Antimicrobial Properties of Snail Mucus Against Neisseria gonorrhoeae

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Research Article

Keywords: Antimicrobial, Resistance, Snail, Mucus, Gram-negative

Posted Date: September 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2028038/v1

Abstract

Background: Antibiotic resistance has escalated worldwide without proportionate production of new antibiotics. One of the new ways is to explore natural sources of treating bacterial infections without acquiring bacterial resistance. Snail type *Achatina fulica* in Japan, the mucus extract has demonstrated immense antibacterial activity against Gram-positive *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa* bacterial pathogens. The type of snail used in this study, *Achatina achatina* is commonly found in West Africa particularly, Ghana can be explored for its antibacterial activity against the fastidious bacterium,*Neisseria gonorrhoeae* which is fast becoming resistant in most hospital settings.

Place of study: The study lasted for six months and was conducted at the Microbiology laboratory of the School of Biomedical and Allied Health Sciences

Aim: The main aim of this study is to assess the possible antibacterial activity of mucus secretions from *Achatina achatina*against *Neisseria gonorrhoeae.*

Methods: The test strain *Neisseria gonorrhoeae* ATCC 49226 was subjected to susceptibility testing using sterile mucus of *Achatina achatina* to perform both well and disc diffusion techniques. Gram stain, catalase, oxidase and fermentation tests were used to assess the identity of the test strain in this study.

Results: The mucus of *Achatina achatina* showed no inhibition against the tested control strain *Neisseria Gonorrhoeae* ATCC 49226 while the reference discs, ciprofloxacin 5 µg and penicillin 1 µg were susceptible for both the wells made by cork-borer and prepared filter paper discs, respectively.

Conclusion: The zones of inhibition from the mucus of *Achatina achatina* demonstrated no activity with the test strain *Neisseria gonorrhoeae* ATCC 49226. The study seems to suggest that the mucus from *Achatina achatina* snail-type used may have an insignificant antibacterial activity to inhibit the test organism used.

Introduction

Antimicrobial agents are used in treating bacterial, parasitic, fungal and viral infections (Gallagher, Adjunct and Macdougall, 2012) while antibiotics are types used in treating and preventing pathogenic bacteria (Penesyan, Gillings and Paulsen, 2015). These agents may either destroy or prevent the growth of bacteria (Davies, 2006). Those which kill the bacteria are called bactericidal while bacteriostatic types prevent growth (Pankey and Sabath, 2004) while before surgical cases, some antibiotics can be given as a prophylactic to prevent especially bacterial infection (Chodak, Plaut and Surg, 1997). Various diseases which were fatal prior to the development of antibiotics have been effectively treated using these antibacterial agents (Soffar, 2018). Some of the antibiotics have been used as cancer therapy (Bhattacharya and Mukherjee, 2015).

At the right dosage, the use of antibiotics has a significant number of benefits to humans. These agents have facilitated the reduction of millions of deaths every year caused by several infectious diseases (Leibovici *et al.*, 2016). Since antibiotics were first developed, clinicians were excited about their success in eliminating pathogens, such that in the 1970, a US Surgeon General made a statement that the agents have won the war against diseases (Aminov, 2010). However, these antibiotics may have side effects if they are overly used (Norris *et al.*, 2013).

Neisseria gonorrhoeae is a highly resistant fastidious organism (Jorgensen and Ferraro, 2000). Currently, the newer antibiotic agents susceptible to this bacterium include ceftriaxone, azithromycin, cefixime, and doxycycline. These agents are expensive and mostly used as combined therapy (Lin, Nishino and Roberts, 2015).

The use of natural medicines as a source of antibacterial substance may help reduce the cost of these antibiotic agents. *Neisseria gonorrhoeae* is a species of fastidious Gram-negative organism with a characteristic coffee bean-shaped diplococci responsible for the sexually transmitted infection gonorrhoea (Towns, Eyi and Andel, 2014). Gonorrhoea first infects the mucous membrane, after which they penetrate and cause acute inflammation (Edwards and Apicella, 2004). Depending on the site of infection, the symptoms of infection with *Neisseria gonorrhoeae* may be different. Infection at the genitalia, the organism can cause a purulent (or pus-like) discharge from the genitals, which may be foul-smelling. Symptoms may include swelling, inflammation, dysuria and redness (Karnath, 2009). The infection found at the urogenital tract may not normally spread to distant organs, specifically the urethra of males and endocervix of females (Fiumara, 1972). The cell wall of *Neisseria gonorrhoeae* has structures called pili that have proteases that digest IgA on the surface of the mucous membrane and this facilitates the attachment of gonococci to the columnar and transitional epithelium of the urogenital tract (Halter, Pohlner and Meyer, 1984). Men have a purulent urethral discharge and dysuria after an incubation period of about 3 to 5 days. With prompt antibiotic treatment, the infection is arrested and the organism is confined to the mucosal surface (Shirtliff and Mader, 2002). This bacterium is a fastidious Gram-negative organism which needs supplementation to grow in the laboratory specifically, chocolate agar under carbon dioxide incubation (Oliveira, Abels and Zbinden, 2013).

Antibiotic resistance may occur as result of several reasons ranging from the hosts' activities to that particular bacteria (Tenover and McGowan, 1996). Most bacteria are naturally resistant to antibiotics due to genetic mutation or by acquiring resistance from another bacterium. Strangely, mutations are rare spontaneous variations of the bacteria's genomic material. Some mutations enable the bacteria to produce potent chemicals (enzymes) which inactivate the antibiotics, while other mutations eliminate the cell target where the antibiotic attacks (Munita, Arias, Unit and Santiago, 2016). Bacteria may also acquire antibiotic resistance genes from other bacteria in several ways, by a simple mating process called "conjugation", transfer genetic material which includes genes encoding resistance to antibiotics (found on plasmids and transposons) from one bacterium to another (Bennett, 2008).

Naturally, antibacterial agents obtained from nature either from animals or plants have been used because these agents are obtainable with low side effects (Sutirta-yasa and Jawi, 2017). Among these antibiotic-like substances are those from Snails which produce mucin abundantly in their mucus secretion referred to as slime, which has been reported to contain antibacterial proteins (Adikwu, 2006). The animal protein content of snail slime has a high biological value in wound healing and inhibition of inflammatory process. Slime of *Achatina fulica* can heal wound twice faster than the normal saline solution (Harti, Sulisetyawati, Murharyati, Oktariani and Wijayanti, 2016). The snail mucus contains Glycosaminoglycans (GAGs), complex polysaccharides which participate in the regulation of physiological processes through the interactions with a wide variety of proteins. (Berniyanti and Waskito, 2007). Snail slime has been reported to contain active substances such as heparan sulfate and calcium (Harti, Kusumawati and Setyaningtyas, 2016). The detached mucus content is used as antibacterial and analgesics, while calcium plays a role in hemostasis (Vieira *et al.*, 2004). A bactericidal glycoprotein known as achcin, obtained from the body surface mucus of African giant snail has been reported to kill both Gram-positive and Gram-negative bacteria by attacking the cytoplasmic membrane of the cell (Etim, Aleruchi and Obande, 2016). The mucus has been reported to be rich in proteins of high and low molecular weight hyaluronic acid and antioxidants (Swapna and Rivender, 2015). The secretion of the snail supposedly has a double function when applied to the human skin; firstly, stimulates the formation of collagen, elastin and dermal components which repair the signs of photoaging, Secondly, minimizes the damage generated by free radicals that are responsible for premature skin aging (Carter *et al.*, 2017).

Materials And Methodology

STUDY DESIGN

A prospective study was conducted on the possible effect of the antibacterial property of *Achatina achatina* mucus on *Neisseria gonorrhoeae* ATCC 49226. *Achatina achatina* is the most common species of snail in Ghana. This species of snail is normally found in the tropical rainforest of Ghana. Most people in the country consume the snail for its nutritional benefits.

STUDY SITE

The study was conducted at the Microbiology laboratory Unit, Department of Medical Laboratory Sciences, School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana, Korle-Bu Campus, Accra.

Procedure for data collection

Collection of isolates

A standard strain of *Neisseria Gonorrhoeae* ATCC 49226 was cultured on Chocolate agar and Thayer-Martin's medium obtained from the MDS-Lancet laboratory (Ghana). The organism was transported to the Microbiology Unit of the School of Biomedical and Allied Health Sciences. The culture isolate was sub-cultured on freshly prepared Chocolate/Thayer-Martin's media and kept at 37°C in CO_2 enriched atmosphere. The organism was sub-cultured every 24 hours until the study was completed.

Antibacterial susceptibility testing

Test isolate identification

Gram staining was performed on the control strain *Neisseria Gonorrhoeae* to ascertain the identity, Gram reaction, Gram-negative diplococci (Figure 3.1). catalase tests., oxidase tests. and sugar utilization (glucose, maltose and sucrose) tests. (Table 3.1).

Oxidase test

A drop of oxidase reagent was applied to a clean Whatman grade 1 filter paper and allowed to diffuse through the filter paper. The edge of a sterile slide was aseptically used to pick a reasonable amount of the bacteria colony and applied to the diffused oxidase reagent on the filter paper and left for a minute. A characteristic violet indicated that the organism is oxidase positive. A negative control was set up following the same procedure using *Escherichia coli* where no colour change was observed indicating oxidase negative (Figure 3.2).

Catalase tests

A drop of catalase reagent (H_2O_2) was placed on a clean grease-free slide. The edge of a second clean grease-free slide was used to pick a confluent amount of the bacteria colony from the culture medium. The bacteria on the edge of the slide was made to come into contact with the catalase reagent. The presence of air bubbles indicated that the organism is catalase positive. A negative control was set up following the same procedure using *Streptococcus pyogenes* which displayed no air bubbles.

Procedure for obtaining *Achatina achatina*

A number of snails specifically, *Achatina achatina* (Figure 3.3) were obtained from “Makola” Market for this study. The name “Makola” Market is known to be one of the biggest markets in Accra. The market is situated at the centre portion of the city, Accra close to a popular Car park known as the Rawlings Park. The market is about 4.1km from the School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana, Korle Bu Campus.

Preparation of mucus sample

The mucus was extracted under sterile conditions by using a sterile sharp-end rod to remove the skin from the shell leaving the hemolymph. The mucus was then aseptically obtained from the soft body into a beaker using a Pasteur pipette. With the aid of 0.22µm sterile Millipore filter and a 10ml sterile syringe was used to filter-sterilized into a sterile Bijou bottle (Figure 3.4) and then stored at 4°C in a refrigerator.

Preparation of Inoculum

An inoculum was made from the pure culture by using a sterile swab to inoculate phosphate buffered saline which was compared with the turbidity standard (0.5 McFarland’s standard). The density of test suspension was adjusted until equal to the turbidity standard was sterile saline. The suspension was used within 15 minutes of preparation.

Agar well diffusion assay

A sterile cotton wool swab was dipped into the prepared inoculum suspension, excess drained off and streaked three-dimensionally on the surface of 90mm Mueller-Hinton Chocolate agar plate to ensure even distribution of the bacteria on the agar plate. A sterile 6mm cork-borer was used to create two equidistant wells in the agar plate. Using a sterile micropipette tip, 100µl of the prepared snail mucus was used to fill one well and the other with distilled water serving as a negative control. Ciprofloxacin and Penicillin (100mg/ml) were used as the positive controls. The plates were left on the bench for 15 minutes to allow diffusion of the agents into the agar and then incubated at 37°C in a CO_2 enriched atmosphere for 24 hours. The incubated plate was then read after 24-hour of incubation.

Paper disc diffusion assay

Circular filter paper discs of 6mm diameter were cut using a perforator. The discs were sterilized by autoclaving, dried, allowed to cool after which 20µl of the sterile snail mucus was aseptically applied to the discs. The discs were air dry at room temperature for 15 minutes in a safety cabinet. The impregnated discs were applied unto the inoculated Mueller-Hinton Chocolate agar plates using sterile forceps, gently pressed to ensure a firm contact to the medium. Discs of Ciprofloxacin (5µg) and Penicillin (1µg) were applied respectively as the positive controls. The plates were left on the bench for 15 minutes to allow the diffusion of the mucus and then incubated at 37°C in a CO_2 enriched atmosphere for 18-24 hours. The plates were read after 24-hour incubation.

Results

The mucus of *Achatina achatina* which was filter-sterilized showed no inhibition to the tested control strain *Neisseria Gonorrhoeae* ATCC 49226 while the reference discs, ciprofloxacin 5 µg and penicillin 1 µg were susceptible for both the wells made by cork-borer and prepared filter paper discs, respectively (Fig. 4.1 and 4 2). The overall results of the susceptibility testing are shown in Table 4.1

Discussion

Although, the study showed no significant results with the mucus suspected to contain the antibacterial properties. This is the first study where a fastidious organism, *Neisseria gonorrhoeae* has been used in such investigative work. The type of snail used in this study, *Achatina achatina* is commonly found in West Africa particularly, Ghana compared with the recent type of snail *Achatina fulica* has been extensively studied in Japan (Pitt *et al*., 2015). In the early 1990s, *Achatina fulica* was found to contain an active substance known as “achin” according to Otsuka-Fuchino *et al*., (1992) which revealed a remarkable antimicrobial property inhibiting some Gram-negative and Gram-positive organisms which include *Bacillus subtillis Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* (Otsuka-Fuchino *et al*., 1992).

In another study the “achin” a glycoprotein of about 140-160000Da found in *Achatina fulica* belonging to the mytimacin family and was called Mytimacin-AF was active against *Candida albicans*, several *Bacillus* species, *Staphylococcus aureus* and *Klebsiella pneumoniae*, however, the activity was most susceptible against *Staphylococcus aureus* (Zhong *et al*., 2013), *Staphylococcus aureus*, and *Staphylococcus epidermidis* (Santana *et al*., 2012) while showing no activity against *Escherichia coli, Salmonella* species, *Fusarium* species and *Candida albicans*.

Interestingly, different snail *Helix aspersa’s* mucus analyzed by Pitt *et al*., (2015) proved to have antimicrobial activity only against *Pseudomonas aeruginosa*. Isolate. The investigation was conducted with various bacteria and control strains such as ‘in-house’ isolates *Serratia marcescens* and *Salmonella abony*, and *Streptococcus pyogenes* NCIMB 13285, *Staphylococcus aureus* NCTC 10788, *Klebsiella pneumoniae* NCTC 11228, *Escherichia coli* NCTC 10385, *Proteus mirabilis* NCTC 10823, *Pseudomonas*

aeruginosa NCTC 8626, *Serratia marcescens, Pseudomonas aeruginosa* NCIMB 10548, *Acinetobacter* species (R4474) and *Candida albicans* ATCC 10231 (Pitt *et al*., 2015).

The main source of antibacterial properties found in different animals are peptides and different peptides are found in different animals. The different snail peptides may be as a result of geographical location, weather conditions, mode of life and type of food substances. According to Wimley (2011), antibacterial peptides act by targeting numerous parts of the microorganism (Wimley, 2011). However, previous study proposed that certain peptides were targeted to the intracellular content only (Zasloff, 2002).

Studies have shown that even though most antibacterial peptidases act by ill-defined mechanisms, these substances often demonstrate certain discrimination between different microorganisms, a common example is comparing with Gram-positive and Gram-negative bacteria, and vulnerability of fungal cells compared with other eukaryotic cells (Mangoni and Shai, 2009). Antibacterial peptides have been known to simulate amphipathic structures, that are able to relate directly with the cell membrane of the microorganism, which quickly destroys the membrane in more than one locations, causing a subsequent leak out of important cellular components (Cole *et al*., 2000). A peptide, pleurocidin studied by Yoshida *et al*., (2001) revealed that the mode of action forms pores and demonstrates effective membrane translocation which reacts with both neutral and acidic anionic phospholipid membranes (Yoshida *et al*., 2001).

In Ghana, no known studies have explored the usefulness of different snails' secretions for both clinical and environmental isolates. This study advocate for further studies into antimicrobial properties of snails in the country due to the high cost of modern antibiotics for treatment of common and serious bacterial infections which are a threat to healthcare delivery systems in most hospitals.

Conclusion

The zones of inhibition demonstrated by the control antibiotics suggest that *Neisseria gonorrhoeae* ATCC 49226 is susceptible to Ciprofloxacin and Penicillin. However, the mucus from *Achatina achatina* from Ghana could not demonstrate any antibacterial activity against *Neisseria gonorrhoeae* ATCC 49226. The study seems to suggest that the mucus from *Achatina achatina* snail-type used may have an insignificant antibacterial activity to inhibit the test organism used. Although, other species of snails were not investigated to ascertain or compare with this study results. There is a possibility of different resistant mechanisms mimicking the effect in the fastidious bacterium, *Neisseria gonorrhoeae* ATCC 49226 which may have played a role in rendering the antibacterial properties ineffective in a different geographical location in Ghana. Indeed, further studies need to be conducted with different types of snails and various bacterial isolates.

Recommendation

The present study recommends that further studies need to be conducted on different kinds of pathogenic organisms using the mucus from *Achatina achatina* and other types of snails in the country, Ghana.

Declarations

With the exception of duly acknowledged references, I, Desmond Takyi-Attobrah, do hereby declare that this project was entirely carried out by me at the Department of Medical Laboratory Sciences, SAHS, College of Health Sciences, University of Ghana under the supervision of Dr. Francis S. Codjoe

ETHICAL APPROVAL

Ethical clearance was sought from the Ethical and Protocol Review Committee of the School of Biomedical Allied Health Sciences, College of Health Sciences, University of Ghana for this project. Permission was sought from the authorities at the MDS-Lancet Laboratory, Accra.

CONSENT FOR PUBLICATION

All authors have given their consent that this manuscript can be published.

COMPETING INTEREST

There is no competing interest whatsoever among the authors

FUNDING

This work did not receive any external funding, all funding came directly from the authors

AUTHORS CONTRIBUTION

Desmond Takyi-Attobrah designed, performed, and analyzed the experiments

ACKNOWLEDGEMENT

I thank the Almighty God, who has been the only reason I am still alive for the strength and wisdom he has given me to undertake this project. My profound gratitude goes to my father, Mr Anthony Takyi-Attobrah and my mother Mrs. Beatrice Attobrah for their love and support.

I also express gratitude to my auntie Mrs. Grace Attieku and my uncles Mr. Andy Ayebeng, Mr. Solomong Ohemeng Attobrah, Mr. Paul Attobrah, Mr. Isaac Attobrah and Rev. Matthew Attobrah for their role in my entire education.

A heartfelt appreciation goes to my supervisor, Dr. Francis S. Codjoe for his constructive criticisms and time in assisting and making all needed corrections. Special thanks go to the staff of MDS Lancet Laboratories (Microbiology unit), Mr. Bentum and Mr. Cosmos for helping me obtain my isolate from their lab. I again thank Mr. Michael Olu-Taiwo (Microbiology laboratory, SAHS) for his immense assistance during my laboratory procedures. I further express gratitude to Ampomaa Ofosu Otchere and all my friends who made my time in school very memorable. God bless you all.

Abbreviations

IgA	Immunoglobulin A
GAG_s	Glycosaminoglycans
WHO	World Health Organisation
MIC	Minimum Inhibitory Concentration
Ppm	Parts per million
ATP	Adenosine Tri Phosphate
BHI	Brain Heart Infusion
ALF	Activated lactoferrin
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
TB	Tuberculosis
MRSA	Methicillin-resistant Staphylococcus aureus
MPC	Mutation prevention concentration
ATCC	American Type Culture Collection
KCTC	Korean Collection for Type Cultures
NCTC	National Collection of Type Cultures
NCIMB	National Collection of Industrial Food and Marine Bacteria
CTA	Cystine tryptic agar
CO_2	Carbon dioxide

C_{max}	Maximum concentration
µl	microliters
ml	milliliters
mg	milligram
mm	millimiter
˚C	degrees Celsius
µm	micrometer
km	kilometer

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Tables

Table 1. Results for susceptibility testing using agar well and disc diffusion methods.

Bacteria Strain	Method used for sensitivity testing	Antibiotic used	Zone of inhibition
Neisseria Gonorrhoeae ATCC 49226	Disc diffusion method	Penicillin	48
	Disc diffusion method	Ciprofloxacin	42
	Disc diffusion method	Snail mucus	0
Neisseria Gonorrhoeae ATCC 49226			
	Well diffusion method	Penicillin	48
	Well diffusion method	Ciprofloxacin	43
	Well diffusion method	Snail mucus	0

Table 2. Results for sugar utilisation test

Organism	Glucose	Maltose	Sucrose
Neisseria meningitidis	+	+	-
Neisseria gonorrhoeae	+	-	-
Neisseria flavescens	-	-	-
Neisseria sicca	+	+	+

Note: += positive, -= negative

Figures

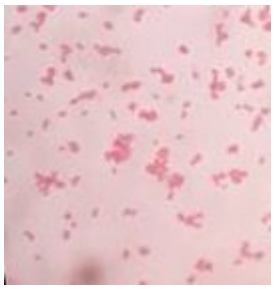

Figure 1

Gram staining of *Neisseria gonorrhoeae* showing Gram-negative diplococcic

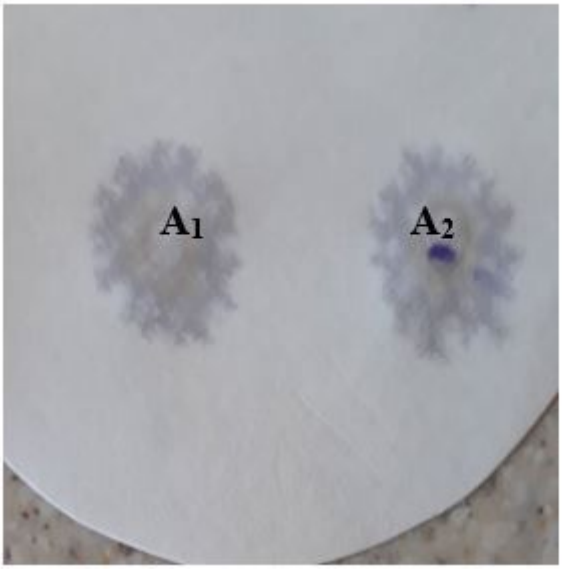

Figure 2

Oxidase test for *Neisseria gonorrhoeae* showing a violet colour

Note: A1= Oxidase negative, A2= Oxidase positive

Figure 3

General overview of *Achatina achatina*

Figure 4

Extracted mucus of *Achatina achatina* showing the characteristic brown colour

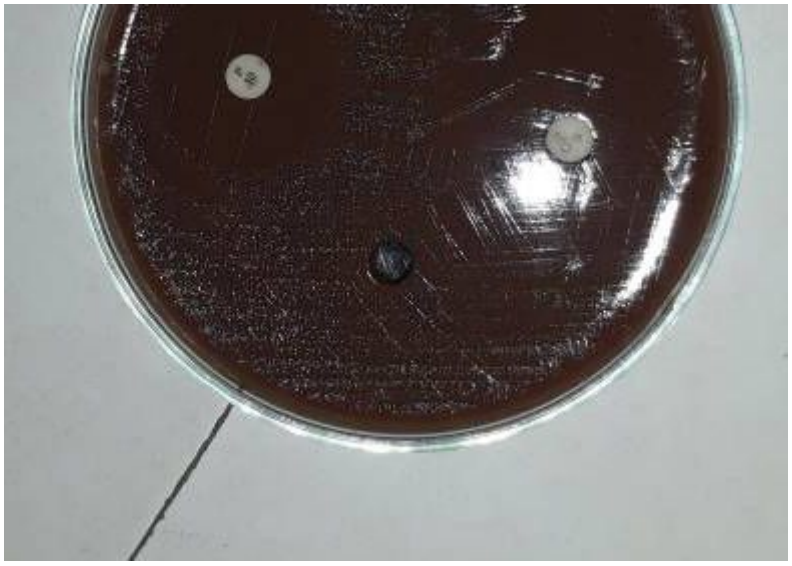

Figure 5

Test strain *Neisseria gonorrhoeae* showing no zones of inhibition.

Note: A= ciprofloxacin 5µg, B=penicillin 1µg, C= Snail mucus in well

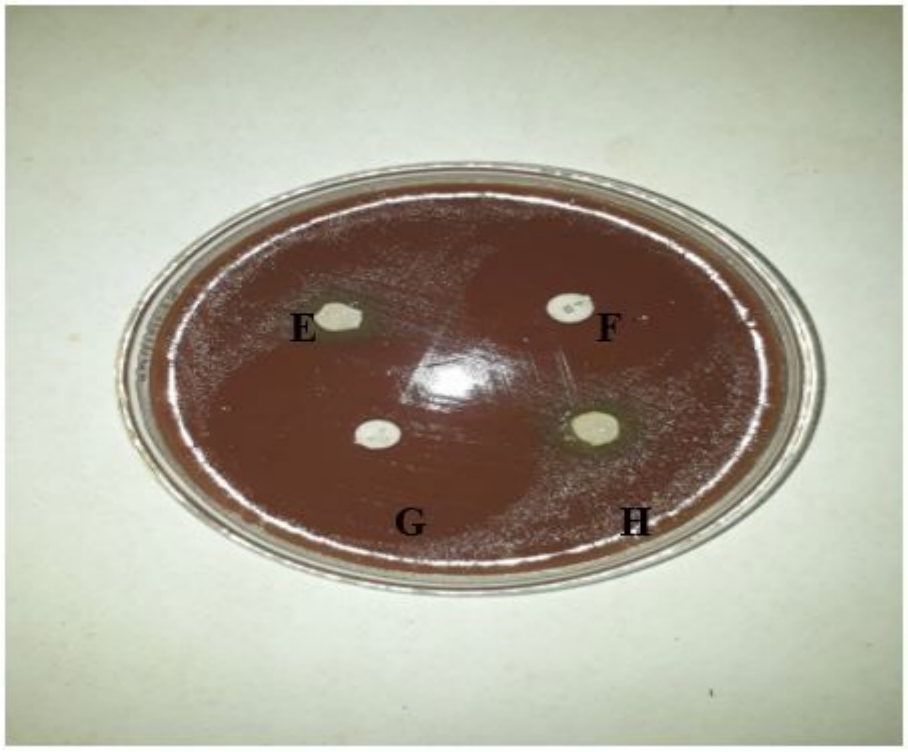

Figure 6

Test strain *Neisseria gonorrhoeae* showing no zones of inhibition with prepared paper discs

Note: E= mucus impregnated paper disc, F= penicillin 1µg, G= ciprofloxacin 5µg, H= mucus impregnated paper disc.