenge. This study focused on ascertaining the level of potability of borehole water in Edo State, Nigeria, for human consumption. Two hundred and forty-three (243) borehole water samples were collected from nine sampling sites and analyzed for physical (pH, total solids, DO, COD and BOD), chemical (NO_3 , SO_4 , PO_4 , chloride and metallic ions) and bacteriological (total bacteria count and total coliform count) properties. The results were compared with standards prescribed by the World Health Organization and Federal Ministry of Environment, Nigeria. The values of all the physicochemical parameters of the borehole water analyzed in the study were below the limits recommended by FME and WHO. The bacteriological analysis of the water revealed that the total bacterial count ranged from 1.78 ± 0.33 to $3.92 \pm 0.32 \times 10^3$ cfu/ml while coliform counts ranged from 0.65 ± 0.32 to $2.75 \pm 0.20 \times 10^3$ cfu/ml. These values were above acceptable limits. Thus, the borehole water from the studied localities across the three senatorial districts are not considered fit for consumption and it is therefore recommended that home water purification such as boiling and filtration should be practiced among the inhabitants before consumption.

Keywords: water, physicochemical, parameters, borehole, potability

Резюме

Както е известно, над 70% от земната кора е покрита с вода, но въпреки това достъпът до питейна вода остава глобално предизвикателство. Това проучване е насочено към установяване нивото на преносимост на сондажната вода в щат Едо, Нигерия за консумация от човека. Двеста четиридесет и три (243) проби от сондажна вода са събрани от девет места и анализирани за техните физични (pH, общо твърди вещества, DO, COD и BOD), химични (NO_3 , SO_4 , PO_4 , хлорид и метални йони) и бактериологични (общ брой бактерии и общ брой колиформи) свойства. Резултатите са сравнени със стандартите, предписани от Световната здравна организация (СЗО) и Федералното министерство на околната среда (ФМОС), Нигерия. Стойностите на всички физикохимични параметри на сондажната вода, анализирани в проучването са под препоръчаните от ФМОС и СЗО граници. Бактериологичният анализ на водата показва, че общият бактериален брой варира от 1.78 ± 0.33 до $3.92 \pm 0.32 \times 10^3$ cfu/ml, докато колиформният брой варира от 0.65 ± 0.32 до $2.75 \pm 0.20 \times 10^3$ cfu/ml. Тези стойности са над допустимите граници. Ето защо, сондажната вода от изследваните находища в трите изследвани сенатски района не се счита за годна за консумация. Поради това се препоръчва на жителите преди консумация домашната вода да се пречиства чрез преваряване и филтриране.

Introduction

Despite the fact that 71% of water makes up the earth surface, a great number of the world's population lack access to potable water, which has contributed greatly to global outbreaks of diseases (Kelmendi *et al.*, 2018; Duressa *et al.*, 2019). According to the WHO (2017), approximately 30% of people all over

the world lack access to potable water. In addition, over six million deaths have been reported in the developing countries, resulting from illnesses caused by water-borne diseases (TWAS, 2002). The United Nations has been tasked with the responsibility of improving water quality but discharge of industrial and domestic effluents into water bodies has

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made it difficult to deliver potable water for man's consumption (Duressa *et al.*, 2019). The WHO and other donor organizations have collaborated with governments of most developing countries using various government intervention strategies to provide potable water through community water supply systems. However, the strategies have not been effective as a result of the ever-increasing human population, has led to inadequacy and inconsistency in the community water supply. The threats posed by the inadequacy and inconsistency in the community water supply have led the public to seek for alternative potable water sources whose availability does not guarantee potability (Emmanuel-Akerele and Francis, 2021). Nigeria is one of the countries in the world that has unsafe water supplies especially in the rural communities. Given the low quality of drinking water in Nigeria, millions of people have been trapped in the cycle of poverty and ill health, often rendering them unable to work or go to school due to water-related diseases, which are preventable (Adeyinka *et al.*, 2014). About 90 million people in Nigeria do not have access to safe drinking water and about 130,000 children under the age of five die every year from preventable waterborne diseases. Therefore, lack of access to safe water supply is a serious threat to the country (Adeyinka *et al.*, 2014). Boreholes, rivers, streams and wells are the water sources in most communities in Edo State. These water sources are posing a threat to public health as a result of source contamination, as fertilizer and pesticides used for farming are washed into the water bodies. Most of the cases of waterborne diseases are not always reported, except outbreaks of infectious diseases from hospital settings. Quality assessment of groundwater in Edo State has indeed been undertaken by some researchers (Adegbite *et al.*, 2018; Ogbeifun *et al.*, 2019). However, these studies have either been limited to specific locations/towns or hand-dug wells and not across the State. The objective of this study was to assess the quality of borehole water across the State with respect to the potable status following the collapse of the public water supply scheme on the basis of the three senatorial districts that make up Edo State in the State and characterizing its potability with regards to WHO guidelines.

Materials and Methods

Study Area

This research work was carried out in Edo state, Nigeria. Edo State is located between latitude 6°50'N, longitude 6.00'E and 152 meters' elevation

above the sea level. It has 18 local government areas (LGAs) in the three senatorial districts. A multi-stage sampling method was adopted with the Senatorial districts as primary. The secondary stage was the random selection of 3 local Government Areas from each Senatorial district, thereby bringing it to a total of 9 sampling stations across the state. The selected local government areas were Etsako west, Etsako Central, Etsako East, Ikpoba-okha, Oredo, Egor, Esan West, Owan West, Esan Central, Etsako East and Etsako West (Fig. 1).

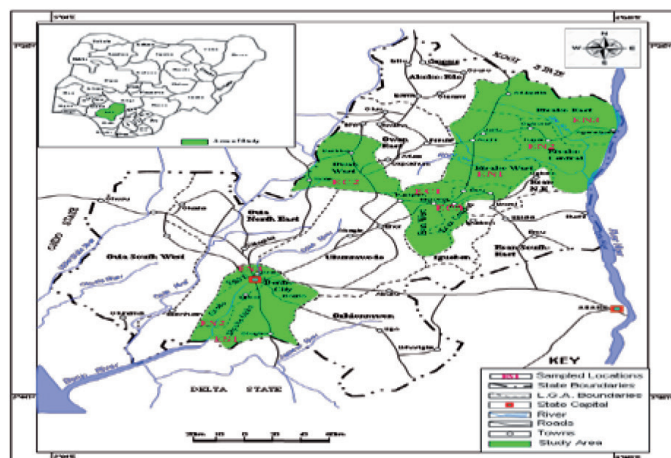


Fig. 1. Map of Edo State showing the sampling sites across the senatorial district

Sample collection

A total of 243 borehole water samples were collected directly from three different sampling borehole locations within the selected local government areas between January and August 2018 using sterile glass reagent bottles (250 ml). The bottles were filled with the borehole water, labelled and transported in an ice-packed container to the laboratory within 4 h of collection for physicochemical and bacteriological analysis.

Physicochemical analysis of the water samples

Physicochemical parameters and heavy metal compositions of the borehole water samples were carried out at the Nigerian Institute for Palm Oil Research (NIFOR) Benin City, Edo State, Nigeria. Samples were analysed following the procedures laid down by the World Health Organization (WHO). The pH, salinity and electrical conductivity, total dissolved solids (TDS), dissolved oxygen (DO), biological oxygen demand (BOD), and chemical oxygen demand (COD) were determined at the point of collection using the HANNA combo (H198130) instrument by connecting the electrodes to each meter submerged in a clean beaker containing the sample according to the manufacturer's instruction. Other parameters such as nitrate,

sulphate chloride, phosphate, calcium, magnesium, sodium, iron, zinc, and copper were analysed at the chemistry laboratory section of the Nigerian institute for palm oil research (NIFOR). The presences of heavy metals were examined in a process whereby samples were digested with a concentration of 55% of nitric acid using an Agilent (240) FS flame atomic-absorption spectrophotometer (USA). This was achieved after the equipment was calibrated with set up standard solutions. Reagents and chemicals used for the study were of laboratory grade and used without additional purification processes (APHA, 1998).

Bacteriological analysis of the borehole water samples

Enumeration of total viable and faecal coliform counts was carried out using membrane filtration technique (Habash and Johns, 2009). A sterilised membrane filter of pore size (0.45µm) was used to filter 100 ml of each sample. The filter paper was then placed on solidified Membrane Lactose Glucoronide agar (MLGA) plate and then inverted. The plates were incubated at 37°C for 20 h and at 44.5°C for 24 h, respectively, for enumeration of total coliform and thermo-tolerant fecal coliforms (ASTM, 2012). Plates showing green and yellow colonies were counted as total coliform while plates showing green with yellow colonies were counted as thermo-tolerant fecal coliforms using a colony counter and expressed as Colony Forming Units (CFU) per 100 ml. Further tests were carried out using morphological, biochemical, and molecular techniques for proper identification of the isolates.

Statistical analysis

All analyses were performed in triplicates. The data were recorded as means $\pm$ standard deviation and analyzed using Microsoft Excel. One-way ANOVA was used to analyze the significant differences between their means. Differences between means at 5% significant level (P -value < 0.05) were considered.

Results and Discussion

The results of the physicochemical analysis of the borehole water samples from the nine sampling sites that cuts across the three senatorial districts in Edo State, Nigeria are presented in Table 1. They showed that there was no significant difference in the pH of all the borehole water in the sampling sites.

This slight acidic pH of all the borehole water samples was below the limits (6.5-8.5) recommended by local and International regulatory bod-

ies. This could be due to the presence of dissolved mineral leached into the water table. Ogbeifun *et al.* (2019) reported similar findings in respect to borehole water in Benin City, Edo State. The recorded values for electrical conductivities and total dissolved solids were below the limit (1000 µS/cm and 500 mg/l) recommended by local and International regulatory bodies. This showed that there is low dissolved inorganic salt in the water table. Electrical conductivity is related to the ionic content of the water, a function of the total dissolved solids concentration. Similar low electrical conductivity and total dissolved solids was reported in samples from Edo State (Iyasele and Idiata, 2011; Ogbeifun *et al.*, 2019). The BOD and COD values recorded in this study were not statistically significant in all the borehole water from the sampling sites (Table 1).

The recorded values were below the recommended limits. The highest mean $\pm$ SE values of BOD (2.93 ± 0.37) and COD (16.24 ± 0.24) were recorded in Edo Central. This can be attributed to the low organic material dissolved in borehole water (Efe *et al.*, 2005; Nduka *et al.*, 2008; Ogbeifun *et al.*, 2019). The results in Table 1 also show that there were significant differences in the nitrate content of all the borehole water in the sampling sites, although the values were generally below the recommended limit (10 – 20 mg/l). This revealed that there is low organic and inorganic material in the area that could contaminate the water table. Such results were in line with Ogbeifun *et al.* (2019) and Okereke *et al.* (2014), who reported low nitrate content in their study. The mean $\pm$ SE of the phosphate and sulphate in this study recorded as 1.46 ± 0.35 and 1.28 ± 0.24 were below the limit (5 mg/l and 2 – 3.5 mg/l) recommended by local and international regulatory bodies. The mean value of the phosphate and sulphate does not show any significant differences in all the borehole water from the sampling sites. The recorded mean $\pm$ SE values of the phosphate and sulphate were 1.46 ± 0.35 and 1.28 ± 0.24 , respectively, which were below the limit recommended by local and International regulatory bodies. This could be due to low contamination levels of these parameters in the source water.

The results in Table 2 showed the mean values of the metals and heavy metal ions contents of the borehole water samples from the nine sampling sites. Moreover, there was no significant difference in the zinc, iron, manganese, magnesium, calcium, chloride, carbonate contents of all the borehole water in the sampling sites. The mean $\pm$ SE values were below the limit recom-

Table 1. Physical and Chemical properties of the borehole water sample

Location/ Source	pH	EC μS/cm	TDS mg/l	DO mg/l	BOD mg/l	COD mg/l	PO ₄ mg/l	NO ₂ mg/l	SO ₄ mg/l
Edo North 1	5.26 ±0.27	286.82 ±0.42	137.42 ±0.14	5.91 ± 0.22	2.13 ±0.23	10.24 ±0.05	0.82 ±0.22	1.39 ±0.16	0.97 ±0.25
2	5.46 ±0.13	287.24 ±0.25	138.23 ±0.44	5.01 ± 0.06	1.63 ±0.41	9.39 ±0.09	0.16 ±0.43	0.72 ±0.26	0.10 ±0.14
3	5.60 ±0.42	286.42 ± 0.56	136.62 ±0.12	6.81 ± 0.51	2.63 ±0.02	11.09 ±0.11	1.48 ±0.11	2.06 ±0.55	1.84 ±0.08
Edo South 1	5.30 ±0.55	170.32 ± 0.42	85.53 ±0.15	5.74 ± 0.32	2.41 ±0.23	15.00 ±0.40	1.56 ±0.47	3.15 ±0.22	1.77 ±0.45
2	5.07 ±0.15	170.71 ± 0.33	86.26 ±0.42	6.24 ± 0.10	2.74 ± 0.25	15.30 ±0.32	1.23 ±0.36	3.61 ±0.56	2.09 ±0.27
3	5.51 ±0.26	170.36 ± 0.42	84.93 ±0.24	5.16 ± 0.18	2.08 ±0.27	14.70 ±0.12	1.89 ±0.43	2.69 ±0.32	1.45 ±0.06
Edo Central 1	5.41 ±0.44	116.83 ± 0.34	56.92 ±0.22	6.03 ± 0.31	2.93 ±0.33	16.24 ±0.02	2.01 ±0.56	3.89 ±0.09	1.11 ±0.40
2	5.86 ±0.38	117.35 ± 0.24	57.52 ±0.54	5.31 ± 0.12	3.54 ±0.22	15.74 ±0.47	2.33 ±0.27	3.04 ±0.29	0.72 ±0.30
3	4.96 ±0.54	116.36 ± 0.37	56.44 ±0.43	6.75 ± 0.23	2.32 ±0.55	16.74 ±0.22	1.69 ±0.30	4.74 ± 0.09	1.50 ±0.17
P- Value	0.8423	0.0934	0.1024	0.4834	0.2643	0.2334	0.0702	0.0000 [*]	0.4543
FME Limits	6.5-8.5	1000	500	3.0	30	60	5.0	10	2.0
WHO Limits	6.5-9.0	1000	500	6.4	40	80	5.0	< 20	3.5

P-value significant at > 0.05

mended by local and international regulatory bodies. This could be due to the low level of dissolved metallic ions leached into the water underground table water since these ions are not known to be present in the study area. This finding was similar to the findings of Enuneku *et al.* (2018), who reported a low value of some trace metals in borehole water proximal to dumpsites, Benin City, Nigeria. The significant value of copper recorded in this study could be attributed to the wearing away of the pipes or brass taps leached from coatings into the water distribution channel. This metallic ion is known to cause serious gastrointestinal disorder and irritations, but its mean $\pm$ SE value of 0.13 ± 0.26 was below the limits (0.5 – 1.5) recommended by local and international regulatory bodies, which makes the water safe for consumption.

This finding was in agreement with Odiana and Edosomwan, 2019, Adekola *et al.* (2012), and Okereke *et al.* (2014), who reported low value of copper in borehole water from Edo State, Yola, Adamawa State and Uturu, Abia State.

Figures 2 and 3 present the bacteriological analysis of the borehole water collected from the different sampling sites in cfu $\times 10^3/100$ ml. The results showed that Edo North 2 had the highest TBC, which ranges from 3.60×10^3 to 4.24×10^3 while Edo South 3 had the lowest TBC, which ranges from 1.45×10^3 to 2.11×10^3 , respectively. Similar-

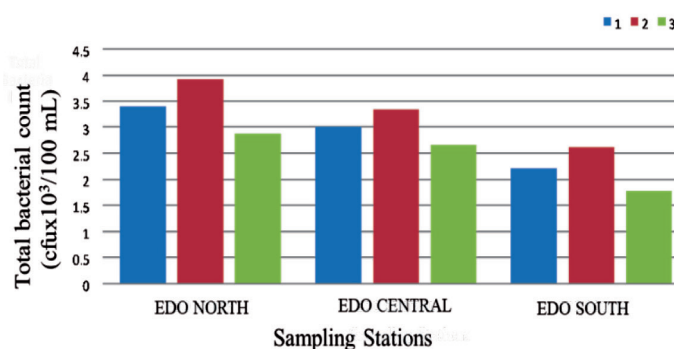


Fig. 2. Total bacterial count of the sampling locations within senatorial districts

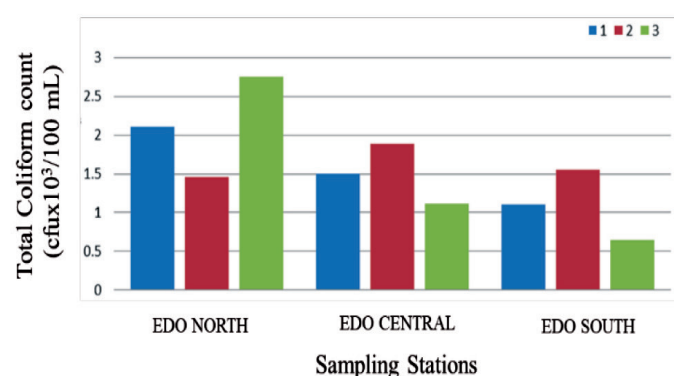


Fig. 3. Total coliform count of the sampling locations within senatorial districts

ly, Edo North 3 had the highest TCC which ranges from 2.55×10^3 to 2.95×10^3 while Edo South 3 had the lowest TCC, which ranges from 0.33×10^3 to 0.97×10^3 respectively.

Table 2. Concentration of metal ions (mg/l) in borehole water by sampling sites (M $\pm$ SE)

Location/ source	Zn	Cu	Fe	Mn	Ca	Na	Mg	Cl	HCO ion
Edo North 1	0.19 ± 0.50	0.03 ± 0.30	0.52 ± 0.23	0.04 $\pm$ 0.32	4.15 ± 0.20	1.83 ± 0.20	1.39 $\pm$ 0.08	56.64 ± 0.05	41.01 ± 0.44
2	0.18 ± 0.44	0.04 ± 0.15	0.42 ± 0.34	0.04 $\pm$ 0.02	3.19 ± 0.23	1.03 ± 0.34	0.41 $\pm$ 0.12	57.43 ± 0.10	41.23 ± 0.33
3	0.20 ± 0.52	0.02 ± 0.42	0.62 ± 0.09	0.05 $\pm$ 0.48	5.11 ± 0.54	2.63 ± 0.52	2.37 $\pm$ 0.02	55.85 ± 0.55	40.79 ± 0.12
Edo South 1	0.26 ± 0.06	0.03 ± 0.34	1.08 ± 0.16	0.17 $\pm$ 0.09	2.29 ± 0.21	1.14 ± 0.25	0.76 $\pm$ 0.30	52.08 ± 0.31	33.84 ± 0.17
2	0.25 ± 0.31	0.04 ± 0.09	1.17 ± 0.51	0.16 $\pm$ 0.16	3.18 ± 0.39	1.04 ± 0.09	0.99 $\pm$ 0.06	51.18 ± 0.07	34.74 ± 0.21
3	0.27 ± 0.25	0.02 ± 0.50	1.00 ± 0.25	0.17 $\pm$ 0.18	1.39 ± 0.22	1.24 ± 0.01	0.53 $\pm$ 0.03	52.98 ± 0.23	32.94 ± 0.16
Edo Central 1	0.44 ± 0.20	0.31 ± 0.35	1.35 ± 0.37	0.05 $\pm$ 0.32	2.36 ± 0.14	0.91 ± 0.48	0.83 $\pm$ 0.27	58.01 ± 0.41	34.90 ± 0.35
2	0.43 ± 0.28	0.08 ± 0.11	3.25 ± 0.50	0.04 $\pm$ 0.28	1.57 ± 0.50	1.31 ± 0.28	0.43 $\pm$ 0.52	57.13 ± 0.44	34.30 ± 0.08
3	0.45 ± 0.09	0.56 ± 0.08	1.44 ± 0.22	0.06 $\pm$ 0.37	3.15 ± 0.09	0.51 ± 0.11	1.23 $\pm$ 0.06	58.89 ± 0.30	35.50 ± 0.03
P- Value	0.0634	0.0000*	0.0902	0.1010	0.1403	0.2975	0.2722	0.9345	0.6732
FME Limits	15	1.5	0.3	0.5	200	100	150	600	< 20
WHO Limits	- 15	0.5 – 1.5	0.1-1.0	0.05- 0.5	- 200	200	- 150	- 600	- 30

P-value significant at > 0.05

These values of the TBC and TCC recorded in this study were above the recommended zero limits for drinking water (WHO, 1985). This could be attributed to contamination of the underground water table by leached sewage as septic tanks, latrines and soakaways are sited in closed proximity to the borehole in the study area. Such a high value of TBC and TCC is of great public health importance as consumption of microbiologically unfit water poses a menace of disease outbreak within a large number of human populations. This finding was in agreement with Onajite *et al.* (2018), Chidinma *et al.* (2016), Olajubu *et al.* (2014), Nwachukwu *et al.* (2013), Emmanuel-Akerele and Francis (2021), and Obi *et al.* (2011), who reported high number of total coliform and pathogenic bacteria in borehole water samples in Edo State and some parts of Nigeria.

Conclusion

From the findings above it can be said that despite the low physicochemical properties of the borehole water across the state, the waters are not safe for consumption because of the risk of water-borne diseases that may be linked to the high TBC and TCC recorded in the study. Thus, it is recommended that home water purification such as boiling and filtration should be practiced among the inhabitants before consumption. Also, sewage dis-

posing tanks soakaways and latrines should be sited at least 50 m away from the borehole in order to reduce sewage contamination of the borehole water.

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Case Report

Epidemiological COVID-19 Data for Eastern Rodopi, Bulgaria

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Abstract

In a brief summary we present a report of the results from rtPCR diagnostic tests of individuals with travel arrangements and border-crossing movement, and the importance of restrictive measures, diagnostic testing and travel limitations in order to contain the COVID-19 pandemic during the summer months of 2020. rtPCR test results were a prerequisite for any planned trip and border crossing, therefore the collected data are cumulative and the statistics extremely helpful and necessary. Our Molecular Diagnostics Unit is licensed within the territory of Bulgaria. Our methods were strict protocols from the guidelines of the National Center for Infectious and Parasitic Diseases, Bulgaria, the kits used were all CE, the overall concept in sync with the global regulations as from the CDC and the WHO. We demonstrated an increase in the number of infected randomly tested travelers during the summer months and thereafter, giving a glimpse of the magnitude of the pandemic that followed. We showed a total increase of infection from 12.5% in July to almost 56% for the month of November. The current report presents retrospective data and makes suggestions that are available to nations and applicable locally; it is of immense importance for the regions located near border-crossing points in a situation of pandemic of such magnitude without immediate access to vaccines or reliable treatment for the novel infectious disease outbreaks.

Keywords: COVID-19, SARS-CoV-2, global pandemic, border-crossing restrictions

Резюме

Настоящата статия представя кратко обобщение на резултатите от rtPCR диагностичните тестове за хора, осъществяващи организирани пътувания и преминаващи през граничните пунктове, както и значението на ограничителните мерки, диагностичните тестове и ограничението на пътуванията с цел овладяване на пандемията от Covid 19 по време на летните месеци на 2020 г. В този период, задължително изискване за планирано пътуване и преминаване през границата беше резултат от rtPCR тест. Това даде възможност за събиране на кумулативни данни, които след статистическа обработка се оказаха изключително полезни и необходими. Те са събрани в Лаборатория за молекулярна диагностика, лицензирана на територията на България. Прилаганите методи са съобразени с протоколите от Националния център по заразни и паразитни болести, България, а използваните китове са разрешени от ЕС. Цялостната концепция на изследването е в синхрон с глобалните разпоредби от Центъра за контрол и превенция на заболяванията и СЗО. Получените резултати демонстрират нарастващия брой на заразените сред тестваните на случаен принцип пътници през летните месеци и дава представа за мащаба на пандемията, която последва. Установено е, че от месец юли до месец ноември инфекцията нараства от 12.5% до почти 56%. Статията представя ретроспективни данни и предлага възможности, приложими на местно ниво и особено в региони с гранично-пропускателни пунктове в ситуация на пандемия с такъв мащаб и без незабавен достъп до ваксини и надеждно лечение за нови инфекциозни огнища.

Introduction

In the beginning of the year 2020, news of a new and deadly disease broke. We only had limit-

ed information of this novel virus of the family of Coronaviruses; some vague specifics were yet to be discovered. Swiftly it became evident that the en-

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tire world would be affected and partake in the fight against it, as well as in describing the symptoms and major manifestations, the treatment of patients and testing, contributing to the global knowledge of its prevention and cure. Our COVID unit testing lab was established within the Microbiology and Virology Division in the main County Hospital in the town of Kardzhali, Eastern Rodopi, Bulgaria. Upon the beginning of our new line of work, we immediately started testing huge amounts of inpatients and outpatients as we did our best to provide prompt service, deliver results the same day, report to physicians and local health authorities for the appropriate measures and prescribe quarantine to whoever tested positive. We worked according to mandatory regulations for border crossings globally [<https://wiki.unece.org/display/CTRBSBC/Bulgaria#page-banner-start>] and locally [https://ec.europa.eu/regional_policy/en/projects/Greece/new-greece-bulgaria-border-checkpoint-makes-for-safer-faster-travel; Pueyo, 2020], and provided standard testing plus verified result certification. Globally, new regulations on migration and border crossing were introduced, while in Europe each country provided a bulletin and informative data with recommendations for migration.

An important aspect was presented by authors, suggesting increased tension in areas with higher rates of migration with population emerging from Wuhan as the main infection source in other cities and provinces. In China some cities with a low number of cases showed a rapid increase in case load. Owing to the upcoming Spring Festival return wave, understanding the risk trends in different regions is crucial to ensure preparedness at both the individual and organization levels and to prevent new outbreaks (Chen *et al.*, 2020).

In the USA since the outbreak, the FBI anticipated a rise in hate crimes across the United States citing examples such as the stabbing of an Asian American family, including children ages 2 and 6, whom the assailant believed were spreading COVID-19. Their prediction was confirmed by the Asian Pacific Policy & Planning Council, with over 1.000 documented reports from Asian people of coronavirus discrimination and hate crimes from March 19th to April 1st of last year. Common incidents included verbal harassment, shunning, and physical assault. These accounts, along with associations between “Chinese” and “virus”, suggest the emergence of a more sinister phenomenon, namely, the personification of COVID-19 as Asian people. This is especially tragic for Asian healthcare workers, who make up 17% of physicians in active practice and are the most

represented ethnic group among foreign-born medical professionals currently in the USA (Ze-Liang Chen *et al.*, 2020).

Pueyo (2020) recently published a paper of particular interest on the development of the pandemic and the importance of coronavirus measures. According to the author, it is enough to introduce strong measures in just a few weeks, which will reduce the peak of coronavirus infection and will save the lives of millions of people at a reasonable cost. Otherwise, tens of millions will be infected, many will die, and the healthcare system will have collapsed.

In our study, the tested patients for the months of the pandemic produced data from July to November and 2020, which we report as we believe they might be an important piece of evidence toward the global understanding of the spread, contagiousness, significance, morbidity and mortality. We believe our data are very important for the region in which we live, not only in terms of treating patients, controlling the spread and guiding the health authorities measures locally, but also contributing to the bigger picture of understanding this new and deadly disease globally.

With the current work, we aim to show COVID results at regional level and potentially help to better understand the territorial patterns of the epidemic and to support the definition of place-based policies to tackle the crisis at local and national level.

Case Report

Our statistical data are based on the results collected directly from patients tested in our hospital on a daily basis. Our testing methods were the rtPCR for COVID-19 according to the global standards of the WHO and the CDC guidelines. Our methodology utilized isolation of RNA from patient samples prior to the rtPCR protocol. The isolation was done manually. The rtPCR was run in the Applied Biosystems StepOne Real-Time PCR System. The supporting methods of testing were the rapid tests with whole blood or plasma, as well as nasopharyngeal antigen testing via swabs. The reagents used were procured from different sources, governmental, official central supply, local grants or purchases. All of our reagents and kits were CE.

For the testing, the following data were collected: (a) key dates, which included the date of onset of disease, date of admission to hospital, date of confirmation of infection, and dates of travel; (b) demographic information about the age and sex of patients/cases; (c) geographic information at the highest resolution available down to district level (information at building level was excluded so that

cases could not be identified, this information was subdivided into administrative units (county, city, and, where available, specific locations); (d) symptoms, (e) any additional information, such as exposure to specific places with higher density of people, record of exposure to infected individuals, if known. All the information collected with the results was entered into the national system for COVID for Bulgaria within the same day. Summaries of the data are shown in Fig. 1.

the epidemiological data are given in Table 1.

As the volume of collected data increased, we can report solid evidence of one very good reason for the spread of the COVID infection in regions near border crossing points. For the Greek authorities to grant entrance to the citizens with Greek passports, rtPCR, negative results were a prerequisite. We also observed percentages of positive cases not in peaks, but rather as a steady increase in their number. The increase in positive tests further

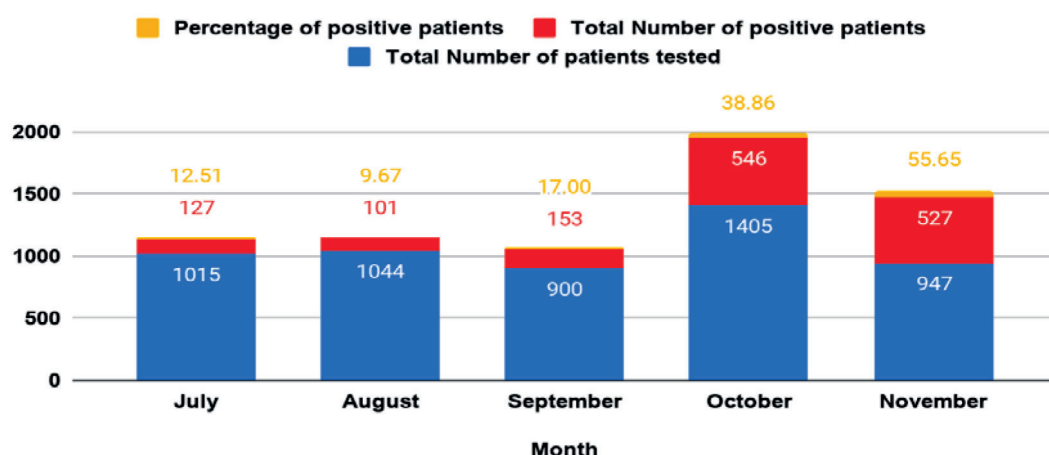


Fig. 1. Percentage of positive and negative for the months from July through November

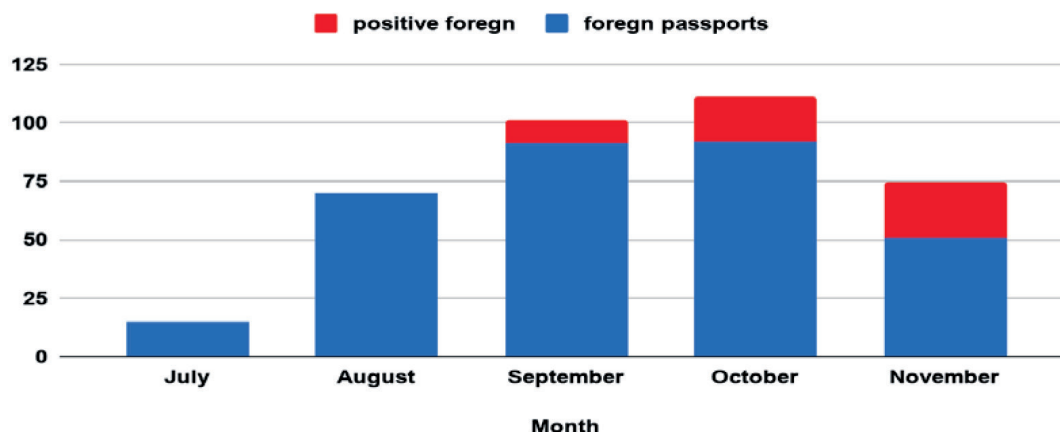


Fig. 2. Distribution of positive test results in foreign passport holders by month

According to the data, there was an increase in the number of patients who tested positive from the beginning of the summer through July until November. During the summer months, travelling across borders was possible only with evidence of a negative PCR test. We recorded mainly negative and healthy individuals traveling through earlier on, but with the shift of the seasons, the number of patients with positive tests increased (Fig. 2).

We have no information about the purpose of people's travels and the need to cross borders, but we speculate the summer months were for family annual vacations, as well as business-related, and the need to travel back home for people with seasonal jobs throughout Europe. The important aspects of

led to an increase in morbidity and mortality (not presented), a large number of patients with a need of hospitalization by far exceeding the numbers of available hospital beds, and exhausting hospital resources and staff. The increase in infected people exposed many doctors and healthcare workers to an immense risk on the job, a rather grave situation, just like anywhere else in the world.

Discussion

While our data were being collected daily and reported to local and national authorities, we continued to study the trends and nuances of the mass number of tested individuals. Although foreign passport holders are listed separately in our database, we firmly believe the reason for testing was for

Table 1. Distribution of patients with foreign passports who tested positive for each month from July through November

Month	Total number of patients tested	Total number of positive patients	Number of patients with foreign passports	Patients with foreign passports tested negative	Patients with foreign passports tested positive
July	1015	127	15	15	0
August	1044	101	70	70	0
September	900	153	91	81	10
October	1405	546	92	73	19
November	947	527	51	28	23

travel across borders. We cannot tell though what percentage of Bulgarian passport-holder healthy individuals subjected to testing had the intention of traveling and therefore we can only speculate based on the data presented. However inaccurate and biased the results are, the trend shows clear evidence of spread due to people's travel and movement, regardless of the mechanisms of spread - via infected patients or simply by mechanical dissemination of the virus particles. Furthermore, apart from the actual rtPCR protocol as such, there is a considerable amount of waiting time before the results become available, so we have no control over the patients' physical contacts during that time; the time they spend at the local grocery store or a restaurant, the lingering at the actual hospital and the like. Based on our evidence, that we observe a higher percentage of infected, tested positive and ultimately sick patients in the larger cities near border, where testing for SARS-Cov2 is done. Based on our evidence, it can be assumed that a higher percentage of infected, positive-testing and ultimately sick patients are observed in larger cities near border crossing points where SARS-Cov2 testing is performed.

As we have experienced several full lockdowns followed by slight removal of restrictions, we can only hope we can follow the scheme outlined by "The Hammer and the Dance". Our greatest hope is that with the growing number of medicinal products now available, more and more peo-

ple will take advantage and, hopefully, will slowly move back to normalcy.

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The authors would like to dedicate this manuscript to all the health care workers, doctors, nurses, patient care personnel, laboratory technicians, receptionists, security services, and to express gratitude for their compassionate work and highly professional approach in this pandemic.

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*Note: Journal names should be abbreviated according to the List of Title World Abbreviations:

<http://www.issn.org/services/online-services/access-to-the-ltwa/>

<https://library.caltech.edu/reference/abbreviations/>

- Reference to a book:

John, R., W. Villiam, G. Wilmington (2011). New approach for purification of enteroviral proteins. In: Peterson, K., A. Smith (Eds.), *Methods in Proteinology*. Elsevier, London, pp. 281-304.

Weinedborner M. (2001) *Encyclopedia of Food Mycotoxins*. Springer Verlag, pp. 294.

Matthews, K., K. Kniel, Montville, T. (2017). *Food microbiology: An introduction*. 4th Edition, ASM Press.

- Reference to a conference paper:

Dikova, B., A. Djourmanski, H. Lambev (2013). Establishment of economically important viruses *Echinacea purpurea* and their influence on the yield. In: *Proceedings of the Internaional Conference 'Innovative approaches to the study of Echinacea'*, Poltava, Ukraine, 25-25 June, pp. 36-45.

- Reference to a dissertation:

Barišić, Z. (2011). Uropatogena *Escherichia coli*: povezanost otpornosti na kinolone s prisutnošću činitelja virulencije [dissertation]. Zagreb: Medicinski Fakultet Sveučilišta u Zagrebu.

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CR 517 (2011). Commission Regulation (EU) No 517/2011 of 25 May 2011 on the reduction of the prevalence of certain salmonella serotypes in laying hens.

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