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Characterization and Determination of Drug Resistance Genes in Bacterial and Fungal Pathogen Co-Infecting Individual with SARS-CoV-2 in Oyo-State, Nigeria

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Being A PhD Thesis Submitted to the Department of Biological Science, Faculty of Natural and Applied Sciences, Lead City University, Ibadan, Oyo State, Nigeria.

In Partial Fulfilment of the Requirement for the Award of Doctor of Philosophy (PhD) in Medical Microbiology

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2022

Certification

This is to certify that Florence Adenike BAMIGBOLA with Matriculation number LCU/PG/001275 carried out this research work titled, "**Characterization and Determination of Drug Resistantance Genes in Bacterial and Fungal Pathogen Co-Infecting Individuals with SARS-CoV-2 in Oyo State, Nigeria"** in the Department of Biological Sciences, Faculty of Applied Sciences, Lead City University, Ibadan, Oyo state, for the award of Doctor of Philosophy Degree in Medical Microbiology and that this has not been previously submitted.

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_____ ----------

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Head of Department Date

Dedication

This research work is dedicated to God Almighty the source of all wisdom.

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“Even though the above-mentioned institutions and persons have assisted in the process of this research work, I alone stand responsible for the errors, if any, found in the work”

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Abstract

Epidemiological data of SARS-C0V-2 indicated that bacterial and fungal complications increased mortality rate and decreased clearance rate of the virus therefore antimicrobial drugs were administered to patients with this disease without susceptibility testing. This study sought to identify co-infecting pathogen(s), determine their antimicrobial resistant pattern, detect macrolide, azole and polyene resistant genes in the identified pathogen(s) from confirmed SARS-CoV-2 individual in Oyo State, Nigeria. Nasopharyngeal samples were collected from 400 symptomatic and asymptomatic infected adults; structured questionnaires were administered to determine predisposing factors to SARS-CoV-2 infection. Isolates were obtained by standard microbiological methods, identified using API 20E, VITEK 2.0 ID cards and MALDITOF MS VITEK. Kirby Bauer disc diffusion methods and VITEK 2.0 AST card kits were used to determine antimicrobial susceptibility testing. Resistant genes in the isolates were amplified using Polymerase Chain Reaction with specific primers, genes, detection was done by Agarose Gel Electrophoresis. Bacterial and fungal coinfection among SAR-CoV-2 infected individuals were detected (7.3%). Many of the identified bacteria were in family of Enterobacteriaceae, *Loddermyces elongisporous* (39.0%) was the most fungi isolated followed by *Aspergillus flavus* 17.5%). The Gram positive bacteria isolated were more resistant (66.6%) to azithromycin) used for palliative treatment of COVID-19 cases. However, bacterial isolates show significant higher susceptibility (89.0%) to quinolones. Only mefA (36.8%) and ermB (26.3%) genes were detected in the bacterial isolates and were more in Gram positive bacteria and no mphA gene was detected. Among fungal isolates, ERG 11 gene was detected among the *Loddermyces elongisporous*, FKS gene was detected in *Aspergillus niger* while CPY gene was present in *Aspergillus niger* and *Aspergillus flavus*. Some of the microbial isolates detected in SARS-CoV-2 infected individuals were multidrug resistant with resistant to Azithromycin inclusive. This finding is of great health concern and should be further looked into.

Keywords: SARS-CoV-2, Bacterial and Fungal co-infection, MALDITOF MS VITEK and Resistant genes.

Word Count: 294

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List of Acronyms

Abbreviation	Meaning
SARS-CoV-2	Severe Acute Respiratory Syndrome Corona Virus 2
COVID 19	Novel Corona Virus Disease 2019
SARS	Severe Acute Respiratory Syndrome
MERS	Middle East Respiratory Syndrome
MRSA	Methicillin Resistant Staphylococcus aureus
MSSA	Methicillin Sensitive Staphylococcus aureus
GPC	Gram positive cocci
GNB	Gram negative bacilli
GPB	Gram positive bacilli
API 20E	Analytical Profile Index 20E
UCH	University College Hospital
PPE	Personal Protective Equipment
NCCLS	National Committee for Clinical Laboratory
EUCAST	European Committee on Antibiotic Susceptibility Testing
CLSI	Clinical and Laboratory Standard Institute
BALF	Broncho- alveolar lavage fluid
IgG	Immunoglobulin
DNA	Deoxy-Ribonucleic acid
RNA	Ribonucleic acid
PCR	Polymerase chain reaction
WHO	World Health Organization

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ACE2	Angiteosin Convertin Enzyme 2
TNF	Tumor Necrotic Factor
CRS	Cytokine Release Syndrome
NGS	Next Generation Sequencing
VAP	Ventilation Associated Pneumonia
ITS	Internal Transcribed Spacer
CAPA	COVID-19 Associated Pulmonary Aspergillosis
ARDS	Acute Respiratory Distress Syndrome
ABPA	Allergic Bronco Pulmonary Aspergillosis
MALDITOF	Matrix Assisted Laser Desorption Ionization Time of Flight
MS	Mass Spectrophotometry
CHCA	& -Cyano-4-hydroxycinnamic acid
TAE	Tris Acetic Ethelyene-diamine-tetra-acetic-acid
TBE	Tris Boric Ethlyene-diamine-tetra-acetic-acid

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Chapter One

Introduction

1.1 Background to the Study

The novel Corona Virus Disease 2019 (COVID-19) is an infectious disease caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) which was first identified in December 2019 in Wuhan, China, and is now currently circulating throughout the whole world[1]. This virus belongs to the Coronaviridae family, in the Nidovirales order. The subgroups of the coronavirus family are alpha (α), beta (β), gamma (γ), and delta (δ) coronavirus. The four 'common human coronaviruses' are 229E (α coronavirus), NL63 (α coronavirus), OC43 (β coronavirus) and HKU1 (β coronavirus)[2]. It was reported that pathogens that cause corona type of pneumonia include; SARS, MERS, and SARS-CoV-2, all these viruses have been observed to be capable of co-infecting in the setting of community-acquired bacterial and fungal pneumonia[3].

In early January 2020, the Chinese Health Authority announced the causative agent of this disease as a novel coronavirus, and its whole genome was published[4]. The disease was declared a pandemic because of the way it spread across the globe[5]. Majority of the confirmed COVID-19 patients in China had mild or moderate infection, some patients developed a severe clinical infection while some developed critically severe infection. Severe clinical course is characterized by multi-systemic and life-threatening manifestation with pneumonia and acute respiratory distress complications including inflammatory storm, coagulation dysfunction, septic shock, and multiple organ failure[6]. The presence of comorbidities, such as old age, hypertension, diabetes, and obesity have been linked with increased COVID-19 severity and mortality. The COVID-19 pandemic caused a large

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number of immunocompromised individuals to be hospitalized and some reports indicated that some COVID-19 patients were diagnosed with secondary infections[7,8]. Some findings indicates that COVID-19 patients often have infections with other respiratory viruses, such as influenza virus[9]. The β-coronaviruses which includes SARS-CoV and MERS-CoV, and other acute-lung-injury causing coronaviruses are of zoonotic origin[10]. Viral, bacteria and fungi coinfection have been reported among patients with the SARS-CoV-2 infection[11]. During a multi-center study in Eastern China, nasopharyngeal swab samples were collected for total RNA extraction, followed by multiplex Polymerase Chain Reaction and mNext Gene Sequensing analysis[12]. According to a results, 11 of 20 co-infections were identified in laboratory-confirmed COVID-19 cases and included regular respiratory viruses, fungi, and bacteria and the most common pathogens detected were Influenza virus, Adenovirus, *Acinetobacter baumannii, Klebsiella pneumoniae, Aspergillus flavus, Candida glabrata,* and *Candida albicans*[11]. In fact, bacteria are known to participate in interactions between host cells and viruses[13]. Although bacteria are typically secondary invaders during influenza infections, they express virulence factors that promote viral pathogenesis. As a result, viral load increases and clearance rates decline[13]. Studies on confirmed SARS cases showed that co-infection with bacterial and fungal infection was one of the major complications encountered especially in those who underwent extended hospitalization and long-term mechanical ventilation[13]. Some conditional pathogens such as *Enterococcus faecalis, Klebsiella pneumonia, Acinetobacter baumannii, Stenotrophomonas maltophilia,* and *Candida albicans* could originate from a hospital environment, or nosocomial infection, but the majority inhabit the oral cavity[14,15]. In a study where nasopharyngeal test were conducted on SARS-CoV-2 infected patients, 38 kind of bacteria, 10 kind of viruses except for severe

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acute respiratory syndrome coronavirus 2 (SARS-CoV-2), nine kind of fungi and three kind of atypical pathogens had been found. They include bacteria such as *Haemophilus* spp*, Corynebacterium* spp*, Prevotella* spp*, Staphylococcus* spp*, Moraxella* spp*, Neisseria* spp*, Streptococcus* spp*, Megaphaera* spp*, Pediococcus* spp and *Dolosigaranulum* spp. Similarly, fungi found include *Candida* spp and *Aspergillus* spp and virus includes parainfluenza virus, respiratory syncytial virus, adenovirus and human metapneumovirus[16]. The test for SARS-CoV-2 involves taking oropharyngeal and nasopharyngeal swab specimens, sputum, and broncho-alveolar lavage fluid[17]. According to a study on 213 confirmed COVID-19 patients, viral Ribonucleic acid could be detected in all Broncho Aleovelar Lavage Fluid samples of severe cases, but not from mild cases and sputum appeared to be a good clinical sample with a high positive rate (74.4-88.9%), followed by nasal swabs (53.6-73.3%) and throat swabs (50-61.3%)[18]. In order to detect bacterial or fungal infections among COVID-19 patients, researchers have documented the use of sputum, nasopharyngeal swabs, and blood samples[19]. A study documented the use of Nasopharyngeal swab samples taken from SARS-CoV-2 infected patients and the samples were also used to further test for fungal and bacterial pathogens in the patients and *Bordetella pertussis, Chlamydophila pneumoniae* and *Mycoplasma pneumonia* were detected[20].

A documentation of different approaches used to screen for fungi and bacteria among individuals infected with SARS-CoV-2, where the use of sputum and endotracheal aspirates was reportedly used for screening[21]. Another study mentioned the use of blood, urine, and respiratory tract sampling to identify co-infections such as bacterial and fungal among individuals with SARS-CoV-2[22]. Symptomatic and asymptomatic individuals infected with SARS-CoV-2 has possibility of presenting with different clinical features, where

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symptomatic patients will always present with a great deal of symptoms greater than asymptomatic individual but the asymptomatic group had a significantly longer duration of viral shedding than the symptomatic group[23]. Also, viral-specific ImmunoglobulinG levels in the asymptomatic group were significantly lower and they exhibit lower levels of 18 pro and anti-inflammatory cytokines that are presented with less bacterial and fungal infections[23]. Antibiogram when properly prepared and interpreted serve as an important source of information for health care providers and is the overall profile of antimicrobial susceptibility results of a microbial species to a battery of antimicrobial agents[24]. Data from antibiograms are most useful when initiating empirical therapy and when tracking antimicrobial resistance trends over time within a hospital or health care system. Most SARS-CoV-2 infected persons are put on empirical antimicrobial treatment, which might lead to an increase in antimicrobial resistance. For example, in a reports of 102 patients from critical and non-critical care in China where all (99%) patients received antibacterial therapy, 87/102 (85%) received quinolone therapy, 34/102 (33%) cephalosporin's, and 25/102 (25%) carbapenems, no bacterial nor fungal co-infection was however reported in this study, because it is difficult to distinguish bacterial or fungal infections with existing viral pneumonia based on clinical and radiological performance[25]. Corroborating this statement, which is a witness of an increasing reports on the co-occurrence of epidemics or pandemic respiratory viruses like influenza virus, Severe Acute Respiratory Syndrome, Middle East Respiratory Syndrome and Severe Acute Respiratory Syndrome Corona Virus-2, secondary bacteria and invasive fungal infection that resulted in poor patients' outcomes with consequently high mortality rates and is a critical reality that demands an urgency for special focus on different aspects of this new disease[6].

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1.2 Statement of the Problem

Epidemiological data on COVID-19 have indicated that bacterial and fungal complications further increased morbidity and mortality of influenza infection. A few challenges exist in diagnosing secondary coinfection in COVID-19 patients. Although, it can be difficult to distinguish bacterial or fungal infection and existing viral pneumonia, based on clinical and radiological examinations, microbiological examination can add great value to diagnoses[26]. However, this approach can pose significant risks to bio-sample collectors and laboratory technicians processing samples from COVID-19 patients because the virus is transmitted via virus-laden aerosols in addition to respiratory droplets and direct contact. To detect infection with bacterial and fungi pathogens, medical institutions generally use traditional smears and cultures and is well known that the sputum smear has low positivity rate and the culture process is very time-consuming[27]. In addition, the fear of an increase in microbial resistance due to anti-microbial prescribing is also an issue among individuals infected. A reseacher reported that majority of infected patients are on anti-microbial drug despite not having any form of infection[28]. However, several issues such as a useful strategy to prevent disease spread, collection of appropriate clinical specimens, transmission route, viral dynamics and effective drug treatments remain unclear, also, possibility of co-infection with other respiratory pathogens remain unknown and this should be an important concern for clinicians in the management of COVID-19 in Nigeria and Africa at large.

1.3 Justification of the Study

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Studies have shown that co-infection with either bacterial or fungal infection in patients with SARS-CoV-2 has been proven to be fatal and is not currently well understood and the knowledge gap has important implications[29]. Proper diagnosis and treatment is needed to prevent mortality as a result of co-infections in-order to provide information for appropriate antibiotic prescription for proper control. Furthermore, the findings from this research will help to mitigate antibiotic overuse, reducing and minimize unintended consequences of antimicrobial resistance among SARS-CoV-2 infected person.

1.4 Aim and Objectives of the Study

The aim of this study is to isolate, identify bacterial and fungal co-infecting individual with SARS-CoV-2, determine their drug resistance pattern, detect macrolides resistant genes, azoles and polyene resistant genes in Oyo state, Nigeria.

The Specific Objectives are to:

i. determine the socio-demographic patterns of the infected individual in the study using structured questionnaires.
ii. isolate and phenotypicallycharacterize bacterial and fungal pathogen from the nasopharyngeal samples of SARS-CoV-2 confirmed individual phenotypically
iii. determine the antimicrobial susceptibility pattern of the pathogenic bacterial and fungal isolates.
iv. detect Macrolide resistant gene among pathogenic bacterial isolated from SAR CoV-2 individuals using specific primers.
v. detect carriage of some Azoles and Polyenes antifungal resistant genes among the fungal isolated from SARS-CoV-2 individuals using specific primers.

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1.5 Significance of the Study

Studies have shown that co-infection with either bacteria or fungi infection in patients with SARS CoV-2 has been proven to be fatal[29]. Frequent use of empirical antibiotics in patients infected with COVID-19 poses a risk of selection for antibiotic empirically[31]. Proper diagnosis and treatment is needed to prevent mortality as a result of co-infections. There is no recommended testing or diagnostic process to identify coinfections among SARS-CoV-2 infected individuals[19]. Some researchers have mentioned the use of various methods which includes blood samples, sputum samples and nasopharyngeal swabs whereas each of this method have safety concerns associated with it and how effective it will be in diagnosing SARS-CoV-2 infection[18]. To this effect, the need for a careful and frequent laboratory analysis in investigating coinfection of bacterial and fungal among COVID-19 individuals cannot be over-emphasized. Therefore, this study will assess the use of nasopharyngeal samples among individuals infected with SARS-CoV-2 to identify bacteria and fungi pathogens in order to provides a theoretical and factual basis for precise intervention, accurate prevention and treatment of infectious complication for effective reduction in mortality rate among coronavirus infected patients.

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1.6 Scope of the Study

This is a hospital-based, cross-sectional, purposeful, experimental study, carried out in all COVID-19 government isolation center in Oyo state, Nigeria from July 2020 to April 2021. Questionnaires and informed consent were employed to gather information from individuals

who participated in releasing their biological samples. The questionnaire consists of pre-formulated written sets of question which were divided into three sections; A; demographic data, B; severity of infection and C; focuses on comorbidity associated with the infection. They were all structured to incorporate the dependent and independent variables.

The study also conducted laboratory analysis to isolate and confirm the presence of some pathogenic bacteria and fungi from their nasopharyngeal samples, phenotypic antibiogram was done on all the isolated organisms and presence of macrolide resistant gene, azoles and polyene resistant gene were all investigated.

1.7 Limitation of the Study

The study was limited within its scope and the limitation therefore include among others:

- Non-compliance of individuals to readily release their samples because of traditional beliefs about any human body fluid; therefore, informed consent was sought for
- The field work was stressful in filling of questionnaires to get actual facts from the subjects.

1.8 Operational Definition of Terms

Pandemic: A disease outbreak that spread across country or continents. It affects and claims more people lives

Epidemic: An outbreak of disease that spreads quickly and affects many individuals spontaneously

Pathogenic: Ability to cause disease in a person, animal or plants

Infection: The invasion and growth of germs in the body, the germs can be bacteria, viruses, fungi, or other microorganisms.

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Nasopharyngeal Sample: A sample collected at the upper part of the pharynx behind and above the soft palate

Symptomatic: Showing signs of a disease or illness

Asymptomatic: Showing no signs of disease or illness

Antimicrobial Agents: Is a natural or synthetic substance that kills or inhibits the growth of microorganisms.

Antifungal Agents: Is a drug that selectively eliminates fungal pathogens from a host with minimal toxicity to the host

Antibacterial Agents: Is a drug that selectively eliminates bacterial pathogens from a host with minimal toxicity to the host

Pneumonia: It is a lung inflammation caused by bacteria or viral infection, when the air sacs fill with pus that can become solid.

Mortality: The condition of being subject to death in a given area or period, or from a particular cause.

Therapy: This is a treatment given to a person to get better from the effect of a disease or injury.

Co-infecting Individual: Co-infection of individual with multiple pathogen species simultaneously.

Bacteria: They are prokaryotes, ubiquitous microscopic, single-celled organisms that exist in their millions, in every environment, both inside and outside.

Fungi: They are eukaryotic organisms that include microorganisms such as yeasts, moulds and mushrooms.

Bacterial: A disease caused by bacteria that are common in the environment

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Fungal: A disease caused by fungi that are common in the environment.

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Chapter Two

Literature Review

2.1 Origin and Evolutionary History of SARS-CoV

The virus is thought to be of natural animal origin, most likely through spillover infection[1,2,3]. The world experienced the outbreaks of coronavirus (SARS) infections in 2002 - 2003, (MERS) in 2012 and SARS-CoV-2 in 2019 caused by SARS-CoV, MERS-CoV and SARS-CoV-2 respectively[4]. There are several theories about where the index case originated and investigations into the origin of the pandemic are ongoing[4]. Phylogenetic estimates that SARS-CoV-2 arose in October or November 2019[5,6,7]. A phylogenetic algorithm analysis suggested that the virus may have been circulating in Guangdong before Wuhan[8]. Evidence suggests that it descends from a coronavirus that infects wild bats, and spread to humans through an intermediary wildlife host[9,10]. The possibility that the virus was accidentally released from a laboratory is also under increasingly active consideration[11]. United. State intelligence agencies found that the virus was not developed as a biological weapon and that it is unlikely for it to have been genetically engineered[12].

The first confirmed human infections were in Wuhan, Hubei, China. A study of the first 41 cases of confirmed COVID- 19, published in January 2020 in The Lancet, reported the earliest date of onset of symptoms as 1 December 2019[13,14,15]. Official publications from the WHO reported the earliest onset of symptoms as 8 December 2019[16]. Human-to-human transmission was confirmed by the WHO and Chinese authorities by 20 January 2020[17,18]. According to official Chinese sources, these were mostly linked to the Huanan Seafood Wholesale Market, which also sold live animals[19]. In May 2020, George Gao, the director of

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the CDC, said animal samples collected from the seafood market had tested negative for the virus, indicating that the market was the site of an early super spreading event, but that it was not the site of the initial outbreak[20]. Traces of the virus have been found in wastewater samples that were collected in Milan and Turin, Italy, on 18 December 2019[21].

By December 2019, the spread of infection was almost entirely driven by human-to-human transmission[22,23]. The number of COVID-19 cases in Hubei gradually increased, reaching 60 by 20 December and at least 266 by 31 December[24,25]. On 24 December, Wuhan Central Hospital sent a Broncho alveolar lavage fluid (BAL) sample from an unresolved clinical case to sequencing company Vision Medicals. On 27 and 28 December, Vision Medicals informed the Wuhan Central Hospital and the Chinese CDC of the results of the test, showing a new coronavirus[26]. A pneumonia cluster of unknown cause was observed on 26 December and treated by the Doctor Zhang Jixian in Hubei Provincial Hospital, who informed the Wuhan Jianghan CDC on 27 December[27]. On 30 December, a test report addressed to Wuhan Central Hospital, from company CapitalBio Medlab, stated an erroneous positive result for SARS, causing a group of doctors at Wuhan Central Hospital to alert their colleagues and relevant hospital authorities of the result. The Wuhan Municipal Health Commission issued a notice to various medical institutions on "the treatment of pneumonia of unknown cause" that same evening[29]. Eight of these doctors, including Li Wenliang (punished on 3 January), were later admonished by the police for spreading false rumours and another, Ai Fen, was reprimanded by her superiors for raising the alarm[29,30]. The Wuhan Municipal Health Commission made the first public announcement of a pneumonia outbreak of unknown cause on 31 December, confirming 27 cases enough to trigger an investigation[31,32,33,34].

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During the early stages of the outbreak, the number of cases doubled approximately every seven and a half days[35]. In early and mid-January 2020, the virus spread to other Chinese provinces, helped by the Chinese New Year migration and Wuhan being a transport hub and major rail interchange. On 20 January, China reported nearly 140 new cases in one day, including two people in Beijing and one in Shenzhen[36]. Later official data shows 6,174 people had already developed symptoms by then and more may have been infected[37,38]. A report in The Lancet on 24 January indicated human transmission, strongly recommended personal protective equipment for health workers, and said testing for the virus was essential due to its "pandemic potential"[39]. On 30 January, the WHO declared COVID-19 a Public Health Emergency of International Concern[38]. By this time, the outbreak spread by a factor of 100 to 200 times[40].

Italy had its first confirmed cases on 31 January 2020, two tourists from China[41]. Italy overtook China as the country with the most deaths on 19 March 2020[42]. By 26 March the United States had overtaken China and Italy with the highest number of confirmed cases in the world[43]. Research on corona virus genomes indicates the majority of COVID-19 cases in New York came from European travellers, rather than directly from China or any other Asian country[44]. Retesting of prior samples found a person in France who had the virus on 27 December 2019 and a person in the United States who died from the disease on 6 February 2020[45,46,47].

RT-PCR testing of untreated wastewater samples from Brazil and Italy have suggested detection of SARS-CoV-2 as early as November and December 2019, respectively, but the methods of such sewage studies have not been optimized, many have not been peer-

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reviewed, details are often missing, and there is a risk of false positives due to contamination or if only one gene target is detected[48]. A September 2020 review journal article said, "The possibility that the COVID- 19 infection had already spread to Europe at the end of last year is now indicated by abundant, even if partially circumstantial, evidence," including pneumonia case numbers and radiology in France and Italy in November and December[49]. As of 1 October 2021, Reuters reported that it had estimated the worldwide total number of deaths due to COVID- 19 to have exceeded five million[50].

Corona virus derive their name from the Latin word “corona” meaning crown. The name refers to the unique appearance of the virus under an electron microscope as round particles with a rim of projections resembling the solar corona. They are enveloped, positive-sense, single-stranded RNA viruses which were first isolated from humans in 1965 [51]. Corona virus belongs to the family Coronaviridae which is known to produce mild respiratory diseases in humans. In recent times, there have been three major corona viruses leading to disease outbreaks, beginning with the severe acute respiratory syndrome corona virus (SARS–CoV) in 2002, followed by the Middle East respiratory syndrome corona virus (MERS–CoV) in 2012, and now the severe acute respiratory syndrome corona virus 2 in 2019 (SARS–CoV2). Severe acute respiratory syndrome corona virus 1 (SARS-CoV-1 or SARS-CoV-2) is a strain of coronavirus that causes severe acute respiratory syndrome (SARS), the respiratory illness is responsible for the 2002–2004 SARS outbreak[52,53]. It is a virus which infects the epithelial cells within the lungs[54]. The virus enters the host cell by binding to angiotensin-converting enzyme-2[55]. It infects humans, bats, and palm civets[56]. A virus very similar to SARS was discovered in late 2019. This virus, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is the

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causative pathogen of COVID-19, the propagation of which started the COVID-19 pandemic[57].

Severe acute respiratory syndrome (SARS) is the disease caused by SARS-CoV-1. It causes an often severe illness and is marked initially by systemic symptoms of muscle pain, headache, and fever, followed in 2–14 days by the onset of respiratory symptoms, mainly cough, dyspnea, and pneumonia. Another common finding in SARS patients is a decrease in the number of lymphocytes circulating in the blood[58,59]. In the SARS outbreak of 2003, about 9% of patients with confirmed SARS-CoV-1 infection died[60]. The mortality rate was much higher for those over 60 years old, with mortality rates approaching 50% for this subset of patients[60].

In March 2003, WHO established a global network of leading laboratories to collaborate in the identification of the causative agent of SARS. Early on, laboratories in the network narrowed the search to members of the paramyxovirus and corona virus families. Early findings shared by the laboratories pointed to corona viruses with increasing consistency. On 21 March, scientists from the University of Hong-Kong announced the isolation of a new virus that was strongly suspected to be the causative agent of SARS[61]. Molecular epidemiological research demonstrated the virus isolated in 2002–2003 in south China and the virus isolated in the same area in late 2003 and early 2004 are different, indicating separate species-crossing events[62]. The phylogeny of the outbreak strains shows that the southwestern provinces including Yunnan, Guizhou and Guangxi compare to the human SARS-Co V-1 better than those of the other provinces, but the viruses' evolution is a product of the host interaction and particularity[63].

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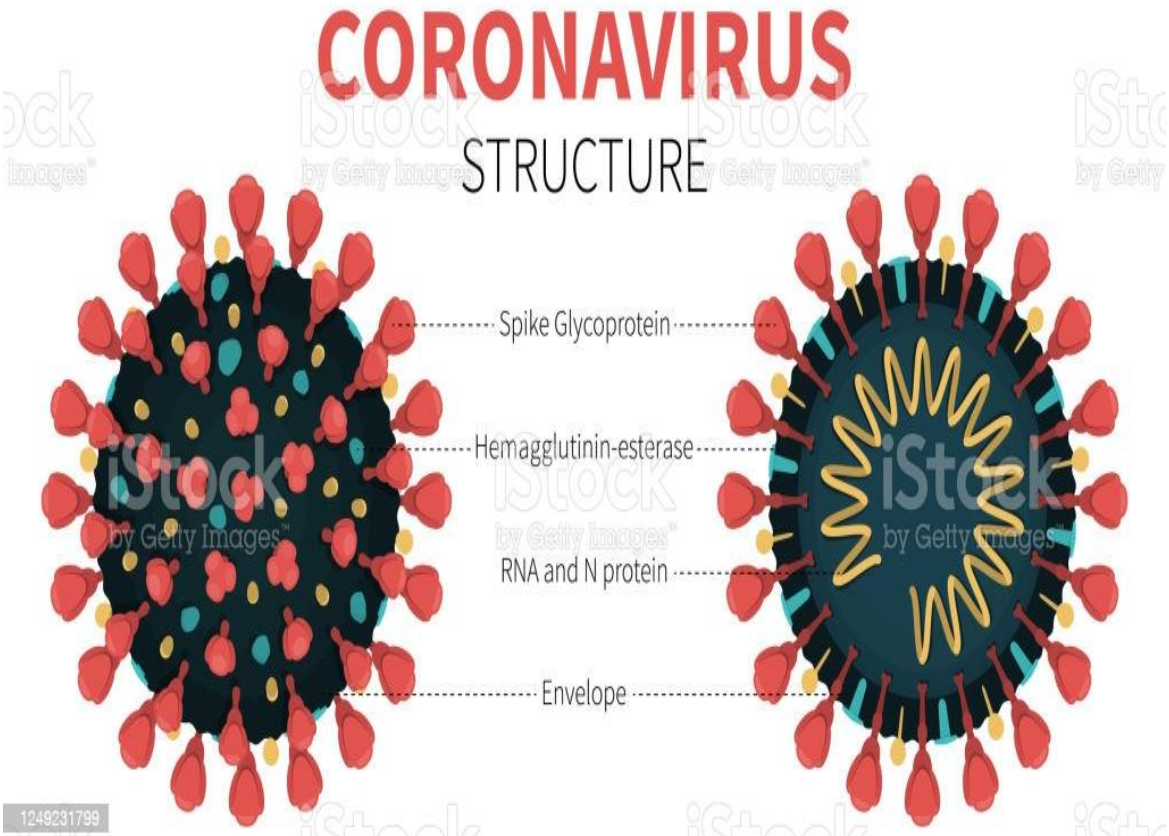

Figure 2.1: Corona virus structures. Source[8]

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2.2 Phylogenetic Tree of SARS-CoV

Bats are likely to be the natural reservoir, that is, the host that harbored the pathogen but that does not show ill effects and serves as a source of infection. No direct progenitor of SARS-CoV was found in bat populations, but WIV16 was found in a cave in Yunnan province, China between 2013 and 2016, and has a 96% genetically similar virus strain. The hypothesis that SARS-CoV-1 emerged through recombination of bat SARS-Co Vs in the Yunnan cave of WIV16 or in other yet-to-be-identified bat caves is considered highly likely[64].

SARS-CoV-1 is one of seven known corona viruses to infect humans. The other six are[65]:

- Human coronavirus 229E (HCoV-229E)
- Human coronavirus NL63 (HCoV-NL63)
- Human coronavirus OC43 (HCoV-OC43)
- Human coronavirus HKU1 (HCoV-HKU1)
- Middle East respiratory syndrome–related coronavirus (MERS-CoV)
- Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

2.3: Signs and Symptoms of COVID-19

Symptoms of COVID-19 are variable, ranging from mild symptoms to severe illness[66,67]. Common symptoms include headache, loss of smell (anosmia) and taste (ageusia), nasal congestion and runny nose, cough, muscle pain, sore throat, fever, diarrhea, and breathing difficulties[68]. People with the same infection may have different symptoms, and their

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symptoms may change over time. Three common clusters of symptoms have been identified: one respiratory symptom cluster with cough, sputum, shortness of breath, and fever; a musculoskeletal symptom cluster with muscle and joint pain, headache, and fatigue; a cluster of digestive symptoms with abdominal pain, vomiting, and diarrhea[68]. In people without prior ear, nose, and throat disorders, loss of taste combined with loss of smell is associated with COVID-19 and is reported in as many as 88% of cases[69,70,71]. Of people who show symptoms, 81% develop only mild to moderate symptoms (up to mild pneumonia), while 14% develop severe symptoms (dyspnea, hypoxia, or more than 50% lung involvement on imaging) and 5% of patients suffer critical symptoms (respiratory failure, shock, or multiorgan dysfunction) [72]. At least a third of the people who are infected with the virus do not develop noticeable symptoms at any point in time[73,74]. These asymptomatic carriers tend not to get tested and can spread the disease[75,76,77,78]. Other infected people will develop symptoms later, called "pre-symptomatic", or have very mild symptoms and can also spread the virus[77].

As is common with infections, there is a delay between the moment a person first becomes infected and the appearance of the first symptoms. The median delay for COVID-19 is four to five days[78]. Most symptomatic people experience symptoms within two to seven days after exposure, and almost all will experience at least one symptom within 12 days[78,79].

Most people recover from the acute phase of the disease. However, some people over half of a cohort of home-isolated young adults continue to experience a range of effects, such as fatigue, for months after recovery, a condition called long COVID; long-term damage to

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organs has been observed. Multi-year studies are underway to further investigate the long-term effects of the disease[80,81,82].

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2.4: Transmission of COVID-19

COVID-19 is mainly transmitted when people breathe in air contaminated by droplets and small airborne particles containing the virus. Infected people exhale those particles as they breathe, talk, cough, sneeze, or sing[83,84,85,86]. Transmission is more likely when people are physically close. However, infection can occur over longer distances, particularly indoors[83,87]. Infectivity can occur 1–3 days before the onset of symptoms[88]. Infected persons can spread the disease even if they are pre-symptomatic or asymptomatic[88]. Most commonly, the peak viral load in upper respiratory tract samples occurs close to the time of symptom onset and declines after the first week after symptoms begin[88]. Current evidence suggests a duration of viral shedding and the period of infectiousness of up to 10 days following symptom onset for persons with mild to moderate COVID-19, and a up to 20 days for persons with severe COVID-19, including immunocompromised persons[88,89].

Infectious particles range in size from aerosols that remain suspended in the air for long periods of time to larger droplets that remain airborne or fall to the ground[90,91,92,93]. Additionally, COVID-19 research has redefined the traditional understanding of how respiratory viruses are transmitted[93,94]. The largest droplets of respiratory fluid do not travel far, and can be inhaled or land on mucous membranes on the eyes, nose, or mouth to infect[92]. Aerosols are highest in concentration when people are in close proximity, which leads to easier viral transmission when people are physically close, but airborne transmission can occur at longer distances, mainly in locations that are poorly ventilated; in those conditions small particles can remain suspended in the air for minutes to hours[92,93,94]. The number of people generally infected by one infected person varies; as only 10 to 20% of people are responsible for the disease's spread[95,96]. It often spreads in clusters, where infections can be

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traced back to an index case or geographical location[97]. Often in these instances, superspreading events occur, where many people are infected by one person[95].

2.4.1 Transmission of COVID-19 on the Human Body

COVID-19 is an RNA virus which is approximately 60-140nm. It is transmitted through respiratory droplets from coughing and sneezing and enters nasal system by inhaling and starts replicating [98]. ACE2 is the main receptor of COVID-19, it is mainly expressed in a small subset of cells in the lung called type 2 alveolar cells. The spike protein on covid-19 surface pinch the host cell binding to the ACE2 receptor. The enzyme furin is present in the host cell and plays a vital role for the virus to enter. The virus propagates with limited innate immune response and reaches the respiratory tract where it faces a robust innate immune response. At this stage, the disease is clinically manifest and an innate response cytokine may be predictive of the subsequent clinical course. For beta and lambda infections, viral-infected epithelial cells are a major source. The disease will be mild for 80% of the infected patients and mostly restricted to the upper and conducting airways [99]. Approximately 20% of the infected patients develop pulmonary infiltrates and some of these develop very severe disease [100]. The mortality rate of severe patients with COVID-19 can be as high as 49%, based on a recent epidemiological by China CDC[101]. In Wuhan, 292 patients with COVID-19 were studied. Age was the risk factor of patients with a severe condition, as shown by the Lasso algorithm. When the age of patients with a severe condition increased by 5 years, the risk increased by 15.15%. Most of the patients with COVD-19 were elderly patients in the severe group, with basic diseases. Chronic obstructive pulmonary disease, hypertension, malignant tumor, coronary heart disease, and chronic kidney disease were more frequent in the severe group than in the mild group. Of 145severe cases, 51 patients died (34.69%), and

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90.2% of the patients who dies were over 60 years old. Forty patients had basic disease out of 51 deaths (78.43%). Reports have demonstrated that patients aged older than 60 years who have co morbidities, especially hyper-tension, are at risk for severe disease and death from SARS-CoV-2 infection [102,103,104].

2.5: Pathophysiology of SARS-CoV-2 and Oral Microbiome on Host Immunity

Change of Cytokines Like SARS-CoV and MERS-CoV, SARS-CoV-2 can also induce excessive and aberrant non-effective host immune responses that are associated with severe lung pathology, and lead to death[105,104]. Most moribund COVID-19 patients are suffering from a cytokines torm, which is manifested by the increased plasma concentrations of IL-2, IL-7, IL-10, G-CSF, CXCL10, CCL2, CCL3, and TNFα[105,106,107]. It has been confirmed that the cellular immunity and cytokines status are closely related to the state of illness, with high levels of IL-6 and IL-10 levels in severe patients[108]. We speculate that changes in cytokines reflect disease status to some extent. The presence of oral microbes such as *Streptococcus, Prevotella and Porphyromonas* cannot only change the microbial composition of the respiratory system, but also promote a series of cytokine responses and affect the immune homeostasis of the lungs. The levels of serum IL-6 and IL-8 were significantly increased in patients with lung dysfunction, and local inflammatory factors spread into systemic circulation[109]. Under certain conditions, *Streptococcus gordonii* can attack host fibronectin, and subsequent cytokine production can induce inflammatory responses[110,111]. The cell-wall-anchored protein Staphylococcal surface protein A (SspA), a key factor in regulating the host innate immunity, is an immune-stimulatory component of *S. gordonii* that promotes bacterial adhesion, and purified SspA can induce IL-6 and monocyte chemotatic protein-1 production from human lung epithelial cells Prevotella from

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microaspiration, can also participate in the immune homeostasis of the respiratory tract[112, 113]. Lung inflammation has been associated with enrichment of the lung microbiome with *Prevotella*, this organism primarily activates toll-like receptor 2 and enhance the expression of inflammatory cytokines, including IL-23 and IL-1[114]. In vitro experiments have shown that *Prevotella* can stimulate the production of IL-8, IL-6, and CCL20 in lung epithelial cells, which promotes mucosal Th17 immune response and neutrophil recruitment[115]. Additional research has shown that Porphyromonas is likely to affect the immune response as well. Animal experiments suggested that *P. gingivalis* can produce Gingipain, which then affect the innate immune response and promote chronic inflammation[116]. Pathological manifestations such as intrapulmonary hemorrhage, necrosis, and neutrophil infiltration occur after infection with *P. gingivalis*. Lung tissue damage is associated with systemic inflammatory response, manifested by elevated levels of TNF, IL-6, IL-17, and C-reactive protein, and these pathological lesions are significantly dependent on the activity of *P. gingivalis*. Besides colonizing the lungs, themselves, DNA from oral microbes can be transmited from the gingival tissue to the pulmonary vasculature this might be a form of distant dissemination causing lung diseases[117]. T Cell Responses to Infections T cells are a type of lymphocyte which reside in the lungs and play a key role in protecting against chronic inflammation. Both the regulatory T cells (T-regs) and T helper cells (Th cells) are important defenders in the immune system. Previous research has shown that a large number of anaerobic bacteria originating from the oral cavity are present in the lungs of pulmonary tuberculosispatients, and bacterial metabolites (short-chainfatty acids) were associated with both the oral anaerobic bacterial load (e.g., Prevotella) and T-regsresponse[118]. A study including 1,099 patients with laboratory-confirmed COVID-19 found that the peripheral

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blood lymphocyte count in severe patients was 95.5% (147/154), which was significantly higher than that of mild cases[119]. It confirmed that the cellular immunity is closely related to the state of illness. In severe patients, CD4+ T and CD8+ T levels presented as low, indicating that T cell subsets can be used as one of the bases to predict the transition from mild to severe[108]. Based on anatomic reports, found that both the peripheral CD4+ T and CD8+ T counts of the COVID-19 patients were significantly reduced, but hyper-activated[120]. Additionally, there was an increase in highly pro-inflammatory CCR6+ Th17 in CD4+ T cells and a high concentration of cytotoxic granules in CD8+T-cells. These results imply that hyper-activation of T cells, manifesting as the increase of Th17 and high cytotoxicity of CD8+ T cells, led to the severe immune injury of the patient to some extent[120].

The Host Factors SARS-CoV-2 is more likely to affect middle-aged and elderly people, especially those with comorbidities, as a result of the weaker immune function soft hesepatients[121]. About 1/4∼1/2 patients infected by SARS-CoV-2 had chronic comorbidities, and the prognosis was strongly associated with the presence and number of comorbidities [121,122]. Malignancy and chronic obstructive pulmonary disease (COPD) appeared to be the main risk factors leading to poorer clinical outcomes, followed by diabetes and hypertension, according to a retrospective study of 1099 COVID-19 cases[122]. Therefore, immune disorders and long-term chronic inflammatory stimulation are likely to be key drivers to poor prognosis, however more mechanism studies are needed to confirm this. Aging Just like other chronic inflammatory conditions, it is crucial to bear in mind that the elderly, though perhaps seemingly healthy, might respond differently to infectious and inflammatory factors. Apart from the increased risk of severe disease, recovery could also be slower and poor, thus increasing the possibility of recrudescence or aggravation as time passes[123]. As human

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beings age, the niche that *Streptococcus pneumoniae* inhabit might change, resulting in a weakening of microbiome resiliency. This is related to the result of the degeneration of the immune system or a low-grade chronic inflammation[124]. The phenomenon of decreased microbial diversity, in conjunction with the presence in the oral cavity of various groups of anaerobic commensals negatively related to the overgrowth of potential lung infection pathogens, was also observed among adults[125]. This not only suggest that these anaerobic commensals play an important role in the level of resilience against pathogen overgrowth and disease, but also emphasizes that the chronic inflammatory process contributes to the changes in microbiota composition potentially making it harder to intervene. Systemic Diseases Alter Composition and Diversity of the Oral Microbiome Compared with healthy individuals, those with chronic diseases have a certain degree of micro-ecological disorder. Here, we will review several comorbidities closely related to COVID19 as examples to illustrate that changes in composition, quantity, and colonization sites may have potential effects on prognosis. Mounting evidence suggests that oral streptococci can be detected from the lungs of COPD patients with increased levels of *P. gingivalis* detected in the subgingival plaque[126,127]. The oral microbiota of cancer patients has also been found to be significantly altered. Veillonella, Streptococcus, Rothia and Aggregatibacter were dramatically increased in non-small cell lung cancer patients and, except for Prevotella, several common oral genera (i.e., *Haemophilus, Neisseria* and *Streptococcus*) decreased in individuals with colitis-associated cancer[128,129]. Moreover, it has been reported that the diversity of oral microbiota in patients with diabetes reduce the number of oral microbiota. Inhibit the biofilm formation of viridans streptococci and the adhesion, proinflammatory effects, and

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immune escape abilities of Candida albicans SARS-CoV, MERS-CoV and potentially SARS-CoV-2 sensitive[130,131].

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2.6. Immune Response to SARS CoV-2 Infection

Clinically, the immune responses induced by SARS-CoV-2 infection are two phased. During the incubation and non-severe stages, a specific adaptive immune response is required to eliminate the virus and to preclude disease progression to severe stages. Therefore, strategies to boost immune responses at this stage are certainly important. For the development of a protective immune response at the incubation and non-severe stages, the host should be in good general health and an appropriate genetic background (e.g. HLA) that elicits specific antiviral immunity[98]. Genetic differences are well-known to contribute to individual variations in the immune response to pathogens. However, when a protective immune response is impaired, virus will propagate and massive destruction of the affected tissues will occur, especially in organs that have high ACE2 expression, such as intestine and kidney. The damaged cells induce innate inflammation in the lungs that is largely mediated by pro-inflammatory macrophages and granulocytes. Lung inflammation is the main cause of life-threatening respiratory disorders at the severe stage[98].

The leading cause for mortality of patients with COVID-19 is respiratory failure from acute respiratory distress syndrome[132]. Secondary hemophagocytic lymphohistiocytosis (sHLH) is characterized by fatal increased cytokine in the blood with multi organ failure. sHLH, resembled by a cytokine profile, is associated with COVID-19 disease severity, characterized by increased interleukin (IL)-2, IL-7, interferon-inducible protein 10, granulocyte-colony stimulating factor, macrophage inflammatory protein 1-, and tumor necrosis factor- (TNF-)[133]. These cytokine release syndrome (CRS) seems to affect patients with severe conditions. Since lymphocytopenia is often seen in severe COVID-19 patients, the CRS caused by SARS-CoV-2 virus has to be mediated by leukocytes other than T cells, a high WBC-count is common, suggesting it, in association with lymphocytopenia,

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as a differential diagnostic criterion for COVID-19. Virally driven hyperinflammation due to raised/elevated IL-6 and ferritin has been recorded and has been associated to mortality in confirmed cases of COVID-19 and in any case, blocking IL-6 may also be effective, while Blocking IL-1 and TNF may also benefit patients[134].

2.7 Epidemiology of COVID-19 Pandemic

Several measures are commonly used to quantify mortality[135]. These numbers vary by region and over time and are influenced by the volume of testing, healthcare system quality, treatment options, time since the initial outbreak, and population characteristics such as age, sex, and overall health[136].

The mortality rate reflects the number of deaths within a specific demographic group divided by the population of that demographic group. Consequently, the mortality rate reflects the prevalence as well as the severity of the disease within a given population. Mortality rates are highly correlated to age, with relatively low rates for young people and relatively high rates among the elderly[137,138,139]. In fact, one relevant factor of mortality rates is the age structure of the countries' populations. For example, the case fatality rate for COVID- 19 is lower in India than in the US since India's younger population represents a larger percentage than in the US[140].

2.7.1 Fatality Rate of COVID-19

The case fatality rate (CFR) reflects the number of deaths divided by the number of diagnosed cases within a given time interval. Based on Johns Hopkins University statistics, the global death-to-case ratio is 1.49% (5,688,009/381,724,054) as of 2 February 2022. The number varies by region[141,142].

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2.7.2: Infection Fatality Rate of COVID-19

A key metric in gauging the severity of COVID- 19 is the infection fatality rate (IFR), also referred to as the infection fatality ratio or infection fatality risk[143,144,145]. This metric is calculated by dividing the total number of deaths from the disease by the total number of infected individuals; hence, in contrast to the CFR, the IFR incorporates asymptomatic and undiagnosed infections as well as reported cases[146].

A December 2020 systematic review and meta-analysis estimated that population IFR during the first wave of the pandemic was about 0.5% to 1% in many locations (including France, Netherlands, New Zealand, and Portugal), 1% to 2% in other locations (Australia, England, Lithuania, and Spain), and exceeded 2% in Italy[147]. That study also found that most of these differences in IFR reflected corresponding differences in the age composition of the population and age-specific infection rates; in particular, the metaregression estimate of IFR is very low for children and younger adults (e.g., 0.002% at age 10 and 0.01% at age 25) but increases progressively to 0.4% at age 55, 1.4% at age 65, 4.6% at age 75, and 15% at age 85[147]. These results were also highlighted in a December 2020 report issued by the WHO[148].

IFR estimate per age group (to December 2020)[147]

Age group	**IFR**
0–34	0.004%
35–44	0.068%
45–54	0.23%
55–64	0.75%
65–74	2.5%
75–84	8.5%
85 +	28.3%

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An analysis of those IFR rates indicates that COVID-19 is hazardous not only for the elderly but also for middle-aged adults, for whom the infection fatality rate of COVID-19 is two orders of magnitude greater than the annualized risk of a fatal automobile accident and far more dangerous than seasonal influenza[147].

2.7.3: Earlier Estimates of IFR

At an early stage of the pandemic, the World Health Organization reported estimates of IFR between 0.3% and 1%[149,150]. On 2 July, The WHO's chief scientist reported that the average IFR estimate presented at a two-day WHO expert forum was about 0.6%[151,152]. In August, the WHO found that studies incorporating data from broad serology testing in Europe showed IFR estimates converging at approximately 0.5–1%[153]. Firm lower limits of IFRs have been established in a number of locations such as New York City and Bergamo in Italy since the IFR cannot be less than the population fatality rate. (After sufficient time however, people can get reinfected) [154]. As of 10 July, in New York City, with a population of 8.4 million, 23,377 individuals (18,758 confirmed and 4,619 probable) have died with COVID-19 (0.3% of the population) [155]. Antibody testing in New York City suggested an IFR of ~0.9% and ~1.4%[156,157]. In Bergamo province, 0.6% of the population has died[158]. In September 2020, the U.S. Centers for Disease Control and Prevention (CDC) reported preliminary estimates of age-specific IFRs for public health planning purposes[159].

2.8: Sex Differences and Impact of the COVID-19 Pandemic

COVID-19 case fatality rates are higher among men than women in most countries. However, in a few countries like India, Nepal, Vietnam, and Slovenia the fatality cases are higher in women than men[140]. Globally, men are more likely to be admitted to the ICU and more likely to die[160,161]. One meta-analysis found that globally, men were more likely to get

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COVID- 19 than women; there were approximately 55 men and 45 women per 100 infections (CI: 51.43–56.58) [162].

The Chinese Center for Disease Control and Prevention reported the death rate was 2.8% for men and 1.7% for women[162]. Later reviews in June 2020 indicated that there is no significant difference in susceptibility or in CFR between genders[164, 165]. One review acknowledges the different mortality rates in Chinese men, suggesting that it may be attributable to lifestyle choices such as smoking and drinking alcohol rather than genetic factors[166]. Smoking, which in some countries like China is mainly a male activity, is a habit that contributes to increasing significantly the case fatality rates among men. Sex-based immunological differences, lesser prevalence of smoking in women and men developing co-morbid conditions such as hypertension at a younger age than women could have contributed to the higher mortality in men[168]. In Europe as of February 2020, 57% of the infected people were men and 72% of those died with COVID- 19 were men[169]. As of April 2020, the US government is not tracking sex-related data of COVID- 19 infections[169]. Research has shown that viral illnesses like Ebola, HIV, influenza and SARS affect men and women differently[169].

2.9: Ethnic Differences and COVID-19

In the US, a greater proportion of deaths due to COVID- 19 have occurred among African Americans and other minority groups[170]. Structural factors that prevent them from practicing social distancing include their concentration in crowded substandard housing and in "essential" occupations such as retail grocery workers, public transit employees, health-care workers and custodial staff. Greater prevalence of lacking health insurance and care of underlying conditions such as diabetes, hypertension, and heart disease also increase their

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risk of death[171]. Similar issues affect Native American and Latino communities[170]. On the one hand, in the Dominican Republic there is a clear example of both gender and ethnic inequality. In this Latin American territory, there is great inequality and precariousness that especially affects Dominican women, with greater emphasis on those of Haitian descent[172]. According to a US health policy non-profit, 34% of American Indian and Alaska Native People (AIAN) non-elderly adults are at risk of serious illness compared to 21% of white non-elderly adults[173]. The source attributes it to disproportionately high rates of many health conditions that may put them at higher risk as well as living conditions like lack of access to clean water[174].

2.10 Comorbidities and COVID-19

Biological factors (immune response) and the general behaviour (habits) can strongly determine the consequences of COVID-19[140]. Most of those who die of COVID-19 have pre-existing (underlying) conditions, including hypertension, diabetes mellitus, and cardiovascular disease[175]. According to March data from the United States, 89% of those hospitalised had preexisting conditions[176]. The Italian Istituto Superiore di Sanità reported that out of 8.8% of deaths where medical charts were available, 96.1% of people had at least one comorbidity with the average person having 3.4 diseases. According to this report the most common comorbidities are hypertension (66% of deaths), type 2 diabetes (29.8% of deaths), Ischemic Heart Disease (27.6% of deaths), atrial fibrillation (23.1% of deaths) and chronic renal failure (20.2% of deaths).

Most critical respiratory comorbidities according to the Centers for Disease Control and Prevention (CDC), are: moderate or severe asthma, pre-existing COPD, pulmonary fibrosis,

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cystic fibrosis[177]. Evidence stemming from meta-analysis of several smaller research papers also suggests that smoking can be associated with worse outcomes[178,179]. When someone with existing respiratory problems is infected with COVID- 19, they might be at greater risk for severe symptoms[180]. COVID- 19 also poses a greater risk to people who misuse opioids and methamphetamines, insofar as their drug use may have caused lung damage[181]. In August 2020, the CDC issued a caution that tuberculosis (TB) infections could increase the risk of severe illness or death. The WHO recommended that people with respiratory symptoms be screened for both diseases, as testing positive for COVID- 19 could not rule out co-infections. Some projections have estimated that reduced TB detection due to the pandemic could result in 6.3 million additional TB cases and 1.4 million TB-related deaths by 2025[182].

2.11 Infections Associated with the 2020 COVID-19 Pandemic

Data regarding secondary respiratory infections in the severe disease caused by the SARS-CoV-2 coronavirus (COVID-19) are limited due to the still ongoing spread of the disease worldwide. However, some reports showed that secondary infections significantly decreased survival of COVID-19 patients, particularly when they were admitted to the Intensive Care Units (ICU). In the studies involving 41 and 191 COVID-19 patients, respectively, performed in Wuhan, China, secondary infections were observed in 10% and 15%, respectively, of patients, with 31% of them requiring mechanical ventilation in ICU care and 0% in no-ICU care[183,184]. A secondary infection was reported in 50% of non-survivors and only 1% of survivors [184]. Respiratory specimens (nasal and pharyngeal swabs, sputum, bronchoalveolar lavages, bronchial aspirates) and blood were tested for routine bacterial and fungal examinations and for common respiratory viruses and COVID-19 virus, using real-

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time PCR or next-generation sequencing (NGS) methods. Secondary infections were diagnosed when patients showed clinical symptoms or signs of pneumonia or bacteremia, and had a positive culture of a new pathogen[183]. In another study which reported that in 221 COVID-19 patients in Wuhan, those with severe illness were 14.2, 18.2 and 2.9 times more likely to have coinfections with bacteria, fungi and other viruses, respectively, than those not severely ill[185]. Furthermore, deaths associated with coinfections by bacteria, fungi and other viruses occurred in 55.6, 44.4 and 44.4%, respectively, of patients in the ICU, and in 26.1, 13.0 and 8.7%, respectively, of patients transferred from ICU to the general wards. In COVID-19 patients coinfected with bacteria in the ICU-death group, carbapenemresistant Acinetobacter baumannii was isolated. This nosocomial, antibiotic-resistant pathogen is known to pose challenges in antibiotic therapy, and to increase the death-risk [185].

2.12 Microorganisms Involved in COVID-19 Co-infections

The species of the microorganisms identified in COVID-19 positive specimens are reported to show that viral coinfections, including mainly influenza virus and rhinovirus/enterovirus, occurred in 17.2% of patients (126/733), while bacterial coinfections due to both Gram-positive and Gram-negative species and Mycoplasma pneumoniae occurred in 11.7% (86/733) patients, and fungal coinfections in 1.8% (13/733) patients. The bacterial species more frequently isolated were, in ranking order, *M. pneumoniae, S. aureus, Legionella pneumophila, Haemophilus spp., Klebsiella spp., Pseudomonas aeruginosa, Chlamydia spp., S. pneumoniae, A. baumannii.* Patients in the ICU were 522/733 and 1.3% of them (7/522) developed nosocomial super-infections with antibiotic-resistant *S. aureus, Klebsiella pneumoniae, P. aeruginosa, or A. baumannii.* Apparently, no antibiotic resistant strains were isolated outside the ICU[186,187,188,102]. Another study also reported bacteremia by clinical

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pathogens in 21/643 blood cultures (3.3%) from COVID-19 patients, with respiratory sources being confirmed in two cases (a community acquired *K. pneumoniae* and a ventilator associated *Enterobacter cloacae*) [189]. All other bacteremias were attributed to non-respiratory sources. No pneumococcal, legionella or influenza infections were detected. Overall, bacterial infections reported in COVID-19 patients were less frequent and different from those causing lower respiratory tract infections in influenza pandemics, with *S. pneumoniae* being rarely isolated[190]. It must be noted that bacteria were mainly cultured from nasopharyngeal samples, while lower respiratory samples were less available also due to safety concern for performing bronchoalveolar lavage[190]. Bacterial diagnosis was performed by routine methods, and by multiplex PCR kits for rapid detection of a wide range of respiratory pathogens, mostly viruses. Thus, coinfections by bacterial species not included in multiplex PCR kits or not searched during emergency, could have been under-estimated, so as to undervalue their contribution in COVID-19 severity and mortality[191]. Early and rapid diagnosis and drug susceptibility testing of mixed bacterial infections by culture-independent approaches such as, for instance, NGS methods and Nanopore metagenomics could better guide or adjust antibiotic therapy so as to prevent fatal outcomes, particularly in case of Multi Drug Resistant (MDR) bacteria[192]. Rapid detection of bacterial infections may also limit development of virus super-spreaders, defined as patients infecting ≥ 10 persons each. For instance, in Singapore, during the SARS-CoV outbreak, two patients hospitalized with bacterial infections were co-infected with SARS-CoV, and caused 76% of SARS-CoV infections in a healthcare facility[192]. Thus, to contain current COVID-19 pandemic it is important to triage and isolate patients with known bacterial infections in

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designated wards, and to apply efficient infection control measures, in order to limit virus super-spreading[192].

2.13 Bacterial Co-Infection with Viral Respiratory Infections

Viral pneumonia and lower respiratory tract infections are well characterized in adult patients, including those diagnosed with severe forms of viral infection [193]. Most viral lower respiratory tract infections seem to be acquired in the community and considered a leading cause of infection in patients who undergo mechanical ventilation. The most common cases diagnosed with bacterial co-infection with viral infections are seen in those infected with influenza virus. The oldest report says bacterial infections that occurred simultaneously or shortly after influenza is related to the 1918 Influenza pandemic, in which most deaths occurred as a result of co-infection with infectious bacteria. Also, the H1N1 Influenza pandemic reported was also complicated by bacterial pneumonia in 4–33% of hospitalized patients [194,195,196]. Bacterial-viral co-infection is not restricted to influenza and also caused by other respiratory viruses, such as parainfluenza virus, respiratory syncytial virus, adenovirus, rhinovirus, and human metapneumovirus [197,198,193]. Despite the discovery of antibiotics and viral vaccines in 1918–1957, the mortality rate, resulting from secondary bacterial pneumonia remained a major problem and reveals that mortality rate seems to be still growing mostly because of the rapid rate of aging in the human population[199,200]. Although, viruses are commonly responsible for the development of acute upper and lower respiratory infections, in most cases patients may be infected by both bacterial and viral pathogens; however, the clinical manifestations at the early stages of the disease would not be nosologically distinguishable for physicians to differentially diagnose viral from a bacterial infection [201]. Recently, a group of respiratory emerging viruses has been identified, such as

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human coronavirus (HCoV), NL63, human bocavirus, influenza viruses' type H1N1 and H5N1, SARS, Middle East Respiratory Syndrome-related coronavirus (MERS), and COVID-19[199]. In children, atypical bacterial pathogens, such as *Legionella pneumophila, Mycoplasma pneumoniae,* and *Chlamydophila pneumoniae* include the majority of infectious agents that cause mild, moderate, or even severe forms of acute respiratory infections[199].

Bacterial co-infections with respiratory viral pathogens are very common, often through synergistic interaction among viruses such as influenza virus, and bacterial pathogens and the host immune system of the human being; nevertheless, the interaction between viruses and unusual bacteria is not yet fully understood. These secondary infections predominantly involve a specific group of bacterial pathogens, such as *S. aureus, S. pneumoniae, S. pyogenes*, and *H. influenzae*[193]. A complete list of bacterial co-infections with viral pathogens is depicted insyncytial virus stimulated the expression of intercellular adhesion molecule 1 (ICAM-1) by primary respiratory tract epithelial cells. ICAM1 acts as a receptor for Type 4 pilus (T4P) of non-typeable *H. influenzae* (NTHI), thus promoting the binding of this pathogen to cells expressing this molecule[193].

2.14 Viral Predisposition to Bacterial Co-Infection in The Respiratory Tract

Commonly, viral infection can destroy histologically and functionally the respiratory tract of individuals upon viral spread[202]. Depending on the type of the virus, the histopathological outcomes could be relatively different from mild types to severe ones. These detrimental changes include altered mucus secretion, cell death, hyperplasia, decreased mucosal clearance, reduced oxygen exchange, and impaired surfactant secretion[202]. Each of these effects is caused by various molecular mechanisms, depending on the virus, bacterial species,

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as well as the degree of the host immune reaction to either a bacterium or virus[202]. It has been noted that viral infections promote bacterial colonization of the airway through a variety of mechanisms and found that influenza viruses can enhance the colonization of the nasopharynx by S. pneumoniae bacterium, however, only particular subtypes were found to mediate the development of bacterial otitis media and sinusitis[202,203]. These data explain why the rate of bacterial infection is high in influenza seasons[202]. The neuraminidase enzyme of the influenza virus has been found to be presented on the host cell receptors, and they are employed for the adherence of bacteria due to its sialidase ability that changes the carbohydrate moieties on the host epithelial cells. This enzyme is also capable of increasing the possibility of bacterial adherence to the host cells through the stimulation of transforming growth factor-beta (TGF-β) which triggers the up-regulation of integrin and fibronectin. Integrin and fibronectin have been shown to act as receptors for bacteria[204]. Also, it has been found that the influenza virus predisposes the host to develop pneumonia caused by S. aureus where viral and bacterial loads are increased during co-infection[205]. It has been hypothesized that viral load is increased following bacterial coinfection because of increased shedding rate of the virus from infected host cells; however, bacterial loads would be elevated as a result of impaired function of alveolar macrophages[206]. Additionally, other upper respiratory tract viruses increase the adherence ability of bacterial pathogens to primary and immortalized epithelial cells with particular differences, such differences are determined by the types of epithelial cells and their response to parainfluenza virus-3, respiratory syncytial virus, and/or influenza virus[206]. It was shown that Adenovirus and respiratory syncytial virus stimulates the expression of intercellular adhesion molecule 1 (ICAM-1) by primary respiratory tract epithelial cells. ICAM1 acts as a receptor for Type 4

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pilus (T4P) of non-typeable *H. influenzae* (NTHI), thus promoting the binding of this pathogen to cells expressing this molecule[207,208]. Also, respiratory syncytial virus infection increases the binding ability of *P. aeruginosa* to normal epithelial cells, as well as cells affected by cystic fibrosis. Such phenomena have been frequently employed by other bacteria to increase their virulence to infect the cells.

2.15 Bacterial and Fungi Infections

A limited number of bacterial species are responsible for the majority of infectious diseases in healthy individuals. Due to the success of vaccination, antibiotics, and effective public health measures, until recently, epidemics were felt to be a thing of the past. Due to the development of antibiotic resistant organisms, this situation is changing rapidly[209]. All humans are infected with bacteria (the normal flora) living on their external surfaces (including the skin, gut and lungs). We are constantly also exposed to bacteria (including air, water, soil and food). Normally due to our host defenses most of these bacteria are harmless. In compromised patients, whose defenses are weakened, these bacteria often cause opportunistic infectious diseases when entering the bloodstream (after surgery, catheterization or other treatment modalities). When initiated in the hospital, these infectious diseases are referred to as nosocomial. Some common bacteria found in the normal flora include *Staphylococcus aureus, S. epidermidis* and Propionibacteriumacnes (found on the skin) and Bacteroides and Enterobacteriaceae found in the intestine (the latter in much smaller numbers). In order to be spread, a sufficient number of organisms must survive in the environment and reach a susceptible host. Many bacteria have adapted to survive in

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water, soil, food, and elsewhere. Some infect vectors such as animals or insects before being transmitted to another human[209].

New species and new variants of familiar species continue to be discovered, particularly as we intrude into new ecosystems. Both Lyme disease and Legionnaire's disease, now well-known to health-care professionals, were discovered as recently as the 1970s. The recent increased prevalence of highly immunosuppressed individuals was both due to AIDS and the increasing use of immunosuppressive drugs as chemotherapy and for transplantation of organs, tissues, and cells, has led to a population of patients highly susceptible to types of bacterial infections that were comparatively rare before[209]. Several factors lead to the development of bacterial infection and disease. First, the infectivity of an organism determines the number of individuals that will be infected compared to the number who are susceptible and exposed. Second, the pathogenicity is a measure of the potential for an infectious organism to cause disease. Pathogenic bacteria possess characteristics that allow them to evade the body's protective mechanisms and use its resources, causing disease. Finally, virulence describes the organism's propensity to cause disease, through properties such as invasiveness and the production of toxins. Host factors are critical in determining whether disease will develop following transmission of a bacterial agent. These factors include genetic makeup, nutritional status, age, duration of exposure to the organism, and coexisting illnesses. The environment also plays a role in host susceptibility. Air pollution as well as chemicals and contaminants in the environment weaken the body's defenses against bacterial infection[210]. Bacterial pathogens can be classified into two broad groups, primary and opportunistic pathogens.

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2.15.1 Primary pathogens: are capable of establishing infection and causing disease in previously healthy individuals with intact immunological defenses. However, these bacteria may more readily cause disease in individuals with impaired defenses.

2.15.2 Opportunistic pathogens rarely cause disease in individuals with intact immunological and anatomical defenses, only when such defenses are impaired or compromised, as a result of congenital or acquired disease or by the use of immunosuppressive therapy or surgical techniques, are these bacteria able to cause disease. Many opportunistic pathogens, e.g. coagulase negative *staphylococcus* and *Escherichia coli*, are part of the normal human flora and are carried on the skin or mucosal surfaces where they cause no harm and may actually have beneficial effects, by preventing colonization by other potential pathogens. However, introduction of these organisms into anatomical sites in which they are not normally found, or removal of competing bacteria by the use of broad-spectrum antibiotics, may allow their localized multiplication and subsequent development of disease[209].

Bacteria are prokaryotic organisms that carry their genetic information in a double-stranded circular molecule of DNA. Some species also contain small circular plasmids of additional DNA. The cell cytoplasm contains ribosomes and there is both a cell membrane and, in all species except Mycoplasma, a complex cell wall. External to the cell wall, some bacteria have capsules, flagella, or pili. Bacteria normally reproduce by binary fission. Under the proper conditions, some bacteria can divide and multiply rapidly. Consequently, some infections require only a small number of organisms to cause potentially overwhelming infection. Bacteria are classified as Gram-positive or Gram negative based on the characteristics of their cell wall, as seen under a microscope after stains have been

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administered, a procedure called Gram staining, that was developed in 1882 by Hans Christian Gram

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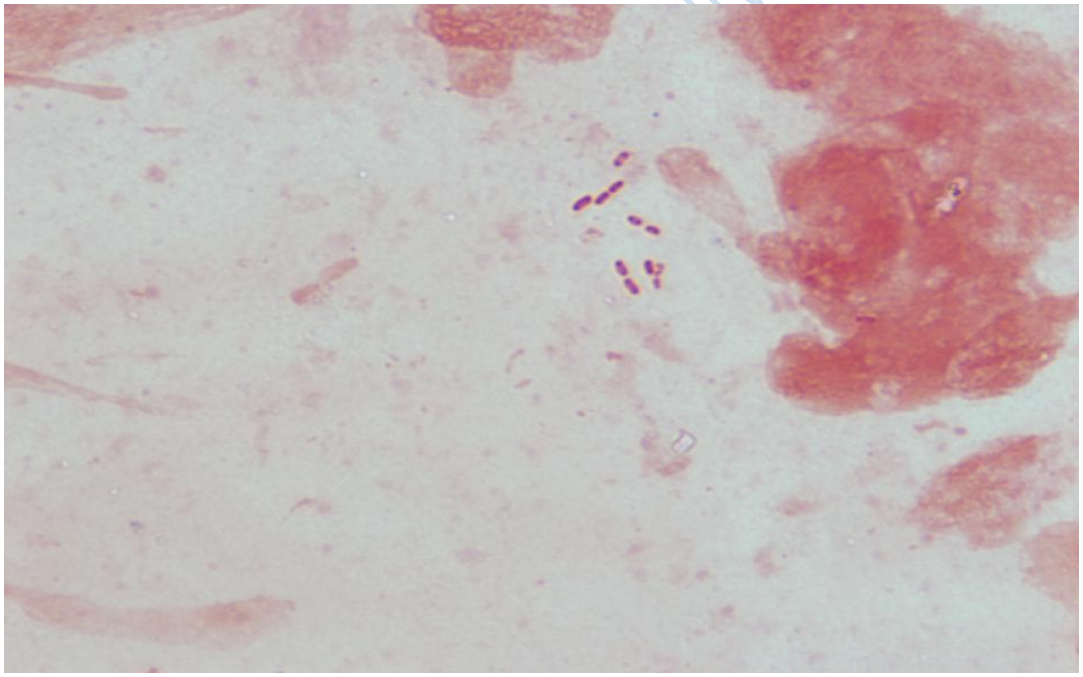

Fig 2.2: Gram stains of Gram Negative Bacteria[28]

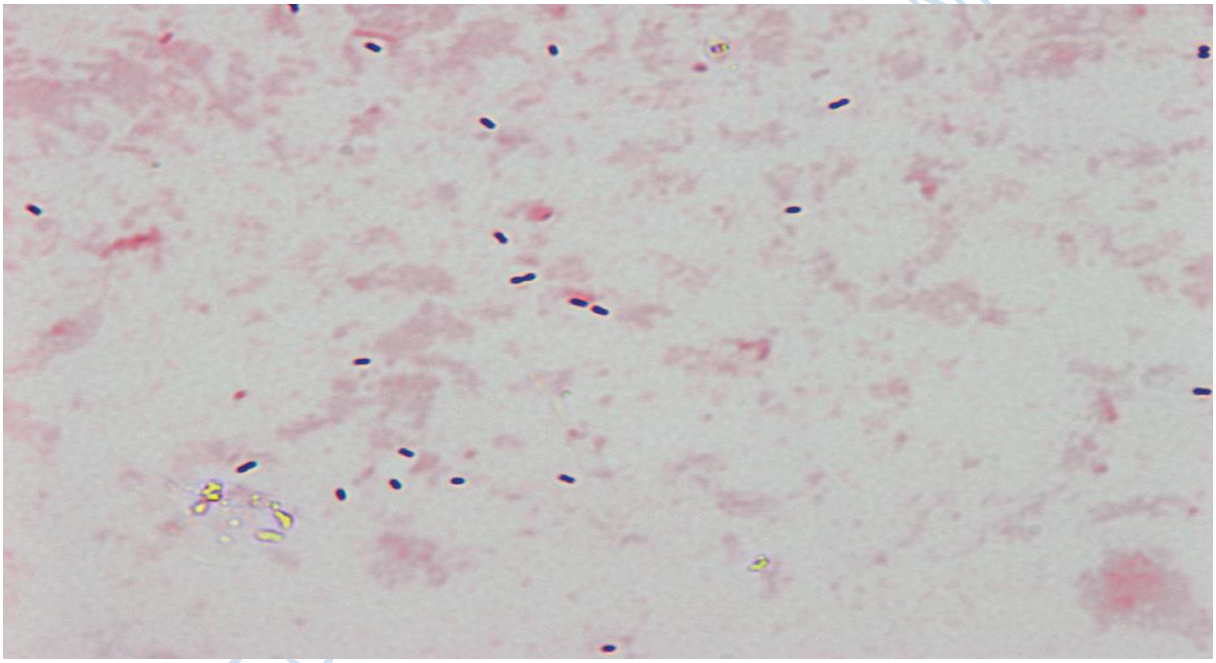

Figure 2.3: Gram stains of Gram-Positive Bacteria[28]

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Most, but not all, bacteria fall into one of these two categories. Clinically, one of the main differences between gram-positive and gram-negative organisms is that gram negative bacteria tend to produce an endotoxin that can cause tissue destruction, shock, and death. The two classes of bacteria differ in their antibiotic susceptibilities as well. Bacteria can also be classified based on their growth responses in the presence and absence of oxygen. Aerobic bacteria, or aerobes, grow in the presence of oxygen. Obligate aerobes such as Bordetella pertussis require oxygen. Facultative organisms can grow in the presence or absence of oxygen. Anaerobic bacteria such as the Clostridia are able to grow in the absence of oxygen and obligate anaerobes require its absence.

Fungi on the other hand are eukaryotic microorganisms that have a cellular wall and do not photosynthesize. They parasitize organisms or exist as spores. In superficial mycoses, fungi einvade keratinized tissue such as the horny cell layer, hair and nails. In deep fungal infection, fungi tend to parasitize the dermis and deeper layers. The frequency and variety of invasive fungal infections have increased greatly over the last three decades as a consequence of changes in medical and surgical care, particularly in intensive care units which utilize invasive catheters for monitoring, coupled with the use of more potent

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immunosuppression and antibiotic agents[211]. Life-threatening fungal infections that invade the blood, and other organs pose a serious risk to millions of immunocompromised people, such as those living with HIV/ AIDS, or receiving chemotherapy. (Despite available antifungal drugs, invasive fungal infections are associated with high mortality rates worldwide, causing an estimated 1.5 million deaths each year, a number comparable to tuberculosis. Fungi are remarkable organisms and they constitute a separate kingdom for purposes of classification. Fungi are eukaryotes; they have a membrane surrounding their nucleus, their cells are much larger than bacteria and their molecular processes closely resemble those of plants and animals. However, unlike mammalian cells, fungi almost always possess a rigid cell wall composed of chitin products that surrounds their plasma membrane[211].

A fungus is a vegetative organism and is definitely not a plant either because fungi do not synthesize chlorophyll. It is non-motile life form and its basic structural unit consists of either a chain of cylindrical cells (hyphae) or a unicellular form, or both. The most common species like Aspergillus and Candida are found everywhere on earth. Gardens, playgrounds, houses, hotels, hospitals and even the skin and mucous membranes have been identified as sources of fungi that caused life-threatening infections. Fungi, like all living things, are recognized and identified on the basis of their shapes, structures and their behavioral properties. Fungi that exist predominantly in the form of independent single cells are usually called yeasts while those based on hyphal threads are called moulds (i.e. hyphal fungi). Hyphae and yeast are nearly always microscopic cell forms. Only a few of the fungi pathogenic for humans are sufficiently virulent to infect a healthy host. Most are relatively harmless unless they encounter an immunocompromised patient, in whom a weakened

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defence system permits them to invade the body. Under normal circumstances, the intact epithelial surfaces of the gastrointestinal tract will prohibit invasion by micro-organisms and the mucociliary barrier of the respiratory tract prevents aspiration of fungal cells and spores, while, in contrast, dead or damaged tissue may turn into a breeding ground for infection. For these reasons invasive fungal infections have to be ranked amongst the typically opportunistic infections[212]. It has been hypothesized that the susceptibility to invasive fungal infections is influenced by genetic variation within key innate or adaptive immune response genes that may lead to a failure in the IL-10 production, Toll-like receptor polymorphism, plasminogen gene polymorphism. Virtually all human fungal infections originate from the environment through skin contact with or without trauma, or via inhalation or ingestion of fungal spores. Candida, the prototype of a colonizing fungus that may stay unnoticed on the surfaces of a body has become the most common genus of fungal pathogens and remains very menacing[213]. Candida species was described as a normal part of the microflora of the oropharynx and the gut which explains why alterations in the equilibrium between the indigenous organisms by the use of broad-spectrum anti-bacteria can lead to overgrowth of Candida species.

The fact that the gut is a major source of disseminated candidosis is increasingly acknowledged. Aspergillus species, notably Aspergillus fumigatus, are undoubtedly prominent and deadly fungal pathogens. Aspergillus species are airborne pathogens that pass through the nose, may penetrate into the paranasal sinuses and will eventually land in the lower respiratory system that constitutes the major entry and in the last decades an obvious change in pathogens has occurred[214,215]. More aggressive treatment modalities can jeopardize the defense mechanisms to an extent that even fungi with a low intrinsic

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virulence enter the body to cause serious disease. Organisms that were previously considered harmless commensals have been held accountable for serious invasive fungal disease[214,215]. Non- Aspergillus moulds like Zygomycetes can generate a clinical picture indistinguishable from Aspergillus. Fusarium, a soil fungus, can enter the body through the respiratory system or via severe onychomycosis but has also been connected with venous access devices, its prevalence being clearly higher in patients who carry a central venous line[216].

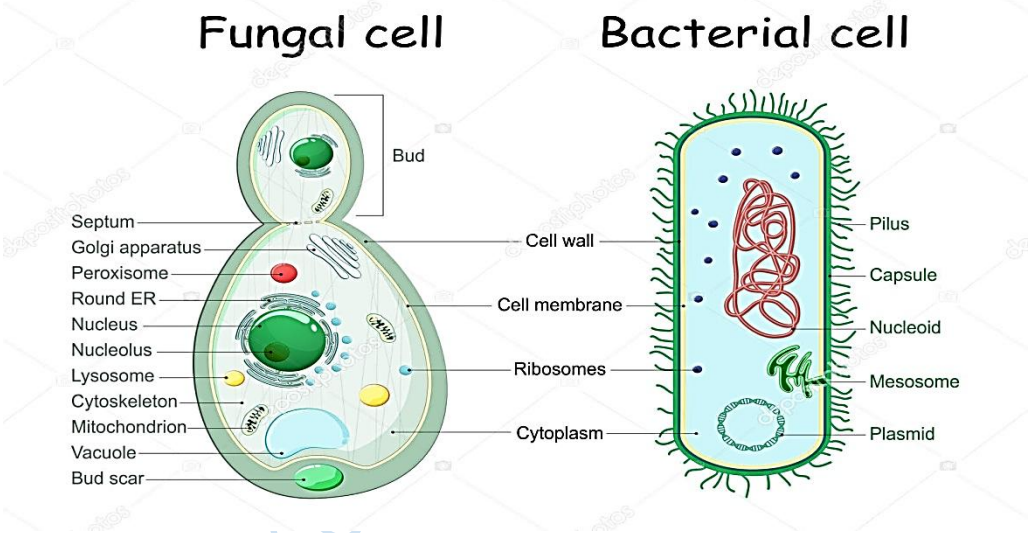

Figure 2.4: Fungal and Bacterial cell[28]

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2.16: Bacterial Co-Infection in SARS-CoV-2 Patients.

The recent community-acquired pneumonia (CAP) guidelines by the American Thoracic Society (ATS) and Infectious Diseases Society of America (IDSA) recommended initial antibacterial treatment for adults with CAP who test positive for influenza, because bacterial co-infections are a common and serious complication of influenza and it is difficult to exclude the presence of bacterial co-infection in a patient with CAP who tested positive for influenza virus[217]. Also, respiratory viral infections predispose patients to co-infections and these lead to increased disease severity and mortality[194]. Most fatalities in the 1918 influenza outbreak were due to subsequent bacterial infection, particularly with Streptococcus pneumonia. Similarly, it has been argued that poor outcomes in the 2009 H1N1 influenza pandemic were also associated with bacterial co-infections, although few studies captured these data[218]. Report from previous studies on severe coronavirus infections, serological evidence among SARS patients indicated incidences of acute or recent Chlamydophila pneumonia (30%) or Mycoplasma pneumoniae (9%) infection, respectively[219]. Furthermore, SARS and human meta-pneumovirus co-infection were reported during a major nosocomial

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SARS outbreak in Hong Kong[220]. Moreover, it was explained and reportedndthe possibility of co-infection of the Middle East respiratory syndrome coronavirus (MERS-CoV) with influenza and tuberculosis[221]. In addition, a multicenter retrospective cohort study of critically ill patients with MERS-CoV demonstrated that 18% and 5% had bacterial and viral co-infections, respectively[104]. In the current coronavirus disease 2019 (COVID-19) pandemic, 50% of patients with COVID-19 who have died had secondary bacterial infections[184]. Similarly, both bacterial and fungal co-infections were also reported[221]. Although 71% of the admitted patients with COVID-19 received antibiotic drugs, no information is available on the antimicrobial sensitivities of the organisms that were identified, or on the type and duration of antimicrobial treatment[221]. In a retrospective study conducted among critically ill SARS COV 2 infected patients to determine secondary bacterial infections it was found that 5 patients out of 101 had SARS-CoV-2 pneumonia complicated with secondary bacterial infection[222]. The bacteria identified were gram-negative nonfermenting bacilli, including B cepacia, S maltophilia, and P China aeruginosa[223].

During a multi-center study in Eastern nasopharyngeal swab samples were collected for total RNA extraction, followed by multiplex PCR and mNGS analysis. According to the results, 11 of 20 co-infections were identified in laboratory-confirmed COVID-19 cases and included regular respiratory viruses, fungi and bacteria[224]. The most common pathogens detected were *Acinetobacter baumannii, Klebsiella pneumoniae, Aspergillus flavus, Candida glabrata*, and *Candida albicans*. In another study where nucleic acids from the BALF of 5 hospitalized patients was extracted for deep sequencing, the results showed that apart from SARS-CoVs, some bacterial pathogens were also detected, including

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Acinetobacter, Pseudomonas, Escherichia, Streptococcus, and *Lactococcus*[224]. It was also reported that *Capnocytophaga* and *Veillonella* were found in the BALF of COVID-19 patients by mNGS[225]. A recent study revealed coinfection with *Moraxella catarrhalis* in laboratory-proven cases of SARS-CoV-2, and quantified increased bacterial burden in some patients over others[226]. Most of above mentioned microbiota can be found in human oral cavity with the elevated level of oral commensal bacteria were found from the BALF of COVID-19 patients it's worth noting that the co-infection occurs between oral microbiota and SARS-CoV-2 in the patients' lungs[227,228]. Recently it was discovered that the intestinal bacterial diversity of patients with COVID-19 is significantly reduced, the relative abundance of opportunistic pathogens such as *Streptococcus, Rothia, Veillonella*, and *Actinomyces* are significantly higher, while the relative of beneficial symbionts abundance, such as *Blautia, Romboutsia, Collinsella*, and *Bifidobacterium*, is lower[229]. In most individuals, SARS-CoV-2 infection is mild, while coinfection can increase the susceptibility of patients to severe disease by affecting the body's immune function, SARS-CoV-2 may enhance colonization and attachment of bacteria to host tissue, and the combined infections may result in increased tissue destruction and pathophysiology[230]. Airwaydysfunction, cytopathology and tissue destruction induced by SARS-CoV-2 infection or duringbacterial co-infection may facilitate systemic dissemination of the virus and/or bacterial co-pathogens, dramatically increasing the risk of blood infections and sepsis[230]. Virus-mediated enhancement of bacterial infection is not unprecedented. Rhinovirus and influenza virus infections increase the invasion of the airway epithelium by respiratory pathogens[231]. Bacterial infections result in dampening of the activation of host defence signaling which may result in increasing susceptibility to SARS-CoV-2 infection and subsequent

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pathology[232]. For example, respiratory pathogens including *Klebsiella pneumoniae* limit the activation of NF-κ B governed responses, which are also part of the host antiviral programme. Additionally, type I and III IFNs produced following bacterial infection may facilitate SARS-CoV-2 infection because the ACE2 receptor used by the virus is an IFN stimulated gene[233]. Although whether IFN-mediated ACE2 up-regulation results in enhanced virus entry and infection is still unknown[233].

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Table 2.1: Common Respiratory Viral-Bacterial coinfections and their associated clinical infections in human

Viral infection	Bacterial coinfection	Clinical infection
Influenza	*Staphylococcus aureus*, MRSA	Community-acquired pneumonia[234]
	Streptococcus pneumonia	Pneumococcal pneumonia, sepsis, meningitis, otitis media[235]
	Streptococcus pyogenes (group A) Streptococci	Sepsis, pleural empyema[236]
	Haemophilus influenza	Pneumonia[237]
	Moraxella catarrhalis	Pneumonia and bacteremia[238]
	Neisseria meningitides	Meningococcemia[239]
	Chlamydophila pneumonia	Pneumonia[240]
	Mycoplasma pneumonia	Pneumonia[240]
	Legionella pneumophila, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii, Burkholderia cepacia, Enterobacter aerogenes	Pneumonia[241]
Metapneumovirus	*Haemophilus influenzae, enterococcus* spp, *N. meningitidis* group B, *Brucella* spp, *Streptococcus pyogenes, Streptococcus pneumonia*	Acute otitis media, pneumonia[242]

Viral infection	Bacterial coinfection	Clinical infection
Respiratory syncytial virus	*Pseudomonas aeruginosa*	Respiratory infections in cystic fibrosis patients[243]
Adenovirus	Non-typeable *Haemophilus influenzae, Chlamydia trachomatis*	Pneumonia or acute otitis media[244]
Parainfluenza	*Streptococcus pneumonia, Streptococcus agalactiae, Haemophilus influenza*	Acute otitis media, pneumonia[245]
Rhinovirus	*Streptococcus pneumoniae, Mycoplasma*	Pneumonia[246]
	Staphylococcus aureus	Respiratory complications[247]
SARS	*Chlamydophila pneumonia Mycoplasma pneumonia*	Pneumonia[248]
	MRSA	Pneumonia[249]
MERS	*Mycobacterium tuberculosis*	Immune suppression and augment the infection of each other[250]
	Mycoplasma spp. *Legionella* *Chlamydia* spp.	Not reported[251]

Table 2.2: The Potential Mechanisms Responsible for the Bacterial coinfection with Viral Respiratory infections

	Description
Elevation in bacterial adherence due to viral infection	Virus can modulate surface membrane receptors, thereby enhancing bacterial adhesion[252]
Cell destruction by viral enzymes	Viral enzymes destroy mucosal glycoproteins, mainly those inhibiting bacterial attachment[253]
Reduction of mucociliary clearance	Virus can reduce mucociliary clearance leading to the decreased production of bactericidal materials[254]
Reduction in chemotaxis	Virus can decrease the chemotactic factors, leading to the reduced cell response to attacking organisms[255]

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	Description
Direct effect on phagocytic and induction of post phagocytic alveolar macrophage functions	Virus hinders or modifies a number of immune functions, such as phagosome-lysosome fusion and intracellular killing[255]
Induction of immature phagocytes	Virus can disrupt macrophages and probably replace them with immature phagocytes[256]
Reduction of surfactant levels	Virus impairs the function of alveolar type-2 pneumocyte[257]
Induction of dysbiosis in lower respiratory tract microbiome	Microbiome dysbiosis can affect the immune response against respiratory viral infection[258]
Dysregulation of the innate and adaptive immune responses	Virus decreases the number of alveolar macrophages through the development of apoptosis[259]
Modulation of apoptosis and inflammation	Autophagy and apoptosis facilitates secondary bacterial pneumonia after viral infection[260]
Reduction of antibacterial immune function at the respiratory epithelium	Respiratory viral infection leads to the predisposition to secondary bacterial infection via the deviation of the respiratory tract immune status[261]
Dysregulation of nutritional immunity	Some viruses can subvert nutritional protection to promote bacterial infection[262]
Immunosuppression	Immunosuppression is induced by several viruses such as HIV[263]
Synergism during viral/bacterial co-infections	Both viruses and bacteria play a role in the immunopathogenicity of co-infection[264]
Release of planktonic bacteria from biofilms	Viruses can manipulate many factors such as chemokines and hydrogen peroxide, thereby leading to the disruption of biofilm structure[265]

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Table 2.3: List of Bacterial Co-infection with COVID-19

Bacterium	Infection
Staphylococcus aureus	Necrotizing pneumonia[266]
Mycoplasma pneumonia	Exacerbate clinical symptoms, increase morbidity and prolonged intensive care unit stay[267]
Legionella pneumophila	Pneumonia[268]
Enterobacter cloacae	Pneumonia[269]
Acinetobacter baumannii	Pneumonia[270]
Klebsiella pneumonia	Pneumonia[271]
Mycoplasma pneumonia	Interstitial pneumonia[272]
Mycoplasma pneumonia	Not reported[273]
Legionella pneumophila	Not reported[274]
Streptococcus pneumonia	Not reported[275]
Prevotella	Not reported[276]
Haemophilus	Not reported[277]
Lautropia	Not reported[278]
Cutibacterium	Not reported[279]

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2.17: Enterobactericeae

Enterobacteriaceae is a large family of Gram-negative bacteria, which now includes over 30 genera and more than 100 species[280].

Family: Enterobacteriaceae[280]

Class: Gammaproteobacteria

Order: Enterobacterales

Phylum: Proteobacteria

Domain: Bacteria

Lower classifications: *E. coli, Salmonella, Klebsiella pneumoniae* and more.

Enterobacteriaceae are a large family of Gram-negative bacteria that includes a number of pathogens such as *Klebsiella*, *Enterobacter*, *Citrobacter*, *Salmonella*, *Escherichia coli*, *Shigella Proteus*, *Serratia* and other species[281]. Enterobacteriaceae is a family of gram-negative bacteria that is rod shaped and called bacillus. Many like salmonella are motile whilst many are not. They are not spore formers. Some bacteria produce endo-toxins that are lethal to humans[281].

These pathogens are present in the human intestinal tract and are a normal part of the gut flora. They are a common cause of urinary tract infections (UTIs), and some species can also cause diarrhoea. These pathogens can spread to the bloodstream resulting in life-threatening complications. Enterobacteriaceae, like all bacteria, can develop resistance to antibiotics, including the carbapenem group of antibiotics, which are sometimes referred to as the last line of antibiotic treatment against resistant organisms. Refer to carbapenem-resistant Enterobacteriaceae (CRE) and carbapenemase-producing Enterobacteriaceae (CPE). These pathogens can also cause healthcare-associated infections (HAIs).

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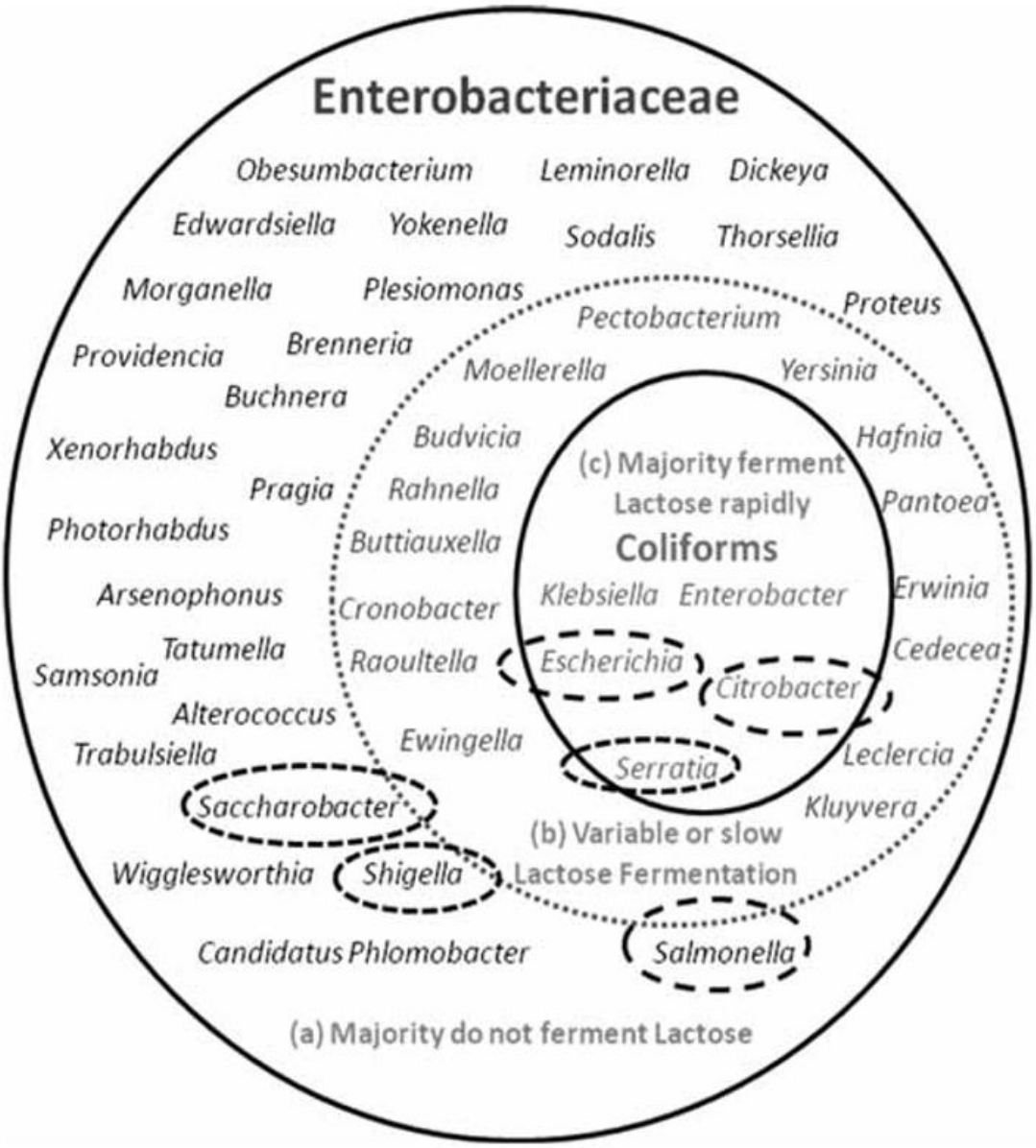

Source: Wikipedia

Figure 2.5: Classes of Enterobactericeae

2.17.1: *Pseudomonas aeruginosa*

The word Pseudomonas means "false unit", from the Greek pseudēs and (Latin: monas, from Greek: The stem word mon was used early in the history of microbiology to refer to germs, e.g., kingdom Monera. The species name aeruginosa is a Latin word meaning verdigris ("copper rust"), referring to the blue-green color of laboratory cultures of the species. This blue-green pigment is a combination of two metabolites of *P. aeruginosa*, pyocyanin (blue) and pyoverdine(green), which impart the blue-green characteristic color of cultures. Another assertion is that the word may be derived from the Greek prefix ae- meaning "old or aged", and the suffix ruginosa means wrinkled or bumpy. The names pyocyanin and pyoverdine are from the Greek, with pyo-, meaning "pus"cyanin, meaning "blue", and verdine, meaning "green". Pyoverdine in the absence of pyocyanin is a fluorescent-yellow color.

Pseudomonas aeruginosa is a common encapsulated, Gram-negative, rod-shaped bacterium that can cause disease in plants and animals, including humans. A species of considerable medical importance, *P. aeruginosa* is a multidrug resistant pathogen recognized for its ubiquity, its intrinsically advanced antibiotic resistance mechanisms, and its association with serious illnesses which may be hospital-acquired infections such as ventilator-associated pneumonia and various sepsissyndromes. The organism is considered opportunistic in so far as serious infection often occurs during existing diseases or conditions most notably cystic fibrosis and traumatic burns. It generally affects the immunocompromised but can also infect the immunocompetent as in hot tub folliculitis. Treatment of *P. aeruginosa* infections can be difficult due to its natural resistance to antibiotics. When more advanced antibiotic drug regimens are needed adverse effects may result. It is citrate, catalase, and oxidase

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positive. It is found in soil, water, skin flora, and most man-made environments throughout the world. It thrives not only in normal atmospheres, but also in low-oxygen atmospheres, thus has colonized many natural and artificial environments. It uses a wide range of organic material for food; in animals, its versatility enables the organism to infect damaged tissues or those with reduced immunity. The symptoms of such infections are generalized inflammation and sepsis. If such colonizations occur in critical body organs, such as the lungs, the urinary tract, and kidneys, the results can be fatal[282]. Because it thrives on moist surfaces, this bacterium is also found on and in medical equipment, including catheters, causing cross-infections in hospitals and clinics. It is also able to decompose hydrocarbons and has been used to break down tarballs and oil from oil spills[283].

2.17.1.1: Pathogenesis of *Pseudomonas aeruginosa*

An opportunistic, nosocomial pathogen of immunocompromised individuals, *P. aeruginosa* typically infects the airway, urinary tract, burns, and wounds, and also causes other blood infections. It is the most common cause of infections of burn injuries and of the outer ear (otitis externa), and is the most frequent colonizer of medical devices (e.g., catheters). Pseudomonas can be spread by equipment that gets contaminated and is not properly cleaned or on the hands of healthcare workers. Pseudomonas can, in rare circumstances, cause community-acquired pneumonias, as well as ventilator-associated pneumonias, being one of the most common agents isolated in several studies. Pyocyanin is a virulence factor of the bacteria and has been known to cause death in C. elegans by oxidative stress. However, salicylic acid can inhibit pyocyanin production. One in ten hospital-acquired infections is from Pseudomonas. Cystic fibrosis patients are also predisposed to *P. aeruginosa* infection of the lungs due to a functional loss in chloride ion movement across

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cell membranes as a result of a mutation. *P. aeruginosa* may also be a common cause of "hot-tub rash" (dermatitis), caused by lack of proper, periodic attention to water quality. Since these bacteria thrive in moist environments, such as hot tubs and swimming pools, they can cause skin rash or swimmer's ear. Pseudomonas is also a common cause of postoperative infection in radial keratotomy surgery patients. The organism is also associated with the skin lesion ecthyma gangrenosum. *P. aeruginosa* is frequently associated with osteomyelitis involving puncture wounds of the foot, believed to result from direct inoculation with *P. aeruginosa* via the foam padding found in tennis shoes, with diabetic patients at a higher risk. antibiotic resistance, rather than the biofilm simply acting as a diffusion barrier to the antibiotic[284].

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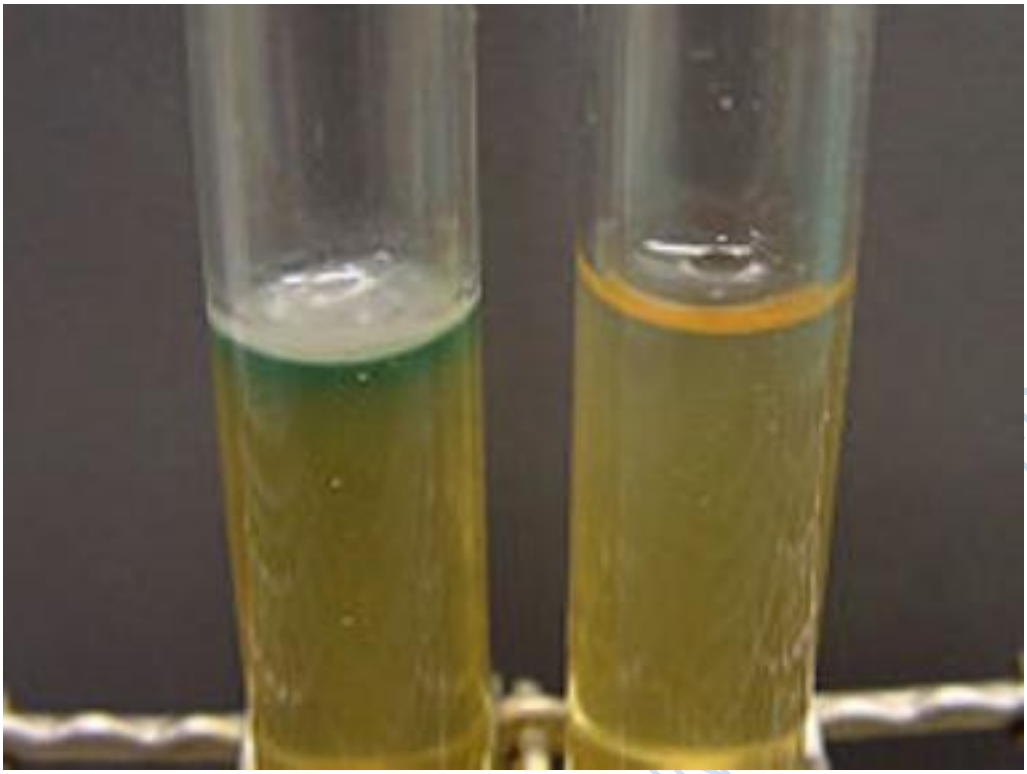

Figure 2.6: Production of pyocyanin, water-soluble green pigment of P. aeruginosa (left tube)[34]

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2.17.1.2 Treatment of Pseudomonas specie

Many *P. aeruginosa* isolates are resistant to a large range of antibiotics and may demonstrate additional resistance after unsuccessful treatment. It should usually be possible to guide treatment according to laboratory sensitivities, rather than choosing an antibiotic empirically. If antibiotics are started empirically, then every effort should be made to obtain cultures (before administering first dose of antibiotic), and the choice of antibiotic used should be selected based on result of culture obtained.

Due to widespread resistance to many common first-line antibiotics, carbapenems, polymyxins, and more recently tigecycline were considered to be the drugs of choice; however, resistance to these drugs has also been reported. Despite this, they are still being used in areas where resistance has not yet been reported. Use of β-lactamase inhibitors such as sulbactam has been advised in combination with antibiotics to enhance antimicrobial action even in the presence of a certain level of resistance. Combination therapy after rigorous antimicrobial susceptibility testing has been found to be the best course of action in the treatment of multidrug-resistant *P. aeruginosa*. Some next-generation antibiotics that are reported as being active against *P. aeruginosa* include doripenem, ceftobiprole, and ceftaroline. However, these require more clinical trials for standardization. Therefore, research for the discovery of new antibiotics and drugs against *P. aeruginosa* is very much needed. Antibiotics that may have activity against *P. aeruginosa* include: aminoglycosides (gentamicin, amikacin, tobramycin, but not kanamycin), quinolones (ciprofloxacin, levofloxacin, but not moxifloxacin), cephalosporins (ceftazidime, cefepime, cefoperazone, cefpirome, ceftobiprole, but not cefuroxime, cefotaxime, or ceftriaxone), antipseudomonal penicillins: carboxypenicillins (carbenicillin and ticarcillin), and ureidopenicillins

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(mezlocillin, azlocillin, and piperacillin)[284]. *P. aeruginosa* is intrinsically resistant to all other penicillins, carbapenems (meropenem, imipenem, doripenem, but not ertapenem), polymyxins (polymyxin B and colistin) and monobactams (aztreonam). As fluoroquinolones are one of the few antibiotic classes widely effective against *P. aeruginosa*, in some hospitals, their use is severely restricted to avoid the development of resistant strains. On the rare occasions where infection is superficial and limited (for example, ear infections or nail infections), topical gentamicin or colistin may be used[284].

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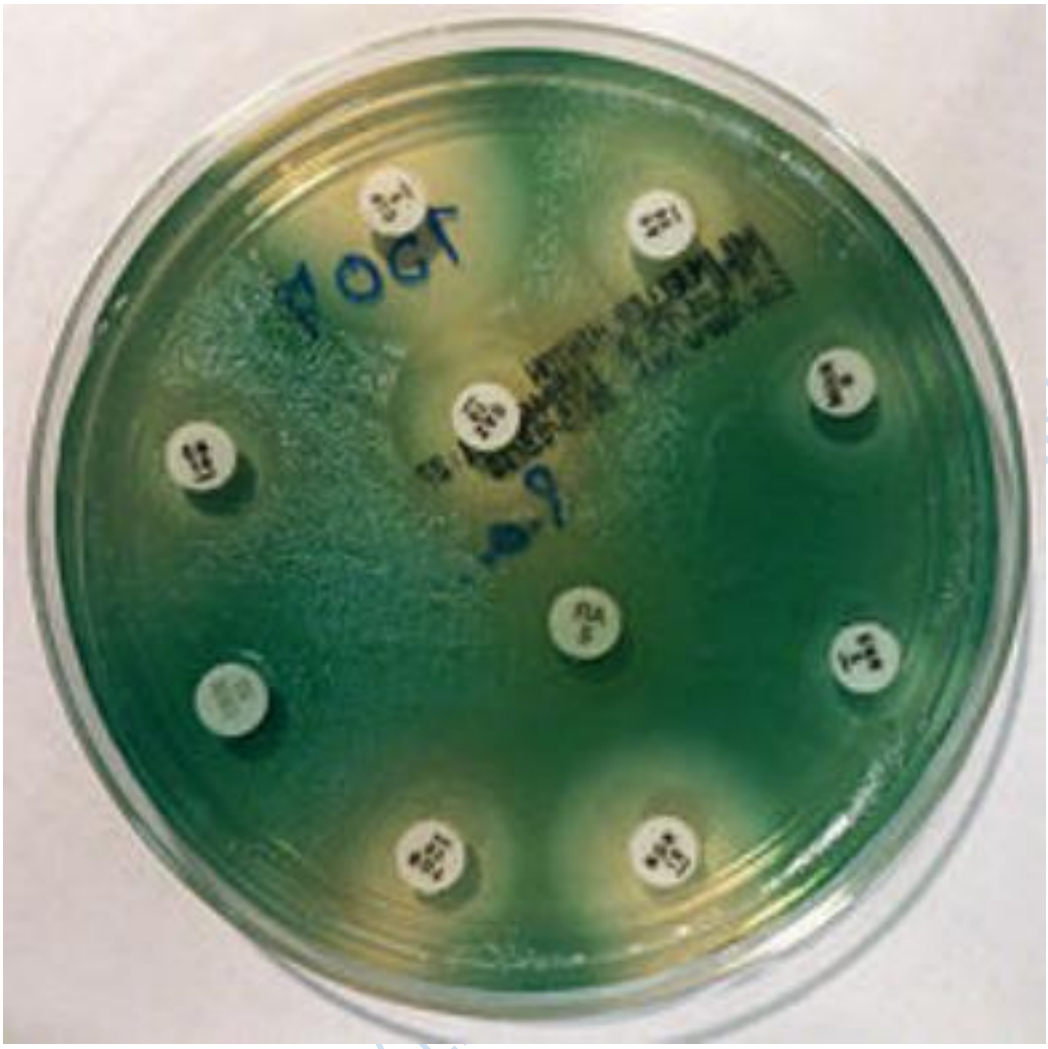

Figure 2.7 The Antibiogram of P. aeruginosa on Mueller-Hinton agar[34]

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2.17.2: *Escherichia coli*

Escherichia coli also known as *E. coli*, is a Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus Escherichia that is commonly found in the lower intestine of warm-blooded organisms (endotherms) [289]. Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for food contamination incidents that prompt product recalls[290]. The harmless strains are part of the normal microbiota of the gut, and can benefit their hosts by producing vitamin K_2, (which helps blood to clot) and preventing colonization of the intestine with pathogenic bacteria, having a symbiotic relationship[291]. *E. coli* is expelled into the environment within fecal matter. The bacterium grows massively in fresh fecal matter under aerobic conditions for 3 days, but its numbers decline slowly afterwards[292].

Escherichia coli and other facultative anaerobes constitute about 0.1% of gut microbiota, and fecal–oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them potential indicator organisms to test environmental samples for fecal contamination. A growing body of research, though, has examined environmentally persistent *E. coli* which can survive for many days and grow outside a host[293]. The bacterium can be grown and cultured easily and inexpensively in a laboratory setting, and has been intensively investigated for over 60 years. E. coli is a chemoheterotroph whose chemically defined medium must include a source of carbon and energy[294]. *E. coli* is the most widely studied prokaryotic model organism, and an important species in the fields of biotechnology and microbiology, where it has served as the host organism for the majority

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of work with recombinant DNA. Under favorable conditions, it takes as little as 20 minutes to reproduce[295].

2.17.2.1: Type and Morphology of *E coli*

Escherichia coli is a Gram-negative, facultative anaerobe (that makes ATP by aerobic respiration if oxygen is present, but is capable of switching to fermentation or anaerobic respiration if oxygen is absent) and nonsporulating bacterium. Cells are typically rod-shaped, and are about 2.0 μm long and 0.25–1.0 μm in diameter, with a cell volume of 0.6–0.7 μm^3. *E. coli* stains Gram-negative because its cell wall is composed of a thin peptidoglycan layer and an outer membrane. During the staining process, *E. coli* picks up the color of the counterstain safranin and stains pink. The outer membrane surrounding the cell wall provides a barrier to certain antibiotics such that E. coli is not damaged by penicillin[296].

2.17.2.2: Culture growth of *E coli*

Optimum growth of *E. coli* occurs at 37 ºC (98.6 ºF), but some laboratory strains can multiply at temperatures up to 49 ºC (120 ºF). *E. coli* grows in a variety of defined laboratory media, such as lysogeny broth, or any medium that contains glucose, ammonium phosphate monobasic, sodium chloride, magnesium sulfate, potassium phosphate dibasic, and water. Growth can be driven by aerobic or anaerobic respiration, using a large variety of redox pairs, including the oxidation of pyruvic acid, formic acid, hydrogen, and amino acids, and the reduction of substrates such as oxygen, nitrate, fumarate, dimethyl sulfoxide, and trimethylamine N-oxide. *E. coli* is classified as a facultative anaerobe. It uses oxygen when it is present and available. It can, however, continue to grow in the absence of oxygen using fermentation or anaerobic respiration. The ability to continue growing in the absence of

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oxygen is an advantage to bacteria because their survival is increased in environments where water predominates[297]. Strains that possess flagella are motile. The flagella have a peritrichous arrangement. It also attaches and effaces to the microvilli of the intestines via an adhesion molecule known as intimin.

2.17.2.3: Role of *E coli* in disease condition

Escherechia coli strains do not cause disease, naturally living in the gut but virulent strains can cause gastroenteritis, urinary tract infections, neonatal-meningitis, hemorrhagic colitis, and Crohn's disease. Common signs and symptoms include severe abdominal cramps, diarrhea, hemorrhagic colitis, vomiting, and sometimes fever. In rarer cases, virulent str ains are also responsible for bowel necrosis (tissue death) and perforation without progressing to hemolytic-uremic syndrome, peritonitis, mastitis, sepsis, and Gram-negative pneumonia. Very young children are more susceptible to develop severe illness, such as hemolytic uremic syndrome; however, healthy individuals of all ages are at risk to the severe consequences that may arise as a result of being infected with *E. coli*[298].

Some strains of *E. coli,* for example O157:H7, can produce Shiga toxin (classified as a bioterrorism agent). The Shiga toxin causes inflammatory responses in target cells of the gut, leaving behind lesions which result in the bloody diarrhea that is a symptom of a Shiga toxin-producing *E. col*i (STEC) infection. This toxin further causes premature destruction of the red blood cells, which then clog the body's filtering system, the kidneys, in some rare cases (usually in children and the elderly) causing hemolytic-uremic syndrome (HUS), which may lead to kidney failure and even death. Signs of hemolytic uremic syndrome include decreased frequency of urination, lethargy, and paleness of cheeks and inside the lower eyelids. In 25% of HUS patients, complications of nervous system occur, which in

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turn causes strokes. In addition, this strain causes the buildup of fluid (since the kidneys do not work), leading to edema around the lungs and legs and arms. This increase in fluid buildup especially around the lungs impedes the functioning of the heart, causing an increase in blood pressure[299].

2.17.2.4: Treatment of *E coli*

The mainstay of treatment is the assessment of dehydration and replacement of fluid and electrolytes. The antibiotic used depends upon susceptibility patterns in the particular geographical region. Currently, the antibiotics of choice are Fluoroquinolones or Azithromycin, with an emerging role for Rifaximin.

2.17.3: *Enterobacter*

Enterobacter is a genus of a common Gram-negative, facultative anaerobic, rod-shaped, non-spore-forming bacteria belonging to the family *Enterobacteriaceae*. Two of its well-known species, *Enterobacter aerogenes* and *E. cloacae* have taken on clinical significance as opportunistic bacteria and have emerged as nosocomial pathogens from intensive care patients pathogenic, especially to those who are on mechanical ventilation[300]. *Enterobacter aerogenes* was originally named *Aerobacter aerogenes*, and was later included in the genus *Enterobacter* in 1960. In 1971, this species was proposed to be renamed *Klebsiella mobilis* due to its motility conferred by peritrichous flagella and its genetic relatedness to *Klebsiella* genus. It is interesting to note that phenotypic dissimilarities between *E. aerogenes* and the genus *Klebsiella* include not only the motility but also the presence of ornithine decarboxylase (ODC) activity and the lack of urease activity in *E. aerogenes*[300]. However, recently, the whole genome sequencing of a multidrug-resistant (MDR) clinical isolate,

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(including colistin) suggested a possible reclassification of the species in the genus *Klebsiella*, under the name *K. aeromobilis E. aerogenes* particular phenotype can be attributed to the horizontal acquisition of additional genes from other *Enterobacteriaceae* species and mobile elements that rapidly integrated and translated as easily as its own ancestral heritage[301]. For example, the flagellar genes and its assembly system have been acquired in bloc from the *Serratia* genus. Plasmid conjugation is a chimera of transposons and genetic elements (conjugation, integration) of various bacterial origins. *E. aerogenes* also contains eight rRNA operons and 87 tRNA associated with the ability to translate imported genes that use different codons, improving its ability to use its integrated foreign genes. *E. aerogenes* has been involved in significant European outbreak between 1993 and 2003 and is considered as the paradigm of opportunistic bacteria[301].

Species of the *E. cloacae* complex are widely encountered in nature, but they are also pathogens: *E. cloacae* and are most frequently isolated from human clinical specimens. Thus, *E. cloacae* is among the most common *Enterobacter* sp. causing only nosocomial infections in the last decade and a lot has been published on the antibiotic-resistance features of these microorganisms. Despite the relevance of *E. cloacae* as a nosocomial pathogen, the pathogenic mechanisms and factors contributing in the disease associated with the *E. cloacae* complex are not understood yet; this could be due to the scarcity and the dispersion of information available. Its ability to form biofilms and to secrete various cytotoxins (enterotoxins, hemolysins, pore-forming toxins) are important for its pathogenicity[300]. Some genotypes and species, have previously exhibited some associations with clinical specimens, in particular urines and sputum, when clonal outbreaks with members of the *E. cloacae* complex were rare[302]. Interestingly, due to the diffusion of most frequent extended spectrum

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β-lactamases (ESBL) and carbapenemases in this species, *E. cloacae* has now become the third broad spectrum *Enterobacteriaceae* species involved in nosocomial infections after *Escherichia coli* and *K. pneumoniae*[303].

2.17.3.1: Epidemiology and Infections

Enterobacter aerogenes is isolated as human clinical specimens from respiratory, urinary, blood, or gastrointestinal tract[304]. Epidemiology of this species has been particular in Europe: it has regularly been involved in nosocomial infections outbreaks since 1993, particularly in the Western Europe *Enterobacter cloacae* is ubiquitous in terrestrial and aquatic environments (water, sewage, soil, and food) [305]. The species occurs as commensal microflora in the intestinal tracts of humans and animals and is also pathogens in plants and insects. This diversity of habitats is mirrored by the genetic variety of *E. cloacae* [300]. Recently, MLST and PFGE epidemiological methods data revealed world circulation of several epidemic clonal complexes [302].

It is also a well-known nosocomial pathogen contributing to bacteremia, endocarditis, septic arthritis, osteomyelitis, and skin/soft tissue infections, and lower respiratory tract- urinary tract and intra-abdominal infections[306]. *Enterobacter cloacae* tends to contaminate various medical, intravenous, and other hospital devices[307]. Nosocomial outbreaks have also been associated with the colonization of certain surgical equipment and operative cleaning solutions[308]. Since a decade, *E. cloacae* has been repeatedly reported as a nosocomial pathogen in neonatal units and several outbreaks of infection have been reported[309]. Today, variability among strains are less frequent and outbreaks due to clonal *E. cloacae* hyper-producing AmpC β-lactamase and ESBL carrier isolates are described from neonate specimens, adult's urines/feces samples or from environmental samples[310].

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Enterobacter cloacae has an intrinsic resistance to Ampicillin, Amoxicillin, first-generation Cephalosporins, and Cefoxitin owing to the production of constitutive AmpC β-lactamase. It exhibits a high frequency of enzymatic resistance to broad-spectrum cephalosporins. Resistance of *Enterobacter* sp. to third-generation cephalosporins is most typically caused by overproduction of AmpC β-lactamases, and thus treatment with third-generation cephalosporins may select for AmpC-overproducing mutants. AmpC overproduction is due to the derepression of a chromosomal gene or by the acquisition of a transferable *ampC* gene from plasmids or other mobile elements. The AmpC plasmid-mediated resistance is distinguished from chromosomal enzyme production because they are not inducible. However, they represent a problem due to its increasing prevalence among clinical isolates. The enzyme confers a resistance to third-generation cephalosporins and ureido- and carboxy-penicillins and is not inhibited by common inhibitors of β-lactamases. Fourth-generation cephalosporins retain reasonable activity against derepressed strains, but if strains are also ESBL producers, they become resistant to this antibiotic class. The prevalence of ESBL and CTX-M producers represented approximatively 5% of the isolates in the recent studies and ESBLs are most often plasmid-mediated. These characteristics, associated with the frequent endogenous intestinal carriage of *E. cloacae*, may result in abnormally high levels in the bowels of hospitalized patients, especially those who have received Cephalosporins[303].

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2.17.3.2: Enzymatic Barrier and Antibiotic Resistance

The production of β-lactamases is the prominent mechanism responsible for β-lactam resistance in most of these species. *E. aerogenes* strains have a broad ability to develop antibiotics resistance mechanisms[311]. They naturally express a chromosomal AmpC β-lactamase type cephalosporinase at low level (group 1 Bush) that induces resistance to first-

generation cephalosporins[312]. Chromosomal acquired β-lactams resistance mechanisms induce the overproduction of chromosomal AmpC cephalosporinase: this results from an induction during a third-generation cephalosporin treatment or by a mutation in the AmpR repressor, and generates a resistance to almost all β-lactams [313]. Moreover, it has been described that *E. aerogenes* strains harboring cephalosporinase AmpC gene, integrated the gene of chromosomal origin (*bla*CMY-10) on a large plasmid (130 kb), contributing to a systematic gene transmission even in the absence of antibiotic pressure[314].

In 1993 appeared the first cases of nosocomial infections caused by strains with resistance to common β-lactam antibiotics due to ESBL[315]. The ESBL TEM- 24 associated to *E. aerogenes* clonal dissemination in France was constantly reported[316]. Other ESBLs of TEM type or CTX-M type (ex CTX-M-2) are often identified but TEM-24 remains associated with preferential conjugative plasmid of this species[317]. Due to the well-described modification of porins expression and recent dissemination of plasmid bearing carbapenemases, a number of imipenem-resistant clinical strains have come up[318]. Carbapenemases of NDM and VIM types are now, as anticipated, reported in *E. aerogenes* in India and those for the serine protease group as KPC or class D β-lactamases possessing carbapenemase properties as OXA-48 types are described in Europe/Asia[319].

Similarly, to *E. aerogenes*, *E. cloacae* is also naturally resistant to ampicillin, amoxicillin–clavulanic acid, cephalothin, and cefoxitin by low production of the natural inducible cephalosporinase of Bush group 1 (class C). They are capable of overproducing AmpC β-lactamases by blocking the repression of a chromosomal gene or by the acquisition of a transferable *ampC* gene on plasmids conferring the resistance to third-generation cephalosporins[320]. Cefepime alone can keep its activity [321]. Clinical AmpC resistance represents 50% of the isolates and frequently co- exists with the expression of ESBL. In

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1989, appeared the first nosocomial isolate cases bearing plasmidic ESBL causing also resistance to third generation cephalosporins except cefamycins[322]. Together, these enzymes are responsible for a global resistance to all β-lactams except carbapenems[315]. In the last decade, *E. cloacae* has emerged as the third most common *Enterobacteriaceae* resistant to third generation cephalosporins with enteric *E. coli* and *K. pneumoniae* [323]. Imipenem remains the most effective molecule for treating *E. cloacae* infections. Since then, various ESBL of TEM, SHV, and CTX -M types have been characterized in *E. cloacae* including resistant TEM inhibitors or IRT (for inhibitor-resistant TEM[317]. However, among ESBL producers, some sub-clones are now identified, associated with CTX-M-3 and 15 productions, when other TEM or SHV (SHV-12 for example) types are also associated with epidemic-episodes-involved isolates. Diffusion of *E. cloacae* producing CTX-M-15 ESBL is the consequence of the wide dissemination of identical or related plasmids harboring the CTX-M-15 gene firstly identified in the epidemic *E. coli* clone, and the CTX-M β-lactamases are now the most prevalent ESBL globally [324].

2.17.4: *Serratia marcescens*

Serratia marcescens is a species of rod-shaped, Gram-negative bacteria in the family Yersiniaceae. It is a facultative anaerobe and an opportunistic pathogen. It was discovered in 1819 by Bartolomeo Bizio in Padua, Italy[325]. *S. marcescens* is commonly involved in hospital-acquired infections (HAIs), particularly catheter-associated bacteremia, urinary tract infections, and wound infections, and is responsible for 1.4% of HAI cases in the United States[326,327,328]. It is commonly found in the respiratory and urinary tracts of hospitalized adults and in the gastrointestinal systems of children. Due to its abundant presence in the environment, and its preference for damp conditions, *S. marcescens* is commonly found growing in bathrooms (especially on tile grout, shower corners, toilet

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water lines, and basins), where it manifests as a pink, pink-orange, or orange discoloration and slimy film feeding off phosphorus-containing materials or fatty substances such as soap and shampoo residue[328].

Once established, complete eradication of the organism is often difficult, but can be accomplished by application of a bleach-based disinfectant. Rinsing and drying surfaces after use can also prevent the establishment of the bacterium by removing its food source and making the environment less hospitable. *S. marcescens* may also be found in environments such as dirt and the subgingival biofilm of teeth. Due to this, and because *S. marcescens* produces a reddish-orange tripyrrole dye called prodigiosin, it may cause staining of the teeth. The biochemical pathway for the production of prodigiosin by *S. marcescens* has been characterized by analyzing what intermediates become accumulated in specific mutants[329].

S. *marcescens* is a motile organism and can grow in temperatures ranging from 5–40 °C and in pH levels ranging from 5 to 9. It is differentiated from other Gram-negative bacteria by its ability to perform casein hydrolysis, which allows it to produce extracellular metalloproteinases which are believed to function in cell-to-extracellular matrix interactions. Since this bacterium is a facultative anaerobe, meaning that it can grow in either the presence of oxygen (aerobic) or in the absence of oxygen (anaerobic), it is capable of nitrate reduction under anaerobic conditions. Therefore, nitrate tests are positive since nitrate is generally used as the final electron acceptor rather than oxygen. *S. marcescens* also exhibits tyrosine hydrolysis and citrate degradation[330]. Citrate is used by *S. marcescens* to produce pyruvic acid, thus it can rely on citrate as a carbon source and test positive for citrate utilization[325]. In identifying the organism, one may also perform a methyl red test, which determines if a microorganism performs mixed-acid fermentation. *S. marcescens* results in a

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negative test. Another determination of S. marcescens is its capability to produce lactic acid by oxidative and fermentative metabolism. Therefore, *S. marcescens* is lactic acid O/F+[331].

Test	**Result**[332]
Gram stain	−
Oxidase	−
Indole production	−
Methyl Red	> 70% −
Voges-Proskaeur	+
Citrate (Simmons)	+
Hydrogen sulfide production	−
Urea hydrolysis	> 70% −
Phenylalanine deaminase	−
Lysine decarboxylase	+
Motility	+
Gelatin hydrolysis, 22 °C	+
Acid from lactose	−
Acid from glucose	+
Acid from maltose	+
Acid from mannitol	+
Acid from sucrose	+
Nitrate reduction	+ (to nitrite)
Deoxyribonuclease, 25 °C	+
Lipase	+
Pigment	some biovars produce red
Catalase production (24h)	+

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Figure 2.8: Morphology and Antibiogram of S. marcescens on Mueller–Hinton agar[34]

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In humans, *S. marcescens* can cause an opportunistic infection in several sites, including the urinary tract, respiratory tract, wounds, and the eye, where it may cause conjunctivitis, keratitis, endophthalmitis, and tear duct infections[333,328,334]. It is also a rare cause of endocarditis and osteomyelitis (particularly in people who use intravenous drugs recreationally), pneumonia, and meningitis[328]. Most *S. marcescens* strains are resistant to several antibiotics because of the presence of R-factors, which are a type of plasmid that carry one or more genes that encode resistance; all are considered intrinsically resistant to ampicillin, macrolides, and first-generation cephalosporins (such as cephalexin) [327].

In Elkhorn coral, *S. marcescens* is the cause of the disease known as white pox disease[335]. In Silkworms, it can also cause a lethal disease, especially in association with other pathogens[336]. In research laboratories employing Drosophila fruit flies, infection of them with *S. marcescens* is common. It manifests as a pink discoloration or plaque in or on larvae, pupae, or the usually starch and sugar-based food (especially when improperly prepared). A rare clinical form of gastroenteritis occurring in early infancy caused by infection with *S. marcescens*. The red color of the diaper can be mistaken for hematuria (blood in the urine), which may cause unnecessary investigations by the physicians was discovered in 1819 by Venetian pharmacist Bartolomeo Bizio, as the cause of an episode of blood-red discoloration of polenta in the city of Padua[338]. Bizio named the organism four years later in honor of Serafino Serrati, a physicist who de veloped an early steamboat; the epithet marcescens (Latin for 'decaying') was chosen because of the dyestuff's rapid deterioration (Bizio's observations led him to believe that the organism decayed into a mucilage-like substance upon reaching maturity)[339]. *Serratia* was later renamed Monas prodigiosus and Bacillus prodigiosus before Bizio's original name was restored in the 1920s[315].

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Since 1950, *S. marcescens* has steadily increased as a cause of human infection, with many strains resistant to multiple antibiotics[326]. The first indications of problems with the influenza vaccine produced by Chiron Corporation in 2004 involved *S. marcescens* contamination[326].

In early 2008, the U.S. Food and Drug Administration issued a nationwide recall of one lot of Pre-Filled Heparin Lock Flush Solution USP[340]. The heparin IV flush syringes had been found to be contaminated with *S. marcescens*, which resulted in patient infections. The Centers for Disease Control and Prevention confirmed growth of *S. marcescens* from several unopened syringes of this product. *S. marcescens* has also been linked to 19 cases in Alabama hospitals in 2011, including 10 deaths[341]. All of the patients involved were receiving total parenteral nutrition at the time, and this is being investigated as a possible source of the outbreak[342]

2.17.5: Staphylococcus aureus

Staphylococcus aureus is a Gram-positive, round-shaped bacterium that is a member of the Firmicutes, and it is a usual member of the microbiota of the body, frequently found in the upper respiratory tract and on the skin. It is often positive for catalase and nitrate reduction and is a facultative anaerobe that can grow without the need for oxygen[343]. Although *S. aureus* usually acts as a commensal of the human microbiota it can also become an opportunistic pathogen, being a common cause of skin infections including abscesses, respiratory infections such as sinusitis, and food poisoning. Pathogenic strains often promote infections by producing virulence factors such as potent proteintoxins, and the expression of a cell-surface protein that binds and inactivates antibodies. The emergence of antibiotic-resistant strains of *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) is a worldwide

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problem in clinical medicine. Despite much research and development, no vaccine for S. aureus has been approved.

An estimated 20% to 30% of the human population are long-term carriers S. aureus which can be found as part of the normal skin flora, in the nostrils, and as a normal inhabitant of the lower reproductive tract of women. *S. aureus* can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, and sepsis[344]. It is still one of the five most common causes of hospital-acquired infections and is often the cause of wound infections following surgery. Each year, around 500,000 patients in hospitals of the United States contract a staphylococcal infection, chiefly by S. aureus. Up to 50,000 deaths each year in the USA are linked with *S. aureus* infections. *Staphylococcus aureus* is catalase-positive (meaning it can produce the enzyme catalase). Catalase converts hydrogen peroxide to water and oxygen. Catalase-activity tests are sometimes used to distinguish staphylococci from enterococci and streptococci. Previously, *S. aureus* was differentiated from other staphylococci by the coagulase test. However, not all *S. aureus* strains are coagulase-positive and incorrect species identification can impact effective treatment and control measures[345].

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2.17.5.1: Role of *Staphylococcus aureus* in health

In humans, *S. aureus* is not part of the normal microbiota present in the upper respiratory tract or gut mucosa or on the skin; rather, when it is prevalent here, it is a colonization. *Staphylococcus aureus*, along with similar species, can colonize and act symbiotically but

can cause disease if they begin to take over the tissues they have colonized or invade other tissues, and as such they have been called "pathobionts"[346].

2.17.5.2: Role of Staphylococcus aureus in disease

Staphylococcus aureus usually acts as a commensal bacterium, asymptomatically colonizing about 30% of the human population, it can sometimes cause disease. In particular, *S. aureus* is one of the most common causes of bacteremia and infective endocarditis. Additionally, it can cause various skin and soft-tissue infections particularly when skin or mucosal barriers have been breached. *Staphylococcus aureus* infections can spread through contact with pus from an infected wound, skin-to-skin contact with an infected person, and contact with objects used by an infected person such as towels, sheets, clothing, or athletic equipment. Joint replacements put a person at particular risk of septic arthritis, staphylococcal endocarditis (infection of the heart valves), and pneumonia. Preventive measures include washing hands often with soap and making sure to bathe or shower daily.

Staphylococcus aureus is a significant cause of chronic biofilm infections on medical implants, and the repressor of toxins is part of the infection pathway. *S. aureus* can lay dormant in the body for years undetected. Once symptoms begin to show, the host is contagious for another two weeks, and the overall illness lasts a few weeks. If untreated, though, the disease can be deadly. Deeply penetrating *S. aureus* infections can be severe. Skin infections are the most common form of *S. aureus* infection. This can manifest in various ways, including small benign boils, folliculitis, impetigo, cellulitis, and more severe, invasive soft-tissue infections[347]. *Staphylococcus aureus* is extremely prevalent in persons with atopic dermatitis, more commonly known as eczema. It is mostly found in fertile, active places, including the armpits, hair, and scalp. Large pimples that appear in those areas

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may exacerbate the infection if lacerated. This can lead to staphylococcal scalded skin syndrome, a severe form of which can be seen in newborns[347].

The presence of *S. aureus* in persons with atopic dermatitis is not an indication to treat with oral antibiotics, as evidence has not shown this to give benefit to the patient. However, topical antibiotics combined with corticosteroids have been found to improve the condition. Colonization of *S. aureus* drives inflammation of atopic dermatitis; *S. aureus* is believed to exploit defects in the skin barrier of persons with atopic dermatitis, triggering cytokine expression and therefore exacerbating symptoms. S. aureus is also responsible for food poisoning. It is capable of generating toxins that produce food poisoning in the human body[348]. Its incubation period lasts one to six hours with the illness itself lasting from 30 minutes to 3 days [349]. Preventive measures one can take to help prevent the spread of the disease include washing hands thoroughly with soap and water before preparing food. Stay away from any food if ill, and wear gloves if any open wounds occur on hands or wrists while preparing food. If storing food for longer than 2 hours, keep the food below 5 or above 63 °C [350]. *S. aureus* is the bacterium commonly responsible for all major bone and joint infections. This manifests in one of three forms: osteomyelitis, septic arthritis, and infection from a replacement joint surgery. *S. aureus* is a leading cause of bloodstream infections throughout much of the industrialized world. Infection is generally associated with breaks in the skin or mucosal membranes due to surgery, injury, or use of intravascular devices such as catheters, hemodialysis machines, or injected drugs. Once the bacteria have entered the bloodstream, they can infect various organs, causing infective endocarditis, septic arthritis, and osteomyelitis[351]. This disease is particularly prevalent and severe in the very young and very old[350].

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Staphylococcus aureus bacteremia has a case fatality rate around 80% when not treated, with the use of antibiotic, the case fatality rates range from 15% to 50% depending on the age and health of the patient, as well as the antibiotic resistance of the *S. aureus* strain. *S. aureus* produces various enzymes such as coagulase (bound and free coagulases) which clots plasma and coats the bacterial cell, probably to prevent phagocytosis. Hyaluronidase (also known as spreading factor) breaks down hyaluronic acid and helps in spreading it. *S. aureus* also produces deoxyribonuclease, which breaks down the DNA, lipase to digest lipids, staphylokinase to dissolve fibrin and aid in spread, and beta-lactamase for drug resistance. Depending on the strain, *S. aureus* is capable of secreting several exotoxins, which can be categorized into three groups. Many of these toxins are associated with specific diseases[352].

2.17.5.3 Classical diagnosis of *Staphylococcus aureus*

Depending upon the type of infection present, an appropriate specimen is obtained accordingly and sent to the laboratory for definitive identification by using biochemical or enzyme-based tests. A Gram stain is first performed to guide the way, which should show typical Gram-positive bacteria, cocci, in clusters. Second, the isolate is cultured on mannitol salt agar, which is a selective medium with 7–9% NaCl that allows *S. aureus* to grow, producing yellow-colored colonies as a result of mannitol fermentation and subsequent drop in the medium's Ph. To determine staphylococcal food poisoning, phage typing is recommended to know if staphylococci were the source of infection. Furthermore, for differentiation on the species level, catalase (positive for all Staphylococcus species), coagulase (fibrin clot formation, positive for *S. aureus*), DNAse (zone of clearance on

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DNase agar), lipase (a yellow color and rancid odor smell), and phosphatase (a pink color) tests are all done.

2.17.5.4: Treatment of *Staphyloccus aureus*

The treatment of choice for *S. aureus* infection is penicillin. An antibiotic derived from some Penicillium fungal species, penicillin inhibits the formation of peptidoglycan cross-linkages that provide the rigidity and strength in a bacterial cell wall. The four-membered β-lactam ring of penicillin is bound to enzyme DD-transpeptidase, an enzyme that when functional, cross-links chains of peptidoglycan that form bacterial cell walls. The binding of β-lactam to DD-transpeptidase inhibits the enzyme's functionality and it can no longer catalyze the formation of the cross-links. As a result, cell wall formation and degradation are imbalanced, thus resulting in cell death. In most countries, however, penicillin resistance is extremely common, and first-line therapy is most commonly a penicillinase-resistant β-lactam antibiotic (for example, oxacillin or flucloxacillin, both of which have the same mechanism of action as penicillin). Combination therapy with gentamicin may be used to treat serious infections, such as endocarditis but its use is controversial because of the high risk of damage to the kidneys. The duration of treatment depends on the site of infection and on severity. Adjunctive rifampicin has been historically used in the management of *S. aureus* bacteraemia, but randomised controlled trial evidence has shown this to be of no overall benefit over standard antibiotic therapy [353]. Antibiotic resistance in *S. aureus* was uncommon when penicillin was first introduced in 1943. Indeed, the original Petri dish on which Alexander Fleming of Imperial College London observed the antibacterial activity of the Penicillium fungus was growing a culture of *S. aureus*. By 1950, 40% of hospital *S. aureus* isolates were penicillin-resistant; by 1960, this had risen to 80%[354]. MRSA, is one of

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a number of greatly feared strains of *S. aureus* which have become resistant to most β-lactam antibiotics. For this reason, vancomycin, a glycopeptide antibiotic, is commonly used to combat MRSA. Vancomycin inhibits the synthesis of peptidoglycan, but unlike β-lactam antibiotics, glycopeptide antibiotics target and bind to amino acids in the cell wall, preventing peptidoglycan cross-linkages from forming. MRSA strains are most often found associated with institutions such as hospitals, but are becoming increasingly prevalent in community-acquired infections[354].

2.17.6: Proteus

Proteus is a member of the Enterobacteriaceae family. The genus of *Proteus* consists of motile, aerobic and facultatively anaerobic, Gram-negative rods. *Proteus* is a member of the tribe Proteeae, which also includes *Morganella* and *Providencia*. The genus Proteus currently consists of five named species: *P. mirabilis, P. vulgaris, P. penneri, P. myxofaciens* and *P. hauseri* and three unnamed genomospecies: Proteus genomospecies 4, 5, 6[355]. However, a recent study indicated that *P. myxofaciens* may represent a separate genus with low similarity to tribe Proteeae, and it has been suggested that this organism be renamed *Cosenzaea myxofaciens*[356].

A striking microbiologic characteristic of Proteus species is their swarming activity. Swarming appears macroscopically as concentric rings of growth emanating from a single colony or inoculum. On a cellular level, swarming results from bacterial transformation from "swimmer cells" in broth to "swarmer cells" on a surface such as agar, in a process involving cellular elongation and increased flagellin synthesis. The genus name *Proteus* originates from the mythological Greek sea god *Proteus*, who was an attendant to Poseidon. *Proteus* could change his shape at will. This attribute reminded early microbiologists of the

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morphologic variability of the Protei on subculture, including their ability to swarm[357]. Members of the genus *Proteus* are widespread in the environment and are found in the human gastrointestinal tract. The most common infections caused by *Proteus* spp. are urinary tract infections (UTIs). *Proteus* spp. can be found to colonize the vaginal introitus prior to onset of bacteruria. Therefore, like *Escherichia coli, Proteu* ssp. causes urinary tract infections by ascending from the rectum to the periurethra and bladder[357].

Proteus mirabilis is by far the most common species identified in clinical specimens. *P. mirabilis* is a common cause of both community-acquired and catheter-associated UTI, cystitis, pyelonephritis, prostatitis, wound infections, and burn infections, and occasionally causes respiratory tract infections, chronic suppurative otitis media, eye infections (endophthalmitis), meningitis, and meningoencephalitis. It is a common cause of bacteremia following catheter-associated UTI, and in rare cases has been reported to cause cellulitis, endocarditits, mastoiditis, empyema, and osteomyelitis. It has also been suggested that *P. mirabilis* could have a role in the etiology of rheumatoid arthritis[358].

2.17.6.1: Clinical manifestations of *Proteus* Specie

The clinical manifestations of infections with Proteus spp. are, in the main, non-specific. However, urinary tract infections involving struvite stones are characteristic by producing urease. Proteus specie can hydrolyze urea into ammonia and carbon dioxide, and therefore raise urinary pH. It also helps in the alkalinization of urine that promotes precipitation of magnesium-ammonium phosphate salts leading to the formation of struvite stones, which may serve as a nidus for the persistence of infection or may directly obstruct the urinary tract, thereby promoting infection[356].

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2.18.6.2: Laboratory Diagnosis of *Proteus* Specie

The members of the genus Proteus are Gram negative, motile facultative anaerobic rods. On culture plates, Proteus species are distinguished by their ability to swarm. Proteus specie have 2-3mm colorless, flat, colonies on MacConkey agar, whereas they swarm in waves to cover blood agar plates and LB agar plates. Proteus specie are identified by the following biochemical characteristics: positive Methyl-red reaction, negative Voges-Proskauer reaction, phenylalanine deaminase production, growth on KCN and urease production. *P. mirabilis* and *P. penneri* are indole-negative, while other *Proteus* species are indole-positive. The Proteus genomospecies (4, 5, and 6) can be distinguished from other Proteus species based on five biochemical characteristics: esculin hydrolysis, salicin fermentation, L-rhamnose fermentation, and elaboration of DNase and lipase.

2.17.6.3: Antibiotic Susceptibility in-vitro and in-vivo of *Proteus* Specie

Proteus specie can be naturally resistant to antibiotics, such as Benzylepenicillin, Oxacillin, Tetracycline, and Macrolides [359]. *Proteus* specie can acquire resistance to Ampicillin through plasmid mediated beta-lactamases, and chromosomal beta-lactamase expression has now been reported[360]. In the last decade there have also been numerous reports of production of extended-spectrum beta-lactamases (ESBLs) by Proteus spp. The ESBLs can confer resistance to third generation cephalosporins such as Cefotaxime, Ceftriaxone and Ceftazidime, as well as the Monobactam, Aztreonam. The Cephamycins (Cefoxitin, Cefotetan and Cefmetazole) and the Carbapenems (Imipenem and Meropenem) are

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generally not hydrolyzed by ESBLs. However, resistance to Carbapenems is starting to be observed in Proteus specie[361]

It should be noted that the minimum inhibitory concentration (MICs) for third generation cephalosporins or aztreonam may not reach widely used breakpoints for resistance with some ESBL producing Proteus isolates. In 2010, there was a change in the CLSI recommendations for susceptibility breakpoints, resulting in many ESBL-producing isolates previously considered to be resistant to these antibiotics now being regarded as susceptible. For instance, 78-97% of ESBL-producing strains tested were considered susceptible to Ceftazidime, Cefepime, and Aztreonam using the new breakpoints[362]. Another change in CLSI recommendations occurred in 2012, and SENTRY data from North America indicates that this change decreased the level of imipenem susceptibility compared to the 2010 criteria (64.5% of 1244 isolates were susceptible by 2012 criteria vs 99.8% by 2010 criteria)[363]. Due to these changes in breakpoints for susceptibility, data concerning resistance to Celphalosporins, Aztreonam, and Carbapenems may be underestimated.

2.17.7: Klebsiella pneumonia

Klebsiella pneumoniae (*K. pneumoniae*) are bacteria that normally live in your intestines and feces. These bacteria are harmless when they are in your intestines. But if they spread to another part of your body, they can cause severe infections. The risk is higher if you are sick. *K. pneumoniae* can infect organs like lungs, bladder, brain, liver, eyes, blood and it can be present in wounds, the location of your infection will determine your symptoms and treatment. Generally, healthy people do not get *K. pneumoniae* infections, but more likely to get it if you have a weak immune system due to a medical condition or long-term

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antibiotic use. *K. pneumoniae* infections are treated with antibiotics, but some strains have developed drug resistance and these infections are very difficult to treat with normal antibiotics[364].

2.17.7.1: Causes of *Klebsiella pneumoniae* Infection

Klebsiella infection is caused by the bacteria *K. pneumoniae*. It happens when *K. pneumoniae* directly enter the body. This usually occurs due to person-to-person contact. In the body, the bacteria can survive the immune system's defenses and cause infection[364].

2.17.7.2: Symptoms of *Klebsiella pneumoniae*

Because *K. pneumoniae* can infect different parts of the body, it can cause different types of infections. Each infection has different symptoms. *K. pneumoniae* often causes bacterial pneumonia, or infection of the lungs. It happens when the bacteria enter your respiratory tract. Community-acquired pneumonia occurs if you get infected in a community setting, like a mall or subway. Hospital-acquired pneumonia occurs if you get infected at a hospital or nursing home.In Western countries, *K. pneumoniae* causes about 3 to 5 percentTrusted Source of community-acquired pneumonia. It's also responsible for 11.8 percent Trusted Source of hospital-acquired pneumonia worldwide. Symptoms of pneumonia include: fever, chills, coughing, yellow or bloody mucus, shortness of breath, and chest pain[364].

2.17.7.3: Treatment of *Klebsiella pneumoniae* Infection

Klebsiella pneumoniae infections are treated with antibiotics. However, the bacteria can be difficult to treat. Some strains are highly resistant to antibiotics. If the infection is drug-resistant, laboratory tests to determine which antibiotic will work best will have to be done.

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Medical advice should be strictly adhered to. If taking antibiotics is stopped too soon, the infection might relapse. Medical advice should be sought when any sign of infection is noticed. If sudden fever or inability to breathe is experienced, medical help should be gotten immediately. Klebsiella infections can quickly spread throughout the body, so it's important to seek medical help[364].

2.17.7.4: Prevention of *Klebsiella pneumonia* infection

Since *K. pneumoniae* spreads through person-to-person contact, the best way to prevent infection is to frequently wash your hands. Good hand hygiene will make sure the germs don't spread. You should wash your hands; before touching your eyes, nose, or mouth, before and after preparing or eating food, before and after changing wound dressings, after using the bathroom, after coughing or sneezing. If you're in the hospital, the staff should also wear gloves and gowns when touching other people with Klebsiella infection. They should also wash their hands after touching hospital surfaces[364].

2.18: Treatment of Bacterial Infections during the COVID-19 Pandemic

The respiratory symptoms of patients with COVID-19 pneumonia admitted to hospital with fever and dry cough can mimic those of atypical bacterial pneumonia, making difficult to distinguish patients with hospital acquired pneumonia (HAP) and ventilator associated pneumonia (VAP). A biomarker used to differentiate bacterial from viral infections is procalcitonin[104,184,193], a peptide whose serum levels increase during bacterial but not viral infections. To decrease chances of VAP in the ICU, most COVID-19 patients were empirically treated with antibiotics. The principles of antibiotic stewardship should be considered, but in the case of severely ill patients, the concern surrounding the pandemic

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forced clinicians to start treatment with antibiotics. A study reported that 88.3% of COVID-infected patients (476/539) were treated with broad-spectrum antibiotics including third-generation cephalosporins, quinolones, carbapenems[184]. The choice of empiric regimens should take into account possible side effects (e.g. QT prolongation, diarrhoea), local epidemiology of drug resistance, and impact of drug resistance on the patient. In some countries, bacteria are resistant to at least one antibiotic class, therefore empiric broad-spectrum therapy could have limited effect particularly in hospital-acquired infections. In case of sepsis, inadequate antibiotic therapy may increase mortality says[365].

Overall, since COVID-19 pandemic is still ongoing, and transfer of patients in the ICU continues, the use of antibiotics will steadily raise and increase development and transmission of MDR strains in the healthcare systems. Thus, when the probability of a bacterial infection is low, antibiotic treatment of COVID-19 patients should be re-evaluated, and stopped if not necessary, therefore, antibiotics should be reserved for patients with the most severe respiratory presentations[189].

2.19: Fungal Co-infection in COVID-19 Patients.

20 of 90 Severe Acute Respiratory Syndrome (SARS) patients had secondary lower respiratory tract infections in 2003, which accounted for 70.6% of those critical SARS patients who underwent an invasive operation[366]. The pathogens causing secondary infections in SARS patients were diverse: negative bacilli were the most common but Candida was also common. Invasive pulmonary aspergillosis was another common complication secondary to influenza. A study isolated Candida specie and unspeciated yeast from respiratory samples taken (24/112; 21.4%). These isolates are likely to represent oropharyngeal thrush or normal flora rather than pulmonary candidiasis. Three patients, all

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requiring critical care admission, developed HA *Candida albicans* bacteraemia, all were attributed to central line associated infections. Three culture positive patients with *Aspergillus fumigatus* were identified; one patient was known to be colonized with this filamentous fungus that was not thought to currently represent a pathogen[267]. Also in 2004, a study found that the incidence of fungal infection in SARS patients was 14.8–27%, which was even higher in severely ill ones, up to 21.9–33%, meanwhile, fungal infection was the main cause of death for SARS patients, accounting for 25–73.7% in all causes of death[267].

In China, a study performed fungal culture on all 99 COVID-19 patients at admission and found five (5%, 5/99) cases of fungal infection, including one case of *Aspergillus flavus*, one case of *Candida glabrata* and three cases of *C. albicans*[104]. Another study reported that (3/52, 5.8%) of patients had fungal co-infection in 52 critically ill patients, including *A. flavus*, *A. fumigatus* and *C. albicans*[188]. In addition, a study carried out among patients in Germany found that COVID-19 was associated with invasive pulmonary aspergillosis (IPA) and was found in five (26.3%) of 19 consecutive critically ill patients with moderate to severe ARDS[364]. In another research, IPA was diagnosed in 83 (19%) of 432 patients admitted with influenza, which was higher in immunocompromised patients (32%), and in the event of IPA, the mortality rate increased from 28 to 51%[368]. It was stated that Aspergillus species could be an important cause of life-threatening infection in COVID-19 patients, especially in those with high risk factors[369]. The potential risk factors for the patients include GC use, prolonged neutropenia, chronic obstructive pulmonary disease COPD), allogeneic hematopoietic stem cell transplant (allo-HSCT), solid organ transplant (SOT), inherited immune-deficiencies, hemopoietic malignancy (HM), cystic fibrosis (CF) [369]. For the severe COVID-19 patients who have more opportunities to be treated with

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broad-spectrum anti-bacterial drugs, parenteral nutrition and invasive examinations, or the patients accompanied with prolonged neutropenia and other immune impairment factors, the risk of infection with Candida species may significantly increase[370]. It has also been reported that COVID-19 patients with trauma, diabetes mellitus, GC use, HM, prolonged neutropenia, allo-HSCT, SOT are more likely to develop mucormycosis[371]. Aithough COVID-19 patients with human immunodeficiency virus (HIV) infection accompanied by CD4 + Tlymphocyte count<200 cells/lL, allo-HSCT, SOT, or other immune impaired are susceptible to cryptococcosis which predominantly present as meningo- encephalitis.

2.20: Fungal Diseases and COVID-19

Symptoms of some fungal diseases can be similar to those of COVID-19, including fever, cough, and shortness of breath.[372] Laboratory testing is necessary to determine if a person has a fungal infection or COVID-19. Some patients can have COVID-19 and a fungal infection at the same time. People with severe COVID-19, such as those in an intensive care unit (ICU), are particularly vulnerable to bacterial and fungal infections and the most common fungal infections in patients with COVID-19 include aspergillosis or invasive candidiasis[373]. These fungal co-infections are reported with increasing frequency and can be associated with severe illness and death, an awareness of the possibility of fungal co-infection is essential to reduce delays in diagnosis and treatment in order to help prevent severe illness and death from these infections[374].

2.20.1: COVID-19 × Aspergillus

Like severe flu, COVID-19 progression leads to the manifestation of acute respiratory distress syndrome (ARDS), which predisposes patients to secondary pulmonary aspergillosis. This is an infection caused by Aspergillus, a worldwide distributed filamentous fungus.

Aspergillus spores are typically present in the environment; so, they can easily enter the airway system and, subsequently, they reach the human lung tissue and/or paranasal sinuses by breathing. Aspergillus causes a wide range of infection with various clinical manifestations ranging from localized to disseminated diseases. For instance, invasive aspergillosis typically affects severely immunocompromised patients occasionally as a result of organ transplant, cancer treatment (due to the chemotherapy and/or radiotherapy), neutropenia and long-term treatment with corticosteroids. In addition, allergic forms of aspergillosis (e.g., allergic bronchopulmonary aspergillosis - ABPA) are implicated in asthma exacerbation and bronchitis in individuals with hyperactive immune responses as well as in cystic fibrosis patients[375]. Invasive aspergillosis caused by Aspergillus species (e.g., *A. fumigatus, A. niger, A. flavus, A. terreus*) carries an overall 30 to 95% mortality rate even if it is early diagnosed and despite antifungal treatment approaches[376]. Some studies from China reported high rates of aspergillosis among COVID-19 patients[377]. A retrospective study from an ICU in Wuhan showed the isolation of *A. flavus* and *A. fumigatus* from respiratory tract secretions in two out of seven (28.6%) patients with hospital acquired pneumonia[378]. In another retrospective study conducted in two hospitals of Wuhan regarding 85 fatal cases of COVID-19, fungal culture from sputum obtained from 9 patients were reported positive in 33.3% of cases with 8 (9.4%), 3 (3.5%) and 2 (2.4%) patients receiving voriconazole, fluconazole and caspofungin [379]. However, in all the studies from China fungal infections were poorly defined and for such reason it appears difficult to make any inference. European countries such as France, Germany, Belgium and The Netherlands have recently reported high rates of chronic pulmonary aspergillosis among COVID-19-positive patients with a prevalence index of 20-35% [380]. A case report from

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Brazil, which diagnosed a patient infected with *A. penicillioides* postmortem, pointed out the importance of considering invasive pulmonary aspergillosis in patients with underlying severe COVID-19[381]. An observational study from Pakistan showed that Aspergillus specie were isolated from tracheal aspirates of 39.1% COVID-19-positive patients and, in this fraction, 21.7% were diagnosed with aspergillosis and 17.4% were only considered colonized[382]. In this scenario, the most commonly used drugs are the new triazoles voriconazole and vuconazole followed by less common cases treated with liposomal amphotericin B and caspofungin[383]. These findings and other previous reports highlighted that many cases may remain undiagnosed, since standard culture methods exhibit limited sensitivity. Consequently, the appropriate therapy is not achieved on time and clinical failure outcomes are usually reported[384].

2.20.2: COVID-19-associated pulmonary Aspergillosis.

Scientists are still learning about Aspergillosis (infections caused by the fungus Aspergillus) in people with severe COVID-19. In the past, scientists thought Aspergillosis occurred almost entirely in people with severely weakened immune systems. However, Aspergillosis has been increasingly reported in patients without weakened immune systems but who have severe respiratory infections caused by viruses, including influenza. Several recent reports described COVID-19-associated pulmonary Aspergillosis (CAPA) [385].

2.20.2.1: Available information that indicates CAPA:

- usually occurs in patients with severe COVID-19 (e.g., patients on ventilators in ICUs) [386]
- can be difficult to diagnose because patients often have non-specific symptoms and testing typically requires a specimen from deep in the lungs[387].

- can cause severe illness and death[388].
- Clinicians should consider the possibility of Aspergillosis in patients with severe COVID-19 who have worsening respiratory function or sepsis, even if they do not have classical risk factors for Aspergillosis.(CDC: Information for Health Professionals About Aspergillus).
- Testing for CAPA usually involves obtaining specimens from patients' lower respiratory tract, which are tested for Aspergillus galactomannan antigen and fungal culture.

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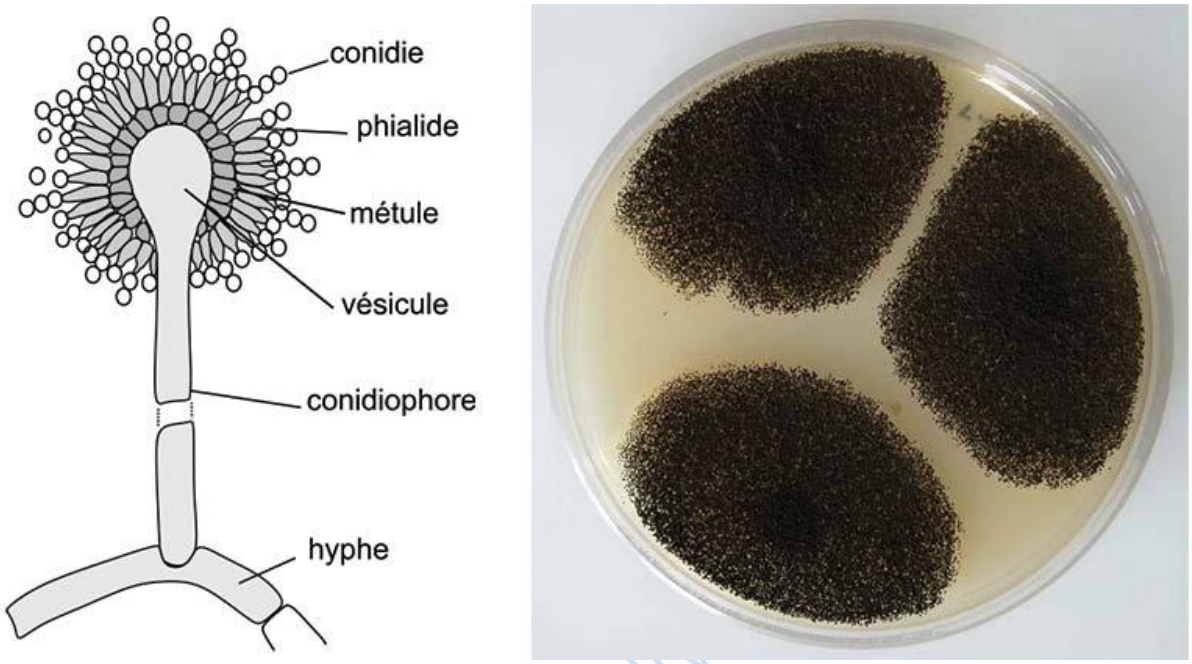

Figure 2.9: KOH Preparation and Morphology of *Aspergillus niger*[34]

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Figure 2.10: Morphology of *Aspergillus parapsilopsis*[39]

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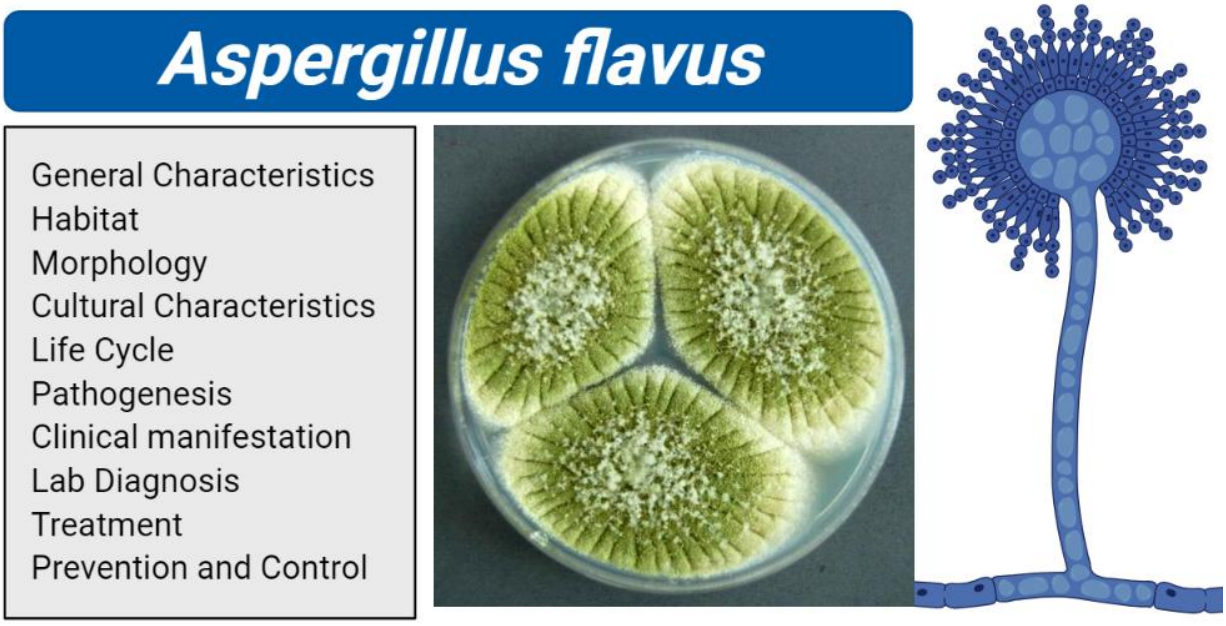

KOH Preparation and Morphology of *Aspergillus flavus*[39]

Figure 2.11: *Aspergillus funmigatus*[39]

2.20.3: COVID-19 × Candida

Fungal infections caused by yeasts can also occur in patients with ARDS, including COVID-19, as a result of impaired immune system functions. Invasive candidiasis is an important health care-associated fungal infection responsible for high mortality rates and it is caused by several opportunistic species belonging to the Candida genus, with *Candida albicans* as the most common species.[389] Data from a hospital in Spain pointed out a rising incidence of invasive candidiasis in COVID-19 positive patients, with an associated mortality of 40%[390]. Invasive candidiasis by *C. albicans* was similarly reported in COVID-19 patients requiring critical care in United Kingdom hospitals[391]. A case report from Austria described a secondary catheter-related candidiasis caused by *C. glabrata* successfully treated with caspofungin for 14 days[392]. Likewise, in another published work, *Candida* spp. was one of the most frequently fungi identified in the bloodstream of patients using central venous catheters during COVID-19 pandemic episodes in New York City, USA[393]. According to recent studies, the majority of *Candida* species recovered from COVID-19 patients were isolated from the oropharynx. Oropharyngeal candidiasis is a localized mucous membrane infection, which is characterized by invasion and damage of oral epithelial cells[394]. *Candida* spp. and other yeasts were isolated from the respiratory tract in 21.4% of positive cases of co-infection during the first pandemic of COVID-19 in two hospitals in the United Kingdom[189]. A retrospective study in Italy evaluated the respiratory specimens of hospitalized COVID-19 patients in ICU. The results showed that almost 52% of cultures were positive for bacteria and fungi (*Candida albicans* and *C. glabrata*). Additionally, in a study conducted in Iran, the authors reported that *Candida albicans* was the most frequent fungus followed by other species isolated from oral lesion of COVID19-positive patients suffering from oropharyngeal candidiasis. Interestingly, those Candida isolates were susceptible to all tested antifungal drugs[395].

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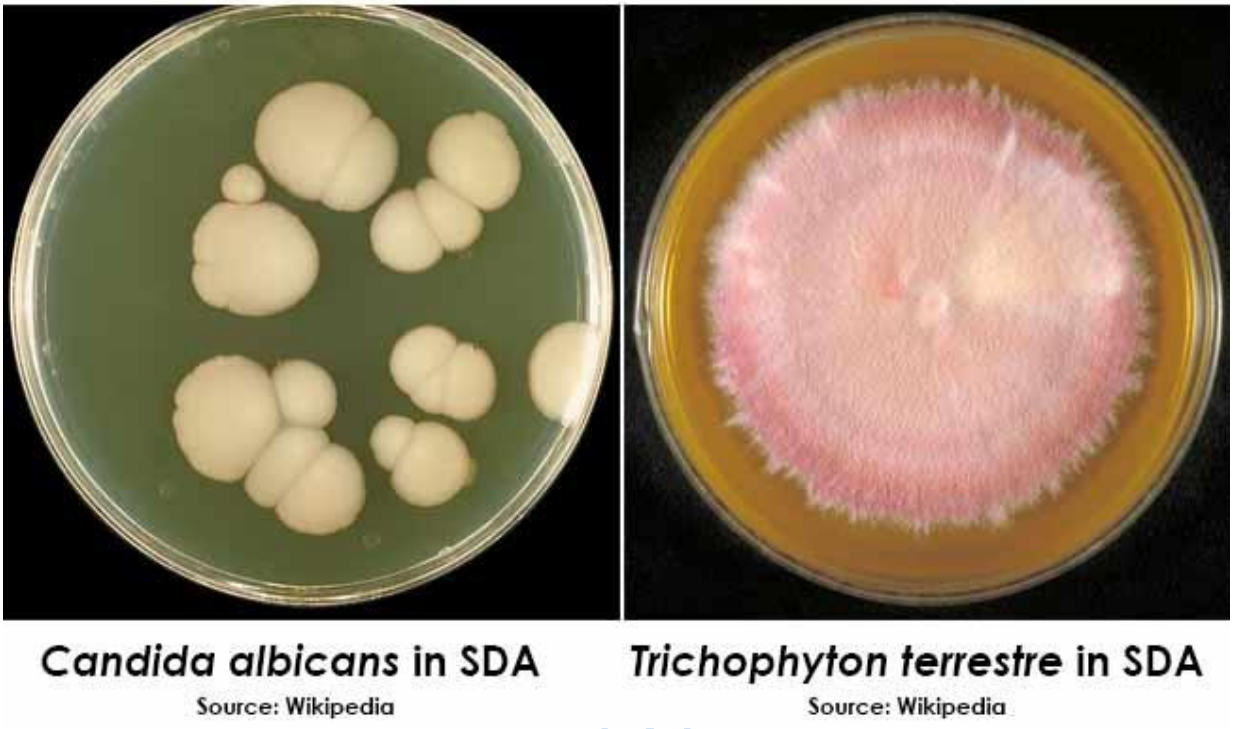

Figure 2.12: Morphology of *Candida albican* and *Trichophyton terrestre* on SDA

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2.20.4: COVID-19 × Saccharomyces

Invasive infection by *Saccharomyces cerevisiae* was reported in two COVID-19 patients hospitalized in ICU after receiving prophylactic supplementation containing Saccharomyces[396]. Initially, both patients were immediately treated with anidulafungin; however, after conclusive identification of species and antifungal performing susceptibility testing, the treatment was changed to fluconazole[396]. First suspected as candidiasis, reported in a patient with secondary mucosal lesions caused by S. cerevisiae resulting from the treatments for COVID-19 or due to the deterioration of the host immune system. The patient received a combination treatment including intravenous fluconazole (for 10 days), oral nystatin (for 30 days) treatment, chlorhexidine digluconate (0.12%) mouth rinses and daily prescription of 1% hydrogen peroxide[397].

2.20.5: COVID-19 × Pneumocystis

Pneumocystis pneumonia, one of the most common associated opportunistic fungal infections in acquired immunodeficiency syndrome (AIDS) patients, has long been associated with other immunodeficiency states as well[398]. Because COVID-19 and Pneumocystis pneumonia may present common clinical features, this fungal infection is often undiagnosed[398]. A case report from the USA described a patient with COVID-19 and pneumonia due to Pneumocystis[399]. Surprisingly, the patient did not have any known immunodeficiency or any classical risk factors for the development of Pneumocystis pneumonia, which resulted in a successful treatment with trimethoprim-sulfamethoxazole and an effective extubating on day 7 of hospital stay. In a case reported of Pneumocystis

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pneumonia that was diagnosed in a German patient after the presence of mild reticular changes visualized by a chest tomography. *Pneumocystis jirovecii* was confirmed in the broncho-alveolar lavage fluid and the patient was treated with intravenous trimethoprim-sulfamethoxazole (20 mg/kg/day of trimethoprim) together with 50 mg of prednisone (a corticoid drug) daily to prevent adverse immune reactions[400].

2.20.6: COVID-19 × other Clinically Relevant Fungi

A rare case report in the USA described a 48-year-old Hispanic male presenting both COVID-19 and chronic pulmonary coccidioidomycosis[401]. The authors revealed that the symptoms of both diseases are extraordinarily similar and include fever, dry cough, dyspnea, myalgia and headache, in addition the authors believed that probably the person contacted pulmonary coccidioidomycosis and subsequently developed COVID-19 and the patient was discharged home without hospitalization[401]. Also a retrospective study in which 94.2% COVID-19 patients were co-infected with one or more different respiratory microbial pathogens. Besides frequent cases of Aspergillus and Candida, six cases of Mucor and one case of Cryptococcus were detected in COVID-19 infected patients. In that report, fungal infection occurred in 29.5% of total co-infected cases[402].

2.20.7: Fungal Pneumonias that resemble COVID-19

Other fungal diseases, such as Valley fever, Coccidioidomycosis, Histoplasmosis, and Blastomycosis, can cause fever, cough, and shortness of breath, similar to COVID-19 and bacterial pneumonias[403]. These fungi live in soil, people become infected by breathing in fungi present in the air. Clinicians should consider fungal pneumonias as a possible cause of

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respiratory illness, particularly if COVID-19 testing is negative. It is important to note that these fungal diseases can occur at the same time as COVID-19[404].

2.20.8: COVID-19-associated Mucormycosis

COVID-19–associated Mucormycosis is less common than other COVID-19–associated fungal infections, emerging reports from India highlight the importance of considering this infection[405]. Some medications used to treat severe COVID-19, including high-dose corticosteroids and tocilizumab, might predispose patients with COVID-19 to Mucormycosis. Mucormycosis has been reported in patients with severe COVID-19 infection who lacked other classical Mucormycosis risk factors, such as diabetes, conditions or medications that weaken the immune system, and cancer[406]. Early diagnosis and treatment are key to improving outcomes for patients with COVID-19–associated Mucormycosis. Clinicians should consider the possibility of Mucormycosis in patients with severe COVID-19 even when patients lack classical risk factors for this disease. Biomarkers for diagnosing invasive Aspergillosis, such as beta-d-glucan and galactomannan, are typically negative in patients with Mucormycosis. The treatment for Mucormycosis frequently involves aggressive surgical intervention and treatment with antifungals, including amphotericin B, Posaconazole, or Isavuconazole. Voriconazole is not recommended for treating Mucormycosis[407].

2.21: Diagnosis of COVID-19

COVID- 19 can provisionally be diagnosed on the basis of symptoms and confirmed using reverse transcription polymerase chain reaction (RT-PCR) or other nucleic acid testing of infected secretions[408,409]. Along with laboratory testing, chest CT scans may be helpful to

diagnose COVID- 19 in individuals with a high clinical suspicion of infection[410]. Detection of a past infection is possible with serological tests, which detect antibodies produced by the body in response to the infection[408].

2.21.1.: SARS-CoV-2 Testing by Taking a Posterior Oropharyngeal Swab (Throat Swab)

Hold the tongue out of the way with a tongue depressor; Use a sweeping motion to swab the posterior pharyngeal wall and tonsilar pillars. Have the subject say "aahh" to elevate the uvula. Avoid swabbing the soft palate and do not touch the tongue with the swab tip because this procedure can induce the gag reflex. Put the swab into Virus Transport Medium(VTM).

2.21.2: SARS-CoV-2 Testing by Taking a Nasopharyngeal Swab (Nasal swab)

Insert a flexible, fine-shafted polyester swab into the nostril and back to the nasopharynx. The swab should be slid straight into the nostril with the patient's head held slightly back. The swab is inserted following the base of the nostril towards the auditory pit and will need to be insert at least 5–6 cm in adults to ensure that it reaches the posterior pharynx. Do not use rigid shafted swabs for this sampling method, a flexible shafted swab is essential. Leave the swab in place for a few seconds. Withdraw slowly with a rotating motion.

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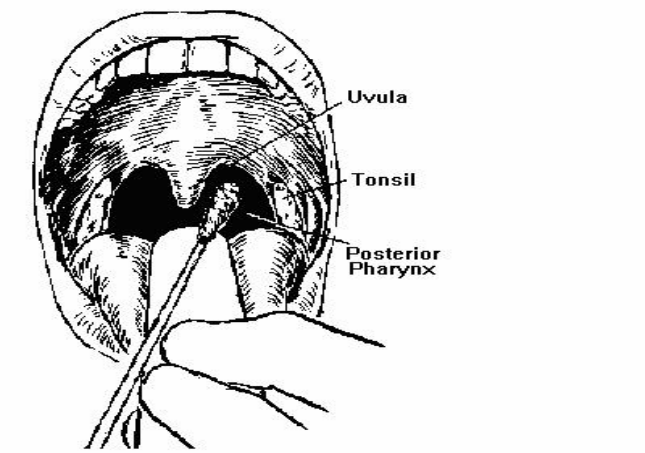

Figure 2.13: Taking a Throat Swab for SARS-CoV-2 testing

Source: WHO/CDS/EPR/ARO/2006

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Figure 2.14: Demonstration of a Nasopharyngeal swab for SARS-CoV-2 testing

SOURCE: http://en.wikipedia.org/wiki/file 2020

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The standard methods of testing for presence of SARS-CoV-2 are nucleic acid tests, which detects the presence of viral RNA fragments[411,412]. As these tests detect RNA but not infectious virus, its "ability to determine duration of infectivity of patients is limited[413]. The test is typically done on respiratory samples obtained by a nasopharyngeal swab; however, a nasal swab or sputum sample may also be used[414,415]. Results are generally available within hours[408]. The WHO has published several testing protocols for the disease[416]. Several laboratories and companies have developed serological tests, which detect antibodies produced by the body in response to infection. Several have been evaluated by Public Health England and approved for use in the UK[417].

2.22: Medium for Fungal and Bacteria Isolation

Sabouraud Dextrose Agar (SDA) is used for the isolation, cultivation, and maintenance of non-pathogenic and pathogenic species of **fungus**. SDA was formulated by Sabouraud in 1892 for culturing dermatophytes. The pH is adjusted to approximately 5.6 in order to enhance the growth of fungi, especially **dermatophytes**, and to slightly inhibit bacterial growth in clinical specimens[416].

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2.22.1: Composition of SDA

Ingredients	In gm/L
Dextrose (Glucose)	40 gm
Peptone	10 gm
Agar	15 gm
Distilled Water	1000 ml

Final pH 5.6 +/- 0.2 at 25ºC.

2.22.2: Principle of SDA

Peptone (Enzymatic Digest of Casein and Enzymatic Digest of Animal Tissue) provide the nitrogen and vitamin source required for organism growth in SDA. Dextrose is added as the energy and carbon source. Agar is the solidifying agent. Chloramphenicol and/or Tetracycline may be added as broad spectrum antimicrobials to inhibit the growth of a wide range of Gram-positive and Gram-negative bacteria. Gentamicin is added to further inhibit the growth of Gram-negative bacteria. The neutral pH of the Emmons modification seems to enhance the growth of some pathogenic fungi, such as dermatophytes.

2.22.3: Uses of SDA

1. SDA is primarily used for the selective cultivation of yeasts, moulds and aciduric bacteria.

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2. The medium is often used with antibiotics for the isolation of pathogenic fungi from material containing large numbers of other fungi or bacteria.
3. This medium is also employed to determine microbial contamination in food, cosmetics, and clinical specimens.

2.22.4: Preparation of SDA

1. Combine all ingredients in ~900 ml of deioinized water.
2. Adjust to pH 5.6 with hydrochloric acid and adjust final volume to 1 liter.
3. Heat to boiling to dissolve the medium completely.
4. Autoclave at 121ºC for 15 minutes.
5. Cool to ~45 to 50°C and pour into petri dishes or tubes for slants.

Sabouraud agar plates can be inoculated by streaking, as with standard bacteriological media, or by exposing the medium to ambient air. Typically, molds are incubated at room temperature (22 to 25°C) and yeasts are incubated at 28 to 30°C or 37°C if suspected of being dimorphic fungi. Incubation times will vary, from approximately 2 days for the growth of yeast colonies such as *Malasezzia,* to 2 to 4 weeks for growth of dermatophytes or dimorphic fungi such as *Histoplasma capsulatum.* Indeed, the incubation time required to acquire fungal growth is one diagnostic indicator used to identify or confirm fungal species.

2.22.5: Result Interpretation on SDA

Identification of fungi is performed by observing various aspects of colony morphology, characteristic microscopic structures, rate of growth, media which supports the organism's growth, and source of specimen. Yeasts are identified by various biochemical tests. Yeasts

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will grow as creamy to white colonies. Molds will grow as filamentous colonies of various colours.

2.22.6: CHROMagar Candida

CHROMagar Candida (CAC, Becton Dickinson, Heidelberg, Germany) is a differential ready-to-use chromogenic medium designed to identify *Candida albicans, Candida tropicalis, and Candida krusei* by colony color and morphology. Colonies of *Candida albicans* appear light to medium green; *Candida tropicalis* colonies appear dark blue to metallic blue; and *Candida krusei* pink with a whitish border[25]. Other yeasts may develop either light to dark mauve or cream colors (e.g., *Candida glabrata*) on isolation media Chromogenic Candida agar (OCCA, Oxoid, Basingstoke, UK) contains a chromogenic substratum for rapid detection and specific identification of such *Candida* spp. Typically, on OCCA, colonies of *Candida albicans* are green; *Candida tropicalis* are dark blue; and pink, irregular and dry colonies indicate *Candida krusei*. OCCA permits the identification of *Candida guilliermondii*, which forms blackberry wine-colored colonies[26]. Other *Candida s*pecies produce bluish-green, beige, yellow, maroon, or violet colonies[26].

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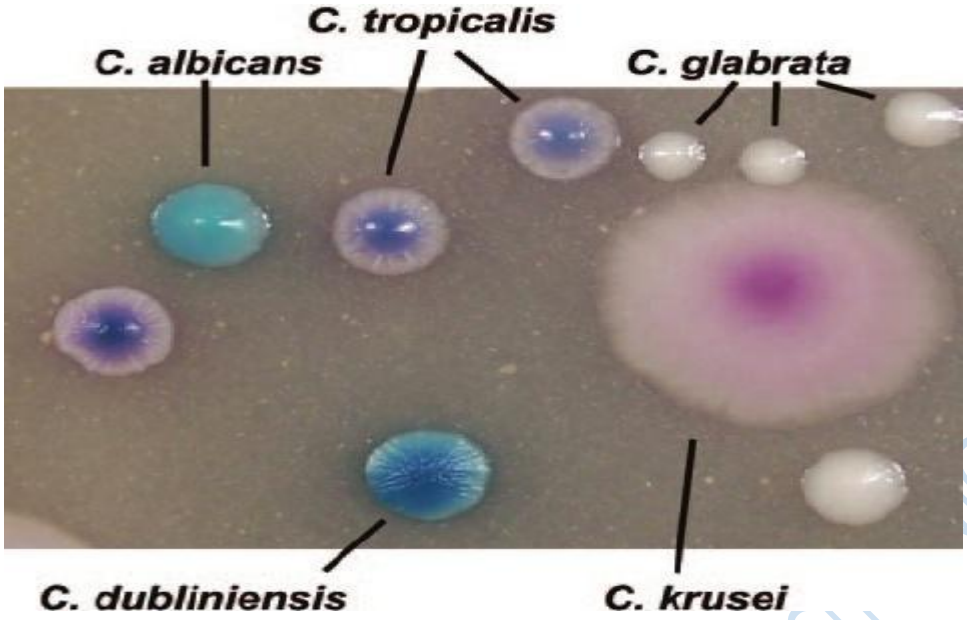

Source: Wikipedia.

Figure 2.15: Chromogenic candida specie Identification

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2.23: Gram Positive Bacteria on Mannitol Salt Agar Medium

Mannitol salt agar or MSA is a commonly used selective and differential growth medium in microbiology. It encourages the growth of a group of certain bacteria while inhibiting the growth of others. This medium is important in medical laboratories as one method of distinguishing pathogenic microbes in a short period of time. [418] It contains a high concentration (about 7.5–10%) of salt (NaCl) which is inhibitory to most bacteria - making MSA selective against most Gram-negative and selective for some Gram-positive bacteria (Staphylococcus, Enterococcus and Micrococcaceae) that tolerate high salt concentrations[419] It is also a differential medium for mannitol-fermenting staphylococci, containing carbohydrate mannitol and the indicator phenol red, a pH indicator for detecting acid produced by mannitol-fermenting staphylococci[420] Staphylococcus aureus produces yellow colonies with yellow zones, whereas other coagulase-negative staphylococci produce small pink or red colonies with no colour change to the medium.If an organism can ferment mannitol, an acidic byproduct is formed that causes the phenol red in the agar to turn yellow[418] It is used for the selective isolation of presumptive pathogenic (pp) Staphylococcus species[421]

Expected results

1. Gram + Staphylococcus: fermenting mannitol: medium turns yellow (e.g. S. aureus)
2. Gram + Staphylococcus: not fermenting mannitol, medium does not change color (e.g. S. epidermidis)

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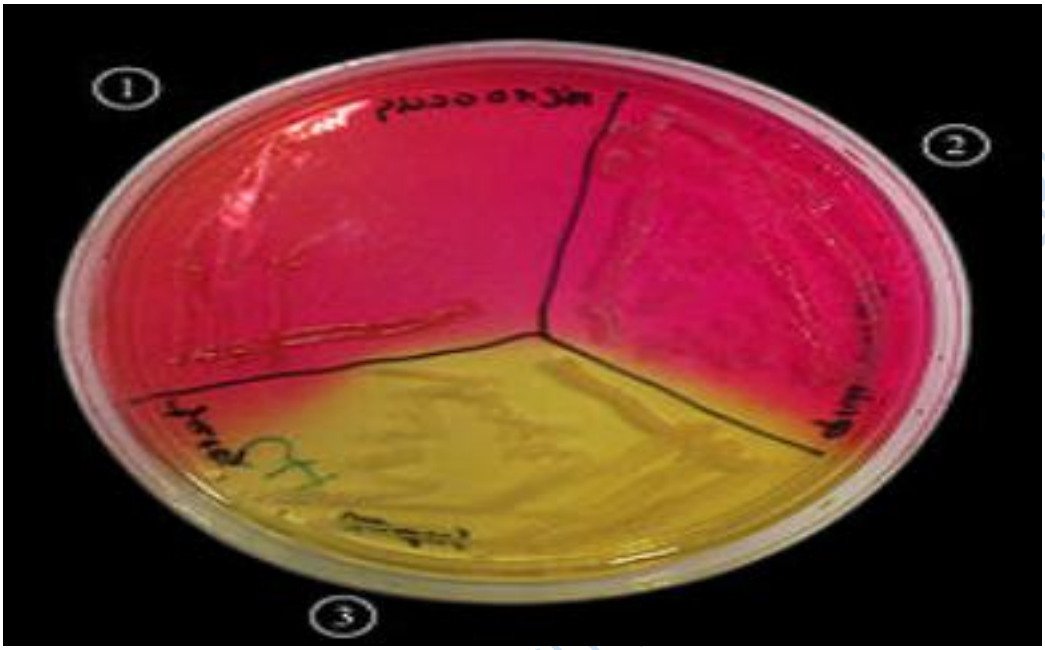

Source: Wikipedia

Figure 2.16: Colour Speciation and Identification on MSA plate with Micrococcus sp. (1), Staphylococcus epidermidis (2) and S. aureus colonies (3).

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2.24: Chocolate Agar (CHOC) or Chocolate Blood Agar (CBA),

This a nonselective, enriched growth medium used for isolation of pathogenic bacteria[422, 423, 424]. It is a variant of the blood agar plate, containing red blood cells that have been lysed by slowly heating to 80°C. Chocolate agar is used for growing fastidious respiratory bacteria, such as *Haemophilus influenzae* and *Neisseria meningitidis*[425]. In addition, some of these bacteria, most notably H. influenzae, need growth factors such as nicotinamide adenine dinucleotide (factor V or NAD) and hemin (factor X),which are inside red blood cells; thus, a prerequisite to growth for these bacteria is the presence of red blood cell lysates. The heat also inactivates enzymes which could otherwise degrade NAD. The agar is named for its color and contains no chocolate products. Chocolate agar with the addition of bacitracin becomes selective for the genus *Haemophilus*. Another variant of chocolate agar called Thayer Martin agar contains an assortment of antibiotics which select for *Neisseria* species[425]

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Source: Wikipedia

Figure 2.17: Chocolate Agar showing *Francisella tularensis* colonies

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2.25: MacConkey Agar

MacConkey agar is a selective and differential culture medium for bacteria. It is designed to selectively isolate Gram-negative and enteric (normally found in the intestinal tract) bacteria and differentiate them based on lactose fermentation[422]. Lactose fermenters turn red or pink on MacConkey agar, and non-fermenters do not change color. The media inhibits growth of Gram-positive organisms with crystal violet and bile salts, allowing for the selection and isolation of Gram-negative bacteria. The media detects lactose fermentation by enteric bacteria with the pH indicator neutral red[423]. It contains bile salts (to inhibit most Gram-positive bacteria), crystal violet dye (which also inhibits certain Gram-positive bacteria), and neutral red dye (which turns pink if the microbes are fermenting lactose).

2.25.1: Composition of MacConkey Agar[424]

- Peptone – 17 g
- Proteose peptone – 3 g
- Lactose – 10 g
- Bile salts – 1.5 g
- Sodium chloride – 5 g
- Neutral red – 0.03 g
- Crystal violet – 0.001 g
- Agar – 13.5 g
- Water – add to make 1 litre; adjust pH to 7.1 +/− 0.2
- Sodium taurocholate

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There are many variations of MacConkey agar depending on the need. If the spreading or swarming of *Proteus* species is not required, sodium chloride is omitted. Crystal violet at a concentration of 0.0001% (0.001 g per litre) is included when needing to check if Gram-positive bacteria are inhibited. MacConkey with sorbitol is used to isolate *E. coli* O157, an enteric pathogen[423].

The medium was developed by Alfred Theodore MacConkey while working as a bacteriologist for the Royal Commission on Sewage Disposal. Using neutral red pH indicator, the agar distinguishes those Gram-negative bacteria that can ferment the sugar lactose (Lac+) from those that cannot (Lac-). This medium is also known as an "indicator medium" and a "low selective medium". Presence of bile salts inhibits swarming by Proteus species.

2.25.2: Lac positive

By utilizing the lactose available in the medium, Lac+ bacteria such as *Escherichia coli*, *Enterobacter* and *Klebsiella* will produce acid, which lowers the pH of the agar below 6.8 and results in the appearance of pink colonies. The bile salts precipitate in the immediate neighborhood of the colony, causing the medium surrounding the colony to become hazy[425,426].

2.25.3: Lac negative

Organisms unable to ferment lactose will form normal-colored (i.e., un-dyed) colonies. The medium will remain yellow. Examples of non-lactose fermenting bacteria are *Salmonella, Proteus species, Yersinia, Pseudomonas aeruginosa* and *Shigella.*

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2.25.4: Lac Slow

Some organisms ferment lactose slowly or weakly, and are sometimes put in their own category. These include *Serratia* and *Citrobacter*[427,428].

2.25.5: Mucoid colonies

Some organisms, especially *Klebsiella* and *Enterobacter*, produce mucoid colonies which appear very moist and sticky and slimy. This phenomenon happens because the organism is producing a capsule, which is predominantly made from the lactose sugar in the agar.

2.25.6: Variant

A variant, sorbitol-MacConkey agar, (with the addition of additional selective agents) can assist in the isolation and differentiation of enterohemorrhagic *E. coli* serotype O157:H7, by the presence of colourless circular colonies that are non-sorbitol fermenting[427]

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Source: Wikipedia

Figure 2.18: MacConkey agar with Lactose Fermenter (pink colour) and non-Lactose Fermenter colonies (colourless or pale colour).

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2.26: API 20E Identification for Bacteria Pathogens

The Analytical Profile Index or API is a classification of bacteria based on experiments, allowing fast identification. This system is developed for quick identification of clinically relevant bacteria. Because of this, only known bacteria can be identified. The API fast identification system combines some conventional tests and allows the identification of a limited number of Gram-negative Enterobacteriaceae or non-Enterobacteriaceae. The test systems are stored in 20 small reaction tubes, which include the substrates. An identification is only possible with microbiological culture. API test strips consists of microtubes (cupules) containing dehydrated substrates to detect the enzymatic activity or the assimilation / fermentation of sugars by the inoculated organisms. During incubation, metabolism produces colour changes that are either spontaneous or revealed by the addition of reagents. When the carbohydrates are fermented, the pH within the cupule changes and is shown by an indicator. Assimilation tests are inoculated with a minimal medium (API AUX medium) and the bacteria grow if they are able to utilize the corresponding substrate: a positive result is indicated by growth. Test results are entered into an online database to determine the bacterial identity.

Following presumptive organism identification using Gram's stain, morphological features and other simple tests, the appropriate API kit should be selected using the table below

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Table 2. 4: BACTERIAL IDENTIFICATION USING BIOMERIEUX API KITS

Presumptive Organism ID	Which API strip to use	Additional notes
Gram negative bacillus • Oxidase positive • Non-fastidious • Non-Enterobacteriaceae	API 20 NE	*Stenotrophomonas* & *Acinetobacter* spp.(oxidase negative) may also be identified using API 20E.
Gram negative bacillus • Oxidase negative • Enterobacteriaceae & other non-fastidious GNB	API 20 E	*Vibrio* spp. and *Aeromonas* spp. (oxidase positive) may also be identified using API 20NE.
Gram positive cocci • Pairs or chains • Catalase negative • Streptococci, Enterococci& related genera	API 20 Strep	*Streptococcus pneumoniae* and groupable beta-haemolytic *streptococci* do not usually require API testing.
Gram negative cocci in pairs Pleomorphic nutritionally demanding Gram negative bacilli or coccobacilli (e.g. *Neisseria, Haemophilus, Moraxella*)	API NH	*Moraxella catarrhalis* can be adequately identified using the trybutyrin test if isolated from a non-sterile site. *Haemophilus influenzae* can be adequately identified by XV-factor dependent growth.

Source: Global Health Network 2018

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2.27: VITEK 2.0 Analysis for the Identification of Bacteria Pathogens.

VITEK 2 system (BioMérieux) is a new automated bacterial identification and susceptibility testing system that uses fluorescence-based technology. This direct-identification rate was slightly lower than that obtained by comparing results of the VITEK 2 systems with those of standard API identification systems on pure isolates[432.] The slower rate of metabolism of nonenteric bacteria may cause weaker fluorescent biochemical reactions in the reaction wells of the VITEK 2 cards; therefore, some nonenteric bacteria may not be identifiable by this system. The VITEK 2 system had an overall reliable performance, except for some minor problems. For example, there were memory button and test card autoloader failures, and the differential centrifugation necessary to obtain the bacterial pellet is quite labor intensive. However, the direct identification results take only 3.3 h, which is much faster than conventional identification results[432].

2.28: MS VITEK Analysis for the Identification of Fungal Pathogens.

Fungal infections can lead to high morbidity and mortality, particularly in immunocompromised patients. An early treatment which considers the fungal species and any potential resistance is required to successfully treat these infections[429, 430, 431]. Optimal therapies are also needed to reduce the overall cost of hospital care for patients with a fungal infection[432]. Using conventional laboratory techniques, identification of the fungal species responsible for infection can take a long time (2 days to several weeks). It also requires specialized laboratory personnel and is costly. Given these issues, Matrix-Assisted Laser

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Desorption Ionization–Time of flight Mass Spectrometry (MALDI-TOF MS)-based systems present an interesting alternative for the routine identification of fungal species. This technique allows accurate species identification, takes only a few minutes, and is cost-effective[433,434]. Moreover, the spectrum of identifiable fungal species gradually spreads. While until recently, this technique involved only yeasts, new developments have allowed the use of MALDI-TOF MS for the identification of filamentous fungi, particularly Aspergillus species[435,436]. The protocol typically used with this MALDI-TOF technology to identify fungal species requires an extraction procedure of fungal proteins before the deposit, as recommended with other mass spectrometry systems[437,434.438.439]. This procedure is time-consuming, because the colonies must be suspended in microtubes, mixed with various solvents, and centrifuged several times. Even if simplified procedures begin to appear, particularly for filamentous fungi[435, 436].

2.29: Antibiotics

An antibiotic is a type of antimicrobial substance active against bacteria. It is the most important type of antibacterial agent for fighting bacterial infections, and antibiotic medications are widely used in the treatment and prevention of such infections[440,441]. They may either kill or inhibit the growth of bacteria. A limited number of antibiotics also possess antiprotozoal activity[431,432]. Antibiotics are not effective against viruses such as the common cold or influenza drugs which inhibit viruses are termed antiviral drugs or antivirals rather than antibiotics[442].

Sometimes, the term antibiotic literally "opposing life", from the Greek roots anti, "against" and bios, "life"is broadly used to refer to any substance used against microbes, but in the

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usual medical usage, antibiotics (such as penicillin) are those produced naturally (by one microorganism fighting another), whereas nonantibiotic antibacterials (such as sulfonamides and antiseptics) are fully synthetic. However, both classes have the same goal of killing or preventing the growth of microorganisms, and both are included in antimicrobial chemotherapy. "Antibacterials" include antiseptic drugs, antibacterial soaps, and chemical disinfectants, whereas antibiotics are an important class of antibacterials used more specifically in medicine and sometimes in livestock feed[443].

Antibiotics have been used since ancient times. Many civilizations used topical application of mouldy bread, with many references to its beneficial effects arising from ancient Egypt, Nubia, China, Serbia, Greece, and Rome. The first person to directly document the use of moulds to treat infections was John Parkinson (1567–1650). Antibiotics revolutionized medicine in the 20th century. Alexander Fleming (1881–1955) discovered modern day penicillin in 1928, the widespread use of which proved significantly beneficial during wartime. However, the effectiveness and easy access to antibiotics have also led to their overuse[i] and some bacteria have evolved resistance to them. [444] The World Health Organization has classified antimicrobial resistance as a widespread "serious threat [that] is no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country"[445].

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2.29.1: Medical uses of Antibiotics

Antibiotics are used to treat or prevent bacterial infections, and sometimes protozoan infections. (Metronidazole is effective against a number of parasitic diseases). When an infection is suspected of being responsible for an illness but the responsible pathogen has

not been identified, an empiric therapy is adopted. This involves the administration of a broad-spectrum antibiotic based on the signs and symptoms presented and is initiated pending laboratory results that can take several days[446].

When the responsible pathogenic microorganism is already known or has been identified, definitive therapy can be started. This will usually involve the use of a narrow-spectrum antibiotic. The choice of antibiotic given will also be based on its cost. Identification is critically important as it can reduce the cost and toxicity of the antibiotic therapy and also reduce the possibility of the emergence of antimicrobial resistance. To avoid surgery, antibiotics may be given for non-complicated acute appendicitis[447].

Antibiotics may be given as a preventive measure and this is usually limited to at-risk populations such as those with a weakened immune system (particularly in HIV cases to prevent pneumonia), those taking immunosuppressive drugs, cancer patients, and those having surgery. Their use in surgical procedures is to help prevent infection of incisions. They have an important role in dental antibiotic prophylaxis where their use may prevent bacteremia and consequent infective endocarditis. Antibiotics are also used to prevent infection in cases of neutropenia particularly cancer-related[448].

2.29.2: Classes of Antibiotics

Antibiotics are commonly classified based on their mechanism of action, chemical structure, or spectrum of activity. Most target bacterial functions or growth processes[449]. Those that target the bacterial cell wall (penicillins and cephalosporins) or the cell membrane (polymyxins), or interfere with essential bacterial enzymes (rifamycins, lipiarmycins, quinolones, and sulfonamides) have bactericidal activities. Protein synthesis inhibitors

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(macrolides, lincosamides, and tetracyclines) are usually bacteriostatic (with the exception of bactericidal aminoglycosides)[450]. Further categorizations are based on their target specificity. "Narrow-spectrum" antibiotics target specific types of bacteria, such as gram-negative or gram-positive, whereas broad-spectrum antibiotics affect a wide range of bacteria. Following a 40-year break in discovering new classes of antibacterial compounds, four new classes of antibiotics have been brought into clinical use in the late 2000s and early 2010s: cyclic lipopeptides (such as daptomycin), glycylcyclines (such as tigecycline), oxazolidinones (such as linezolid), and lipiarmycins (such as fidaxomicin)[451].

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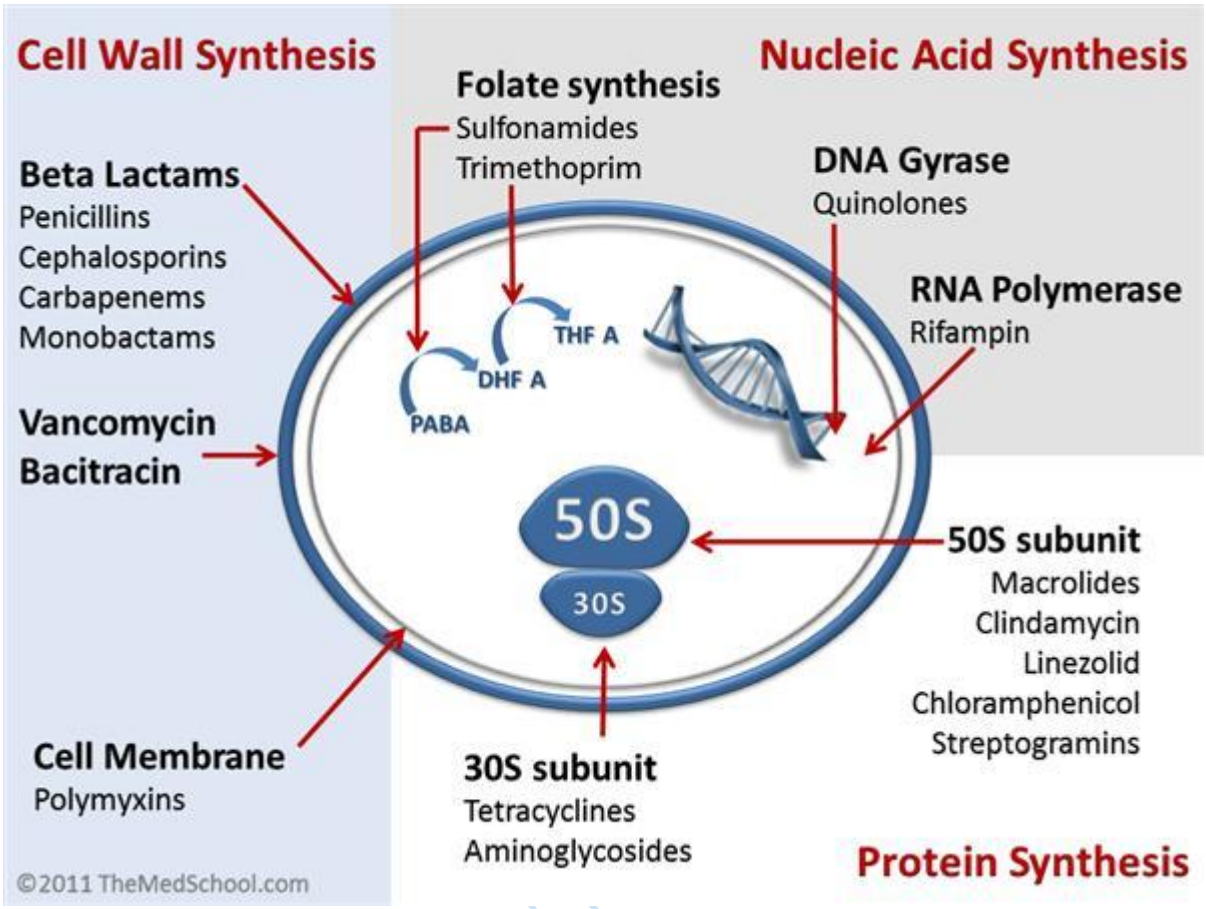

Source: Wikipedia

Figure 2.19: Bacteria cell wall

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2.30: VITEK 2.0 Analysis to Determine the Antibiogram of Isolated Pathogens.

According to hospitals routinely perform antimicrobial susceptibility testing for bacterial pathogens which then summarizes the results in a table called an antibiogram[452]. Clinicians refer to antibiograms to guide optimal empiric antibiotic therapy and reduce inappropriate antibiotic usage. Antibiograms can also be used to track changes in antibiotic resistance over time, perform surveillance for emergence of drug-resistant organisms, and identify areas for intervention by antimicrobial stewardship programs[453]. The Clinical and Laboratory Standards Institute (CLSI) developed consensus guidelines for the collection, storage, analysis, and presentation of antimicrobial susceptibility data[454]. Creating an antibiogram is typically a labor-intensive, manual process, however creating a digital form of antibiogram has been given priority. An example is the VITEK which is used to perform organism identification and antimicrobial susceptibility testing in conjunction with disk diffusion and gradient strip testing per CLSI guidelines[454].

2.31: Post COVID-19 andAntimicrobial Resistance Bacteria

It has been noted during the current pandemic, the antibiotic administration has been frequently used for COVID19 patients who were admitted to the intensive care unit[455]. While scientists attempt to understand and control the COVID-19 pandemic, it would be also critical to prepare for the effect of the current and future viral pandemics on secondary bacterial infections, resulting in antimicrobial resistance in the near future. In combination with using an antimalarial drug, hydroxyl-chloroquine, azithromycin has become a popular therapeutic option for COVID-19 patients[456]. Reports demonstrated that a combination of hydroxyl-chloroquine and azithromycin was effective for a large proportion of Covid-19

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patient[456]. It is hard to estimate how often this combination is prescribed, but such a rate would be high enough to cause a shortage of azithromycin. However, 30–40% of common types of bacterial agents are already resistant to azithromycin, and overuse could render this or other antibiotics even less effective[457]. The findings could help experts' advice on using the antibiotics in COVID-19 patients and help them to better understand the spread of co-infections in hospitals and the mechanism of bacterial viral coinfection. One factor that involves in the antibiotic resistance in bacterial co-infection is the widespread use of antibiotics in COVID-19 patients. Emerging data show that more than 90% of COVID-19 patients receive antibacterial drugs[458]. This rapid increase in antibiotic administration can cause a strong selective pressure on bacterial pathogens to evolve resistance leading to the increased incidence of drug-resistant bacterial infections in the years subsequent to the COVID-19 pandemic. It was estimated that 10 million people could die from an antibiotic-resistant bacterial infection in the year of 2050, but such prediction may be altered and shortened because of the devastating impact of the COVID-19 pandemic on the usage of antibiotics, so this timeline will almost have to be modified[459]. Nevertheless, concerted efforts must be made to better understand antibiotic administration in COVID-19 patients.

Antibiotics do not directly act on viral infections but viral respiratory infections often lead to bacterial co-infections[460]. The current pandemic highlights the necessity for understanding the complex relationship between viral and bacterial infections. Of note, in patients who have treated with high dose antibiotics may have more co-infections with drug resistant bacteria. Additionally, a recent clinical trial conducted, demonstrated that the use of broad-spectrum antibiotics (which led to depleting gut microbiota) decreased and impaired the immune system's ability to generate antibodies[461]. Also, the current study shows that the use

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of antibiotics perturbed bile acid metabolism and induced inflammatory responses[462]. Hence, improved functional therapies, including antibiotics and alternative therapies as well as the prediction of bacterial respiratory infections using vaccines, should be regarded as potential therapeutic approaches[462]. Besides, standard guidelines should also be established for the administration of the antibiotics. In addition to the direct effect on drug-resistant bacteria as a result of enhanced antibiotic administration, the transmission of drug-resistant bacteria through the medical system should be taken into account. The COVID-19 pandemic has highlighted the importance of vaccination, the need for functional antimicrobials, as well as the necessity for supporting research into the understanding and control of co-infections. Rapid characterization of co-infection is essential in the treatment of the most COVID-19 patients, and could help to save lives, and will improve antimicrobial stewardship during the pandemic[462]. Additionally, mixed bacterial-viral infections can result in antibiotic treatment failure. These observations show that a better understanding of the underlying mechanisms will enable researchers to design effective preventive and therapeutic options[462].

2.32: Ranges of Macrolide

Macrolide antibiotics are mainly active against Grampositive bacteria such as Staphylococcus aureus. Acquired resistance to macrolide antibiotics has been extensively studied in these bacteria and is generally due to N-6-dimethylation of a specific adenine residue in 23S rRNA1>2). Members of the family Enterobacteriaceae, like most Gram-negative organisms, are intrinsically resistant to low levels of erythromycin A, probably by efflux pump[463]. Macrolides are antibacterial substances which have a central lactone ring as their basic structure. Lincosamides are structurally different from macrolides, but their binding sites overlap. Streptogramins consist of two types of molecules, A and B, acting in

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synergy. The binding site of streptogramin B overlaps that of macrolides and lincosamides. Modification of the bacterial target site of these molecules typically leads to cross-resistance between macrolides, lincosamides and streptogramin B (MLSB resistance phenotype).

Macrolides are used for treatment of diseases that are common in food producing animals and for medication of large groups of animals (mass medication). Lincosamides are more limited in indications, and the number of products is lower. Macrolides have been categorised as critically important and lincosamides as highly important for veterinary medicine in the list of antimicrobials of veterinary importance[464]. Macrolides and streptogramins are classified as critically important in human medicine[61].

Macrolides and Streptogramins are classified as Critically Important in Human Medicine

Macrolides			Lincosamides	Streptogramins (A and B)
	15-membered ring	16-membered ring	Clindamycin* Lincomycin* Pirlimycin	Pristinamycin Quinupristin/Dalfopristin Virginiamycin
14-membered ring				
Clarithromycin Erythromycin* Oleandomycin Roxithromycin Telithromycin	Azithromycin	Josamycin Mideacamycin Miocamycin Rokitamycin Spiramycin* Tildipirosin*** Tilmicosin* Tylosin* Tylvalosin		

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Macrolides are classified according to the number of atoms which comprise the lactone ring, reaching from 12 to 16 members[465].To this ring, two or more sugar moieties can be attached. Macrolides with a 12-member ring are no more in use. The first macrolide discovered in the early 1950ies was erythromycin, which is an organic substance produced by the actinomycete Saccharopolyspora erythraea (formerly Streptomyces erythraeus)[466]. The first macrolide intended for animal use was spiramycin, which was introduced in the early 1960ies, followed by erythromycin and tylosin. A chemically modified tylosin, tylvalosin (acetylisovalery ltylosin), was authorized for pigs in the EU in 2004. In early 1990ies the semi-synthetic, new generation macrolides were introduced into human medicine[467].

2.33: Classes of Macrolides and related compounds.

Azithromycin and other macrolides have been largely used to treat infections from Gram-positive microorganisms, including *Streptococcus pneumoniae*, methicillin-sensitive *Staphylococcus aureus,* and group A, B, C, and G *Streptococcus,* but Azithromycin also possesses satisfactory activity against different gram-negative microorganisms, including *Haemophilus* spp., *Moraxella catarrhalis, Escherichia coli, Salmonella* spp*., Yersinia enterocolitica, Shigella* spp, *Campylobacter jejuni, Vibrio cholerae, Neisseria gonorrhoeae,* and *Helicobacter pylori*[468]. In fact, Azithromycin is active against atypical pneumonia pathogens, including *Legionella pneumophila, Chlamydia pneumoniae,* and *Mycoplasma pneumoniae*[468]. Nevertheless, acquired macrolide resistance is an increasingly recognised problem. The development of acquired resistance towards Azithromycin and other related macrolides is associated with active macrolide efflux pumps produced by the bacteria[469]. Active macrolide efflux pumps are encoded by the macrolide efflux genes MSRA and

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MSRB. These efflux pumps are part of the bacterial systems involved in the extrusion of molecules from bacteria to the environment, including bacterial products such as siderophores, as well as toxic compounds and macrolide antibiotics[469].

2.34: Macrolides Resistance Gene

There are different mechanisms of resistance: the first is efflux of the drug from the bacteria encoded by mefA, second is alteration of the ribosomal target by a ribosomal methylase encoded by the erm gene, and third mphA, mutations occurring in ribosomal protein L4 or L22 or 23S rRNA confer macrolide resistance (mutations in the genes encoding ribosomal proteins L4 and L22 and in 23S rRNA) [470]. Chromosomal efflux pumps are bacterial systems involved in the extrusion of molecules from bacteria to the environment, including bacterial products such as siderophores as well as toxics and antibiotics[471]. In this line chromosomal efflux pumps are involved in intrinsic and acquired Azithromycin resistance[472]. Additionally, target amino acid substitutions in the L4 (rplD) and L22 (rplV) ribosomal proteins and in 23S rRNA (rrlH) have also been involved in macrolide resistance[473]. Nonetheless, the most relevant mechanisms of Azithromycin resistance in Enterobacteriaceae are those encoded in mobile elements. Different Macrolide Resistance Genes (MRGs) have been described, leading to resistance through different pathways such as target modifications produced by rRNA methylases encoded in erm genes or macrolides-inactivation, mediated by esterases such as those encoded by ere(A) or ere(B) genes or by phosphorylases such as those encoded in the mph(A) and mph(B) genes. Additionally, transferable genes such as msr(A), mef(A) or mef(B) have been reported to encode macrolide-efflux pumps[472].

2.35: Ranges of Antifungal Agents

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Antifungal treatment selection is a factor of the causative agent identification and in vitro susceptibility testing which provide valuable information for patient management. In resource-limited settings, Echinocandins, voriconazole and liposomal formulations of amphotericin B are either unvailable or if available, not affordable[474]. Fungal infections are a major cause of morbidity and mortality despite the latest developments of diagnostic tools and therapeutic options. Early initiation of the correct antifungal therapy has been demonstrated to have a direct impact on the patient's outcome. More severe infections affect mainly immunocompromised patients but other populations are also infected[475]. New chronic lung infections have been described with a huge impact on the patient's quality of life, and a high cost of treatment and care. Besides, some skin fungal infections involving mucosa and subcutaneous tissues cause substantial morbidity[476]. *Cryptococcus, Candida, Aspergillus,* and *Pneumocystis* are the main etiologic agents of fungal infections. The burden and mortality associated with these diseases depend on the region and the affected population. Thus, it has been estimated that *Cryptococcal meningitis* affects nearly one million people per year. Despite treatment, mortality rates can reach 55 to 70% in AIDS patients in Latin America and sub-Saharan Africa, the estimated number of deaths per year being over 620,00045[475].

Cryptococcus, Candida, Aspergillus, and Pneumocystis affect mainly immunocompromised individuals, however endemic dimorphic fungi such as *Histoplasma, Blastomyces, Coccidioides* and *Paracoccidioides* affect immunocompetent patients as well, and endemic areas include several regions of Latin America[475]. Nowadays, three main families of antifungals are used in the clinical setting to treat fungal infections: polyenes represented by amphotericin B (and its different formulations); azoles with several derivatives such as

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itraconazole, fluconazole, voriconazole, posaconazole, isavuconazole; and the echinocandins: caspofungin, micafungin and anidulafungin. The availability of new antifungals in recent years has provided clinicians with more options, increasing the use of these compounds not just for treatment when the infection has been diagnosed, but also as prophylactic, empirical or preemptive treatment. The increased use of antifungals has induced a higher selective pressure on fungal strains and resistance has emerged in two main ways: several species have developed secondary resistance and susceptible species have been replaced by resistant ones, changing the epidemiology of fungal infections[475]. Antifungal susceptibility testing methods are available to detect antifungal resistance and to determine the best treatment for a specific fungus. Clinical microbiology relies on these methods to select the agent of choice for a fungal infection, and to know the local and the global epidemiology of antifungal resistance[475].

Susceptibility of Candida species to different antifungal agents are also a common practice, however, different methods are employed to achieve the same goal. Some of the different techniques include: Disc diffusion method, Epsilometre test (E-test), Candfast, Microdilution technique among others. Fluconazole, Voriconazole, Itraconazole and Amphotericin B are some of the antifungal agents commonly used for treatment of infections due to Candida species. Most of these antifungals are also covered in this review. The commonly encountered Candida species in clinical settings include: *C. parapsilosis, C. tropicalis, C. albicans, C. krusei,* and *C. glabrata*. This review also gave some insight into their variable susceptibility pattern, as it affects different methods of susceptibility testing.

Presently, fluconazole and amphotericin B deoxycholate are the drugs most commonly used in some nations. Several studies indicated that Candida species distribution and in vitro drug

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susceptibility vary among nations and even in different regions of the same country, even though most of these researches were done in the United States and/or in Europe, they revealed a consistently decreased levels of susceptibility to fluconazole by both Candida albicans and non-albicans candida species and such is expected even in other parts of the world unless proved otherwise[475]. It has been observed that limited information is available from many parts of the world, which means no large studies that evaluated species distribution and antifungal susceptibility pattern of yeast isolates in some infections. The only limited information at hand mostly suggests that non-*C. albicans* species are in most cases the aetiologic agents of blood stream infections (BSIs) in some countries than expected[477]. The information may have serious implications for infection treatment against the Candida species, because *Candida glabrata* is also considered a fluconazole acquired resistant species, *Candida parapsilosis* may display high MIC values for echinocandins while *C. krusei* is also considered to be a resistant specie to fluconazole irrespective of its MIC[478]. In a swift effort, the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing, the (AFST-EUCAST) has developed a standard broth microdilution protocol for the determination of antifungal MICs for fermentative yeast species [479].

2.35.1: Azole

Azole antifungals are a group of medicines that contain an azole ring and inhibit the growth of a wide range of fungi. They are classified into two groups: those with two nitrogens in the azole ring (the imidazoles; examples include clotrimazole, econazole, ketoconazole, miconazole, and tioconazole) and those with three nitrogens in the azole ring (the triazoles; examples include fluconazole, itraconazole, posaconazole, and voriconazole).Azole

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antifungals work by inhibiting the cytochrome P450 dependent enzyme lanosterol 14-alpha-demethylase, which converts lanosterol to ergosterol, the main sterol in the fungal cell membrane. Depletion of ergosterol damages the cell membrane resulting in cell death. Azole antifungal agents can be used to treat fungal infections of the body and skin, including athlete's foot, onychomycosis (fungal nail infections), ringworm, and vaginal candidiass [478].

2.35.2: Medical conditions associated with azole antifungals:

- Aspergillosis, Aspergilloma
- Aspergillosis, Invasive
- Blastomycosis
- Bone Marrow Transplantation
- Candida Urinary Tract Infection
- Candidemia
- Chromomycosis
- Chronic Mucocutaneous Candidiasis
- Coccidioidomycosis
- Coccidioidomycosis, Meningitis
- Cryptococcal Meningitis, Immunocompetent Host
- Cryptococcal Meningitis, Immunosuppressed Host
- Cryptococcosis
- Cutaneous Fungal Infection
- Dermatophytosis
- Esophageal Candidiasis

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- Eumycetoma
- Febrile Neutropenia
- Fungal Infection Prevention
- Fungal Infection Prophylaxis
- Fungal Infection, Internal and Disseminated
- Fungal Meningitis
- Fungal Peritonitis
- Fungal Pneumonia
- Fusariosis
- Histoplasmosis
- Microsporidiosis
- Mucormycosis, Invasive
- Ocular Fungal Infection
- Onychomycosis, Fingernail
- Onychomycosis, Toenail
- Oral Thrush
- Paracoccidioidomycosis
- Pseudoallescheriosis
- Sporotrichosis
- Systemic Candidiasis
- Systemic Fungal Infection
- Tinea Capitis
- Tinea Versicolor

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Vaginal Yeast Infection

Data sources include IBM Watson Micromedex (updated 6 Dec 2021), Cerner Multum™ (updated 1 Dec 2021), ASHP (updated 13 Dec 2021) and others.
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2.35.3: Polyene

The polyene antifungals include Nystatin and Amphotericin B. They have the advantage of being fungicidal by binding to ergosterol in the fungal cell wall, creating pores in the membrane. Nystatin is too toxic to administer parenterally and is not absorbed orally. Its main use in veterinary medicine is a topical ophthalmic preparation for the treatment of fungal keratitis.Amphotericin B is used only sporadically in equine medicine. It is also frequently used to treat Fusarium spp. in humans. No pharmacokinetic data are available on amphotericin B in the horse, but it has been given as an IV infusion at doses of 0.38 to 1.4 mg/kg.[480] Its use is often limited by its toxicity. Infusion reactions and nephrotoxicity are common.

Amphotericin B (AMB) generally remains the cornerstone of therapy for disseminated and deep organ Candida infection, especially those infections that may be rapidly fatal or refractory to azoles or to echinocandins. In the overall longitudinal experience of treatment of Candida infections, most of the experience has been with amphotericin B deoxycholate (AMB-D). However, lipid formulations of amphotericin B, including liposomal amphotericin B (LAMB) and amphotericin B lipid complex (ABLC), have been approved by regulatory agencies and are used extensively now. While evidence does not exist that LAMB or ABLC is more efficacious than AMB-D for the management of Candida infections, their use has become very popular, due mainly to their lower level of renal toxicity.[481] The polyene antifungals that are in current clinical use are Nystatin, Natamycin, and Amphotericin B. They are fungicidal, and their mode of action is disruption of the fungal cell membrane by binding to ergosterol, the main sterol in the

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membrane. This results in pore formation and leakage of cellular cations and anions, leading to fungal cell death[478].

Although the presence of ergosterol in the fungal cell membrane confers some selectivity to the action of polyenes, there are notable toxicities associated with their systemic use. Nystatin and Natamycin are currently only used topically and, although initial studies with a liposomal formulation of nystatin (Nyotran) demonstrated good efficacy, they were halted due to infusion-related toxicity. Amphotericin B is used systemically, but as it is not absorbed orally, it must be administered intravenously (IV). It has a broad spectrum of activity against yeasts and molds and has been widely used over several decades to treat a range of diseases, including invasive Aspergillosis, Cryptococcosis, Candidosis, the endemic mycoses, and Leishmaniasis.

Agent Name	
Amphotericin B	A polyene antifungal, Amphotericin B is used primarily as a systemic treatment for candidiasis. It can also be used topically.
Nystatin	Nystatin is a polyene antifungal that is normally used topically for candidiasis. It is sometimes used orally for intestinal candidiasis.
Natamycin	Natamycin is a polyene antifungal that is occasionally used topically to treat candidiasis.
Flucytosine	Flucytosine is a pyrimidine analogue used in treating systemic Candida infections.
Voriconazole	Voriconazole is an antifungal triazole used systemically for the treatment of candidiasis.
Ketoconazole	Ketoconazole is an imidazole antifungal used systemically to treat candidiasis.

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Agent Name	
Clotrimazole	Clotrimazole is an imidazole antifungal used topically to treat candidiasis.

2.35.4: Amphotericin B

Amphotericin B is an amphoteric polyene antifungal produced by *Streptomyces nodosus*. It is a highly lipophilic molecule that is prepared as a colloidal suspension with sodium dexoxycholate for intravenous injection. Amphotericin B binds to ergosterol, a component of the fungal cell membrane. Upon binding, amphotericin alters the permeability of the cell by forming pores in the fungal cell membrane. The pores allow leakage of intracellular ions and macromolecules, leading to cell death. Amphotericin B has a broad spectrum of activity against clinically relevant fungi, including most Candida and Aspergillus species. It may be fungicidal or fungistatic depending on the drug concentration and sensitivity of the organism[483].

Until the early 1990s, amphotericin B was the only antifungal available for the treatment of serious, systemic fungal infections. As a result, clinical experience in treating fungal IE (Infective Endocarditis) has been almost exclusively with amphotericin B[480]. It is still considered the preferred treatment option despite its propensity to cause nephrotoxicity. Administration of amphotericin B causes a decline in glomerular filtration rate and renal blood flow in addition to impaired proximal and distal tubular reabsorption of electrolytes. Patients with fungal IE commonly require high doses and prolonged treatment courses, placing them at high risk for amphotericin B related nephrotoxicity. Newer lipid-associated formulations of amphotericin B are considered equally efficacious and have lower rates of renal toxicity. For these reasons, the lipid-associated formulations may be preferable for the

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treating fungal IE. Nephrotoxicity may be minimized by reversing sodium depletion with normal saline boluses administered before and after each infusion[480]. Close monitoring for hypokalemia and hypomagnesemia is also warranted, with repletion when necessary. Amphotericin B infusion-related reactions such as chills, fevers, and phlebitis at the injection site are also common. Premedication with meperidine and/or hydrocortisone has been shown to be helpful in alleviating these symptoms[481]. Acetaminophen and diphenhydramine are also frequently prescribed as part of the pretreatment regimen, but clinical evidence does not support this practice.

2.36: Anti-fungal Resistant Genes

2.36.1: ITS1 and ITS4 Fungal Detection and Identification Gene

The diagnosis of fungal infection is often delayed because of the poor availability of infected material from the affected site and the slow growth of a large number of fungi in routinely used culture media, and therefore, early intervention is not always possible and the patient's intervention is often lost or delayed. In general, the diagnosis of fungal infection is dependent on Gram and Giemsa staining, which have low sensitivities of about 50 to 80%[483]. Recent advances in molecular biology techniques have opened the door for culture-independent diagnostic methods. Immunological detection and identification by use of distinctive metabolites and nucleic acid probes are the tools most often used for diagnosis[484,485,486,487]. One such technique is PCR, which has been shown to be useful for the culture-independent diagnosis of various microbial infections, including mycoses[488,489,490]. To date, a few cases of mycotic keratitis have successfully been diagnosed by PCR[491,492]. Ribosomal DNA is the most conserved region in the genome, with capabilities of phylogenetic divergence[493]. The whole rRNA gene contains a small subunit (SSU) 18S

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rRNA, 5.8S rRNA, and a large subunit (LSU) 28S rRNA. Internal transcribed spacer (ITS) region I (ITSI) and ITSII are more variable than the rest of the ribosomal gene subunits and are found between SSU rRNA and 5.8S rRNA and between 5.8S rRNA and LSU rRNA, respectively. Besides this, intergenic spacer (IGS) region I (IGSI) and IGSII are found between the end of the LSU and start of the next SSU sequence[494]. Many workers (9,15–17) have used the single-stranded conformation polymorphism (SSCP) technique to identify sequence variations in a single strand of DNA due to its adoption to a unique conformation under nondenaturing conditions[495]. Here we report on the experimental proof and the clinical laboratory diagnosis of three cases of corneal ulcer by PCR by the ITS SSCP technique, in which useful vision could be restored due to prompt diagnosis and specific antifungal therapy[495].

2.36.2: ERG 11 Anti-fungi Resistant Gene

In *Candida albicans*, the ERG11 gene encodes lanosterol demethylase, the target of the azole antifungals. Mutations in ERG11 that result in an amino acid substitution alter the abilities of the azoles to bind to and inhibit ERG11, resulting in resistance[496]. Although ERG11 mutations have been observed in clinical isolates, the specific contributions of individual ERG11 mutations to azole resistance in *C. albicans* have not been widely explored. The strains that were homozygous for the single amino acid substitutions Y132F, K143R, F145L, S405F, D446E, G448E, F449V, G450E, and G464S had a ≥ 4-fold increase in FLC MIC. The strains that were homozygous for several double amino acid substitutions had decreased azole susceptibilities beyond those conferred by any single amino acid substitution. These findings indicate that mutations in ERG11 are prevalent among azole-resistant clinical isolates and that most mutations result in appreciable changes in FLC and

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VRC susceptibilities. Studies in yeast have revealed several molecular mechanisms of azole resistance [496, 497,498,499, 500,501] :(1) decreased affinity of azoles for the target enzyme CYP51A1 caused by point mutations in the ERG11 gene, (2) increases in CYP51A1 copy number through upregulation of ERG11 expression, (3) metabolic modifications, and (4) decreased intracellular azole accumulation by upregulation of multidrug transporters or drug sequestration.Many studies have identified point mutations in the ERG11 gene in azole-resistant *C. albicans* isolates. Such mutations can alter the affinity of CYP51A1 for an azole if the resultant amino acid substitutions lead to changes in the tertiary structure of the enzyme. Mutations in the ERG11 gene encoding more than 160 distinct amino acid substitutions have been reported [502, 503, 504]. However, only 10 of these amino acid substitutions have been confirmed to cause fluconazole (FLZ) resistance. Of these, four substitutions (T315A, Y118A, Y18F, and Y118T) were created in the laboratory and have not yet been detected in clinical isolates[505,506].

2.36.3: FKS Anti-Fungi Resistant Gene

Mutations in two specific regions of the Fks1 subunit of 1,3-β-D-glucan synthase are known to confer decreased caspofungin susceptibility on *Candida* spp. Caspofungin is an echinocandin that inhibits 1,3-β-D-glucan synthesis in several fungal species involved in human infections, including *Candida* spp. and *Aspergillus* spp[507]. Caspofungin is used for the treatment of invasive candidiasis and aspergillosis as well as for oropharyngeal and esophageal candidiasis[508]. The target of caspofungin is the enzyme 1,3-β-D-glucan synthase, encoded by one or several fks genes, depending on the species[508]. It has been shown that in laboratory mutants as well as in some clinical isolates, mutations in the fks1 gene resulting in amino acid changes in the protein were necessary and sufficient to confer reduced

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susceptibility to caspofungin[509,510]. These mutations, associated with reduced susceptibility to caspofungin, have been observed only within two "hot spot" (HS) regions of the Fks protein (located at amino acid positions 640 to 650 and 1345 to 1365 in *Candida albicans*) [511,512]. Several clinical studies have shown that the presence of an *FKS* mutation is the most important independent risk factor in predicting echinocandin therapeutic responses among patients with IC (Invasive Candidiasis) [513,514]. Further, the presence of an *FKS* mutation was found to be superior to MIC (Minimum Inhibitory Concentration) values in predicting clinical responses, especially when caspofungin (CAS) was used for testing[515]. The underlying molecular mechanisms promoting development of resistance in *C. gla*brata to multiple drug classes, including azoles and echinocandins, are multifactorial. Recently, MSH2 DNA mismatch repair (MMR) gene deletions in *C. glabrata* resulting in a mutator phenotype that facilitates rapid acquisition of fluconazole (FLU), echinocandins, and amphotericin B (AMB) resistance were detected in clinical isolates of *C. glabrata*[516]. Over half of the isolates of *C. glabrata* collected in the United States and other countries carried mutations in MSH2 that conferred a partial hypermutable phenotype with significantly increased frequency of FKS mutations[516]. MSH2 alleles were subsequently shown to be dependent upon sequence type. Additionally, a more recent study evaluated antifungal resistance in clinical isolates of *C. glabrata* in a large cohort of patients in Saint-Louis Hospital, Paris, France, and demonstrated that the mutator phenotype was not associated with FLU resistance but instead corresponded to rare and specific genotypes[516].

2.36.4: CPY Anti-Fungi Resistant Gene

Part of the metabolic armory harbored by Aspergillus species is over 100 CPYs encoded in the genome[517]. These enzymes participate in a variety of physiological activities that allow

the fungi to adapt to new ecological niches. Soil is a hostile and competitive environment, so these CPYs play a role in the synthesis and degradation of various toxic compounds. *Aspergillus nidulans* contains 119 predicted CPYs, for which the functions of 13 have been determined experimentally, and 32 are positioned near key secondary metabolite synthases, suggesting their potential biosynthetic role[518]. Therefore, a large number of CPYs have no known or predicted function. The white rot fungus Phanerochaete chrysosporium has an outstanding capability for degrading and/or mineralizing high-molecular-weight PAHs and contains an extraordinarily large repertoire (over 150) of CPYs in its genome[519]. An excellent study by Syed and colleagues identified and characterized six CPYs in *P. chrysosporium* (Pc-PAH1 to Pc-PAH6) capable of oxidizing different PAHs. These CPYs were inducible by naphthalene, phenanthrene, pyrene, and BaP. Expression of each of the six Pc-PAH CPYs in the yeast Pichia pastoris in conjunction with the homologous P450 oxidoreductase led to identification of Pc-PAH1 and Pc-PAH3 as CPYs with the ability to oxidize BaP to 3-hydroxybenzo[a]pyrene[520]. This was the first report to identify a set of specific fungal CPYs having catalytic activity toward BaP. However, the functions of these CPYs have not been studied in vivo due to the limited ability of genetic manipulation in this organism, and hence further metabolism and the resulting products remain a mystery. Likewise, many reports about BaP-degrading fungal species isolated from contaminated sites lack systematic study due to limited genetic tools[521]. Filamentous fungi harbor many more CPYs relative to their genome size than animals and bacteria, yet the functions of many remain unknown. The diversity of CPYs in fungi could be due to their need to metabolize many different carbon sources found in soils, including large cyclic compounds like lignin and plant polymers. It is also feasible that fungi, like animals, may

need detoxification systems reliant on CPY activity to avoid toxic compounds produced by competing microbes and plants. Our results demonstrate that the regulation of bapA is governed by response to carbon starvation, rather than exposure to the toxicant BaP. Regulation of bapA also demonstrates a novel understanding of how Aspergillus species respond to organic contaminants like BaP. Humans and fungi have evolved different strategies to deal with exposure to xenobiotics, yet both employ CPYs. Humans do not invest energy into utilizing carbon sources more complex than various sugars and a few of their polymers, so CPY transformation of BaP yields more polar metabolites that can then be excreted. Regulation of encoded BaP-metabolizing CPYs is predominantly governed by the aryl hydrocarbon receptor, yet BaP and its metabolites also activate NF-κB[522,523].

2.37: Prevention of COVID-19 Disease

Preventive measures to reduce the chances of infection include getting vaccinated, staying at home, wearing a mask in public, avoiding crowded places, keeping distance from others, ventilating indoor spaces, managing potential exposure durations, washing hands with soap and water often and for at least twenty seconds, practicing good respiratory hygiene, and avoiding touching the eyes, nose, or mouth with unwashed hands[524,525, 526]. Those diagnosed with COVID- 19 or who believe they may be infected are advised by the CDC to stay home except to get medical care, call ahead before visiting a healthcare provider, wear a face mask before entering the healthcare provider's office and when in any room or vehicle with another person, cover coughs and sneezes with a tissue, regularly wash hands with soap and water and avoid sharing personal household items[527, 528]. The first COVID- 19 vaccine was granted regulatory approval on 2 December 2020 by the UK medicines regulator MHRA[529]. It was evaluated for emergency use authorization (EUA) status by the US FDA, and in

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several other countries[530]. Initially, the US National Institutes of Health guidelines do not recommend any medication for prevention of COVID-19, before or after exposure to the SARS-CoV-2 virus, outside the setting of a clinical trial[531,532]. Without a vaccine, other prophylactic measures, or effective treatments, a key part of managing COVID-19 is trying to decrease and delay the epidemic peak, known as "flattening the curve"[533]. This is done by slowing the infection rate to decrease the risk of health services being overwhelmed, allowing for better treatment of active cases, and delaying additional cases until effective treatments or a vaccine become available[534].

2.37.1: COVID-19 Vaccine

A COVID-19 vaccine is a vaccine intended to provide acquired immunity against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes coronavirus disease 2019 (COVID-19). Prior to the COVID-19 pandemic, an established body of knowledge existed about the structure and function of coronaviruses causing diseases like severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS). This knowledge accelerated the development of various vaccine platforms during early 2020[535].The initial focus of SARS-CoV-2 vaccines was on preventing symptomatic, often severe illness[536]. On 10 January 2020, the SARS-CoV-2 genetic sequence data was shared through GISAID, and by 19 March, the global pharmaceutical industry announced a major commitment to address COVID-19[537]. The COVID-19 vaccines are widely credited for their role in reducing the severity and death caused by COVID-19[538,539]. Many countries have implemented phased distribution plans that prioritize those at highest risk of complications, such as the elderly, and those at high risk of exposure and transmission, such as healthcare workers[540].

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As of 1 February 2022, 10.1 billion doses of COVID- 19 vaccines have been administered worldwide based on official reports from national public health agencies[541]. By December 2020, more than 10 billion vaccine doses had been preordered by countries, with about half of the doses purchased by high-income countries comprising 14% of the world's population[542,543].

2.37.2: Face Masks and Respiratory Hygiene

Masks with an exhalation valve. The valves are a weak point that can transmit the viruses outwards. The WHO and the US CDC recommend individuals wear non-medical face coverings in public settings where there is an increased risk of transmission and where social distancing measures are difficult to maintain[544,545]. This recommendation is meant to reduce the spread of the disease by asymptomatic and pre-symptomatic individuals and is complementary to established preventive measures such as social distancing[545,546]. Face coverings limit the volume and travel distance of expiratory droplets dispersed when talking, breathing, and coughing[170,171]. A face covering without vents or holes will also filter out particles containing the virus from inhaled and exhaled air, reducing the chances of infection[547]. But, if the mask include an exhalation valve, a wearer that is infected (maybe without having noticed that, and asymptomatic) would transmit the virus outwards through it, despite any certification they can have. So the masks with exhalation valve are not for the

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infected wearers, and are not reliable to stop the pandemic in a large scale. Many countries and local jurisdictions encourage or mandate the use of face masks or cloth face coverings by members of the public to limit the spread of the virus[548].

Masks are also strongly recommended for those who may have been infected and those taking care of someone who may have the disease[549]. When not wearing a mask, the CDC recommends covering the mouth and nose with a tissue when coughing or sneezing and recommends using the inside of the elbow if no tissue is available. Proper hand hygiene after any cough or sneeze is encouraged. Healthcare professionals interacting directly with people who have COVID- 19 are advised to use respirators at least as protective as NIOSH-certified N95 or equivalent, in addition to other personal protective equipment[550].

2.37.3: Indoor Ventilation and Avoiding Crowded Indoor Spaces

The CDC recommends that crowded indoor spaces should be avoided[551]. When indoors, increasing the rate of air change, decreasing recirculation of air and increasing the use of outdoor air can reduce transmission[552].The WHO recommends ventilation and air filtration in public spaces to help clear out infectious aerosols[553, 554 555]. Exhaled respiratory particles can build-up within enclosed spaces with inadequate ventilation. The risk of COVID- 19 infection increases especially in spaces where people engage in physical exertion or raise their voice (e.g., exercising, shouting, singing) as this increases exhalation of respiratory droplets. Prolonged exposure to these conditions, typically more than 15 minutes, leads to higher risk of infection[551].

Displacement ventilation with large natural inlets can move stale air directly to the exhaust in laminar flow while significantly reducing the concentration of droplets and particles.

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Passive ventilation reduces energy consumption and maintenance costs but may lack controllability and heat recovery. Displacement ventilation can also be achieved mechanically with higher energy and maintenance costs. The use of large ducts and openings helps to prevent mixing in closed environments. Recirculation and mixing should be avoided because recirculation prevents dilution of harmful particles and redistributes possibly contaminated air, and mixing increases the concentration and range of infectious particles and keeps larger particles in the air[556].

2.37.4: Hand-washing and Hygiene

Thorough hand hygiene after any cough or sneeze is required[557]. The WHO also recommends that individuals wash hands often with soap and water for at least twenty seconds, especially after going to the toilet or when hands are visibly dirty, before eating and after blowing one's nose[558]. When soap and water are not available, the CDC recommends using an alcohol-based hand sanitiser with at least 60% alcohol[559]. For areas where commercial hand sanitisers are not readily available, the WHO provides two formulations for local production. In these formulations, the antimicrobial activity arises from ethanol or isopropanol. Hydrogen peroxide is used to help eliminate bacterial spores in the alcohol; it is "not an active substance for hand antisepsis." Glycerol is added as a humectant[560].

2.37.5: Social Distancing

Social distancing measures related to the COVID-19 pandemic**.** Social distancing (also known as physical distancing) includes infection control actions intended to slow the spread of the disease by minimizing close contact between individuals. Methods include quarantines; travel restrictions; and the closing of schools, workplaces, stadiums, theatres, or

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shopping centers. Individuals may apply social distancing methods by staying at home, limiting travel, avoiding crowded areas, using no-contact greetings, and physically distancing themselves from others. Many governments are now mandating or recommending social distancing in regions affected by the outbreak[561]. Outbreaks have occurred in prisons due to crowding and an inability to enforce adequate social distancing.[187, 188] In the United States, the prisoner population is aging and many of them are at high risk for poor outcomes from COVID- 19 due to high rates of coexisting heart and lung disease, and poor access to high-quality healthcare[562].

2.37.6: Surface Cleaning

After being expelled from the body, coronaviruses can survive on surfaces for hours to days. If a person touches the dirty surface, they may deposit the virus at the eyes, nose, or mouth where it can enter the body and cause infection[563]. Evidence indicates that contact with infected surfaces is not the main driver of COVID- 19, leading to recommendations for optimised disinfection procedures to avoid issues such as the increase of antimicrobial resistance through the use of inappropriate cleaning products and processes[564,565,566,567,568]. Deep cleaning and other surface sanitation has been criticized as hygiene theater, giving a false sense of security against something primarily spread through the air[569, 570]. The amount of time that the virus can survive depends significantly on the type of surface, the temperature, and the humidity[571]. Coronaviruses die very quickly when exposed to the UV light in sunlight[571]. Like other enveloped viruses, SARS-CoV-2 survives longest when the temperature is at room temperature or lower, and when the relative humidity is low (<50%)[571].

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On many surfaces, including glass, some types of plastic, stainless steel, and skin, the virus can remain infective for several days indoors at room temperature, or even about a week under ideal conditions[572]. On some surfaces, including cotton fabric and copper, the virus usually dies after a few hours[571]. The virus dies faster on porous surfaces than on non-porous surfaces due to capillary action within pores and faster aerosol droplet evaporation[573]. However, of the many surfaces tested, two with the longest survival times are N95 respirator masks and surgical masks, both of which are considered porous surfaces[571].

The CDC says that in most situations, cleaning surfaces with soap or detergent, not disinfecting, is enough to reduce risk of transmission[574,575]. The CDC recommends that if a COVID- 19 case is suspected or confirmed at a facility such as an office or day care, all areas such as offices, bathrooms, common areas, shared electronic equipment like tablets, touch screens, keyboards, remote controls, and ATM machines used by the ill persons should be disinfected[576]. Surfaces may be decontaminated with 62–71 percent ethanol, 50–100 percent isopropanol, 0.1 percent sodium hypochlorite, 0.5 percent hydrogen peroxide, and 0.2–7.5 percent povidone-iodine. Other solutions, such as benzalkonium chloride and chlorhexidine gluconate, are less effective. Ultraviolet germicidal irradiation may also be used[553]. A datasheet comprising the authorized substances to disinfection in the food industry (including suspension or surface tested, kind of surface, use dilution, disinfectant and inoculum volumes) can be seen in the supplementary material[567].

2.37.7: Self-isolation

Self-isolation at home has been recommended for those diagnosed with COVID- 19 and those who suspect they have been infected. Health agencies have issued detailed instructions

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for proper self-isolation[577]. Many governments have mandated or recommended self-quarantine for entire populations. The strongest self-quarantine instructions have been issued to those in high-risk groups[578]. Those who may have been exposed to someone with COVID- 19 and those who have recently travelled to a country or region with the widespread transmission have been advised to self-quarantine for 14 days from the time of last possible exposure[579].

2.37.8: Healthy Diet and Lifestyle

The Harvard T.H. Chan School of Public Health recommends a healthy diet, being physically active, managing psychological stress, and getting enough sleep[580]. Consistently meeting scientific guidelines of 150+ minutes per week of exercise or similar physical activity was shown to be associated with a smaller risk of hospitalisation and death due to COVID- 19, even when considering likely risk factors such as elevated Body mass index (BMI) [581,582]. A meta-analysis, published online in October 2021, concluded that "Vitamin D supplementation in SARS-CoV-2 positive patients has the potential to positively impact patients with both mild and severe symptoms[583].The largest observational study on the subject using online questionnaires, with over 6,000 participants and a dosage regime near the RDI, is set to conclude in July 2021[584,585]. One of the collaborators in the study is Synergy Biologics Ltd, a manufacturer of vitamin D3 supplements[585].

2.37.9: International Travel-related Control Measures

A 2021 Cochrane rapid review found that based upon low-certainty evidence, international travel-related control measures such as restricting cross-border travel may help to contain the spread of COVID- 19[586]. Additionally, symptom/exposure-based screening measures at borders may miss many positive cases. While test-based border screening measures may be

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more effective, it could also miss many positive cases if only conducted upon arrival without follow-up. The review concluded that a minimum 10-day quarantine may be beneficial in preventing the spread of COVID-19 and may be more effective if combined with an additional control measure like border screening[586].

2.38: Treatment of COVID-19

There is no specific, effective treatment or cure for coronavirus disease 2019 (COVID-19), the disease caused by the SARS-CoV-2 virus[587,588]. One year into the pandemic, highly effective vaccines have now been introduced and are beginning to slow the spread of SARS-CoV-2; however, for those awaiting vaccination, as well as for the estimated millions of immunocompromised persons who are unlikely to respond robustly to vaccination, treatment remains important[589]. Thus, the lack of progress developing effective treatments means that the cornerstone of management of COVID-19 has been supportive care, which includes treatment to relieve symptoms, fluid therapy, oxygen support and prone positioning as needed, and medications or devices to support other affected vital organs [590, 591, 592]. Most cases of COVID-19 are mild and in these, supportive care includes medication such as paracetamol or NSAIDs to relieve symptoms (fever, body aches, cough), proper intake of fluids, rest, and nasal breathing[593,594,595]. Good personal hygiene and a healthy diet are also recommended[596]. The U.S. Centers for Disease Control and Prevention (CDC) recommend that those who suspect they are carrying the virus isolate themselves at home and wear a face mask[597].

People with more severe cases may need treatment in hospital. In those with low oxygen levels, use of the glucocorticoid dexamethasone is strongly recommended, as it can reduce

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the risk of death[598,599,600]. Non-invasive ventilation and, ultimately, admission to an intensive care unit for mechanical ventilation may be required to support breathing[601]. Extracorporeal membrane oxygenation (ECMO) has been used to address the issue of respiratory failure, but its benefits are still under consideration[602, 603]. Some of the cases of severe disease course are caused by systemic hyper-inflammation, the so-called cytokine storm[604].

Several experimental treatments are being actively studied in clinical trials[605]. These include fluvoxamine, a cheap and widely available antidepressant; and the antivirals molnupiravir (developed by Merck), and nirmatrelvir/ritonavir (developed by Pfizer)[606,607]. Others were thought to be promising early in the pandemic, such as hydroxychloroquine and lopinavir/ritonavir, but later research found them to be ineffective or even harmful[608, 609]. Despite ongoing research, there is still not enough high-quality evidence to recommend so-called early treatment[608, 609]. Nevertheless, in the United States, two monoclonal antibody-based therapies are available for early use in cases thought to be at high risk of progression to severe disease[609]. The antiviral remdesivir is available in the U.S., Canada, Australia, and several other countries, with varying restrictions; however, it is not recommended for people needing mechanical ventilation, and is discouraged altogether by the World Health Organization (WHO), due to limited evidence of its efficacy[610,587]. In November 2021, the UK approved the use of molnupiravir as a COVID treatment for vulnerable patients recently diagnosed with the disease[611].

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Chapter Three

3.0 Methodology

3.1 Study Area

The study was carried out in Ibadan, the capital of Oyo state in South West Nigeria. Oyo state covers a total land area of about 27,249 square kilometers and it is bounded in the South by Ogun State and in the North by Kwara State. To the West, it is bounded partly by Ogun State and partly by the Republic of Benin while in the East, it is bounded by Osun State. The State consists of 33 Local Government Councils. The State is homogenous, comprising mainly people of Yoruba ethnic group who speak pure and undiluted form of Yoruba language. Oyo State is one of the most urbanized States in the Federation. There are 2 tertiary health facilities, 41 secondary health facilities, and 315 Primary Health Centres (PHCs). The region has a typical tropical continental climate and south-western savannah

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vegetation with alternating humid to wet and cool to dry seasons with different educational and socio-economic background living in neighborhood with distinctly different levels of sanitations. Boreholes and well water are the major source of drinking water in these areas. All the state headquarters have Government run hospitals, private clinics and Primary Health Centers (PHC s) that provide health services to the population. (Information obtained from State Secretariat, Ibadan. Oyo- State)

3.2 Study Setting

The study was carried out in different selected government hospitals in Ibadan, Oyo State Nigeria. They were selected based on the fact that each represents a primary, secondary or a tertiary health institution and because they are either a specialized isolation hospital or have specialized screening department as well as having a high population of patients visiting the hospitals.

Infectious Disease Centre, Olodo, Ibadan, Oyo-State, has 56 bed spaces, 5 different wards which are emergency treatment resuscitation ward, oral rehydration treatment ward, ward 1, ward 2, intensive care unit. The Hospital attends to about 568 critically and severe comorbidity positive SARS-CoV-2 patients since the onset of COVID 19 pandemic in Nigeria. (Information obtained from records unit of the hospital).

University College Hospital, Oritamefa has 850 bed spaces and bed occupancy rates range from 55-60%, attends to about 180 SARS-CoV-2 severely positive patients at their infectious disease unit since the onset of COVID-19 pandemic in Nigeria. (Information obtained from records unit of the hospital.).

Agbami Infectious Disease Centre, Jericho, Ibadan, Oyo-state has about 50 bed spaces, 5 different wards which are emergency treatment resuscitation ward, oral rehydration

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treatment ward, ward 1, ward 2, intensive care unit. The Hospital attends to about 68 critically and severe comorbidity positive SARS-CoV-2 patients since the onset of COVID 19 pandemic in Nigeria. (Information obtained from records unit of the hospital).

3.3 Selection Criteria:

3.3.1 Inclusion Criteria:

- All symptomatic and asymptomatic client presented at any SARS-CoV-2 isolation and screening center within Oyo-state.
- A client who is ready to consent to the study.
- A client that is 18years and above

3.3.2 Exclusion Criteria:

- Any client that want to screen for travelling purposes.
- A client that did not want to consent.
- A client that is >18years old.

3.4 Sample Size Determination

The sample size was obtained by employing the statistical formula for population >10,000[1].

$n=z^2 p q/d^2$

Where n = the desired sample size when population is >10,000

Z=the standard normal deviate set at 1.96 which corresponds to the 95% confidence level

P= Prevalence of bacteria and fungi pathogen 34.8% among SARS-CoV-2[2].

q=1-p= 0.65

d=the desired degree of accuracy, set at 0.05

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From $n=z^2pq/d^2$

$n= (1.96^2) (0.35) (0.65)$

(0.0025^2)

$n=3.84 \times 0.23$

0.0025

n= 354 x 1.11 (Attrition rate)

392 patients.

Therefore, approximate sample size of SARS-CoV-2 samples to be collected in this study is 400.

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3.5 Sample Collection

Nasopharyngeal and oropharyngeal swab samples were collected from all patients with suspected COVID-19 and tested at Bio-respiratory Laboratory, University College Hospital. Ibadan. Oyo-state, using real-time reverse transcriptase PCR (RT-PCR) (proprietary Public Health England Assay) for the positive confirmation of the virus in an infected individual. Then 400 Nasopharyngeal samples were collected from confirmed SARS-CoV-2 infected individual into a sterile Amie's Transport medium in a cryo-vial plastic containers/bottles labelled with the name, sex and age of the clients for Microbiological investigation at all the designated isolation and treatment center in Oyo- state. Nigeria

3.6 Processing of Samples

3.6.1 Culturing Method

All 400 samples collected were inoculated and cultured on Chocolate Biotech medium agar, MacConkey Biotech medium agar and Saboraud's Dextrose Biotech medium agar,

incubated aerobically and anaerobically at 37ºc. Chocolate and MacConkey plates were read after 48hours, while Saboraud's Dextrose plates were read for 7days consecutively. All organisms that grew were stained with Gram's staining reagents to confirm whether they are Gram positive bacteria, Gram Negative bacteria, Yeast, Moulds or Contaminant.

3.6.2 Identification of the Bacteria Isolates

Performed by Standard systematic microbiological techniques methods.

Colony morphology, Gram staining, catalase test, coagulase test, optochin sensitivity were performed for specific isolates.

Coagulase and Catalase tests determination were done on all the Gram positive bacteria. All Coagulase and Catalase tests positive were cultured on Mannitol salt agar for color speciation for different Staphylococcus specie. Confirmatory identification of the isolates with the use of VITEK 2.0 Identification kits cards for Gram Positive Cocci and antibiotic susceptibility testing was done on all the confirmed Gram Positive Bacteria isolates using Kirby baver disc diffusion and VITEK 2.0 Antibiotic Susceptibility Testing kits cards.

All the Gram Negative Bacteria was cultured on CHROM Orientation agar for colour differentiation. Confirmatory identification of the isolates with the use of Analytical Profile Index 20E and antibiotic susceptibility testing was done on all the confirmed isolates using Kirby bauer disc diffusion and VITEK 2.0 Antibiotic Susceptibility Testing kits cards.

Yeasts were identified by employing conventional biochemical and assimilation test procedures[3]. Using CHROM agar Candida culture medium (Becton Dickinson) as per the instruction of the manufacture, germ-tube formation in human serum. Mould were identified microscopically with Lactol Phenol cotton blue, view with 40 objectives lens. All the fungi isolates were confirmed with MALDITOF MS VITEK system.

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3.7: Gram's Staining Techniques for all Organisms Isolated

Principle: When the bacteria is stained with primary stain Crystal Violet and fixed by the mordant Lugol's Iodine, some of the bacteria are able to retain the primary stain and some are decolorized by alcohol. The cell walls of gram positive bacteria have a thick layer of protein-sugar complexes called peptidoglycan and lipid content is low. Decolorizing the cell causes this thick cell wall to dehydrate and shrink which closes the pores in the cell wall and prevents the stain from exiting the cell. So the ethanol cannot remove the Crystal Violet-Iodine complex that is bound to the thick layer of peptidoglycan of Gram positive bacteria and appears blue or purple in color. In case of gram negative bacteria, cell wall also takes up the Crystal Violet-Iodine complex but due to the thin layer of peptidoglycan and thick outer layer which is formed of lipids, CV-Iodine complex gets washed off. When they are exposed to alcohol, decolorizer dissolves the lipids in the cell walls, which allows the crystal violet-iodine complex to leach out of the cells. Then when again stained with safranin, they take the stain and appear red in color.

Procedure: Each heat-fixed sample slide was covered with crystal violet solution and allowed to stand for 1 minute after which the stain was washed off with water and then mordant with Iodine for 1 minute, washed with water then covered with acetone for decolourisation for 30 seconds. After a wash, the slide was covered with safranin and left to stand for 30 seconds to 1 minute. It was washed again with water and gently blot dry. The stained smear was then examined microscopically (Olympus Binocular Microscope, USA) using oil immersion (X 100 Objective lens)[7].

Interpretation of Result:

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GramPositive:**Purplecolour**

Gram Negative: **Red colour**

Yeast**: Dark purple oval shaped**

3.9: Catalase Technique

Principle: The test determines enzyme producing ability of bacteria which protects them from accumulating Hydrogen peroxide (H_2O_2), which can occur during aerobic metabolism, if hydrogen peroxide accumulates it becomes toxic to the organism. Catalase breaks down H_2O_2 into water and oxygen.

Procedure: One or two colonies of 24 hours' pure culture was smeared on a clean grease free slide with a drop of hydrogen peroxide.

Result: Effervescence or bubbles will indicate catalase positive reaction while no effervescence will indicate catalase negative reaction.

3.9: Coagulase Technique

Principle: Coagulase reacts with prothrombin in the blood. The resulting complex is called staphylothrombin, which enables the enzyme to act as a protease to convert fibrinogen, a plasma protein produced by the liver, to fibrin. This results in clotting of the blood. Coagulase is tightly bound to the surface of the bacterium *S. aureus* and can coat its surface with fibrin upon contact with blood. The fibrin clot may protect the bacterium from phagocytosis and isolate it from other defenses of the host.

The fibrin coat can therefore make the bacteria more virulent. Bound coagulase is part of the larger family of MSCRAMM adhesin proteins. The coagulase test has traditionally been used to differentiate *Staphylococcus aureus* from coagulase negative staphylococci. *S.*

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aureus produces two forms of coagulase (i.e., bound coagulase and free coagulase). Bound coagulase, otherwise known as "clumping factor", can be detected by carrying out a slide coagulase test, and free coagulase can be detected using a tube coagulase test.

Procedure A: A slide coagulase test is run with a negative control to rule out auto-agglutination. Two drops of saline are put onto the slide labeled with sample number, Test (T) and control (C). The two saline drops are emulsified with the test organism using a wire loop, straight wire, or wooden stick. A drop of plasma (rabbit plasma anticoagulated with EDTA is recommended) is placed on the inoculated saline drop corresponding to test, and mixed well, then the slide is rocked gently for about 10 seconds[8].

Interpretation of Result:

Positive: Macroscopic clumping was observed in the plasma within 10 seconds, with no clumping in the saline drop.

Negative: No clumping was observed.

Note: When the slide coagulase test is negative, a tube test was done as a confirmation. Clumping in both drops is an indication of auto-agglutination, so a tube test should be carried out.

Procedure B: The tube test uses rabbit plasma that has been inoculated with a staphylococcal colony (i.e., Gram-positive cocci which are catalase positive). The tube is then incubated at 37 °C for 1.5 hours. If negative, then incubation is continued up to 18 hours.

Interpretation of Results:

Positive: The plasma will coagulate, resulting in a clot (sometimes the clot is so pronounced, the liquid will completely solidify). The suspect colony is *S. aureus*

Negative: The plasma remains a liquid. The negative result may be *S. epidermidis* but only a more detailed identification test can confirm this, using biochemical tests as in analytical profile index tests methods. A false negative can be perceived if the sample is not allowed to cool for about 30 minutes at room temperature or 10 minutes in the freezer, given that the serum can melt. If truly negative, the serum will remain liquid after cooling.

List of coagulase-positive staphylococci: *Staphylococcus aureus subsp. anaerobius, S. aureus subsp. aureus, S. delphini, S. hyicus, S. intermedius, S. lutrae,* and *Staphylococcus schleiferi subsp. coagulans.*

List of coagulase-negative staphylococci of clinical significance: *S. saprophyticus, S. cohnii subsp. cohnii, S. cohnii subsp. urealyticum, S. captitus subsp. captitus, S. warneri, S. hominis, S. epidermidis, S. caprae,* and *S. lugdunensis*

3.10: Mannitol Salt Agar Techniques for Gram Positive Bacteria

Principle: It contains peptones and beef extract, which supply nitrogen, vitamins, minerals and amino acids essential for growth. The 7.5% concentration of sodium chloride results in the partial or complete inhibition of bacterial organisms other than staphylococci. It contains a high concentration (about 7.5–10%) of salt (NaCl) which is inhibitory to most bacteria - making MSA selective against most Gram-negative and selective for some Gram-positive bacteria. It is used for the selective isolation of presumptive pathogenic (pp) Staphylococcus species[9].

Procedure: All Gram positive cocci bacteria were inoculated into Mannitol Salt agar Biotec Medium Plate incubated aerobically at 37^0C for 48hours to differentiate the color of all organisms that gives specific coloration

Expected results: 1. Gram + Staphylococcus: fermenting mannitol: medium turns yellow (e.g. S. aureus)**, 2.** Gram + Staphylococcus: not fermenting mannitol, medium does not change color (e.g. S. epidermidis)**, 3.** Gram + Streptococcus: inhibited growth

3.11: Culture on CHROM Orientation Agar for Gram Negative Bacilli

Procedure: All Gram negative bacilli bacteria were sub-cultured into CHROM Orientation Biotec Medium Plate incubated aerobically at 37^0C for 48hours to differentiate the color of all Enterobacteriacea that gives specific coloration.

Interpretation of Results:

Escherichia coli: dark pink to reddish.

Klebsiella, Enterobacter, Citrobacter, serratia: metallic blue (+/- reddish halo).

Proteus, Morganella, Providencia: brown halo.

Proteus vulgaris: blue with brown halo.

Pseudomonas: translucent (+/- natural pigmentation cream to green).

Acinetobacter: cream.

3.12: Oxidase Test

All the Gram negative bacteria isolates were identified using API 20E Identification System. Oxidase test was first performed on the isolates.

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Principle: This test is based on the ability of certain organisms to oxidize artificial electron acceptor tetra methyl dimethyl -P- phenylenediamine.

Procedure: Few drops of oxidase reagent were added to filter paper and the colony of isolate was smeared on it. A positive reaction was indicated by the appearance of purple coloration after 5 minutes. A standard strain of *Pseudomonas aeruginosa* ATCC 2384 was used as a positive control.

Purple: Positive

No colouration: Negative

3.13: Analytical Profile Index (API 20E)

Purpose: is a standardized identification system for gram positive cocci, Enterobacteriae and other non-fastidious gram negative rods which uses 21 miniaturized biochemical tests and a database.

Principle: The API 20E strip consists of 20 microtubes containing dehydrated substrates. These tests are inoculated with a bacterial suspension that reconstitutes the media. During incubation metabolism produces color changes that are either spontaneous or revealed by the addition of the analytical profile index or using the identification software.

Specimen: The microorganisms to be identified must first be isolated on a culture medium which is an 18-24 hours' pure culture of true organism to be identified was obtained from growth. Culture medium adapted to the culture of Enterobacteriacea and/or non-fastidious Gram negative rods according to standard microbiological techniques.

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Procedure: An 18-24 hours' pure culture of the organism to be identified were obtained from the agar media appropriate for the organism. Oxidase test was performed on the organism to be identified before carrying out the procedure.

2-3 colonies of the organism were emulsified in 5mls sterile normal saline. 4 drops of the suspension were dropped into each well with sterile pipette incubated at 35ºc±2ºc for 18-24 hours. Colour change was read and recorded after incubation. Additional tests such as Glucose, TDA, Indole and VP was done and color change was recorded

Reporting: The strip was removed from the incubator, peel back the sealing tape. The reactions were evaluated as positive or negative by comparing them with the color chart. The results were recorded under the appropriate heading on the report form.

Interpretation of Results: Identification is obtained with the numerical profile. By adding the values corresponding to positive reactions within each group, a 7-digits profile number is obtained for the 20 tests of the API 20E strip. The oxidase reaction constitutes the 21st test and has a value of 4 if it is positive. Identification is performed using the database version 5.0 with the analytical profile index. Enter the 7digits numerical profile manually into the computer package and the name of the organisms is revealed.

3.14: Culture on CHROM Candida Agar for Yeast Speciation

Procedure: All Gram yeast isolates were sub-cultured into CHROM Candida Biotec Medium Plate incubated aerobically at 37^{0}C for 48hours to differentiate the color of all yeast that gives specific coloration.

Interpretation of Results:

Bluish: *Candida Krusei*

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Bluish-purple: *Candida tropicalis*

Creamy: *Candida specie*

Greenish: *Candida albican*

Pinkish: *Candida glabrata, Candida kefur*

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3.15: Confirmation of Fungi Isolates using MS- VITEK (MALDI-TOF)

Easy sample preparation of spotting organism onto slide was done. Applying sample matrix 1.0 ul CHCA, plus 0.5ul of formic acid for yeasts was done. MS VITEK carrier was loaded with prepared slides and introduced into the instrument with 48 sample spots per target slide, 192 isolates can be tested per run. LASER Ionizes the sample. RING ELECTRODE Protein "cloud" was released and accelerated by an electric charge. TIME-OF-FLIGHT Proteins travelled up the flight tube before hitting the detector. A long flight tube helps to deliver high resolution spectra for increased accuracy. DETECTOR Structural proteins are detected to create a spectrum that represents the protein makeup of each sample. SPECTRUM A spectra from a sample is carefully digitalized using Advanced Spectra Classifier and Weighted Bin Matrix. Results were reviewed by selection, batch, specimen, slide, or acquisition group. Sort and filter results with customizable fields such as operator, confidence level, bench name, accession number, or setup technician/operator. Displayed of new icons provides additional information during result review, including a duplicate isolate alert.

3.16: Susceptibility Testing

Antibiotic susceptibility testing was done on all the confirmed bacteria isolates using Kirby Bauer disc diffusion method[4] and VITEK 2.0[4] while anti-fungal susceptibility testing of all fungi isolates were done using Kirby-bauer disc diffusion method[5].

3.16.1: Inoculums Preparation for Antibiotic Susceptibility Testing

All confirmed isolates were inoculated into sterile peptone water incubated for 2 hours to adjust turbidity of suspension to that of 0.5 McFarland standard ($1.5x10^8$ cfu/ml) inoculum. A sterile cotton swab, was dipped into the inoculums and drained by the side of the tube and

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was used to streak sterile Mueller-Hinton agar plate for confluent growth. After 5 minutes of streaking, the antibiotic disks were mounted, incubated at 37^0C for 24 hours. Plates were examined for zones of inhibition and the diameter of positive plates was measured to the nearest millimeter (mm). The Antibiotic susceptibility testing results were compared with standard organisms *Escherichia coli* NCTC12241 and *Staphylococcus aureus* NCTC 12981 obtained from Department of Medical Microbiology and parasitology UCH, Ibadan. Oyo-State.

3.16.2: Inoculum Preparation for Anti-Fungal Susceptibility Testing

Yeast inoculum were prepared by suspending five colonies of each isolate from 24-hour old pure culture in sterile saline and suspensions were then adjusted to the turbidity of a 0.5 McFarland standard to obtain 1×106 - 5 ×106 cells per Ml.

Aspergillus inoculum were prepared by harvesting the fungal spore cells on the SDA plates with the aid of sterile cotton wool swab (Professional Hospital supply, Temecula CA, China) which was first moistened in 1ml sterile double- distilled water and was then rolled on the Saboraud Dextrose Agar (SDA) plates to collect the fungi spores. The swab was then immersed in the sterile water dispensed in each of the test tubes to form a suspension. Then a drop of sterile Tween 80 is added to homogenize the spore, covered the tubes and mixed by inverting for 30 secs. The suspension was now taken carefully to another sterile test tube and prepared for inoculum testing.

Yeast and Aspergillus suspensions were then streaked onto Saboraud dextrose agar respectively. (Oxoid, Basingstoke, UK). Antifungal disks were placed on Saboraud dextrose agar seeded with yeast isolates after the plates were dried at ambient temperature for 15minutes. Diameter zone of inhibition was measured by a metal caliper after 24hrs and

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48hrs of incubation at 35∘C. Filter paper disks soaked in respective solvents without antifungal agent were placed on culture plate to serve as control. Standard strains of *C. albicans* ATCC 10231 were used for quality control. Criteria of susceptibility and resistance of antifungal agents were determined according to International Journal of Microbiology.

3.16.3: Zone Diameter Interpretation of Antibiotic for Bacteria Isolates.

The sensitivity of isolates to AMC (20/10µg) = ≥28-36, CRO (30µg) = ≥29-35, CXM (60µg) = ≥20-26, CN (40µg) = ≥19-26, AZM(80µg) = ≥18, CIP (10µg) = ≥29-38, and AK (33µg) = ≥19-26, CAZ (30µg) = ≥25-32, LEV (5µg) = ≥29-37, E (15µg) = ≥22-30, DA (2µg) = ≥24-30, OX(1µg) = ≥18-24, FOX (30µg) = ≥23-29, VA (30µg) = ≥17-21 [4].

3.16.4: Zone Diameter Interpretation of Antifungal for Fungi Isolates.

Drug Potency Zone diameter in mm. Sensitive, Intermediate and Resistant. Fluconazole (25μg/disk) = ≥19, 15–18, ≤19, Ketoconazole (10μg/disk) = ≥28, 21–27, ≤20 Itraconazole (10μg/disk) = ≥20, 12–19, ≤11, Nystatin (50μg/disk) = ≥15, 10–14, No zone diameter**,**

Interpretation of Results for Bacteria and Fungi (CLSI, 2020).

3.17: Identification and Antibiotic Susceptibility Testing for Bacteria and Fungi Isolates using VITEK 2.0 System

Procedure: All isolates are first sub-cultured in Nutrient agar and thereafter an appropriate differential and/or selective medium, MacConkey Agar was used. Pure isolated colonies are then piced for AST using the VITEK 2.0

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3.17.1 Calibrating the DensiCHEK

The acceptable ranges for densiCHEK standards are:

Standard	Acceptable Range	
0.5 McF	0.44	0.56

The DensiCHEK was switched on and set tube setting to 'glasses by pressing the Menu key and SEL. Then, displayed a flashing triange under current tube type setting. The read/enter key was then used to move the triangle to correct setting-glass in this case. The outside of standard tubes was then clean with non-linting tissue and inverted severally without shaking. The tube was inserted, blank and press the '0' key. The value for the standard was displayed and repeated severally, until standards are within acceptable ranges.

3.17.2: Starting and loading the VITEK 2.0 Machine

Cards were selected based on Gram reactions of test isolates, For AST, 145ul or 280ul was transferred for Gram negative and Gram positive organisms respectively from adjusted organism suspension for identification(ID) into fresh tube containing 3mL saline. Capillary tube was inserted and attached to appropriate VITEK card into tube in the cassette. Load the suspension into the machine within 30 minutes of preparation of suspension.

3.17.3: Data Entry into Flexi-prep of VITEK Machine

The username and password was entered to log onto the computer, then clicked on the V2C icon and entered the same username and password again. When the V2C is initialized, an icon screen appeared. Then entered the test microbe/QC organism information in the application screen and double clicked the Manage Cassette View icon, circled below, then clicked on Maintain Virtual Cassette icon in the left view bar of the Setup Test Post Entry Window. Clicked again to Create New Virtual Cassette icon in the upper right view bar also

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called the Action Bar. The Maintain Virtual Cassette window appeared and Virtual Cassette stores the data scanned into the computer. Entered the cassette information by choosing the number from the drop down window labeled "cassette" and entered the card data by scanning the car code on the card. The Cursor must be in the Bar Code space to be entered. You may either hit ENTER and the cursor will move to the next line to be scanned or use the mouse button to move the cursor to the next Bar Code space. Verify that the slot in which the card is located in the cassette matches the corresponding slot in the Virtual Cassette.

3.18: Detection of Antimicrobial Resistance Genes in Bacteria Isolates

Thirty-eight (38) multi-drug resistance confirmed colonies of Gram negative and Gram positive bacterial isolated from the 400 samples of SARS-CoV-2 positive individuals were used. All these samples were collected within a period of six months.

3.18.1: DNA Extraction of Bacteria using NIMR BIOTEK CORP KIT

DNA were extracted from the suspended bacterial cells using a commercially prepared bacterial DNA extraction kit according to the manufacturer's instruction **(NIMR BIOTEK CORP KIT)** as described below:

3.18.2: Genomic DNA Isolation of Bacteria Isolates (Using NIMR BIOTEK CORP KIT)

Specimen: An 18-24 broth culture of the thirty-eight (38) multi-drug resistant bacteria isolates obtained from SARS-CoV-2 positive individual samples were used.

3.18.3: Preparation of Lysates from Cell of Organism and Purification of Genomic DNA from the Lysate

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100µLof specimen was transferred into a 1.5 mL micro-centrifuge tube. 500µL of the Lysis Buffer was added, Vortex and incubate at 56°C for 10 min. It was then centrifuged at 10000RPM for 1 minute to collect cells and the supernatant was discarded. After spinning, 200µL of Absolute ethanol was added to the cell pellet in the tube and transfer the mixture into the Spin Column gently. Then centrifuge at 10,000RPM for 30 seconds, the flow-through was discarded and blot the collection tube on a tissue paper.

3.18.4: Washing Bond DNA

500µL of Wash Solution I was applied to the Spin column and centrifuged for 30 seconds at 10,000RPM. The flow-through was discarded and blot the collection tube on a tissue paper. The spin column was reassembled with its collection tube. 500µL of Wash Solution II was applied to the spin column and centrifuged for 1 minutes at 10,000RPM. The flow through was discarded and blot the collection tube on a tissues paper reassembled the spin column with its collection tube. The Spin column was centrifuge again for 3 minutes in order to thoroughly dry the column at 12,000-14000RPM to remove all traces of ethanol and the collection tube was discarded.

3.18.5: Elution of Clean DNA

The Spin column was placed into another micro-centrifuge tube (1.7mL elution tube) provided. 50µL of Elution Buffer or nuclease-free water was added to the Centre of the column, incubated at room temperature for 1 to 2 minutes and centrifuged for 1 minutes at 10,000RPM for 1 minute to elute the DNA and this step was repeated twice.

3.18.6: Storage of DNA

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The Purified DNA samples were stored at -20°C or -80°C for long term storage but at 4°C for a few days.

Table: 3.1: Compositions of the Bacteria DNA Extraction Kit

Buffers	**Contents for 40 Preparations (Specimens)**
Cell lysis solution	6.4ml
Cell re-suspension solution	6.4ml
Proteinase k (20mg/ml)	0.8mg
Protein precipitation solution	2.2ml
Washing buffer (Final volume 60ml)	Add 48ml ethanol
DNA hydration solution	2.2ml
RNase A	0.16mg

Sources: NIMR BIOTEK CORP KIT, Yaba, Lagos. Nigeria.

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3.19: Measurement of DNA Concentration and Purities

The concentration and purities of DNA extracted from each sample were determined by using Nanodrop Spectrophotometer (Thermo Scientific).

Objectives: To quantify the amount of DNA in a genomic DNA sample and their Plasmid make up.

Principle: Nucleic acid absorbs light at a wavelength of 260nm. If a 260nm light source shines on a sample which can be measured and the amount of light absorbed by the sample can be inferred.

Procedures: The computer attached to the Nanodrop was opened and turned on. The Nanodrop machine was initialized by clicking on the "Nucleic Acid" button in the Nanodrop Software. 20µl of purified water to the lower pedestal, then lower the upper arm and lower arm, click "okay". The computer was on and waited for 20 seconds, then the Nanodrop initialized 2µL of the elution buffer was added. The upper arm of the Nano Drop was lowered and clicks the Blank button in the software, waited for 20 seconds for the blank to be made. When it was done, the upper arm was lifted and dried the pedestal with a wipe tissue. 2µL of the sample was added to the lower pedestal then the upper arms lowered, clicked the "measure" button on the software and waited for 20sec for measurement.

When it was done, the upper arm was lifted and dried the pedestal. The measurement was written down, the cursor was moved to check the absorbance number at various wavelength

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4-5µL. Purified water was added to the lower pedestal, then the arm was lowered and waited for 30-60 seconds, lifted up and tissues was used to wipe both upper and lower pedestal.

3.20: Polymerase Chain Reaction (PCR) for Bacteria

3.20.1 Principle and Application of Polymerase Chain Reaction

PCR is a revolutionary method developed by Kary Mullis in the 1980s. It is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. It is an extremely sensitive technique and possible to achieve a sensitivity of down to one DNA molecule in a clinical specimen.

3.20.2: Component of PCR reaction includes:

DNA template: the sample DNA that contains the target sequence.

DNA polymerase: a type of enzyme that synthesizes new strands of DNA complementary to the target sequence.

Primers: short pieces of single-stranded DNA that is complementary to the target sequence.

The polymerase begins synthesizing new DNA from the end of the primer.

Nucleotides (dNTPs or deoxynucleotide triphosphates): single units of the bases A, T, G and C which are essentially "building blocks" for new DNA strands.

Buffer solution: this provides a suitable chemical environment for optimum activity and stability of the DNA polymerase.

Divalent Cations: this could be Magnesium or Manganese ions.

Monovalent Cations: Potassium ions.

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3.21: Multiplex and Simplex Primers used for Bacteria Isolates.

Three sets of primers (forward and reverse) were used for PCR assay from multi-drug resistant bacteria isolates from confirmed COVID 19 individuals. All primers used in this project were obtained from a commercial company Jena Bioscience company (Germany) and they were as shown in the table below. These primers were also used by [10] and [11].

3.21.1: Dilution of the Primers

The Three primers were supplied in a small vial in lyophilized form. The primers were diluted with PCR graded water (Teknova, Cat No.103340). The dilution factor was described in the synthesis report by the manufacturer.

3.21.2: Preparation of Primers Working Solution.

DNA graded water (180µl) was pipetted with the aid of adjustable pipette into microtubes. Twenty microliter (20µl) of the primer stock was added and vortex for 1minute. A final concentration of 10 pm/ µl was obtained. This now gives a working strength of 10 Pico moles. This is as shown in the table below.

Note: It was important to know the working strength in order to know the amount of Primer to be added. To calculate the total volume of reagent to be use depends on the number of sample to run for PCR. Using the formula, $C_1V_1 = C_2V_2$ the required volume was calculated. Therefore, by multiplying each of the volume per number of reaction given by the total number of sample to be ran to obtain total volume. For example, to run 38 samples for Multiplex ermB and mefA PCR, PCR mix = 4µl x 38 samples. Total volume = 152µl. Primers = 38 x 0.1µleach for forward and backward Primers =0.4µl x 38 = 15.2µl and DNA template = 38 x 2µl each = 76µl. Water = 13.6µl x 38 = 516.8µl of water for the total volume. Therefore, for each PCR reaction ran, 4µl of PCR master mix (Jena Bioscience,

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Germany) was pipetted with the aid of adjustable pipette into PCR tubes, then 0.1 µl each of Primers forward and reverse was also pipette and added and 2µl each of DNA template was added and 13.6µl of water was added to make up 20µl reaction. All the tubes were labeled with the corresponding numbers.

To run 38 samples for Simplex mphA PCR, PCR mix = 4µl x 38 samples. Total volume = 152µl. Primers = 38 x 0.2µl each for forward and backward Primers =0.4µl x 38 = 15.2µl and DNA template = 38 x 2µl each = 76µl. Water = 13.6µl x 38 = 516.8µl of water for the total volume. Therefore, for each PCR reaction ran, 4µl of PCR master mix (Jena Bioscience, Germany) was pipetted with the aid of adjustable pipette into PCR tubes, then 0.2 µl each of Primers forward and reverse was also pipette and added and 2µl each of DNA template was added and 13.6µl of water was added to make up 20µl reaction. All the tubes were labeled with the corresponding numbers.

Note: Negative control containing the same reaction constituents as above without a DNA template was used to check contamination. The PCR tubes was arrange on the Thermal cycler by (AB Applied Bio system PCR 9700, Gene Amp, Germany).

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Table 3.2: The Working Strength of PCR Reaction mix used for Multiplex ermB and mefA

Reagent	Concentration	Concentration	Volume in
Total	In stock volume	in reaction	Reaction
PCR mix	5x	1x	4µl
multiply by number			
Of samples			
Primers	10picomoles	0.4picomoles	0.1µl each
Water			13.6µl
DNA template			2µl each

Sources; (Jena BioScience, Germany)

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Table 3.3: The Working Strength of PCR Reaction mix used for Simplex mphA

Reagent	Concentration In stock volume	Concentration in reaction	Volume in Reaction Total
PCR mix multiply by number of samples	5x	1x	4µl
Primers	10picomoles	0.4picomoles	0.2µl each
Water			13.6µl
DNA template			2µl each

Sources; (Jena BioScience, Germany)

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3.21.3: Principle of Multiplex PCR

Multiplex PCR is a modification of PCR that uses multiple pairs of primers in a single PCR mixture with primer pairs being specific to different DNA sequences. By targeting multiple genes at once, a single PCR can provide the information that otherwise would require several times the amount of reagents and take longer to perform.

Procedures:

2µL of the prepared cDNA of each sample was used in the multiplex reaction using PCR master mix from Inqaba biotech and 1st round primers for mefA, ermB and mphA to make a 50 µL reaction mix as described in table. The PCR master mix contains a premix of PCR buffer, Magnessium chloride, dNTPs, and Taq Polymerase enzyme in optimized concentrations. Nucleotide sequence of the primers is as shown in table. Micro amps tubes containing the PCR reaction mixes were placed in a thermal cycler (Master cycler gradient Eppendoff, Hamburg, Germany) programmed to run as follows:

3.21.4: Amplification of Multiplex ermB and mefA Amplicon for PCR

PCR was performed in 20µl reaction mixtures, containing 10pm of the multiplex primers (Table 3.2). Approximately 20ng DNA and PCR master mix (Jena Bioscience, Germany) was carried out under the following conditions; 5 min at 95˚C, followed by 35 cycles of denaturation at 95˚C for 30 secs, then annealing at 47˚C for 1 min, and extension at 72˚C for 1 min followed by a final extension step at 72˚C for 10 min.

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3.21.5: Amplification of mphA Amplicon for PCR

PCR was performed in 20µl reaction mixture, containing 10pm of each primer (mphA F and mphA R) (Table 3.3), 20ng DNA and PCR master mix (Jena Bioscience, Germany). PCR was carried out under the following; 5 min at 95˚C, followed by 35 cycles of denaturation at 95˚C for 30 secs, then annealing at 58˚C for 1 min, and extension at 72˚C for 1 min, followed by a final extension step at 72˚C for 10 min.

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Table 3.4: Multiplex Primers used for ermB and mefA Macrolides Resistance Genes

Primers	Sequences(5′ to 3′)	Size	References
ermB	F: GAAAAGGTACTCAACCAAATA R: AGTAAGGGTACTTAAATTGTTTAG	639	[10]
mefA	F: AGTATCATTCACTAGTGC R: TTCTTCTGGTACTAAAAGTGG	348	[10]

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Table 3.5: Simplex Primer used for mphA Macrolide Resistance Gene

Primers	Sequence (5' to3')	Size	References
mphA	F: GTGAGGAGGAGCTTCGCGAG	403	[11]
	R:TGCCGCAGGACTCGGAGGTC		

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3.22: Agarose Gel Electrophoresis for Bacterial DNA

To characterize the DNA of the isolates, electrophoresis of DNA was carried out on 2% agarose slab gels in Tris-Acetic-EDTA buffer, (PH 8.0). A dye solution consisting of bromophenol blue

(0.25%) and sucrose (40% w/v) in water was added to DNA samples prior to electrophoresis

Electrophoresis was carried out in a horizontal slab gel apparatus. Ethidium bromide (0.5ug/ml) stain was also added to gel prior to electrophoresis[12].The gels were run for 35minutes at a constant voltage of 70V. The stained gel was visualized with a shortwave ultraviolet light trans-illuminator and the photographs of the DNA base pair bands on gel were taken

3.22.1: Preparation of Reagents and Identification of the Amplified Products by Gel Electrophoresis

(A) Tris Acetate EDTA (TAE) Buffer

TAE buffer was used both as running buffer and in preparation of Agarose in Agarose gel electrophoresis. A stock solution of TAE buffer is prepared by dissolving 242g TRI base in deionised water, adding 57.1ml glacial acetic acid and 1000ml of 500mM EDTA (the pH should be 8.0). The final volume was made up to 1 litre by adding the appropriate volume of deionised water. The stock solution was then diluted 1:50 with deionised water to make a working solution of 1X. This working solution contains 40Nm Tris, 20nM acetic acid and 1M EDTA.

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(B) Ethidium Bromide

Ethidium bromide is a common dye used to make DNA bands visible for agarose gel electrophoresis. It fluoresces under UV light when intercalated into the major groove of DNA. It was prepared by dissolving 10mg of the ethidium bromide powder supplied by Sigma in 1ml of double distilled water. Five microliter of the prepared ethidium bromide was added to the melted Agarose gel. This is usually mixed with DNA samples for use is agarose gel electrophoresis. It contains a dye to assess how fast the gel is running and a reagent to make the DNA sample denser than the running buffer. It was prepared by adding 3ml of 30% glycerol, 25mg of bromophenol blue and 10ml of deionized water. One microliter of the loading buffer was added to each 5µl of PCR amplicons.

(C) Agarose Gel

Two grams of Agarose was diluted in 100ml of 1X working solution of TAE buffer to prepare a 2% Agarose gel. The agar was melted in microwave for 2 minutes and allowed to cool to 45^{o}C. Five microliter of ethidium bromide was added to the melted agar before being poured into a gel plate containing template comb mounted vertically. The agar was allowed to solidify for 20 to 30 minutes before the template was carefully removed. The gel was placed in the electrophoresis unit with TAE buffer added to submerge the gel. Five microliters of each PCR amplicons was added to 1µl of loading buffer dye was loaded into each well and 5µl of standard DNA marker was also added to the first and last wells of each gel.

The electrical leads were attached to the electrophoresis unit and 120 volts and 500 mill amperes was applied for 30 minutes to allow the DNA to migrate towards the anode. At the

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completion of the electrophoresis resulting DNA band was examined and photographed under the ultraviolet light using the Trans-illuminator. Positive samples showed the expected DNA band of 348, 639 and 403 base pairs for mefA, ermB and mphA gene respectively. Samples that showed no band were regarded as negative.

3.23: Detection of Antimicrobial Resistance Genes in Fungal Isolates

Twenty (20) multi-drug resistant confirmed colonies of Aspergillus spp. and candida spp out of 63 fungi isolated from the 400 samples of SARS-CoV-2 positive individuals were used. All these samples were collected within a period of six months.

3.23.1: Re – culturing of Specimen from SDA Slant and those stored in Sterile Water in Cryovial at 4°C

Sterile powdered free gloves and laboratory coat were worn and under a biosafety cabinet (Labcono purifier class ii model 36213 delta series). All the isolates stored on SDA slant and sterile water in 1.8ml cryovial tubes were inoculated onto SDA plates (LAB M) and incubated at 37°C for 7days according to aseptic techniques with daily inspection.

3.23.2: Harvesting Aspergillus Fungal Mycelium

The isolates were harvested using method described by[13] Distilled water was collected into a sterile autoclave bottle (500ml) and this was autoclaved at 121°C for 15 minutes in an autoclave (Systec VE- 65 model) and was allowed to cool down. With the aid of 1ml adjustable pipette, fixed with 1millilitre sterile filtered tips (Axygen Scientific, Germany), 1millilitre of the autoclaved sterile water was pipetted into sterile labeled 1.8ml cryovial tubes arranged in a rack.

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The fungal cells on the SDA plates were harvested with the aid of sterile cotton wool swab (Professional Hospital supply, Temecula CA, China) which was first moistened in 1ml sterile water and was then rolled on the SDA plates to collect the fungi spores. The swab was then immersed in the sterile water dispensed in each of the cryo-vial tubes to form a suspension. The tubes were covered and mixed by inverting for 30 sec.

3.24: Method of DNA Extraction of Fungi using Jena Bioscience, (Germany)

DNA were extracted from the suspended fungal cells using a commercially prepared fungal DNA extraction kit according to the manufacturer's instruction Jena Bioscience, (Germany) as described below:

Procedure: The 1ml of the fungi suspensions in the cryovial tubes were transferred into 1.5ml of nuclease free eppendorff tubes (Coster Cat No 3620) and centrifuged using micro centrifuge (Model 5418) at 15,000g for 1minute and the supernatant were discarded. The cell deposit was then re-suspended in 300µl cell re-suspension solution Jena Bioscience, (Germany) provided in the extraction kit. The fungi cell wall was then digested by adding 1.5µl proteinase K solution (Jena BioScience, Germany) and the content was mixed by inverting the micro tube several times to ensure homogenization. The micro tube was incubated on heating block (model VWR digital heater block) set at 65˚C for 3 h.

At the end of the incubation period, the suspension was further centrifuged at 15,000g for 1minute in the micro centrifuge and the supernatant was discarded into the discard jar. The deposit (pellets) was then re-suspended in 300µl cell lysis solution.

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Table: 3.6: Compositions of the fungal DNA Extraction kit

Buffers	Contents for 20 Preparations (Specimens)
Cell lysis solution	6.4ml
Cell re-suspension solution	6.4ml
Proteinase k (20mg/ml)	0.8mg
Protein precipitation solution	2.2ml
Washing buffer (Final volume 60ml)	Add 48ml ethanol
DNA hydration solution	2.2ml
RNase A	0.16mg

Sources; (Jena BioScience, Germany)

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3.24.1: Precipitation of Protein from Fungi DNA

Protein precipitation solution (100µl) (Jena BioScience, Germany) was added to the cell lysate in the micro tube above and the solution was mixed by inverting for 20 seconds using vortexing mixer (model Lab net) this was centrifuged at 15,000g for 3 minutes. The precipitated protein was seen as a tight pellet. The supernatant was then transferred to fresh labeled 1.5ml micro tube. With the aid of a sterile pipette 300µl of 99% isopropanol (Sigma company, Germany) was added to each micro tube to precipitate the DNA. The sample was mixed by inverting it gently for 50 times and centrifuged at 15,000g for 1minute. The supernatant was discarded and the tube was drained briefly on clean absorbent paper laid in the biosafety cabinet. After which 500 µl of washing buffer (Jena Bioscience, Germany) was added to the tube. The tube was inverted several times to wash the DNA pellets. It was again centrifuged at 15,000g for 1minute and the wash buffer was carefully discarded and the tube was allowed to dry at room temperature for 30 minutes to remove the ethanol which can inhibit the PCR.

3.24.2: Hydration of DNA

The DNA in the micro tube was then hydrated by adding 50µl of DNA hydration solution (Jena Bioscience, Germany) to the dried DNA pellet. 1.5µl of RNASE A was added to the cell lysate, the sample was mixed by inverting the tube and incubate at 37˚C for 30-60 minutes and DNA was hydrated by incubating samples at 65˚C for 1hours. After which the DNA was stored at -20˚C or -80˚C in a laboratory freezer before use.

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3.25: Polymerase Chain Reaction for Fungi

3.25.1: Principle of PCR

Polymerase Chain Reaction is a revolutionary method made while working at Cetus Corporation in USA[14,15,16]. PCR is a DNA template dependent, oligonucleotide primer directed, polymerase enzymes catalyzed amplification of a gene in an exponential manner. Most PCR methods can amplify DNA fragment of between 0.1 and 10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40kb in size[17]. PCR is based on the way cells replicate their DNA. During DNA replication, the two strands of each DNA molecules separate[18].

3.25.2: Components for PCR Reaction

1) **DNA Template**: the DNA template contains the DNA region to be amplified. The DNA templates are obtained from the target fungi, bacterial or virus.
2) **Primers:** a primer is a sequence of single stranded DNA that anneals or binds to the template by specific base pairing.[18] Two primers that are complementary to the 3′ (three prime) ends of each of the sense and anti-sense strand of the DNA target are needed. These primers are also known as short synthetic oligonucleotide primers[18,19].
3) **DNA Polymerase:** the DNA polymerase is an enzyme that synthesizes new strands of DNA complementary to the target sequence[18]. The DNA polymerase isolated from Thermopillus aquaticus was reported to be stable at high temperature remaining active even after DNA denaturation.

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4) **DNTPs:** these are nucleotides containing triphosphate groups. They are also called deoxyribonucleic bases. They include the dCTP, dGTP, dATP and dTTP. The triphosphate groups form the building blocks from which the DNA polymerase synthesized a new DNA strand[11].
5) **Buffer Solution:** the buffer contains Tris HCL 10 milimolar, potassium chloride 5 milimolar at pH 8.3. The buffer solution provides a suitable chemical environment for optimum activity and stability of the DNA polymerase.
6) **Divalent cations such as Mgcl:** Magnesium chloride is needed in PCR reactions which help to stabilize DNA and serves as a co – factor for the enzymes Taq polymerase. The magnesium chloride comes in stock of 15 milimolar[20].
7) **Monovalent cations:** Potassium ions.

3.25.3: Primers used for Fungi Isolates

Four sets of primers (forward and reverse) were used for PCR assay from the candida and aspergillus species isolated from confirmed COVID 19 individuals. All primers used in this project were obtained from a commercial company Jena Bioscience company (Germany) and they were as shown in the table below and the method used was adopted from[21]. These primers were also used by[22, 23, 24, 25]

3.25.4: Dilution of the Primers

The Three primers were supplied in a small vial in lyophilized form. The primers were diluted with PCR graded water (Teknova, Cat No.103340). The dilution factor was described in the synthesis report by the manufacturer.

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Table3.7:Primer used for ITS1 and ITS4 Amplification in Taxonomy of Fungi Isolates

Primers	Sequence (5' to3')	Size	References
ITS1	F: TCCGTAGGTGAACCTGCGG	500-600	[22]
ITS4	R: TCCTCCGCTTATTGATATGC		

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Table 3.8: Primer used for FKS 1 & 2 Resistance Gene in Fungi to Echinocandins and Polyenes Antifungal Agents.

Primers	Sequence (5' – 3')	Size	References
FKS2HS1	F: GCTTCTCAGACTTTCACCG	600	[23]
FKS2HS2	R: CAGAATAGTGTGGAGTCAAGACG		

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Table 3.9: Primer used for ERG11 Resistance Gene in Candida to Azole Antifungal Agents.

Primer	Sequence (5′ – 3′)	Sizes	References
ERG11	F: CCGAGTACAAGGAGGCCTTC R: CCGATAGAGGTCATAACGTGG	1300	[24]

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Table 3.10: Primers used for CPY Resistance Gene in Aspergillus Species to Azoles Antifungal Agents.

Primers	Sequence (5' to3')		Size	References
CPY	F: TACACCTATTCCGATCACACCA	100	[25]	
CPY	R: GTCTCTCATTCGTCCTTGTCCT			

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3.26: To Prepare the Working Solution of Primers

DNA graded water (180µl) was pipetted with the aid of adjustable pipette into micro tubes. Twenty microliter (20µl) of the primer stock was added and vortex for 1minute. A final concentration of 10 pm/ µl was obtained. This now gives a working strength of 10 Pico moles. This is as shown in the table below.

Note: It was important to know the working strength in order to know the amount of Primer to be added. To calculate the total volume of reagent to be use depends on the number of sample to run for PCR. Using the formula, $C_1V_1 = C_2V_2$ the required volume was calculated. Therefore, by multiplying each of the volume per number of reaction given by the total number of sample to be ran to obtain total volume. For example, to run 5 samples for PCR, PCR mix = 5µl x 5 samples. Total volume = 30µl. Primers = 5 x 1µleach for forward and backward Primers =2µl=2 x 5 = 10µl and DNA template = 5 x 5µleach = 25µl. Water = 13µl x 5 = 65µl of water for the total volume. Therefore, for each PCR reaction ran, 5µl of PCR master mix (Jena Bioscience, Germany) was pipetted with the aid of adjustable pipette into PCR tubes, then 1 µl each of Primers forward and reverse was also pipette and added and 5µl each of DNA template was added and 13µl of water was added to make up 25µl reaction. All the tubes were labeled with the corresponding numbers.

Note: Negative control containing the same reaction constituents as above without a DNA template was used to check contamination. The PCR tubes was arrange on the Thermal cycler by (AB Applied Bio system PCR 9700, Gene Amp, Germany).

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Table 3.11: The Working Strength of PCR Reaction Mix, used for ITS1 and ITS 4

Reagent	Concentration In stock volume	Concentration in reaction	Volume in Reaction Total
PCR mix multiply by number Of samples	5x	1x	3µl
Primers	10picomoles	0.4picomoles	1µl each
Water			7µl
DNA template			3µl each

Sources; (Jena BioScience, Germany)

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Table 3.12: The Working Strength of PCR Reaction Mix used FKS, ERG11 and CPY

Reagent	Concentration In stock volume	Concentration in reaction	Volume in Reaction Total
PCR mix multiply by number Of samples	5x	1x	3µl
Primers	10picomoles	0.4picomoles	0.5µl each
Water			9µl
DNA template			2µl each

Sources; (Jena BioScience, Germany)

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3.27: Amplification of ITS 1& 4 Amplicon for PCR

A ribosomal internal transcribed spacer (ITS) region was amplified using ITS primers. PCR was performed in 25µl reaction mixtures, containing 10pm of each primer ITS1 and ITS4 (Table 3.7). Approximately 20ng DNA and PCR master mix (Jena Bioscience, Germany) was carried out under the following conditions; 2 min at 94˚C, followed by 40 cycles of denaturation at 94˚C for 30 secs, then annealing at 44˚C for 30 secs, and extension at 72˚C for 1 min followed by a final extension step at 72˚C for 10 min.

3.28: Amplification of FKS1&2 Amplicon for PCR

PCR was performed in 25µl reaction mixture, containing $10p^m$ of each primer (Act F and Act R) (Table 3.8), 20ng DNA and PCR master mix (Jena Bioscience, Germany). PCR was carried out under the following; 2 min at 94˚C, followed by 35 cycles of denaturation at 94˚C for 1 min, then annealing at 54˚C for 1 min, and extension at 68˚C for 1 min, followed by a final extension step at 68˚C for 10 min.

3.29: Amplification of ERG11 Amplicons for PCR

A partial ERG11 gene was amplified using Primers cmd5 and cmd6 (Table 3.9), then all PCRs were performed in 25µl reaction mixture, containing $10p^m$ of each Primer. Approximately 20ng DNA and PCR was carried out under the following; 94˚C at 10 min, 35 cycles of a denaturation step at 94˚C for 50 secs, then annealing at 55˚C for 50 secs, and elongation at 72˚C for 1 min followed by a final elongation at 72˚C for 7min.

3.30: Amplification of CPY Amplicon for PCR

PCR was carried out in a 25µl reaction mixture, containing 10pM of each forward and reverse primer, approximately 20ng DNA and PCR master mix (Jena Bioscience, Germany).

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Primer CPYF and CPYR (Table 3.10) were used for CPY amplification. Negative controls containing the same reaction but without DNA template were used to check contamination. PCR was run on a (Apply bio system 9700) Gene Amp, Germany) under the following condition; denaturation at 94˚C for 5 min, followed by 45 cycles of denaturation at 94^0C for 30 secs, then annealing at 60^0C for 30 sec and extension at 72^0C for 2 min, followed by a final extension step at 72^0C for 10 min.

3.31: Preparation of Reagents and Identification of the Amplified Products by Gel Electrophoresis

3.31.1: Tris Borate EDTA (TBE) Buffer

TBE buffer was used both as running buffer and in the preparation of Agarose in gel electrophoresis. Commercially available 10x TBE buffer by Jena Bioscience (Germany) was used to prepare 1 litre of 1x TBE buffer. The final volume was made up to 1Litre by adding 100µl of 10x TBE buffer to 900 ml of distilled water and mixed gently.

3.31.2: SYBR DNA Stain

Sybr DNA stain is a common dye used to make DNA band visible for Agarose gel electrophoresis. It fluoresces under UV light when intercalated into the major groove of DNA. One microlitre of the Sybr stain was mixed with three microliters of each amplicon before loading on the prepared agarose gel.

3.31.3: Preparation of 2% Agarose Gel

1) 2g of Agarose powder (Geneon Biological Science) was weighed with a weighing balance (Easy way medical JT2101N, England) and added to a beaker.

2) 100 mls of 1x TBE buffer was then added to the beaker.

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3) The mixture was heated in a microwave oven until it becomes a clear solution for 2 minutes.

4) The melted agar was then poured into the horizontal gel tray (Cs Cleaver Scientific Ltd nano pac - 300) with the appropriate comb inserted. The agar was allowed to solidify for 20 to 30 min in the tank

Note. It was ensured that there were no bubbles inside the tank and if there is, the bubble was removed by dissolving it.

6) Upon solidification of the gel, the gel was placed in the electrophoresis tank with the remaining 200ml of 1x TBE buffer solution prepared above and was poured into the tank to sub merge the gel.

7) Three microliter of each PCR amplicons was mixed with one microliter of sybr stain and loaded into each well created by the comb.

8) Five microliter of the standard DNA marker 100 -3kb DNA ladder (Jena Bioscience, Germany) was also added to the last wells of each gel to serve as a marker when analyzing the run.

9) The electrical leads were attached to the electrophoresis unit and the tank was run at 120 volts, 400 amperes for 30 min to allow the DNA to migrate towards the anode.

Note: DNA is negatively charged, thus migrating from negatively charged anode to positively charged pole (cathode).

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10) At the completion of the electrophoresis, the resulting DNA band was examined and photographed under the ultraviolet light and then visualized on a UV Trans- illuminator. Positive samples should show the expected DNA band of expected base pairs.

3.32 Data Analysis

Analysis of all obtained data from the questionnaires and others data's generated in the study was performed using SPSS version 20.0.

Descriptive analysis was used to present prevalence and frequencies of outcomes

Chi-square was used to determine the association between two categorical variables. P values -< 0.05 at 95% confidence interval will be considered significant.

3.33 Ethical Approval

Approvals were sought from;

- Oyo State Ethical Review Committee, Ministry of Health, Ibadan, in Oyo State. Nigeria (Appendix 1).

- UI/UCH Ethical Review Committee, Ibadan, Oyo- State. Nigeria. (Appendix 2).

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Chapter Four

Results and Discussion of Findings

4.1 The Demographic Characteristics of SARS-CoV-2 Positive Individuals

The overall sample distribution among the 3 isolation center where COVID-19 samples were collected is represented in Table 4.1. University College hospital had highest 221(55.3%, followed by Olodo 141(35.3%) and Agbami center was the least of 38(9.5%) samples.

The age distribution of the SARS-CoV-2 individuals for this research study in Table 4.2 shows mean age of 48.14±14 and the highest frequency were adults in the age brackets 60years and above 110(27.5%), this represents the elderly group of the society whose immunity might have been distorted or compromised which can be a supportive reason of been infected with this particular virus.

The distribution of the gender among the SARS-CoV-2 individual presented in Table 4.3 shows that, out of the 400 individuals enrolled in the study, 190(47.5%) were male and 210(52.5%) were female.

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Table 4.1: Sample Distribution among 3 Isolation Center

Variable	Frequency (n=400)	Percentage
Location		
Olodo	141	35.3
UCH	221	55.3
Agbami	38	9.5
Total	400	100.0

Source: Author's Laboratory Analysis

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Table 4.2: Age Distribution of SARS-CoV-2 Infected Individuals

Variable	Frequency (n=400)	Percentage
Age(years)		
<21	7	1.8
21-30	55	13.8
31-40	109	27.3
41-50	71	17.8
51-60	48	12.0
>60	110	27.5
Total	400	100.0
Mean ±SD	48.14±14	

Source: Author's Laboratory Analysis

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Table 4.3 Gender Distribution of SARS-CoV-2 Infected Individuals.

Variable	Frequency (n=400)	Percentage
Female	210	52.5
Male	190	47.5
Total	400	100.0

Source: Author's Laboratory Analysis

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Relationship between SARS-CoV-2 individual and their educational qualification in Table 4.4 shows nearly all of them 315(78.8%) were literate and had formal education while

85(21.3%) were illiterate and these were able to to give the researcher their concise and in-depth observable reports about their susceptibility to the infection.

The distribution of the marital status of SARS-CoV-2 infected individual in Table 4.5 shows 318(79.5%) married, 55(13%) singled and 27(6.8%) to have divorced to determine how healthy and strong will be their immunity.

Table 4.6 shows that majority of the SARS-CoV-2 infected individual were symptomatic 335(83.8%) while 65(16.3%) were not showing any symptoms indicating SARS-CoV-2 infection but were confirmed positive from RT-PCR result with the infection and this determine their level of immuno-suppression of each individual patients to the virus.

The case status of all the Asymptomatic and Symptomatic positive SARS-CoV-2 individual indicated in Table 4.7 shows that 60(15.0%) experienced mild infection, 139(34.8%) presented with serious infection and those in critical condition were the highest 141(35%)and 60(15.0%) were those individual in the severe state.

The clinical profiles of all SARS-CoV-2 infected individuals used for this research work shows in Table 4.8 various comorbidities status to the infection. Most of the patients were hypertensive 175(43.8%), followed by Diabetes condition 95(23.8%). Chronic respiratory disease 35(8.8%) like asthma that can also subject individual to this virus infection, Obesse individual 14(3.5%) which can posse some chances of getting infected with the virus. Immuno-compromised respondents 9(2.3%) with certain ailment like HIV, Hepatitis, Cancer were also possible, Chronic renal failure 7(1.7%), venous thromboembolism 3(0.7%) and atrial fibrillation 1(0.2%) were also possible comorbidities.

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Various symptoms experienced by SARS-CoV-2 infected individual were analyzed in this figure 4.1, majority of them experienced difficulty in breathing 25%, purulent coughing 17%, terrible fever 21% that mimic malaria infection, diarrhea 10% were experienced by some individual, joint body ache 14% was displayed, watery running nose 7% by some and finally 6% experienced itching throat.

Most of the infected SARS-CoV-2 individual whose samples were taking have been treated with malaria drugs 33.30%, followed by used of empirical antibiotics 20.00%, then cough drug 10.30% and finally 5.50% have used pain relief drugs. (Figure 4.2)

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Table 4.4: Educational Qualification of SARS-CoV-2 Infected Individual

Variable	Frequency (n=400)	Percentage
Educated	315	78.8
Not Educated	85	21.3
Total	400	100.0

Source: Author's Laboratory Analysis

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Table 4.5: Marital Status of SARS-CoV-2 Infected Individual

Variable	Frequency(N=400)	Percentage
Single	55	13.8
Married	318	79.5
Divorce	27	6.8
Total	400	100.0

Source: Author's Laboratory Analysis

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Table 4.6 Health Status of SARS-CoV-2 Infected Individual

Variable	Frequency (N=400)	Percentage
Asymptomatic	65	16.3
Symptomatic	335	83.8
Total	400	100.0

Source: Author's Laboratory Analysis

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Table 4.7: Case Status of SARS-CoV-2 Infected Individual

Variable	Frequency(N=400)	Percentage
Mild	60	15.0
Serious	139	34.8
Critical	141	35.3
Severe	60	15.0
Total	400	100.0

Source: Author's Laboratory Analysis

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Table 4.8: Comorbidities Status of SARS-CoV-2 Infected Individual

Variable	Frequency (N=400)	Percentage
Main comorbidities	Frequency	Percentage
None	61	15.2
Hypertension	175	43.8
Diabetes	95	23.8
Obesity	14	3.5
Atrial fibrillation	1	0.2
Venus thromboembolism	3	0.7
Chronic renal failure	7	1.7
Chronic respiratory disease	35	8.8
Immunocompromised	9	2.3
Total	400	100.0

Source: Author's Laboratory Analysis

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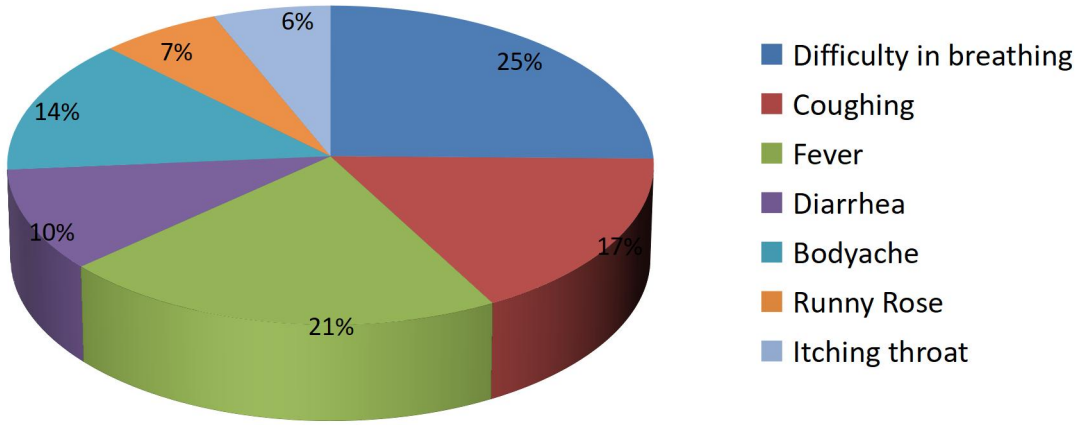

Source: Author's Laboratory Analysis

Figure 4.1: Symptoms Experienced by Symptomatic SARS-CoV-2 Infected Individual

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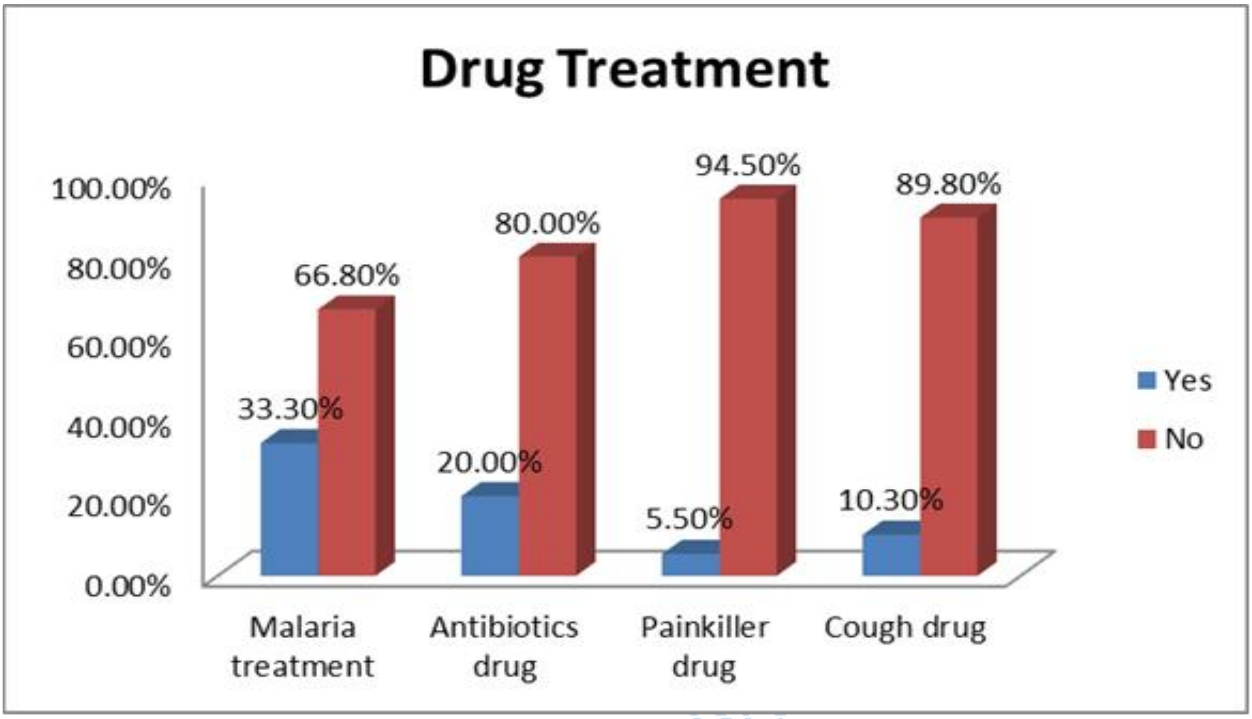

Source: Author's Laboratory Analysis

Figure 4.2 Underlined Drug Treatment of SARS-CoV-2Infected Individual

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4.2 Presentation of Data for Bacterial and Fungal Isolates from SARS-CoV-2 Infected Individual

The experimental and characterization research results of isolated pathogen from the 3 different isolation centers were also presented. The antimicrobial susceptibility pattern of bacterial and fungal were also presented. Further results of molecular analysis were performed to check the multidrug resistant gene/strain in some selected isolates.

Out of all the 400 nasopharyngeal swab samples collected from the SARS-CoV-2 infected individuals which were cultured on MacConkey agar medium, Chocolate agar medium and Sabourauds Dextrose agar medium, 240(60.0%) yielded growth of bacteria, 63(15.8%) yielded fungi growth and 97 had either growth of contaminant or no growth after respective normal incubation periods and condition (Table 4.9).

The prevalence of bacteria and fungi that is together co-infecting individual with SARS-CoV-2 in Table 4.10 shows 7.3% while 92.8% were not having mixed growth of both bacteria and fungi together.

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Table 4.9: Nasopharyngeal Swab Culture Results of SARS-CoV-2 Infected Individual

Variable	Frequency (N=400)	Percentage
Bacteria	240	60.0
Fungi	63	15.8
No growth/ Contaminant	97	22.2
Total	400	100.0

Source: Author's Laboratory Analysis

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Table 4.10: Prevalence of Bacteria and Fungi co-infection with SARS-CoV-2 Infected Individual

Variable	Frequency (n=400)	Percent
Co-Infection		
Yes (Bacteria & Fungi)	29	7.3
No	371	92.8

Source: Author's Laboratory Analysis

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The frequency and percentage distribution of Gram reaction of the various bacterial isolates in Table 4.11 shows that out of 240 (60.0%) bacterial, 124(51.7%) were Gram negative bacilli, 86(35.8%) were Gram positive cocci, 30(12.5%) were combine growth of Gram negative bacilli and Gram positive cocci.

Out of the 124 Gram negative bacteria isolates sub-cultured on CHROM Orientation agar medium for colour speciation 26(16.9%) demonstrated pinkish colouration, 97(63.0%) displayed bluish, 21(13.6%) shows creamy and 10(6.5%) shows greenish colouration. (Table 4.12)

The frequency and percentage of all isolated Gram negatives organisms identified by API 20E is displayed in Figure 4.3. The isolates identified accounted for; *Acinetobacter baumanii* 7(4.5%), *Citrobacter freundii* 1(0.6%), *Citrobacter koseri* 3(1.9%), *Cronobacter* specie 1(0.6%), *Enterobacter aerogenes* 23(14.9%), *Enterobacter cloacae* 27(17.5%), *Escherichia coli* 8(5.1%), *Klebsiella oxytoca* 5(3.3%), *Klebsiella pneumoniae* 35(22.7%), *Kluyevera* specie 3(1.9%), *Proteus mirabilis* 4(2.6%), *Proteus vulgaris* 1(0.6%), *Pseudomonas aeruginosa* 10(6.5%), *Pseudomonas luteola* 3(1.9%), *Raoultella ornithinolytica* 6(3.9%), *Serratia ficaria* 6(3.9%), *Serratia liquefaciens* 2(1.3%), *Serratia marscensces* 6(3.9%) and *Serratia rubidea* 3(1.9%) respectively.

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Table 4.11: Gram Results of Bacteria Isolates from Samples of SARS-CoV-2 Infected Individual

Variable	Frequency(N=240)	Percentage
GRAM (n=240)		
GNB	124	51.7
GPC	86	35.8
GNB + GPC	30	12.5
Total	240	100.0

Source: Author's Laboratory Analysis

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Table 4.12: Initial Identification of Gram Negative Bacteria on CHROM Orientation Agar

Variable	Frequency(N=154)	Percentage
CHROM O (n=154)		
Pinkish	26	16.9
Bluish	97	63.0
Creamy	21	13.6
Greenish	10	6.5
Total	154	100.0

Source: Author's Laboratorty Analysis

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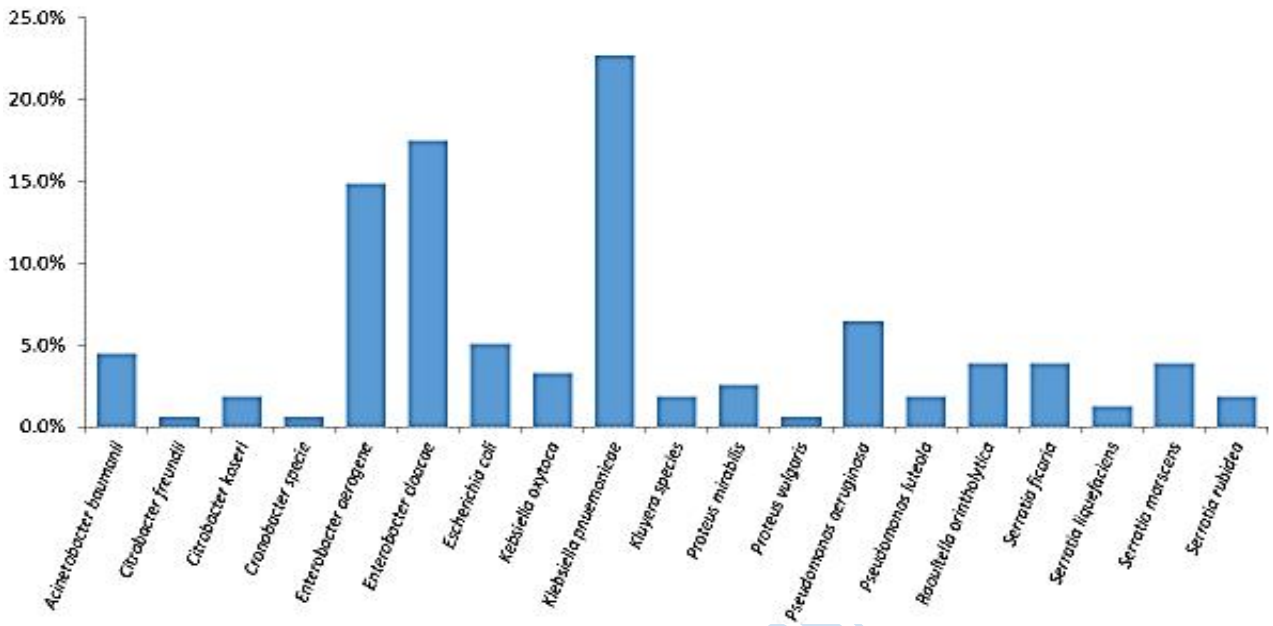

Source: Author's Laboratory Analysis

Figure 4.3: API 20E Confirmatory Identification of all Gram Negative Bacilli

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Table 4.13 shows that, out of all 111(35.8%) Gram postive cocci isolated from the samples of SARS-CoV-2 infected individuals which were subcultured on Mannitol Salt agar medium,

62(54.7%) displayed golden yellow colouration and 49(45.3%) accounted for pinkish colouration.

The frequency and percentage distribution of all Gram positive cocci organisms identified with VITEK 2.0 is displayed in Figure 4.4. The isolates include; *Enterococcus faecalis* 4(3.6%), *Staphylococcus xylosus* 1(0.9%), *Staphylococcus aureus* 36(32.4%), Methicillin Resistant *Staphyloccus aureus* 1(0.9%), *Staphylococcus equorum* 4(3.6%), *Staphylococcus gallinarium* 1(0.9%), *Staphylococcus lentus* 16(14.4%), *Staphylococcus saprophyticus* 35(31.5%), *Staphylococcus scuiri* 11(9.9%) and *Streptococcus agalactiae* 2(1.9%) respectively.

Table 4.14 shows the frequency and percentage make-up of 1(2.7%) of MRSA and 36(97.3%) MSSA from all 37 *Staphylococcus aureus* identified with VITEK 2.0 compact system

The frequency and percentage distribution of all isolated fungi cultured on Sabouraud's Dextrose agar morphologically shows on Table 4.15 indicated that 38(60.3%) were yeast and 25(39.7%) Aspergillus specie respectively.

The colour pigmentation on CHROM Candida agar displayed in Table 4.16 by all the yeast isolates confirmed by Gram reaction accounted for 2(5.3%) purplish, 5(13.2%) bluish-purple, 11(29.0%) creamy, Greenish 19(50.0%) and 1(2.6%) pinkish respectively.

The VITEK MS identification of all the fungal isolates from SARS-CoV-2infected individual samples were displayed on Figure 4.5 confirmed 11(17.5%) to be *Aspergillus flavus*, 9(14.3%) *Aspergillus niger,* 7(11.1%) *Candida albicans*, 2(3.2%) *Candida*

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guillermondii, 2(3.2%) *Candida parapsilosis*, 2(3.2%) *Candida famata,* 5(7.9%) *Candida tropicalis* and lastly 25(39%) *Lodderomyces elongisporus* having highest frequency.

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Table 4.13: Initial Identification of Gram Positive Cocci on Mannitol Salt Agar

Variable	Frequency (N=111)	Percentage
GPC on MSA(n=111)		
Golden Yellow	62	54.7
Pinkish	49	45.3
Total	111	100.0

Source: Author's Laboratory Analysis

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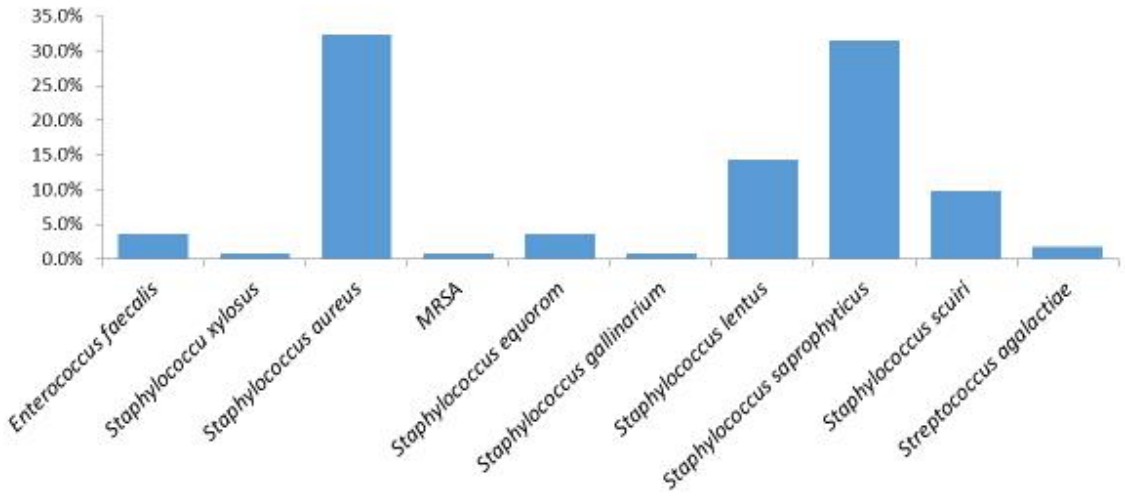

Figure 4.4: VITEK 2.0 Confirmatory Identification of all Gram Positive Cocci

Source: Author's Laboratory Analysis

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Table 4.14: Identification of MRSA and MSSA among the Isolates using VITEK 2.0

Variable	Frequency(N=37)	Percentage
Isolates		
MRSA	1	2.7
MSSA	36	97.8
Total	37	100.0

Source: Author's Laboratory Analysis

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Table 4.15: Gram and Morphological Identification of Fungal Isolates.

Variable	Frequency(N=63)	Percentage
Fungi		
Yeast (Candida specie)	38	60.3
Aspergillus specie	25	39.7
Total	63	100.0

Source: Author's Laboratory Analysis

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Table 4.16: Initial Identification of Yeast Isolates on CHROM Candida Agar medium

Variable	Frequency(N=38)	Percentage
Yeast on CHROM Candida		
Purplish	2	5.3
Bluish-purple	5	13.2
Creamy	11	29.0
Greenish	19	50.0
Pinkish	1	2.6
Total	38	100.0

Source: Author's Laboratory Analysis

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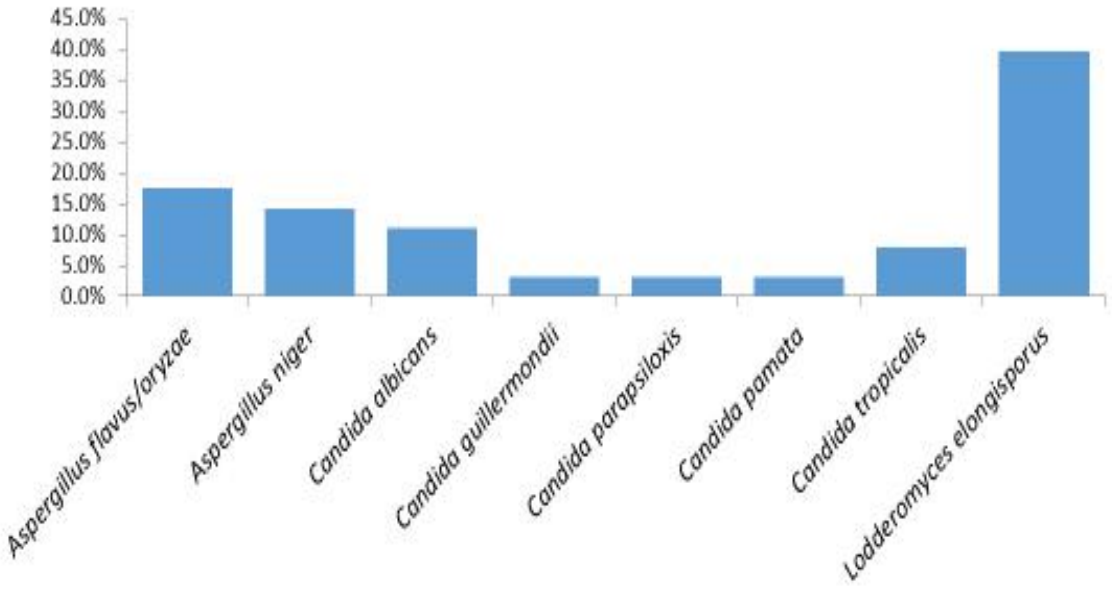

Source: Author's Laboratory Analysis

Figure 4.5: MS VITEK (MALDITOF) Confirmatory Identification of all Fungal Isolates

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The percentage distribution of antibiotics susceptibility pattern of Gram negative bacilli in Figure 4.6 shows that most Gram negative bacilli isolates were mostly sensitive to Levofloxacin (89%), Amikacin (88%), Ceftazidime (79%), Ciprofloxacin (66%), Azithromycin (51%), Cefuroxime (11%), Sulbactam (11%), Ceftriaxone (8%), Gentamycin (6%) respectively.

The susceptibility pattern in Figure 4.7 shows that most Gram positive cocci isolates were sensitive to Vancomycin (74.10%), Ciprofloxacin (71.30%), Levofloxacin (70.40%), Cefoxitin (69.40%), Gentamycin (45.40%), Erythromycin (42.60%), Azithromycin (33.3%), Clindamycin (29.60%) and Oxacillin (23.10%).

The antifungal susceptibility pattern in Figure 4.8 shows that most fungal were sensitive to Nystatin (84.1%), Itraconazole (76.2%), Fluconazole (49.2%) and Ketoconazole (39.7%) respectively.

Table 4.17 shows the frequency distribution of Gram negative bacilli among the symptomatic and asymptomatic SARS-CoV-2 infected individuals with P-value of 0.99 indicates non-significant value. The results indicated that nearly all the Gram negative bacilli isolates were recovered from symptomatic samples, while some asymptomatic samples show growth of 2(11.1%) *Enterobacter aerogenes*, 3(14.3%) *Enterobacter cloaca*e, 1 (12.5%) *Escherichia coli*, 1(25.0%) *Klebsiella oxytoca,* 3(10.3%) *Klebsiella pneumoniae,* 1(20.0%) *Proteus mirabilis,* 3(30.0%) *Pseudomonas aeruginosa*, 1(20.0%) *Raoultella ornithinolytica* and lastly 1(16.7%) *Serratia marscences* respectively.

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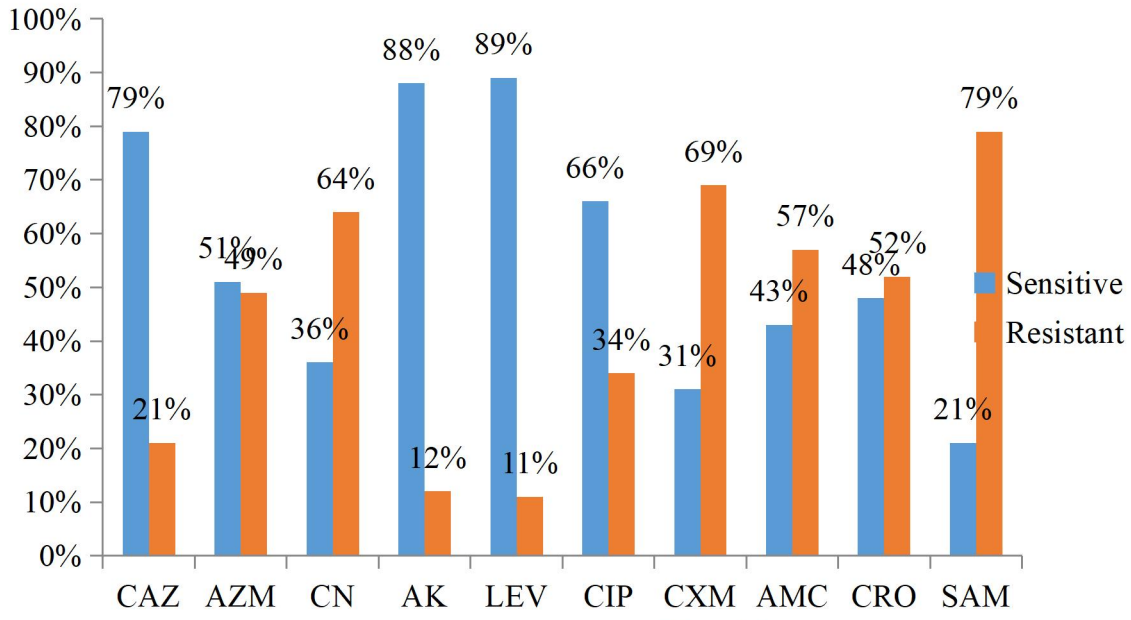

Source: Author's Laboratory Analysis

Figure 4.6: Antibiogram of Gram Negative Bacteria Isolates using Kirby Bauer Disc Diffusion and VITEK 2.0 System

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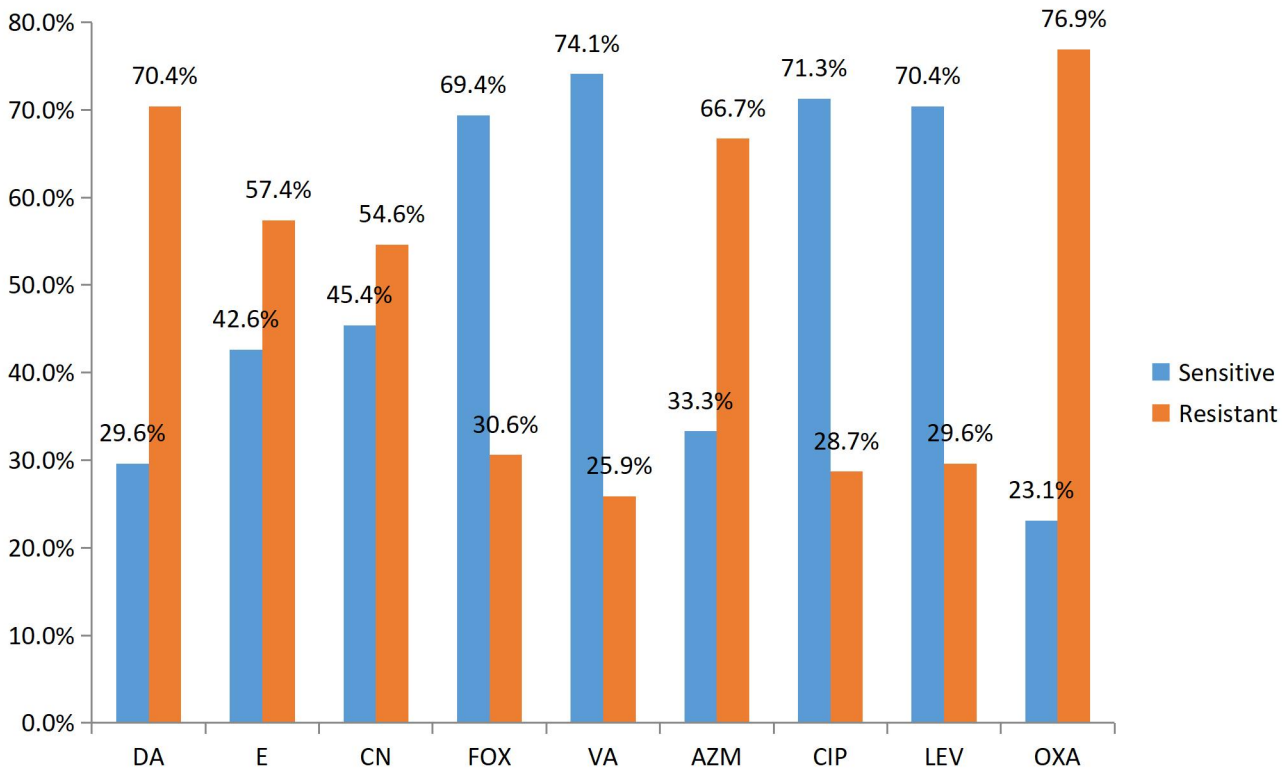

Source: Author's Laboratory Analysis

Figure 4.7: Antibiogram of Gram Positive Bacteria Isolates using Kirby Bauer and VITEK 2.0

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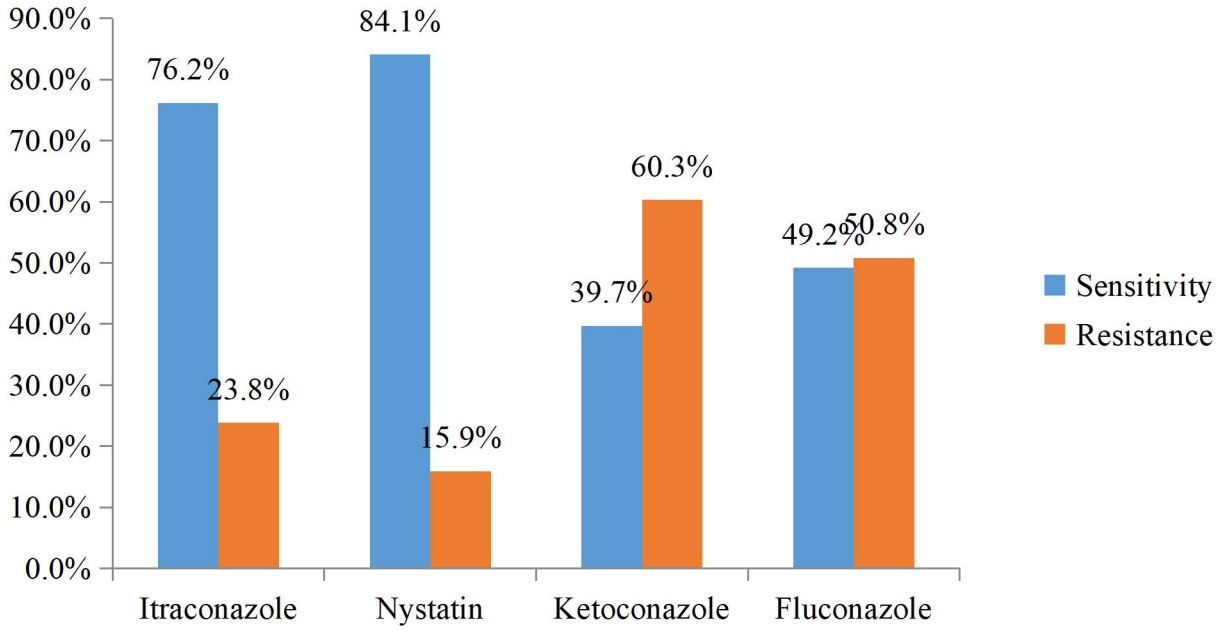

Source: Author's Laboratory Analysis

Figure 4.8: Antibiogram of Fungi Isolates using Kirby Bauer Disc Diffusion.

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Table 4.17: Gram Negative Bacterial Pathogens Recovered from Symptomatic and Asymptomatic SARS-CoV-2 Infected Indivduals

Variable	Health Status		Chi Square	P-value
	Asymptomatic(%)	Symptomatic(%)		
Bacteria pathogen (GNB)				
Acinetobacter baumanii	0(0)	7(100)	8.86	0.99
Citrobacter freundii	0(0)	1(100)		
Citrobacter koseri	0(0)	3(100)		
Cronobacter specie	0(0)	1(100)		
Enterobacter aerogene	3(13.0)	20(87.0)		
Enterobacter Cloacae	4(14.8)	23(85.2)		
Escherichia coli	2(25.0)	6(75.0)		
Kebsiella oxytoca	1(20)	4(80)		
Klebsiella pnuemonieae	3(8.6)	32(91.4)		
Kluyera species	0(0.0)	3(100.0)		
Proteus mirabilis	0(0.0)	2(100.0)		
Proteus vulgaris	0(0.0)	1(100.0)		
Pseudomonas aeruginosa	3(30.0)	7(70.0)		
Pseudomonas luteola	0(0.0)	3(100.0)		
Raoultella orintholytica	1(16.7)	5(83.3)		
Serratia ficaria	1(16.7)	5(83.3)		
Serratia liquefaciens	0(0.0)	2(100.0)		
Serratia marscens	1(20.0)	4(80.0)		
Serratia rubidea	0(0.0)	3(100.0)		

Source: Author's Laboratory Analysis

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Table 4.18 shows the frequency distribution of Gram positive cocci among the symptomatic and asymptomatic SARS-CoV-2 infected individuals with P-value 0.81(not significant). The results indicated that nearly all the Gram positive cocci isolates were recovered from symptomatic samples, while some asymptomatic samples also show growth of 12(46.2%) *Staphylococcus aureus*, and 2(6.9%) *Staphylococcus saprophyticus* respectively.

Table 4.19 shows the frequency distribution of all the fungi isolates among the symptomatic and asymptomatic COVID-19 individuals with p-values of 0.14% (Not significant). The results indicated that most of the fungi isolates were recovered from symptomatic samples, while some asymptomatic samples also show growth of 3(42.9%) *Candida albican*,1(20.0%) *Candida tropicalis* and 3(12.0%) *Lodderomyces elongisporusis* respectively.

Table 4.20 shows the molecular recovery of macrolide resistance gene among all the bacteria isolates that were phenotypically multi-resistant to some antimicrobial agents used. Out of all the 38 multi-drug resistant isolates subjected to the 3 specific macrolides primers, 20 (52.6%) isolates were positive for either mefA or ermB, and none (0.0%) possess mphA gene. 14 (36.8%) isolates had mefA gene, 10 (26.3%) isolates carried ermB gene.

Table 4.21 represents the molecular characteristic of some fungi isolates to specific antifungal resistance gene. Out of all the 20 fungal isolates, 19 (95%) isolates had ITS 1&11 confirmatory taxonomy gene, 18 (90%) fungi isolates had carriage of either of the resistant gene. 10 (50%) isolates possessed CPY2 gene, 7 (35%) isolates carried FKS gene, 9(45%) isolates had ERG11 gene.

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Table 4.18: Gram Positive Cocci Recovered from Symptomatic and Asymptomatic SARS-CoV-2 Infected Individuals

Variable	Health Status		Chi Square	P-value
	Asymptomatic(%)	Symptomatic(%)		
Bacteria pathogen (GPC)				
Enterococcus faecalis	0(0.0)	4(100.0)	6.93	0.81
Staphylococcus xylosus	0(0.0)	1(100.0)		
Staphylococcus aureus	12(33.3)	24(66.7)		
MRSA	0(0.0)	1(100.0)		
Staphylococcus equorom	0(0.0)	4(100.0)		
Staphylococcus gallinarium	0(0.0)	1(100.0)		
Staphylococcus lentus	5(31.3)	11(68.7)		
Staphylococcus saprophyticus	6(17.1)	29(82.9)		
Staphylococcus scuiri	3(27.3)	8(72.7)		
Streptococcus agalactiae	0(0.0)	2(100.0)		

Source: Author's Laboratory Analysis

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Table 4.19: Fungal Pathogens Recovered from Symptomatic and Asymptomatic SARS-CoV-2 Infected Individuals

Variable	Health Status		Chi Square	P-value
	Asymptomatic(%)	Symptomatic(%)		
Fungal pathogen				
Aspergillus flavus	0(0.0)	11(100.0)	10.81	0.14
Aspergillus niger	0(0.0)	9(100.0)		
Candida albicans	3(42.9)	4(57.1)		
Candida guillermondii	0(0.0)	2(100.0)		
Candida parapsiloxis	0(0.0)	2(100.0)		
Candida pamata	0(0.0)	2(100.0)		
Candida tropicalis	1(20.0)	4(80.0)		
Lodderomyces elongisporusis	3(12.0)	22(88.0)		

Source: Author's Laboratory Analysis

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Table 4.20: Macrolide Resistant Gene among Multi-Drug Resistant Bacterial Isolates

Variable	mefA positive(%)	ermB positive(%)	mphA Positive(%)
Enterococcus faecalis	1(14.3)	2(11.1)	0(0)
Stapylococcus saprophyticus	3(42.9)	3(11.1)	0(0)
Staphylococcus lentus	1(0)	1(11.1)	0(0)
Pseudomonas aeruginosa	2(0)	1(11.1)	0(0)
Staphylococcus aureus	3(28.6)	2(33.3)	0(0)
Enterobacter cloacae	1(14.3)	1(0)	0(0)
Klebsiella pneumoniae	0(0)	2(11.1)	0(0)
Staphylococcus xylosus	1(0)	0(0)	0(0)
Serratia marscenses	0(0)	0(0)	0(0)
Enterobacter aerogene	0(0)	0(0)	0(0)
Staphylococcus xylosus	0(0)	0(0)	0(0)
Klebsiella oxytoca	0(0)	0(0)	0(0)
Raoultella ornithinolytica	0(0)	0(0)	0(0)
Serratia ficaria	0(0)	0(0)	0(0)
Staphylococcus eqorum	0(0)	0(0)	0(0)
Total	14(36.8%)	10(26.3)	0(0)

Source: Author's Laboratory Analysis

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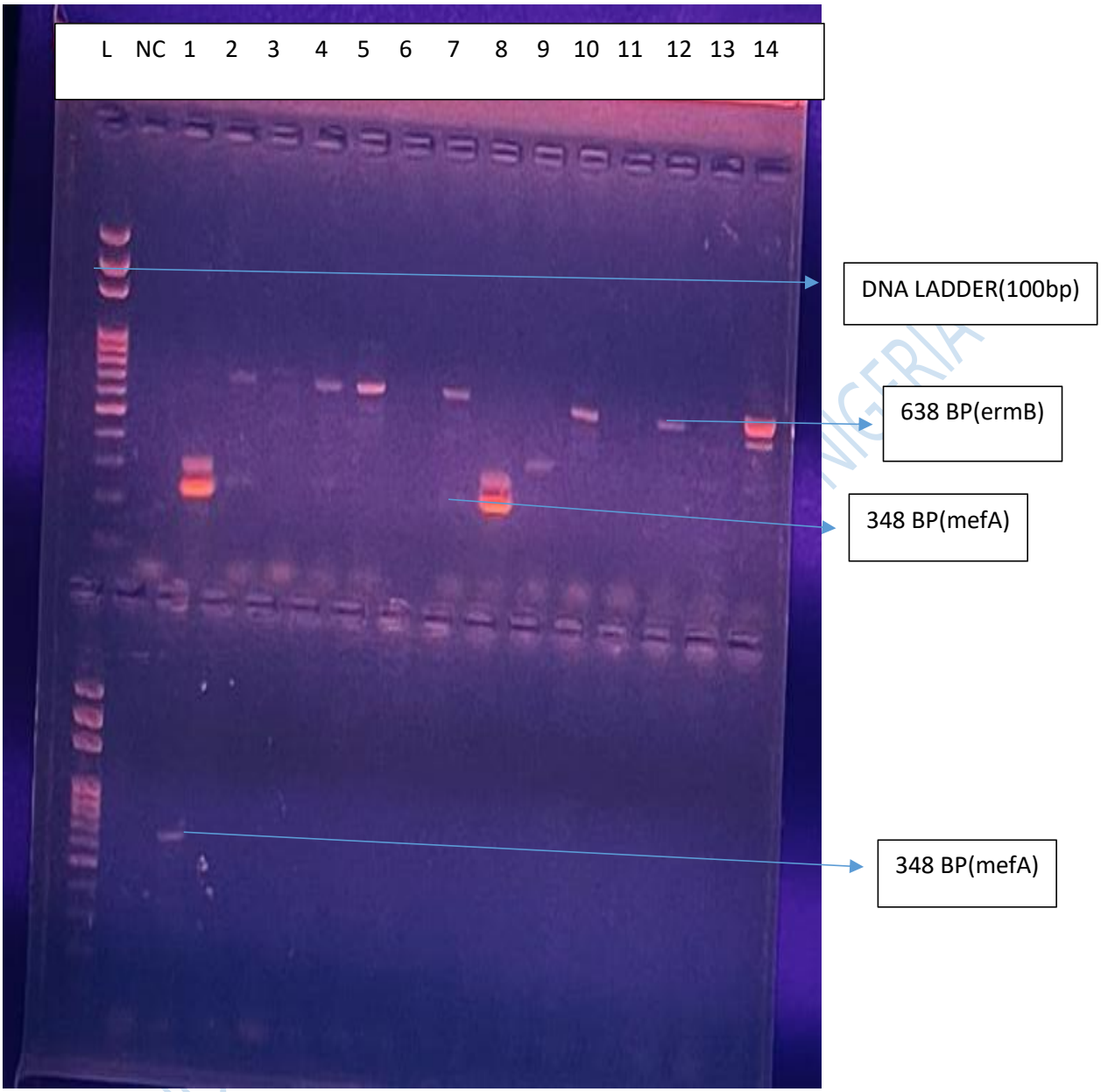
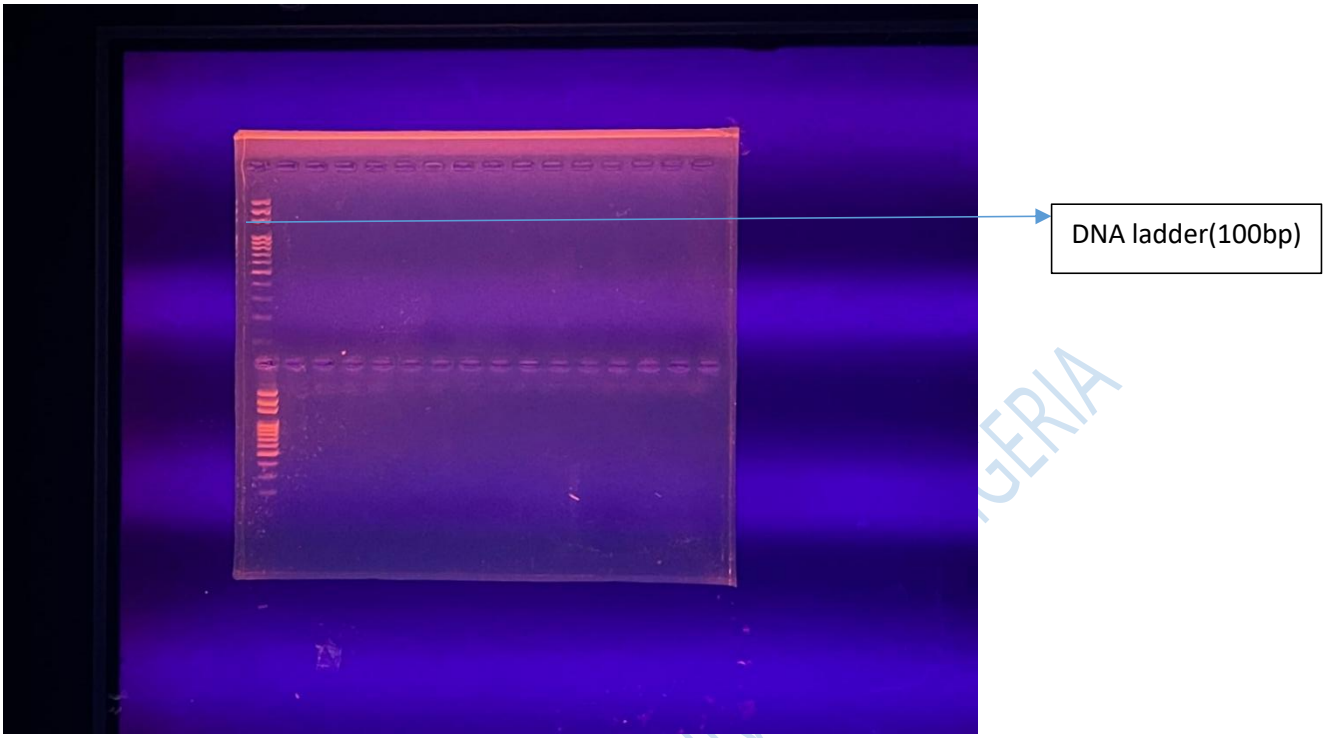


Source: Author's Laboratory Analysis

Figure 4.9: Agarose Gel Electrophoresis of Multiplex Macrolides Resistance mefA and ermB Primers Gene.

Key: Lane1=Ladder (100bp), Lane2= Negative Control, Lane3-14= Bacteria isolates



Source: Author's Laboratory Analysis

Figure 4.10: Agarose Gel Electrophoresis of Simplex Macrolides Resistance mphA Primer Gene.

Key: Lane1=Ladder (100bp), Lane2= Negative Control, Lane3-14= Bacteria isolates

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Table 4.21: Antifungal Resistant Gene with Specific Primers among Fungal Isolates.

Variable	ITS1&4 Positive(%)	CPY2 positive(%)	FKS1&2 Positive(%)	ERG11 Positive(%)
Aspergillus niger	6(30.0)	4(20.0)	4(20.0)	1(5.0)
Aspergillus flavus	6(30.0)	3(15.0)	2(10.0)	1(5.0)
Lodderomyces elongisporusis	6(30.0)	2(10.0)	0(0)	6(30.0)
Candida tropicalis	0(0)	(0)	0(0)	(0)
Candida famata	1(5.0)	1(5.0)	1(5.0)	1(5.0)
Total	19(95)	10(50)	7(35)	9(45)

Source: Author's Laboratory Analysis

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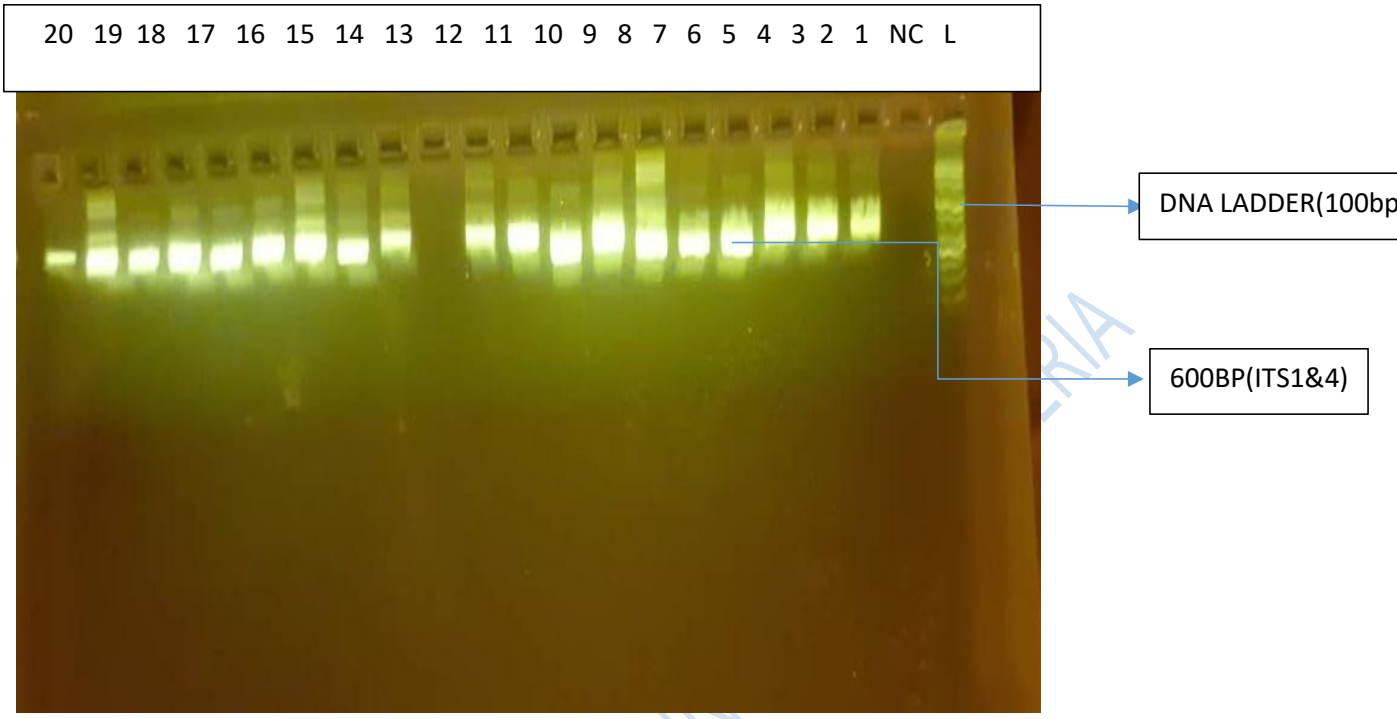

Source: Author's Laboratory Analysis

Figure 4.11: Agarose Gel Electrophoresis of ITS1 and ITS4 gene

Key: Lane1=Ladder (100bp), Lane2= Negative Control, Lane3-20= Fungi isolates

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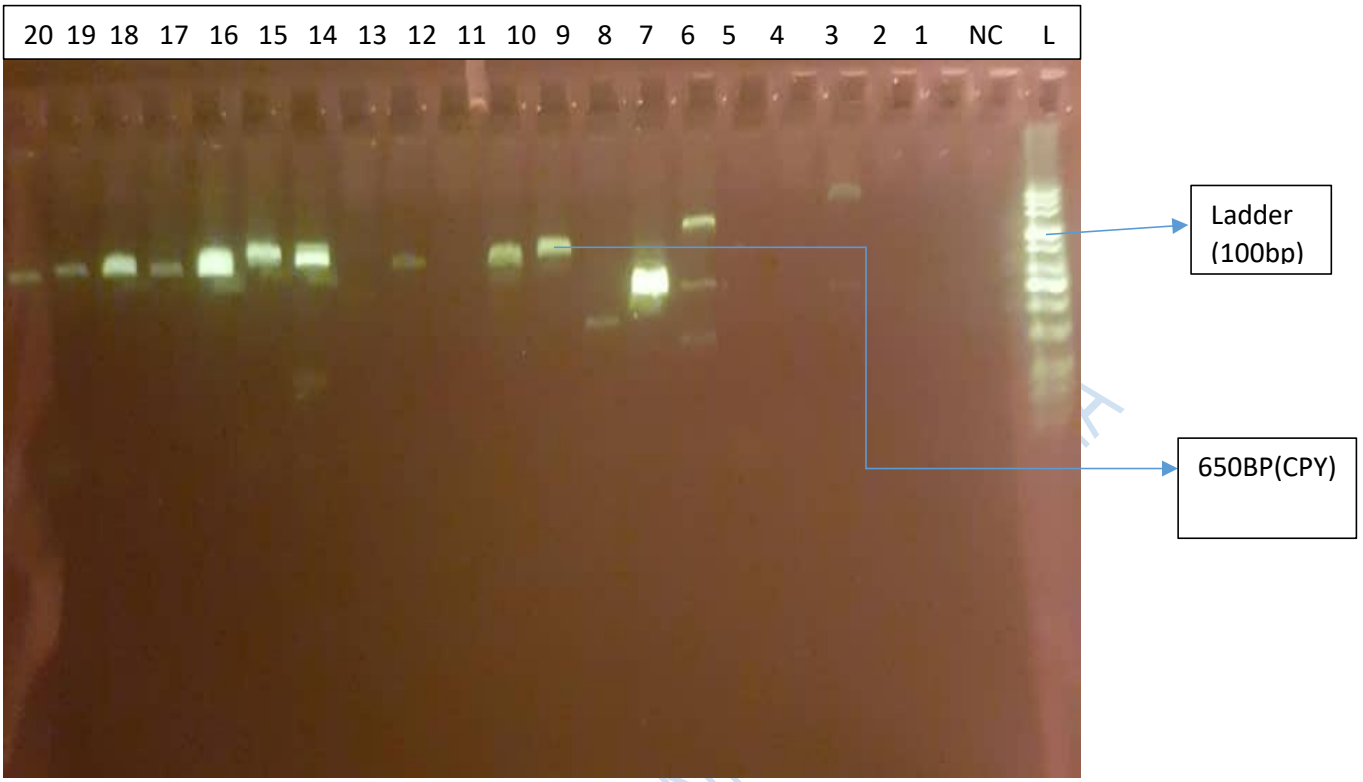

Source: Author's Laboratory Analysis

Figure 4.12: Agarose Gel Electrophoresis of CPY gene.

Key: Lane1=Ladder (100bp), Lane2= Negative Control, Lane3-20 = Fungi isolates

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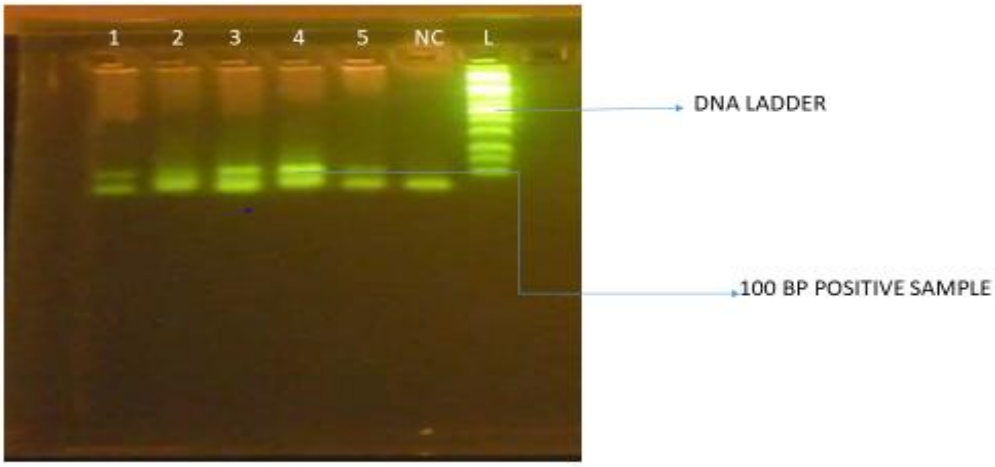

Figure 4.13: Agarose gel of FKS2 gene for fungal isolates to polyene antifungal agent.

Source: Author's Laboratory Analysis

Figure 4.13: Agarose Gel Electrophoresis of FKS 1 and FKS 2.

Key: Lane1=Ladder (100bp), Lane2= Negative Control, Lane3-5 = Fungi isolates

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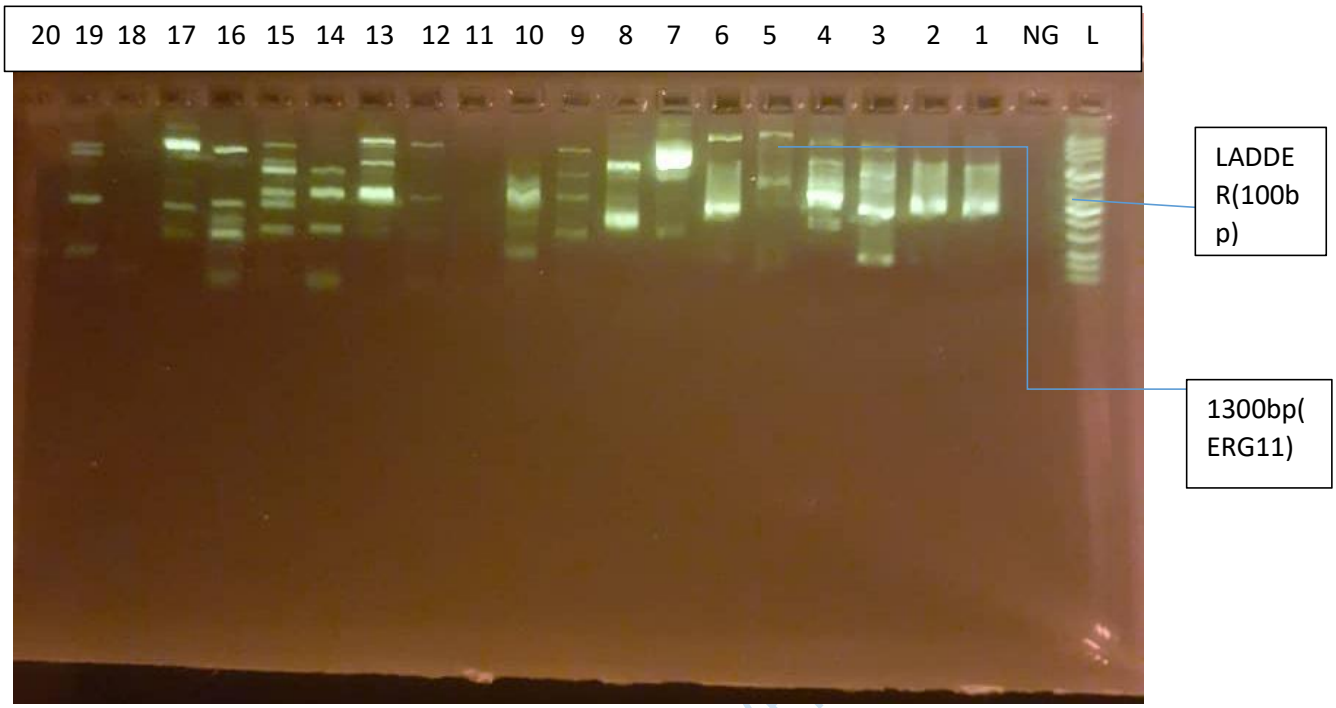

Source: Author's Laboratory Analysis

Figure 4.14: Agarose Gel Electrophoresis of ERG11.

Key: Lane1=Ladder (100bp), Lane2= Negative Control, Lane3-20= Fungi isolates

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4.3 Discussion of Findings

The distribution of samples collection among the 3 isolation centre showed highest frequency of samples from Infectious Disease Unit of University College Hospital (221), followed by Infectious Disease Centre, Olodo (141) and lastly 38 samples from Agbami Infectious Disease Centre, Jericho. The 41.14% median age of SARS-CoV-2 infected individual reported in this study was not in accordance with patients median age of 69.5% reported by a researcher[1]. Majority of the respondent are adults in the age brackets 60 and above which represents the elderly group in the society.

Out of the 400 SARS-CoV-2 infected individuals enrolled in the study, the distribution of the gender showed that 190(47.5%) were male and 210(52.5%) were female supported by a researcher from Indian, Nepal, Vietman and Solvenia[2] but in contrast with reports of some other researchers where the gender status was more of male 62% than female[1]. This outcome result might be because of type of the research study, a cross-sectional design and also a purposeful sampling technique targeting a particular subject at a specified period of time and place. Also, it might be also that female are more concern about their health which made them to report voluntarily and promptly to some of the isolation centre where samples were being collected. Relationship between SARS-CoV-2 infected individual and their educational qualification showed that majority of respondent 315(78.8%) had formal education while 85(21.3%) were not educated and this proved that elite individual in the society are more prone and susceptible to SARS-CoV-2 infection than the illiterate.

The distribution of the marital status of SARS-CoV-2 infected individual showed that majority of the respondent were married 318(79.5%), 55(13%) singled and 27(6.8%) had been divorced but indirectly were married also and this confirmed that most of respondents

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were adults with marriageable age which corroborate the median age of 48.14% recorded from this study. The majority of the SARS-CoV-2 infected individual used for this study were symptomatic 335(83.8%) while 65(16.3%) were not showing any symptoms indicating SARS-CoV-2 infection but were confirmed positive with RT-PCR screening kits. The case status of all the symptomatic positive SARS-CoV-2 infection reported as 60(15.0%) experienced mild infection, 139(34.8%) presented with serious infection while those in critical condition were the highest in frequency 141(35%) and those individual in the severe state were 60(15.0%) and this may be supportive and cogent reasons for increase number of pathogenic isolates recorded from this study.

Additionally, from the on-going COVID-19 pandemic, viral, bacterial and fungal coinfection has been reported among the infected patient which is in concordance with the results of various pathogen recorded in this research study[3]. Also, in this study out of 400 SARS-CoV-2 infected individual samples collected, mixed growth of bacterial and fungal pathogen co-infected with SARS-CoV-2 was 7.3% in aggrement with 8% reported by some researchers[4]. The rates of bacterial coinfection reported in this study appeared to be a little above the percentage reported by some researcher 240(60%) compared with 62(8%) reported[4], which might be due to climatic changes, adaptation measures, environmental condition and immune status in this part of the continent.

Early detection of bacterial infection in 3.2% of all hospitalized patients, 13.5% of those requiring critical care supports the isolation of most pathogen from symptomatic individual in this study population[1]. A reports of bacterial co-infection within 48 hours of admission to ICU in 8% of patients with SARS-CoV-2 is also in agreement with outcome results in this study that most of bacteria isolates were recorded from symptomatic individuals. In the US,

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higher early bacterial co-infection rates (16.6%) were identified by a researcher[5] isolated from respiratory cultures for oral bacteria flora which constituted 15/25 of these cases and is in agreement to this research study that relied predominantly on culture-based techniques[6]. Bacterial co-infection within 48 hours of hospital admission for COVID-19 infection in adults was common and this confirm that bacterial are secondary invader, able to participate in interactions between host cells and viruses by expressing virulence factors that promote viral pathogenesis which helps viral load to increase and clearance rates to decline[7]. The proportion of pathogens detected were noted to increase with duration of ICU stay and symptoms exhibited which consisted largely of Gram negative bacteria, where most of the bacterial pathogens isolated were majorly classes of Enterobacteriacea such as *Klebsiella pneumonia, Enterobacter cloacae, Enterbacter aerogenes, Pseudomonas aerogenes, Pseudomonas luteola, Acinetobacter baumanni, Escherichia coli* and this is in agreement with the isolated pathogen reported by a researcher[8, 9,10].

However, the rate of co-infection is markedly higher than what was observed during the pandemic of influenza, suggesting that, it is more of a significant issue with SARS-CoV-2 infection having coinfection with bacterial and this is relatively agreed with outcome result from this study of 32.4% *Staphylococcus aureus* and predominant pathogens observed among Gram-negative bacteria, particularly *K. pneumonia* 22.7%, *E. cloacae* 17.5% and *E aerogenes* 14.9%, but in contrast to wide range of bacterial pathogen that includes *Heamophilus, Lautrlopia, Prevotella* been detected as coinfection in Brazil, China and USA[10]. Also, isolation of some pathogen like *Klebsiella pneumonia, Pseudomonas aeruginosa, Enterococcus, Acinetobacter baumanii,* which were all in conformity with some of the isolates identified in this research work[8].

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There was emerging evidence report of fungal co-infection among the SARS-CoV-2 infected individual from this study which is in agreement with a report from a reseacher and these cases help to highlight the importance of being vigilant about possibility of opportunistic fungal pathogen among the subject[11]. All the classical risk factors for developing fungal infection like candidemia in a critically ill patients are present in SARS-CoV-2 patients like use of mechanical ventilation, parenteral nutrition, broad spectrum anti-bacterial treatment, indwelling central nervous or bladder catheter intervention, older age, comorbidities, lymphopenia corticosteroids etc. that make a possibility occurrences of co-fungal infections among SARS-CoV-2 infected individuals. Fungal infection occurrences in 29.5% of total coinfected cases with SARS-CoV-2 in China reported by a researcher is not in concordance with 15.8% of fungal infection identified among SARS-CoV-2 infected individual in this study[15]. Invasive aspergillosis caused by Aspergillus species like*: A. funmigatus, A. niger, A. flavus, A. terreus* carries an overall 30-95% mortality rate even if it is early diagnosed and despite antifungal treatment approaches says this statement is a great concern because of evidence based result recorded from this resaerch work[12].

Report of 33.3% of fungal culture among SARS-CoV-2 cases in 2 hospitals in Wuhan, China[17]. Some researchers also reported a prevalence index of 20-35% high rates of chronic pulmonary Aspergillosis among COVID-19 positive patients in some European countries like France, Germany, Belgium and The Netherland[13,14]. An observational study from Pakistan also shows the isolation of 39.1% *Aspergillus* species from COVID-19 positive patients[15]. Some studies from China reported high rates of Aspergillosis among COVID-19 patients[16,17].

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All the aforedmentioned reports were higher than 15.8% fungal prevalent recorded in this study which might be due to awareness and knowledge about possibility of fungal infection among immune-compromised individual. Yeast infection can also occur in patients with SARS-CoV-2 as a result of impaired immune system functions. Data from a hospital in spain pointed out a rising incidence of invasive candidias in SARS-CoV-2 positive patients with an associated mortality of 40%[18]. Invasive candidiasis by *Candida albicans* was similarly reported in COVID-19 patients requiring critical care in United Kingdom hospitals[18]. A published work also reported that Candida specie was one of the most frequently fungi identified in the bloodstream of patients using central venous catheters during COVID-19 pandemic episodes in New York city[19]. Also a report that Candida species and other yeast were isolated from the respiratory tracts in 21.4% of positive cases of coinfection during the first pandemic of COVID-19 in 2 hospitals in the United Kingdom, another study conducted in Iran reported that *Candida albicans* was the most frequents fungus isolated followed by other species from oral lesion COVID-19 positive patients but in contrast with *Lodderomyces elongisporus* with highest frequency among different fungi isolated in this research work and this might be due to newly improved means of fungi identification employed[20].

A researcher also reported multiple organisms of fungal and bacterial coinfection among SARS-CoV-2 positive individuals like *Candida albicans, Acinetobacter baumanii, Klebsiella pneumonia, Aspergillus funmigatus* which are relatively agreed with multiple-microbial isolates recorded in this research study[10]. Isolation of 50% *Enterobacter cloacae*, 40% *Candida albicans* and 20% *Acinetobacter baumanii* among SARS-CoV-2 patients and this confirm that nearly all the SARS-CoV-2 infected individual samples recorded pathogen[9].

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A researcher reported 8% cases of bacterial/fungal coinfection among COVID-19 positive patients which is very low when compared to outcome result of this reseach work[12]. A retrospective study in Italy evaluated the respiratory specimens of hospitalized COVID-19 patients in ICU and the results showed that almost 52% of cultures were positive for bacteria and fungi[11]. While a researcher from China reported no significant growth among COVID-19 positive patient's sputum samples which is in contrast to the outcome results from this study although, it might be differences in the nasopharyngeal sample used instead of sputum sample.

The rapid diagnosis of a broad ranges of potential pathogen and antimicrobial resistance for subsequent monitoring of coinfection which is very crucial and a concern with the rapid expansion of critical cases in managing SARS-CoV-2 that can potentially increase the rate of nosocomial infection within the hospital environment[11]. A researcher reported that those candida isolates were susceptible to all tested antifungal drugs but not in agreement with the outcome of multidrug resistance gene recorded from this research study[21].

Phenotypic detection of 1.2% methicillin resistant *Staphylococcus aureus* (MRSA) among SARS-CoV-2 infected individual was recorded with carriage of both mefA and ermB macrolide resistance genes (that could lead to community acquired pneumonia).

From this study Gram negative bacteria had 49% resistant while Gram positive bacteria has 66.7% phenotypical resistant to Azithromycin drug of choice for empirical management of COVID-19 disease which corroborate highest detection of mefA and ermB macrolides resistance genes among Gram positive bacteria isolates.

Additionally, there was a high frequency of newly emerging detection of 39.7% *Lodderomyces elongisporus* and 17.5% *Aspergillus flavus* from fungal isolates co-infecting

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SARS-CoV-2 infected individuals from this study and these incidence cases highlighted the importance of possible opportunistic invasive or systemic fungal infection among the subject which is in contrast to fungal recorded by some reseacher[18]. Most of the Aspergillus specie that were phenotypically resistant to azole antifungal agents also possessed CPY azole resistant gene. Molecular detection of ERG11 azole resistance gene was high (55.6%) among *Lodderomyces elongisporus* than other *candida* specie. Detection of FKS resistance gene of fungal to polyene was more among *Aspergillus* specie.

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Chapter Five

Conclusion

5.1 Summary of Findings

In the ongoing COVID-19 pandemic, the rising frequency of coinfection of bacterial and fungal with SAR-CoV-2 infected individual as well as increasing reports of resistance to some antimicrobial agents is imperative. This work investigated and detected the phenotypes and genotypes of macrolide, azole and polyenes resistance genes among the pathogenic organisms isolated from nasopharyngeal samples of SARS-CoV-2 infected individual in Oyo-State, Nigeria.

In summary, report from this research study confirms the presence of bacterial and fungal co-infecting agents among individual with COVID-19 infection in our setting. Meanwhile, the occurrence of these coinfections was high when compared to reports from other researchers in other countries setting. Also the predominance of Gram-negative pathogens, presence of methicillin resistance *Staphylococcus aureus*, emergence of Candidiasis and Aspergillosis infection, and incidence of bacterial and fungal isolates harboring macrolide, azole and polyene resistant strain are of great alertment and concern.

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Additionally, this study demonstrated *Lodderomyceses elongisporus* as the most frequently isolated species from COVID-19 patients that are symptomatic. Infections with Candida are almost endogenous; therefore, identification of the species and corresponding susceptibility patterns to antifungal agents can be helpful for the management of these infections optimally. As demonstrated in this study, resistance to azole drugs such as fluconazole and ketoconazole, which are the most frequently antifungal used in the country, could be suggestive of the need for routine fungal culture and in-vitro drug susceptibility pattern of fungus in medical centers in order to manage more efficiently the invasive candidiasis and invasive aspergilliosis among SARS-CoV-2 infected individuals.

5.2: Conclusion

In conclusion this study recorded:

1) SARS-CoV-2 infection among female than male, among educated than non-educated with highest frequency in age 60years and above.
2.) Bacterial and fungal isolates as co-infecting pathogen among SARS-CoV-2 infected individual.
3) Drug resistance genes among bacterial and fungal pathogen isolated from SARS-CoV-2 infected individuals phenotipically.
4) Macrolide resistance genes in bacteria isolates with carriage of mefA and ermB gene but no detection of mphA gene.
5) Azole and polyene resistance genes in fungal isolates with carriage of ERG11, CPY and FKS genes.

5.3 Recommendations

Based on the findings of this research study, it is recommended that:

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i. Routine detection and characterization of bacterial and fungal co-infecting agents among SARS-CoV-2 patients should be integrated.

ii. Streamlining and prescribing of antibiotic stewardship intervention must based on routine determination of Antibiotic Susceptibility Testing.

iii. Determination of bacteria specific-biomarker like Procalcitonin among SARS-CoV-2 infected individual that will increase the serum level due to bacterial infection inorder to prevent prolong antibiotics usage.

iv. Serum assay is needed for all COVID-19 patient undergoing Azithromycin therapy to ascertain the in-vivo sensitivity.

v. Need for routine fungal culture and in-vitro drug susceptibility pattern in medical centers in order to manage more efficiently the invasive Candidiasis and invasive Aspergilliosis among SARS-CoV-2infected individuals.

5.4 Contribution to Knowledge

i. The data obtained updated and affirmed the presence of microbial pathogens in the nasopharyngeal sample of SARS-CoV-2 infected individual.

ii. Confirmation and carriage of ermB and mefA macrolides resistance genes were detected among bacterial isolates.

iii. Isolation of MRSA among bacteria isolates coinfecting SARS-CoV-2 infected individual

iv. Possibility of invasive Candidiasis and Aspergillosis among SARS-CoV-2 infected individual in Ibadan, Oyo-State. Nigeria.

v. Detection of ERG11, CPY, FKS antifungal resistance genes among fungal isolates.

5.5. Suggested Areas for Further Research

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i. Inclusion of some samples like blood, urine, sputum, BAL from SARS-CoV-2 infected individual for comprehensive diagnosis.

ii. Cohort study to determine the concise prevalence and the predictors of coinfection along with its prognostic impact in SARS-CoV-2 infected individual

iii. Above all, more research work in Nigeria and Africa at large is needed to determine how the widespread use of empirical antimicrobial prescription can affect the microbiome and contributes to AMR.

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Appendix

Questionnaires for SARS CoV-2 Screening Test

This questionnaire is designed to obtain information on demographic characteristics and factors affecting the prevalence of Bacteria and Fungi Co-infecting pathogen among individuals infected with SARS-CoV-2 in Ibadan, Oyo-State. Nigeria.

By: **Florence Adenike BAMIGBOLA**. Lead City University, Ibadan. Oyo –State.

Please tick appropriate options. All information supplied will be treated as confidential and used for research purpose only.

Thank you.

1. Name:
2. Age(years): 0-18 19-34 35-59 60 and above
3. Sex: Male ☐ Female ☐
4. Educational Qualification/Background: Educated ☐ Not Educated ☐
5. State of Origin:
6. Marital Status: Single ☐ Married ☐ Divorce ☐
7. Health Status: Asymptomatic ☐ Symptomatic ☐
8. If symptomatic,which one: i. Fever ☐ ii. Bad Headache: ☐ iii. Running Nose: ☐ iv. Itching Nose: ☐ v. Difficulty in Breathing: ☐ vi. Diarrhea: ☐ vii. Coughing: ☐
9. Any Drug Treatment (Thick appropriate):(Malaria drug ☐ , Antibiotics drug ☐ Painkiller drug ☐
10. Is it the first time of doing the test or repeat: Yes ☐ No ☐
11. Have you been isolated recently: Yes ☐ No ☐
12. If yes for above question, Was it- self isolation or government isolation center: Yes ☐ No ☐
13. Case Status: Mild ☐ Serious ☐ Critical ☐ Severe ☐
14. Patient Occupation: Health Care Worker ☐ Artisan ☐ Student ☐ Retiree ☐
15. Main Comorbidities: Obesity ☐ Hypertension ☐ Diabetes mellitus ☐ Cardio-vascular Disease ☐ Atrial Fibrilation ☐ Venous Thromboembolism ☐

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Chronic Renal failure ☐ Chronic Resipioratory Disease ☐ Immuno compromised ☐

16. Sample collected: Nasopharngeal swab ☐ Orophagarngeal swab ☐

Informed Consent (Form to be translated for illiterate parent)

My name is Florence Adenike BAMIGBOLA. I am a student of Lead City University Post Graduate School Ibadan, Oyo-State. I am interviewing SARS-CoV-2 infected individual between 18years and above in Ibadan, Oyo-State. I will need to ask some questions which you may find difficult to answer. Please note that your answer will be kept very confidential, the information you and other people give will be used to know how to monitor and give proper management and administering drugs by clinicians.

During this exercise, nasopharngeal samples will be collected for our private investigations and you will be guided appropriately.

Your honest answers to the questions will help better understand what and will be highly appreciated.

You are free to refuse to take part in the program. You have the chance to withdraw at any given time if you choose to.

Consent

Now that the study has been explained well to me, I…….

voluntarily agree to be enrolled into the study.

Signature/Thumbprint of participant ……

Witness signature/Thumbprint (If required) …….

Interview date……

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Formulas and Preparation of Reagents

Media

Media used in this study includes: MacConkey Agar (Biotec), Blood Agar Base(Biotec) Sabouraud Dextrose Agar (Lab M), Peptone water (Lab M), Nutrient agar (Biotec), Tryptone Soy broth, CHROM agar TM Orientation, CHROM Candida agar.

Reagents, Stains and Solution

Reagents, Stains and Solution used for this study are: API 20E Gram-Negative Identification, VITEK 2.0 System ID& AST Cards, Crystal Violet Solution, Grams Iodine, Acetone, Safranin, and Normal Saline

Antimicrobial Agents

Levofloxacin, Amikacin, Ceftazidime, Ciprofloxacin, Azithromycin, Cefuroxime, Sulbactam, Ceftriaxone, Gentamycin, Clindamycin, Oxacillin, Vancomycin, Cefoxitin, Erythromycin.

Equipments

The following equipments were used in this study: Incubator, Autoclave, Weighing Balance,

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Refrigerator, Hot air oven, Microscope, Bunsen burner, Wire loop, Nose Masks

Preparation of Reagents and Stains

Crystal Violet Stain

Crystal Violet (85% dye content) 20g

Ethanol 95% 95ml

Ammonium oxalate 9g

Water 1 litre

The Crystal violet was weighed and transferred into a brown bottle, the methanol was added and mixed until the dye completely dissolved. Ammonium oxalate was weighed, dissolved in 200L distilled water and added to the stain. It was made to 1 litre with distilled water and well mixed, labelled and store at room temperature for use.

Lugol's Iodine solution:

G zIodine 10g

Potassium iodide 20g

The weighed potassium iodide was dissolved in 25ml of distilled water and mixed until it dissolved completely. It was then made up to 100ml with distilled water, kept in dark place at room temperature until use.

Safranin:

Safranin 5g

Water 100ml

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The safranin was weighed into a brown bottle premarked to hold 1 litre, dissolved in distilled water, well mixed, labeled and stored at room temperature for use.

Absolute Acetone 1 litre

This was measured into a brown bottle premarked to hold 1 litre, and stored at room temperature for use.

Normal saline

Sodium Chloride 0.85g

Distilled water 100ml

Mixed to dissolve in a clean screw capped bottle.

Mueller Hinton agar (Biotec)

Beef, dehydrated infusion 300g

Casein hydrolstate 17.50g

Starch 1.50

Agar Bacteriological 17.00g

Distilled water 1000.00ml

19gramms of the powder was weighed into a media bottle containing 500milliter of distilled water, autoclaved at 121Oc for 15minutes, allowed to cool to a temperature of about 45Oc, then poured into sterile petridishes with a volume of about 25 – 30Ml each and allowed to set.

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MacConkey agar without salt (Biotec)

Mixed Peptone 20.0

Lactose 10.0

Bile 5.0

Neutral red 0.05

Agar

Disperse 48.5g in 1l of deonised water soak for 10 minutes, swirl to mix and sterilize by autoclaving for 15 minutes at 121˚C. Cool to 47˚C and mix before pouring into petri dishes and then dry the agar surface.

Chrom agar Tm Orientation

Agar 15.00g

Peptone and Yeast Extract 17.00g

Chromogenic Mixture 1.0

pH 7.0≠0.2

Tryptone Soy broth

Tryptone Casein digests 17.0

Soy peptone 3.0

Sodium Chloride 5.0

Dipotassium hydrogen phosphate 2.5

Dextrose 2.5

Direction: Weigh 30grams of powder and dispense in 1 litre of deionised water. Allow

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soaking for 10 minutes, Swirl to mi and warm to dissolve, if necessary. Dispense into tubes or flasks, then sterilize by autoclaving at 121˚C for 15 minutes. Do not exceed stated temperature.

Appearance: Straw coloured, clear

Peptone Water

Peptone 5.0g

Trytone 5.0g

Sodium chloride 5.0g

pH 7.2

Weigh 15grams of powder; add to 1 Litre of deionised water. Allow to dissolve then dispense into final containers Sterilise by autoclaving at 121 o C for 15 minutes.

AGAROSE GEL (0.8%) Preparation

0.8g of agarose powder was weighed and mixed with 100mls of electrophoresis buffer, then heated in a microwave oven until completely melted; Ethidium bromide is added to the gel (final concentration 0,5g/ml) at this point to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60Oc, it is then poured into a casting tray containing a sample comb and allowed to solidify at room temperature.

Equipment Supplies and Reagents for API (20 E)

1. 1 Holden Tray

2. Technical Product Insert.

3. Organism ID Report forms including colour Interpretation chart.

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4. API Kit 20E

5. Sterile Normal Saline.

6. Kovac's Reagent for Indole Tests

7. API Reagent VP1

8. API Reagent VP11

9. API Reagent TDA

10. API Reagent Nitrate A

11. API Reagent Nitrate B

12. API Mineral Oil

13. API Oxidase Strips

Genomic DNA Isolation (USING NORGENS BIOTEK CORP KIT)

Reagents Supplied:

Lysis solution

Wash solution I

Wash solution II

Elution Buffer

Proteinase K

Spin columns

Collection Tubes

Elution Tubes (1.7ml)

Product Inser

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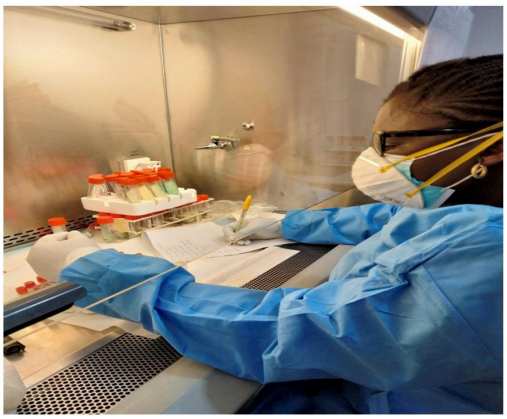
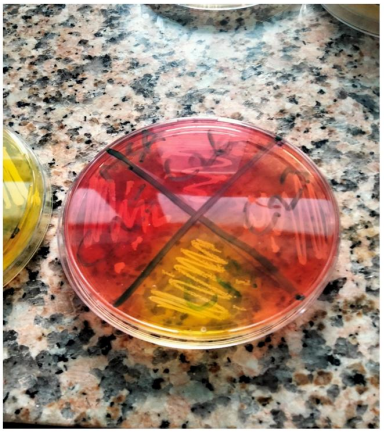
Collection and processing of samples

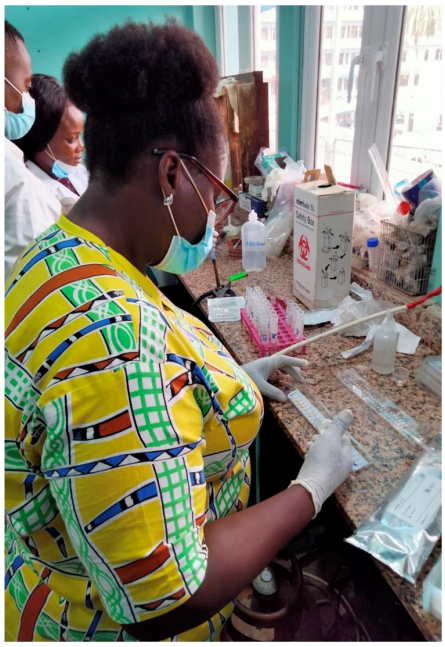
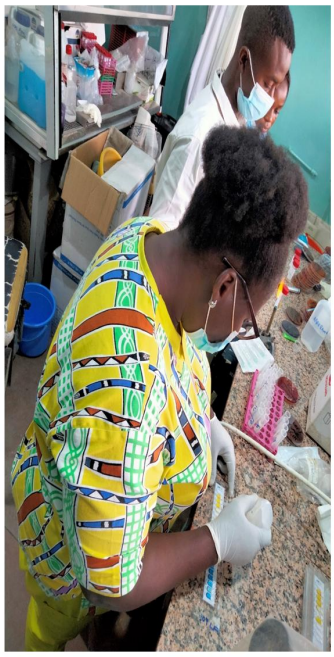


Identification of organisms on MSA, CHROM Orientation and CHROM candida



Bacteria culture and susceptibility testing




Aspergillus flavus, Aspergillus niger and Candida specie susceptibility testing



Molecular Analysis



Bio-data

Personal Data

Full Name:	Florence Adenike BAMIGBOLA
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Date and Place of Birth:	25th April, 1974. Iseyin
Nationality:	Nigeria
State of Origin:	Oyo-state
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	No 12, Adams Street, Akingbile, Moniya, Ibadan, Oyo-state.
Phone No and Email of Next of Kin:	+2348054362305, bamadek2004@yahoo.com ,
Educational Background:	MSc, BSc, FMLSCN, AMLSCN, NIST, LTTS.

Educational Qualifications Obtained with Dates

1.Master of Science in Medical Microbiology.	OOU. Ago-Iwoye	2016
2.Bachelor of Science in Microbiology	Ododuwa University Ipetumodu	2017
3.Fellow of Medical Laboratory Science	Obafemi Awolowo Teaching Hospital	2010
4. Associate of Medical Laboratory Science.	University College Hospital, Ibadan.	2003
5. Final Diploma in Science Laboratory Technology	University of Ibadan, Ibadan.	2000
6. Nigeria Institute of Science and Technology,	University of Ibadan, Ibadan.	2000
7. West Africa Examination certificate (WAEC)		Nov./Dec.2010
8. West Africa Examination certificate (WAEC)		May./Jun. 1993
9. Primary School Leaving Certificate	St Mary's Primary School Iseyin.	1986

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Membership of Research Bodies with Dates

1. Nigeria Fungi Diseases Surveillance Capacity Building (NIFUSCAB), Lagos State University Teaching Hospital, Ikeja. Lagos 2022 Till date
2. University College Hospital COVID 19 Volunteer Swabbing Team 2020 Till date
3. Oyo State Covid-19 Volunteer Team 2020 Till date
4. Severe Typhoid in Africa January, 2017 Till date
5. SHUGA Research Team May, 2015 – Jan 2016
6. University College Hospital Response to Outbreak of Diseases and Emergencies Team (UCHROADs). 2011 Till dates

Working Experience with Date:

1. University College Hospital (UCH), Ibadan. Medical Laboratory Scientist 2010-Till date
2. Antimicrobial Resistant Unit, Department of Medical Microbiology and Parasitology University College Hospital. (2022)
3. Medical Mycology Unit**,** Department of Medical Microbiology and Parasitology University College Hospital. (2021)
4. Main Culture Laboratory**,** Department of Medical Microbiology and Parasitology, University College Hospital. (2020)
5. Media Preparation Unit**,** Department of Medical Microbiology and Parasitology University College Hospital. (2019)
6. Blood Culture and Cerebrospinal Fluid Culture Unit, Department of Medical Microbiology and Parasitology University College Hospital. Ibadan. (2018)
7. Urine Culture Unit**,** Department of Medical Microbiology and Parasitology University College Hospital. Ibadan. (2017)
8. General Out Patient Laboratory, Department of Medical Microbiology and Parasitology University College Hospital. Ibadan. (2016)
9. Accident and Emergency Unit, University College Hospital, Ibadan. January, 2010 – December. (2010-2015).
10. Obafemi Awolowo University Teaching Hospital **(Clinical student)** 2009-2010

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11. Life-Long Biomedical Diagnostic Centre, Opp. Police Barrack Playing Ground, Yemetu, Ibadan. Oyo-State. Nigeria. (**Medical Laboratory Scientist**) 2004-2009
12. MOVIV Specialist, Hospital, Ologuneru, Ibadan. Oyo-State. Laboratory Unit, **(Medical Laboratory Scientist)**. 2004
13. First Bank of Nigeria PLC. Bank Road, Dugbe, Ibadan. Oyo-State. (**National Youth Service Corps)** 2001
14. St Mary and Joseph Nursery and Primary School **(Class Teacher**) 1991 -1992

Registration with Professional Body:

1.) Registered member of Medical Laboratory Sciences Council of Nigeria- **MLS/31/03287**
2.) Member of Society for Scientist in Infection Diseases. 2022 till date. SSID/251
3.) Health and Care Professional Council, UK 2022 till date. RN-BS077004
4.) Member of African Society for Laboratory Medicine. 2021 till date.
5.) Member of American Society of Microbiology Association. 2014 till date. 52585641

Present Place of Work:

1. **Medical Laboratory Scientist** in charge of NIFUSCAB Project titled "Diagnosis and Management of invasive Fungal Diseases. **BASIC MYCOLOGY**. 2022 till date.
2. **Member** of National Quality Control for Antimicrobial Resistant Sentinel Survey University College Hospital, Ibadan, Oyo-State. Nigeria 2020 – till date.
3. **Member** of Quality Management System of Department of Medical Microbiology and Parasitology, University College Hospital, Ibadan, Oyo-State. Nigeria. 2020 – till date.
4. **Acting Team Lead to** COVID-19 Swabbing Team, Disease Surveillance Unit. University College Hospital, Ibadan, Oyo-State. Nigeria 2020 till date.
5. Officer in Charge of Medical Mycology and Parasitology Laboratory, Department of Medical Microbiology and Parasitology University College Hospital Ibadan Oyo-State. Nigeria.
6. Assisting Officer in Charge of Monthly Call-Duty Roaster of Medical Laboratory Scientists, Department of Medical Microbiology and Parasitology, University College Hospital. Ibadan. Oyo-State. Nigeria

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7. Handling of MALDITOF MS VITEK for Identification of Microbiological Pathogens and Research work.
8. Handling of VITEK MS COMPACT for IDENTIFICATION, ANTIBIOTIC SUSCEPTIBILITY TESTING and ANTIFUNGAL SUSCEPTIBILITY TESTING of Microbiological Pathogens and Research isolates.
9. Handling of MICROBACT and API IDENTIFICATION KITS on Microbiological and Research isolates
10. Preparation of special Culture Media, Reagents and Chemical for Isolation of Microbiological Pathogens and Research work**.**
11. Processing of Human Sample Collected from both outpatients and inpatients Clients

Research Completed and On-Going with Dates

1. Characterization and Determination of Drug Resistance Genes in Bacterial and Fungal Pathogen Coinfecting individuals with SARS-CoV-2 in Oyo-State, Nigeria. (PhD 2022)

2. Prevalence and Characterization of Enterohaemorrhagic *Escherichia coli* O157/H7 among Diarrheic children aged 0-5 years in Ibadan, Oyo State, Nigeria. (October, 2016)

3. Distribution of Bacteria Pathogen among Diarrheic children aged 0-5 years in Ibadan, Oyo State, Nigeria. (November, 2016)

4. Investigation of the Occurrence of Syphillis among Blood Donors at University College Hospital. Ibadan. (October, 2010).

5. Prevalence of *Trichomonas vaginalis* among Pregnant Women in Oluyoro Catholic Hospital, Ibadan. Oyo-State. Nigeria. (August, 2003)

6. Nutritional Analysis of Fresh and Fermenting Maize Grain (*Zea Mays*) (April, 2000)

Award Obtained with Dates

Best Graduating Student Award for 2000 Academic Session at Laboratory Technology Training School (Microbiology option) University of Ibadan, Ibadan. Oyo State. Nigeria.

Abstracts and Published Papers to Date:

Deji-Agboola, Anotu Mopelola ; **Bamigbola, Florence Adenike** & Osinupebi, Olubunmi Adetokunbo(2020) Prevalence and Characterization of Enterohaemorrhagic Escherichia Coli O157:H7, Shiga Toxin Genes and Antibiotic Resistance in Children aged 0-5 years with Diarrhoea in Ibadan, Oyo State, African Journal of Science & Nature. Vol 10. June 2020, 171-178.

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Update Training with Dates

1. Initiating Effective Capacity Building for the Diagnosis and Management of Invasive Fungal Diseases in Nigeria. Lagos State Teaching Hospital, **(June 6-10, 2022).**
2. Quality Management System in Pandemics: Medical Laboratory Science Perspective. Medical Laboratory Science Educating Unit, UCH. Ibadan. (**May 16-18, 2022**).
3. Fleming Fellowship Step-down Training in Bacterial Isolation, Identification and Antimicrobial Susceptibility. University College Hospital, Ibadan**. (October 27-29, 2021**).
4. Strengthening Inter-professional Education for HIV STRIPE HIV-Ibadan (Online**). July. 2021**
5. National AMR Surveillance National Reference Laboratory Refresher Training and Mentorship on Quality Management Systems. Supported by the Fleming Fund Country Grant. Microbiology Laboratory, UCH, Ibadan. **May 17-25, 2021**.
6. Infection Prevention Practices: Response to Emergency and Outbreak of Diseases. University College Hospital, Ibadan. **(March 10 2020)**
7. Global Health Project Management. University of Washington, (Online). **September, 2019**
8. Introduction to Epidemiology for Global Health University of Washington, (Online). **April, 2019**
9. Leadership and Management in Health Professional Development**.** University of Washington, (Online). **June, 2018**
10. Severe Typhoid in Africa (SETA) Project. Medical Microbiology Department, UCH. Ibadan. **January 16-25, 2017**
11. Impact Evaluation Study of the SHUGA TV Drama in Nigeria. The World Bank in Collaboration with University College Hospital Ibadan, Oyo-State. Nigeria**. May 2015**

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Signature Date

University Compliance Certification

This is to certify that, this Thesis written by Florence, Adenike BAMIGBOLA with Matric No LCU/PG/001275 in the Department of Biological Science, Faculty of Natural and Applied Sciences, Lead City University, Ibadan is in full compliance with the approved University format and style.

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Signature Date

2.)

THESIS TITLE: Characterization and Determination of Resistance Genes in Bacteria and Fungi Pathogen Co-Infecting Individual with SARS-CoV-2 in Oyo-State, Nigeria.

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Abstract

Epidemiological data of SARS-C0V-2 indicated that bacterial and fungal complications increased mortality rate and decreased clearance rate of the virus therefore antimicrobial drugs were administered to patients with this disease without susceptibility testing. This study sought to identify co-infecting pathogen(s), determine their antimicrobial resistant pattern, detect macrolide, azole and polyene resistant genes in the identified pathogen(s) from confirmed SARS-CoV-2 individual in Oyo State, Nigeria. Nasopharyngeal samples were collected from 400 symptomatic and asymptomatic infected adults; structured questionnaires were administered to determine predisposing factors to SARS-CoV-2 infection. Isolates were obtained by standard microbiological methods, identified using API 20E, VITEK 2.0 ID cards and MALDITOF MS VITEK. Kirby Bauer disc diffusion methods and VITEK 2.0 AST card kits were used to determine antimicrobial susceptibility testing. Resistant genes in the isolates were amplified using Polymerase Chain Reaction with specific primers, genes, detection was done by Agarose Gel Electrophoresis. Bacterial and fungal coinfection among SAR-CoV-2 infected individuals were detected (7.3%). Many of the identified bacteria were in family of Enterobacteriaceae, *Loddermyces elongisporous* (39.0%) was the most fungi isolated followed by *Aspergillus flavus* 17.5%). The Gram positive bacteria isolated were more resistant (66.6%) to azithromycin) used for palliative treatment of COVID-19 cases. However, bacterial isolates show significant higher susceptibility (89.0%) to quinolones. Only mefA (36.8%) and ermB (26.3%) genes were detected in the bacterial isolates and were more in Gram positive bacteria and no mphA gene

was detected. Among fungal isolates, ERG 11 gene was detected among the *Loddermyces elongisporous*, FKS gene was detected in *Aspergillus niger* while CPY gene was present in *Aspergillus niger* and *Aspergillus flavus*. Some of the microbial isolates detected in SARS-CoV-2 infected individuals were multidrug resistant with resistant to Azithromycin inclusive. This finding is of great health concern and should be further looked into.

Keywords: SARS-CoV-2, Bacterial and Fungal co-infection, MALDITOF MS VITEK and Resistant genes.

Word Count: 294

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