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Frequency and Antimicrobial Susceptibility Pattern of isolated Enterococci Species from Different Clinical Specimens at Almak Nemer Hospital, River Nile State, Sudan

التردد ونمط الحساسية لمضادات الميكروبات لأنواع المكورات المعوية المعزولة من العينات السريرية المختلفة بمستشفى المك نمر، ولاية نهر النيل السودان

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الآيـــة

بسم الله الرحمن الرحيم

*قـال تعالى :- وَأَوْحَىٰ رَبُّكَ إِلَى النَّحْلِ أَنِ اتَّخِذِي مِنَ الْجِبَالِ بُيُوتًا وَمِنَ الشَّجَرِ وَمِمَّا يَعْرِشُونَ (68) ثُمَّ كُلِي مِن كُل ِّالثَّمَرَاتِ فَاسْلُكِي سُبُلَ رَبِّكِ ذُلُلًاۚ يَخْرُجُ مِن بُطُونِهَا شَرَابٌ مُّخْتَلِفٌ أَلْوَانُهُ فِيهِ شِفَاءٌ لِّلنَّاسِۗ إِنَّ فِي ذَٰلِكَ لَآيَةً لِّقَوْمٍ يَتَفَكَّرُونَ (69)

صدق الله العظيم

النحل الآية (68-69)

DEDICATION

This Project is dedicated to
To our teacher prophet Mohammed
Who is sent for all population as a mercy
To My mother
Who have given us endless love
To My father
Who have given us encouragement and strength
To My sisters and my brothers
Who have given us advices
To My colleagues and To My teachers
Who have supported us
And to every one
Has participated in this project

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Firstly we are deeply grateful for Allah favors, who has been the source of energy throughout this study; without Allah none of this would be possible. I would like to express my sincere gratitude and appreciation to my supervisor **Dr. Hind Haidar Ahmed,** for her excellent guidance. My thanks also to all staff of Almak nemer Hospital**.** Last but not leas I would like to thank all my friends, and my colleagues for their unlimited support and strong advice throughout this study.

ABSTRACT

Enterococci are opportunistic bacteria that become pathogenic when they colonize niches where they are not normally found. This is a descriptive cross sectional study carried out at Almak Nemer Hospital Shendi city, Sudan, during the period from April 2020 to May 2022.

The aim of this work to study the frequency of Enterococcus species among bacterial infected patients, by using conventional cultural methods and to study the antimicrobial susceptibility pattern.

A total of 362 patients with symptoms of urinary tract infection, wound infection and bacteremia were enrolled in this study.

The age of infected patients ranging from 16 to 70 years with mean age of (42.03 ± 22.6) years. Regarding the gender of patients 140 (38.7%) were males and 222 (66.7%) were females.

The frequency of Enterococcus isolates was 15 (4.1%), out of these 15 Enterococcus isolates, 14 (93.3%) were *E.faecalis* and only one (6.7%) was *E.faecium.* Considering the type of infections, 10/15 (66.7%) of Enterococcus isolates were from UTI patients, 5/15 (33.3%) were from wound infection patients and no Enterococci isolate was detected from bacteremic patients. Eight out these 15 Enterococcus isolates were from males and seven were from females, while 12/15 (80%) of Enterococcus isolates were from inpatients and 3 (20%) were from outpatients.

Enterococcus isolates show high resistance rate to Chloramphenicol (13) (86.7%) Gentamycin 12 (80%), Ciprofloxacin 10 (66.7%) and no Vancomycin Resistant Enterococci was isolated. while 10/15 (66.7%) of Enterococcus isolates were multi drug resistant. The only one *Enterococcus faecium* isolate was resistant to Ampicillin 1 (6.7%).

This study conclude that the frequency of isolated Enterococci among different clinical specimens is moderate and the most predominant species was *Enterococcus*

faecalis. This study showed an association between Enterococcus infections and type of infections, hospital admission and age.

Enterococcus isolates in this study show high rate of multi drug resistant and they showed high resistance rate to Chloramphenicol, Ciprofloxacin, and Gentamycin, while no Vancomycin Resistant Enterococci (VRE) was isolated.

المستخلص

المكورات المعوية هي بكتيريا انتهازية تصبح ممرضة عندما تستعمر مناطق لا توجد فيها عادة. هذه دراسة مقطعية وصفية أجريت في مستشفى الملك نمر بمدينة شندي, ولاية نهر النيل, السودان في الفترة من أبريل 2020إلى مايو 2022.

الهدف من هذا العمل دراسة تردد أنواع المكورات المعوية بين المرضى المصابين بالبكتيريا باستخدام طرق الاستزراع التقليدية ودراسة نمط الحساسية لمضادات الميكروبات.

تم تسجيل ما مجموعه 362 مريضا يعانون من أعراض التهاب المسالك البولية وعدوى الجروح والتسمم البكتيري في هذه الدراسة, تتراوح أعمار المرضى المصابين من 16 إلى 70 سنة بمتوسط عمر (42.03 ± 22.6) سنة. فيما يتعلق بجنس المرضى ، كان 140 (38.7٪) من الذكور و 222 (66.7٪) من الإناث.

بلغ معدل تكرار عزلات المكورات المعوية 15 عزلة (4.1٪)، 14 (93.3٪) عزله كانت بكتريا *المكورات المعوية البرازية*, وواحدة فقط (6.7٪) كانت بكتريا *المكورات المعوية الغائطية*.

وبالنظر إلى نوع عدوى المكورات المعدية، فإن 15/10 (66.7٪) عزله من عزلات المكورات المعوية كانت من مرضى المسالك البولية ، و 15/5 (33.3٪) عزله كانت من مرضى عدوى الجروح ولم يتم الكشف عن عزلات معوية من مرضى التسمم البكتيري.

ثمانية عزلات من 15 عزلة من المكورات المعوية كانت من الذكور و سبعة عزلات من الإناث. في حين أن 15/12 (80٪) من عزلات المكورات المعوية كانت من المرضى المنومين و 3 (20٪) كانت من مرضى العيادات الخارجية.

أظهرت عزلات المكورات المعوية نسبة مقاومة عالية للكلورامفينيكول (13) (86.7٪) والجنتاميسين 12 (80٪) والسيبروفلوكساسين 10 (66.7٪) في حين أن 15/10 (66.7٪) من عزلات المكورات المعوية اظهرت المقاومة المتعددة للأدوية, وكانت عزلة *المكورات المعوية الغائطيه* الوحيدة المقاومة للأمبيسيلين (1) (6.7٪), هذا ولم يتم عزل اي من المكورات المعوية المقاومة للفانكومايسين.

خلصت هذه الدراسة إلى أن تواتر المكورات المعوية المعزولة بين العينات السريرية المختلفة متوسط. وكانت الأنواع الأكثر انتشارًا هي المكورات المعوية البرازية ، وأظهرت هذه الدراسة ارتباطًا بين عدوى المكورات المعوية والتهابات المسالك البولية والمرضى الذين يتلقون العلاج في المستشفى والعمر.

هذا وقد أظهرت عزلات المكورات المعوية في هذه الدراسة ارتفاع معدل المقاومة المتعددة للأدوية وأظهرت نسبة مقاومة عالية لكلورامفينكول وسيبروفلوكساسين وجنتاميسين ، بينما لم يتم عزل المكورات المعوية المقاومة لفانكومايسين.

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LIST OF ABBREVIATIONS

AS	Aggregation substance
BSI	Enterococcal Bloodstream Infections
C.L.E.D	Cystine Lysine Electrolyte Deficient
CAUTI	Catheter Associated Urinary Ttract Infections
CDC	Centers for Disease Control and Prevention
D.W	Distilled Water
Esp	Enterococcus Surface Protein
IE	Infective Endocarditis
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
N.S	Normal Saline
NCTC	National Collection of Typing Culture
PCR	Polymerase Chain Reaction
PYR	Pyrrolidonylarylamid
UTI	Urinary Tract Infection
VRE	Vancomycin Resistant Enterococcus

CHAPTER I

INTRODUCTION

CHAPTER I
INTRODUCTION

1.1 Introduction

The Enterococci have the group D group-specific substance and were previously classified as group D streptococci (Brooks *et al*., 2012).

Because the group D cell wall‑specific antigen is a teichoic acid, it is not an antigenically good marker; Enterococci are usually identified by characteristics other than immunologic reaction with group-specific antisera. They are part of the normal enteric microbiota. They are usually non hemolytic but are occasionally α-hemolytic. Enterococci are PYR positive (Brooks *et al*., 2012).

Enterococcal infections comprise a wide range of diseases with increasing importance due to the growing frequency of health-care-associated infections and increasing incidence of antimicrobial resistance. Vancomycin-resistant Enterococcus is an emerging drug-resistant organism responsible for increasing numbers of nosocomial infections in both adults and children (Armin *et al*., 2019).

Enterococcus faecalis and *Enterococcus faecium* are both human intestinal colonizers frequently used in medical bacteriology teaching laboratories in order to train students in bacterial identification (Armin *et al*., 2019). In addition, hospitals within the United States and around the world commonly isolate these bacteria because they are a cause of bacteremia, urinary tract infections, endocarditis, wound infections, and nosocomial infections. Given that Enterococci are becoming more of a world health hazard (Armin *et al*., 2019).

1.2. Rationale

Enterococci are opportunistic bacteria that become pathogenic when they colonize niches where they are not normally found. Of recent, they have become major cause of nosocomial infections, especially of the bloodstream, urinary tract and surgical sites (Olawale *et al*., 2011).

Treatment of Enterococcal infections could be difficult due to increasing resistance of Enterococci to antimicrobial agents such as b lactams, high-level resistance to aminoglycosides and more recently to glycopeptides (Miller *et al*., 2016).

There is a few data regarding the prevalence and resistance pattern of Enterococcus species in Sudan. Their increasing importance is Longley due to the resistance of antimicrobial

This study aim to collect information about the prevalence and antimicrobial resistance of Enterococcus species in River, Nile State, Sudan.

1.3 Objectives

1.3.1 General objectives:

To study the frequency and antimicrobial susceptibility pattern of isolated Enterococcus species from different clinical specimens in River Nile State-Sudan

1.3.2 Specific objectives:

1-To isolate and identify Enterococci spp from different clinical specimens by conventional culture methods.

2-To determine the frequency of Enterococcus spp.

3-To determine the antimicrobial susceptibility pattern of isolated Enterococci species.

4-To correlate between Enterococci infections and possible risk factors (age, gender, and duration of hospital admission).

CHAPTER II

LITERATURE REVIEW

CHAPTER II

LITERATURE REVIEW

2 Literature review

2.1 Background

The Enterococci are Gram-positive, spherical, oval or coccobacillary and are arranged in pairs and short chains. Most of the species are non-motile and non-capsulated (Parija.2014).

2.2 Classification of Enterococci

In broth containing 6.5% sodium chloride and survived heating to 60°C for 30 minutes. Based on acid formation from mannitol, sorbitol, and sorbose broth and hydrolysis of arginine, genus Enterococcus is classified into five groups. Group I *Enterococcus avium* group, II *Enterococcus faecalis, Enterococcus faecium, Enterococcus gallinarum* , group III *Enterococcus durans*, group IV *Enterococcus sulfurous and Enterococcus cecorum*, group V variants of *Enterococcus faecalis, Enterococcus gallinarum* groups species (Parija, 2014).

The genus Enterococcus has16 species. *Enterococcus faecalis* and *Enterococcus faecium* are two important species known to cause human infections (Parija, 2014).

2.3Hapitat

The Enterococci are commonly found in gastrointestinal and genital tract of humans and animals. Enterococci are non-pathogenic but now are emerging as important agents of nosocomial infection (Brooks *et al*., 2012).

2.4 Physiological properties

Enterococcus species grow well in 6.5% NaCl, grow well between 10C° and 45C°, streptococci generally grow at a much narrower temperature range (Brooks *et al*., 2012).

2.5 Cultural characteristics

Enterococcus species grow at a temperature range of 35–37°C. Colonies on blood agar media are 1–2 mm in diameter and alpha-hemolytic (actually non hemolytic; appearance of alpha-hemolysis is due to the production of the enzyme peroxidase rather than hemolysins).Some cultures are beta-hemolytic on agar containing rabbit, horse, or human blood but not on agar containing sheep blood *Enterococcus durans*is beta-hemolytic on agar containing sheep blood as well. On MacConkey agar they produce tiny and magenta- colored colonies. On potassium tellurite agar they produce black colonies. Bile-esculin-azidemedium and Columbia colistin-nalidixic acid agar are used as selective media (Parija, 2014).

2.6 Biochemical characteristics

Enterococcus species are catalase negative and are resistant to optochin and bile. They ferment sucrose, sorbitol, mannitol, and esculin. They are PYRase test positive, CAMP test negative, resistant to bacitracin, and they do not hydrolyze hippurate (Parija, 2014).

2.7 Differentiation between *E.faecalis* and *E.faecium*

Penicillin can be rapidly employed to distinguish strains and clinical isolates of *E.faecalis* from *E.faecium*, *E.faecalis* is susceptible to Ampicillin, and that Vancomycin resistance in Enterococci shows no sign of abating. Additionally, we show that *E.faecalis* can grow on Mannitol salt agar and ferment Mannitol while *E.faecium* lacks these phenotypes. These data reveal that we now have rapid, cost effective ways to identify Enterococci to the species, and not just genus, level and have significance for patient treatment in hospitals (Quiloan and Carvalho., 2012).

2.8 Antigenic structure

Classically, *E.faecalis* has been linked to increased virulence, where as *E.faecium* commonly exhibits multi resistance characteristics (Sharifi *et al.* 2012).

Recent studies have associated Enterococcal virulence with different factors, such as gelatinase production, *Enterococcus* surface protein (Esp), aggregation substance (AS) and biofilm formation (Chuang *et al*. 2008).

2.9 Pathogenesis

Gelatinase is a zinc metalloprotease, encoded by *gelE*, with hydrolytic capacity (Lindenstrau *et al*., 2011), aggregation substance, encoded by a plasmid gene, mediates binding to the host epithelium and it appears to mediate bacterial aggregation during conjugation, facilitating plasmid exchange (Schlievert *et al*. 2010). The Esp protein, encoded by the *Esp* gene, seems to contribute to the colonization and persistence of *E.faecalis* strains in ascending infections of the urinary tract. In addition, Esp may mediate the interaction with primary surfaces and participate in biofilm formation (Chuang-Smith *et al*., 2010), which substantially enhances bacterial survival in biopolymers and may also be involved in antimicrobial resistance (Ballering *et al*., 2009).

For Enterococci to cause disease several barriers must first be overcome. An initial barrier is the ability to overcome colonization resistance provided by competing microbes, and host defenses such as gastric acid and bile, and colonize the intestinal tract. From this reservoir the bacteria can amplify in number and spread to sites vulnerable to infection. A basic prediction from such a model is that the probability of infection should be a function of the intestinal burden of bacteria in the gut reservoir – the more bacteria; the greater the probability of contamination of a potential infection site in numbers large enough to overcome host defenses. Indeed, colonization of the gastrointestinal tract has been shown to be directly associated with risk of infection (Taur *et al*., 2012).

Infection occurs when Enterococci over host defenses, replicate at rates that exceed clearance, and when pathologic changes result through direct toxin activity, or indirectly by damage from the inflammatory response (Garsin *et al*., 2014).

2.9.1 Enterococcal bloodstream infections (BSI)

Enterococcal bloodstream infections (BSI) are associated with a high level of mortality. In a recent large survey study of Canadian hospitals (Billington *et al.*, 2014).

The incidence of Enterococcal BSI of hospitalized patients was 6.9 per 100,000, with most cases due to *E.faecalis* (4.5 per 100,000). Incidence of Enteroccocal BSI increased between 2008–2014 (9–14 per 100,000) in Switzerland (Buetti *et al.*, 2017), suggesting that Enterococcal BSI are on the rise. Overall mortality from nosocomial Enterococcal BSI is quite high, ranging from 25–50% (Pinholt *et al.*, 2014).Most cases of Enterococcal BSI are thought to result from translocation of Enterococci from the gut into the bloodstream. Other routes of infection include along intravenous lines, Endocarditis, urinary tract infections and other abscesses (Erich and Murray ., 2012).

Of the infections caused by Enterococci, infective endocarditis (IE) is one of the most therapeutically challenging (Munita *et al.*, 2012).

2.9.2 Enterococcal urinary tract infections

In a nosocomial population surveyed between 2011 and 2014, Enterococci were the most commonly isolated Gram-positive bacteria from catheter associated urinary tract infections (CAUTI), with over 20,000 cases reported to the CDC National Healthcare Safety Network between 2011 and 2014. Of these cases, just over 50% were caused by *E.faecalis*, followed by "other *Enterococcus* species" (~30%) and *E.faecium* (~20%). Most worrisome was the fact that ~85% of *E.faecium* isolates were vancomycin-resistant, with the fraction of resistant isolates increasing each year (Weiner *et al.*, 2016).

More recently, several studies have shown that Ebppili are important for adhesion to the urinary epithelium and urinary catheters through the binding of fibrinogen released into the bladder during catheterization (Flores-Mireles *et al.,* 2015).

In a mouse model of CAUTI, *E.faecalis* infection could be blocked by the administration of antibodies raised against the tip subunit of the Ebppili, EbpA (Flores-Mireles *et al*., 2014).

2.9.3 Enterococcus wound infection

Wounds presented by patients vary from one setting to another, ranging from acute surgical wounds, traumatic wounds such as those that occur following an accident, burn wounds or chronic wounds such as diabetic foot, leg and pressure ulcers. All wounds are contaminated with microorganisms that are part of the saprophytic microflora of the skin and the type and quantity of these microorganisms vary from one wound to another. Some important factors such as origin, body location, size and duration of the wound should be taken into account in the wound management because of their impact on wound colonization and infection. Microbial colonization of wounds is characterized by the presence of multiplying microorganisms on the surface of a wound, but with no immune response from the host and with no associated clinical signs and symptoms (Bessa *et al*,. 2013).

Differently, wound infection depends on the pathogenicity and virulence of the microorganisms and on the immune competency of the host and it is determined by the presence of clinical signs of infection such as erythema, pain, tenderness, heat, edema, cellulites and abscess/ pus therefore, wound infection results in active disease that is likely to delay the wound healing process. Moreover, despite these common criteria to identify wound infection, clinicians should be aware that each wound type may present different clinical signs of infection. Thus, the presence of microorganisms per se is not indicative of wound infection, however, the probability that a critical microbial load may directly contribute to the non-healing outcome in both acute and chronic wounds. (Bessa *et al*, 2013).

Soft tissue and wound infections due to *Enterococcus* spp. are increasing worldwide with current need to understand the epidemiology of the Enterococcal infections of wounds (Mathur *et al.,* 2014).

Wound and soft tissue infections due to *Enterococcus* spp. are also rising steadily. Risk factors for colonization and infection include previous antimicrobial therapy. However, data regarding the soft tissue and wound infections due to *Enterococcus* spp. and also its resistance pattern among trauma patients are scarce (Mathur *et al*,. 2014).

2.9.4 Other Enterococcus infections

Enterococcus cause intra-abdominal and pelvic infections. They also cause surgical wound infections, bacteremia, endocarditis, neonatal sepsis and rarely meningitis (Sood *et al.*, 2008).

2.10 Transmission

Enterococcus is one of the nosocomial pathogens which they can be transmitted through person to person, environment or contaminated water and food, infected individuals, contaminated healthcare personnel's skin or contact via shared items and surfaces (Ahmad *et al*., 2015).

2.11 Enterococcus prevalence

The prevalence of *Enterococci* among different clinical samples was 3.5%. This prevalence rate was consistent with the findings of other authors who found the prevalence rate in Egypt (3.3%), in Bangladesh (3.2%), in India (2.3%) and in Asian pacific (3.6%). On the other hand, the prevalence in the present study was higher than the report from Kenya (0.22%). and different hospitals of Ethiopia including Jimma, Felege Hiwot and University of Gondar Teaching Hospital that accounted 0.59%, 0.64% and 2.13% respectively. However, it was lower than the

prevalence study done in USA and Canada, 18.0% and 21.2%, respectively (Ferede *et al*., 2018).

2.12 Laboratory diagnosis

Enterococci are identified in the laboratory by many tests they are Gram-positive cocci to singles, pairs, or short chains especially when grown in broth may be more rod-shaped if grown on solid media. They are catalase negative, oxidase negative and Facultatively anaerobic (Leboffe and Pierce, 2012).

Lactic acid, but no gas, is the sole end product of fermentation of sugar, Grow in 6.5% NaCl broth, and in bile esculin (Leboffe and Pierce, 2012).

Most of Enterococci species are PYR positive. G+C content within the genus ranges between 34 and 42%.Most express the Lancefield Group D antigen. Most species are commensals or opportunistic pathogens. *Enterococcus* species reduce litmus milk by enzyme action as shown by decolorization of the litmus (Leboffe and Pierce 2012).

2.12.1 Gram stain

The Enterococci are Gram-positive, spherical, oval or coccobacillary and are arranged in pairs and short chains (Parija, 2014).

2.12.2 Biochemical identification

2.12.2.1 Catalase test

Enterococcus species are catalase negative (Parija, 2014).

2.12.2.2 Bile Esculin hydrolysis Test

Bile is the selective agent added to separate the Streptococcus bovis group and Enterococci from other Streptococci. However, among the Streptococci, typically only Enterococci and members of the Streptococcus bovis group (Leboffe and Pierce, 2012).

2.12.2.3 PYR Test

Group A streptococci (*S. pyogenes*) and Enterococci produce the enzyme L-pyrrolidonylarylamidase (Leboffe and Pierce, 2012).

2.12.4 Salt tolerance test

Salt acts as a selective agent for bacteria and interferes with membrane permeability and osmotic equilibrium. A high salt concentration thus inhibits a range of bacteria but allows salt-tolerant organisms such as Enterococci to grow (Bailey and Tille, 2014).

2.12.3 Molecular detection

A multiplex PCR was devised for the routine molecular identification of *E.faecium* and *E.faecalis* by primers targeted at specific sequences in the *ddl* (D-Ala–D-Ala ligase) chromosomal genes of the two species and in the glycopeptide resistance ligase genes *vanA*, *vanB*, and *vanC*. The *vanC* gene is present in the motile, low-level constitutive glycopeptide-resistant species *E.gallinarum (vanC-1)* and *E.casseliflavus* and *E.flavescens (vanC-2/3)*; thus, demonstration of its presence indicates the presence of one of the aforementioned species (Angeletti *et al*., 2001). The use of a PCR with primers for *ddl E.faecalis* and *ddl E.faecium*, together with primers for *vanC-1*, *-2*, and *-3*, may be the simplest molecular approach for both rapid and precise identification of Enterococci (Angeletti *et al*., 2001).

Among the proposed molecular methods for enterococcal species identification the amplification and sequencing of the 16S ribosomal DNA (rDNA) gene (Angeletti *et al*., 2001).

2.13 Treatment

Enterococcus faecalis, particularly vancomycin-resistant strains are an important cause of nosocomial infections such as bacteremia, sepsis, endocarditis, urinary tract infection (UTI) and wound infection (Heidari *et al*., 2017).

Combination of a cell wall active agent (ampicillin, penicillin, or vancomycin) and an aminoglycoside, typically gentamicin has been used frequently for treatment of serious Enterococcal infections (Miller *et al*., 2014).

Infections caused by β-lactamase–producing Enterococci can be treated with combination penicillin and β-lactamase inhibitors or Vancomycin (and streptomycin) when in vitro susceptibility has been demonstrated (Brooks *et al*., 2012).

Enterococci often show susceptibility to trimethoprim–sulfamethoxazole by in vitro testing, but the drugs are not effective in treating infections. This discrepancy is because Enterococci are able to utilize exogenous foliates available in vivo and thus escape inhibition by the drugs (Brooks *et al*., 2012).

2.14 Antibiotic resistance

2.14.1 Ampicillin/ penicillin

Ampicillin resistance, mediated by a β-lactamase that inactivates the antibiotic through the cleavage of the β-lactam ring, has been described in both *E.faecalis* and *E.faecium* (Miller *et al*., 2014).

2.14.2 Cephalosporin Resistance

Although the natural resistance of Enterococci to cephalosporins is a well-known feature, the molecular basis of this phenotype is not completely understood. A common observation is that intrinsic resistance is associated with a decrease in binding affinity of cephalosporins for the enterococcal Penicillin-binding proteins (Miller *et al*., 2014).

2.14.3 Glycopeptide Resistance

Glycopeptides (vancomycin and teicoplanin) bind to the terminal d-alanine-d-alanine (d-Ala-d-Ala) moiety of peptidoglycan precursors, thus preventing cross-linking of peptidoglycan chains and inhibiting synthesis of the cell wall. Nine distinct vancomycin resistance clusters have been described in enterococci (*vanA,*

vanB, vanC, vanD, vanE, vanG, vanL, vanM and *vanN*). In general, these clusters consist of three groups of genes encoding two component systems enzymes necessary for the synthesis of new peptidoglycan precursors and enzymes that destroy the normal d-Ala-d-Ala-ending precursors. (Miller *et al*., 2014)

2.14.4 Streptogramins, Macrolides, and Lincosamides Resistance

E.faecalis possess a chromosomal gene named *lsa* (for lincosamide and streptogramin A resistance), which encodes for a putative protein with an ATP-binding cassette motif of transporter proteins but not the trans-membrane region that would be expected for an efflux pump (Miller *et al*., 2014).

Resistance to macrolides, lincosamides and streptogramin B (known as the MLS_B phenotype) is prevalent in Enterococci. Cross-resistance with all macrolides arises from modification of the 23S rRNA target (A2508, as opposed to modification of A2503 by *cfr* in linezolid resistance) by a variety of methylase genes, most commonly *ermB* (Miller *et al*., 2014).

2.14.5 Oxazolidinones Resistance

Linezolid is a bacteriostatic agent with broad activity against gram-positive bacteria. It binds to the 23S rRNA and disrupts the docking of the aminoacyl-tRNA in the A site of the ribosome, thus inhibiting the delivery of peptides and the subsequent elongation of the polypeptide chain. Mutations in genes encoding the 23S rRNA, which is an important part of the drug-binding site at the ribosome, are the most common mechanisms of linezolid resistance. Of note, Enterococci, as many other bacteria, carry multiple copies of the 23S rRNA gene and the number of mutated alleles correlates with the resistance phenotype (Miller *et al*., 2014).

2.14.6 Quinolones Resistance

Enterococci demonstrate low levels of intrinsic resistance to the quinolones, but can acquire high-level resistance through several mechanisms. Mutations in the target genes, specifically *gyrA* and *parC*, have been described in *E.faecium* and *E.faecalis* (Miller *et al*., 2014).

2.14.7 Rifampicin Resistance

Rifampicin resistance arises from a variety of mutations in the *rpoB* gene that encodes for the β-subunit of the RNA polymerase. Interestingly, a specific mutation in *rpoB* (H486Y) in both *E.faecalis* and *E.faecium* was shown to increase resistance to broad-spectrum cephalosporins (Miller *et al*., 2014).

2.15 Previous studies

In a study conducted in Ethiopia by Ferede *et al* (2018) found that from a total of 422 samples processed, 15 Enterococcus species were isolated (3.5%).

Different study was conducted in india by Sreeja *et al* (2012)A number of 128 Enterococcus species were isolated in a period of one year and the rate of the infection was estimated to be 2.3%. Among the isolates, those of Enterococcus *faecalis* were 97and the remaining 31were of *Enterococcus faecium.*

Another study was conducted in India by Paul *et al* (2017) found that the prevalence of Enterococcus species were (8.5%).

Different study was conducted in Kolkata India by Saha *et al* (2016) found that the *prevalence of Enterococcus* species was (4.9%).

Another study was conducted in India by Lall and Basak (2014) found that the Vancomycin, shows 100% susceptibility by disc diffusion method

A study was conducted in tertiary care hospital in India by Suresh *et al* (2013) whom found that the Vancomycin, shows 100% susceptibility by disc diffusion method.

Different study was conducted in Iran by Armin *et al* (2019) found that the findings indicated that 20.7% of the isolates were collected from hospitalized patients in the ICU. Among all the isolates, 254 (30%) were identified as VRE strains.

Another study was conducted in Africa by Alemayehu and Hailemariam (2020) found that the prevalence of Vancomycin Resistant Enterococcus (VRE) isolates were (26.8%).

Different study was conducted in Asia by Shrestha *et al* (2021) found that prevalence of Vancomycin Resistant Enterococcus (VRE) in Asia was (8.1%).

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

3. Materials and methods

3.1 Study design

This was a descriptive cross-sectional hospital base study

3.2 Study area and duration

This study was conducted at Almak Nemer Hospital located in Shendi locality, River Nile State, Sudan during April 2020 to May 2022

Shendi is a town in Northern Sudan on the East bank of the River Nile 150 km northeast of Khartoum.

3.3 Study population

All Patients attending to Almak Nemer Hospital and, suffering from wound infection, bacteremia and urinary tract infection.

3.3.1 Inclusion criteria

All Patients attending with urinary tract infection, bacteremia, wound infection and agreed to participate in this study.

3.3.2 Exclusion criteria

Patients under antimicrobial treatment or refuse to participate in this study were excluded.

3.4 Sampling technique

This study based on non-probability convenience sampling technique.

3.5 Sample size

A total of 362 patients (n=362) were included randomly in this study.

3.6 Ethical considerations

This study was approved by Ethical Committee of College of Medical Laboratory Science, Sudan University of Science and Technology.

3.7 Data collection

Data were collected from the patients using structural questionnaire containing all study variables.

3.8 Specimen processing

3.8.1 Collection of the specimens

Under a septic condition, wound swabs were collected using sterile cotton swabs; Mid-stream urine sample was collected in sterile screw capped universal containers. Blood was collected and dispensed with great care to avoid contamination of the specimen and culture medium. Using a pressure cuff, suitable vein in the arm was located the cuff was Deflated while disinfecting puncture site. Wearing gloves, thoroughly puncture site was disinfected first by 70% ethanol, cleaning an area about 50 mm in diameter. The area was left to dry then 2% tincture of iodine was used by a circular action the area was left to dry the protective cover from the top of the culture bottle was removed, The top of the bottle was wiped using an ethanol-ether swab, Using a sterile syringe and needle, about Ten ml of blood was collected. And the needle was inserted through the rubber liner of the bottle cap and 10ml of blood was dispensed into the diphasic culture medium bottle containing broth. The broth was incubated for 24 hours at 37 C^{o}

3.9.2 Cultivation of the specimens

Specimens were inoculated in Blood agar and MacConkey agar and incubated for 24 hours at 37 C^{o} and used for isolation and identification of clinical isolates, Blood culture bottles were inoculated blindly and incubated for 24 hours at 37 C^{o} and in Blood agar and MacConkeys Agar, incubated for 24 hours at 37 C^{o} for isolation and identification of clinical isolates

3.9.3 Interpretation of culture growth

The plates were examined for any significant bacterial growth. The isolated bacteria were then identified by colonial morphology, Gram stain and biochemical tests.

3.9.4 Identification of the isolates

The isolated organisms were fully identified by Gram stain and the appropriate followed by performing biochemical tests.

3.9.4.1 Colonial Morphology

The cultures morphologically examined for size, color, fermentation of lactose on MacConckey Agar and hemolysis on blood agar. Enterococci show small, 0.5-1mm magenta colored colonies. On MacConckey Agar and circular, translucent, smooth, convex colonies of 1-2mm in diameter, with regular margins showing either alpha or non-hemolytic colonies on blood agar.

3.9.4.2 Gram stain

Three drops of sterile normal saline was dispensed on a clean dry glass slide using sterile wire loop. From pure culture of the tested organism one colony was touched by a sterile wire loop, mixed with normal saline and spread evenly on an area of about 15-20 mm. Fixed the dried smear by heating using the flame, flooded the fixed smear with crystal violet stain for 1 minute. Rapidly was washed off the stain with clean tab water. The smear was flooded with Lugol's iodine for 1 minute, then washed. Decolorized rapidly (few seconds) with alcohol, then washed, flooded the smear with safranine stain for 2 minutes. the smear was left to air dry, drop of immersion oil was added and examined microscopically using X100 (Cheesbrough, 2006).

3.9.4.3 Biochemical tests

3.9.4.3.1 Catalase test

Poured 2–3 ml of the hydrogen peroxide solution into a test tube, using a sterile wooden stick, good growth of the test organism was removed and immersed it in the hydrogen peroxide solution and observed for an immediate bubbling (Chessbrough, 2006).

3.9.4.3.2 Bile Esculin hydrolysis test

Bile Esculin agar plate is obtained, the plate was labeled, inoculated with a single colony from the tested organism, incubated for 24 hours at 37°C**.** The plate was Examined for any darkening of the medium (Leboffe and pierce,.2012).

3.9.4.3.3 Litmus Milk decolorization test

Using a sterile loop 0.5 ml of a sterile litmus milk broth was inoculated with the tested organism. A heavy inoculums of the tested organism was used, incubated at37°C for up to4 hours, Examined for a reduction reaction at half hour interval, as shown by a change in color from mauve to white or pale yellow (Chessbrough,.2006).

3.10 Antimicrobial Susceptibility

3.10.1 Preparation of standard bacterial suspension

Clinical isolates were isolated from different samples in a sterile sloe of Nutrient. Ten ml of normal saline were distributed in a test tubes and sterilized in autoclave at 121ºC for 15mintes, a loop full of purified bacterium was inoculated in a sterile normal saline. Inoculums density was compared with 0.5 McFarland standard suspension.

3.10.2 Disc diffusion method

Using disc diffusion method according to modified Kirby Baur technique Three to five well isolated colonies of the organism were touched using sterile straight wire loop and emulsified in sterile distilled water and adjusted to 0.5 McFarland's

standard .Sterile cotton swab was dipped into suspension optimally several times and pressed firmly into the inside the wall of the tube above the fluid level to remove the excess inoculums from the swab. The dried surface of Muller Hinton agar plate was inoculated by streaking the swab over the entire sterile to more times rotating the plate. After five minutes, antibiotic discs Ciprofloxacin, Ampicillin, Chloramphenicol, Gentamycin and Vancomycin applied using a sterile forceps, then he plates were incubated at 37ºCfor overnight incubation.

3.10.3 Reading and interpretation of the results

The diameter of the zone of inhibition around each disc was measured by using ruler. *E.faecalis* is susceptible to Ampicillin, where is *E.faecium* show resistance pattern to Ampicillin.

3.11 Data analysis

Data were entered, checked and analyzed using Microsoft Excel 2004 and SPSS (Statistical Package of Social Science) Soft program version 11.5 proportional data were presented as frequencies and percentages. Chi squire test were used to detect the possible association. *P*.value were set as < 0.5 for all study results.

CHAPTER IV

RESULTS

CHAPTER IV

RESULTS

A total of 362 patients with symptoms of urinary tract infection, wound infection, and bacteremia were enrolled in this study. The distribution of clinical specimen as followed 232 (64%) were urine samples, 121 (33.5%) were wound swabs and 9, (2.5%) were blood samples as shown in table (4-1).

The age of population ranged from 16 years to 70 years with mean age of (42.03 ± 22.6) were divided into 5 groups as follow:

Out of these 362 patients, 117 (32.3%) patients were from (16-26) years, 20/362 (6.1%) patients were from (27-37) years, 117/362 (%) patients were from (38-48) years, 144/362 (39.7%) patients were from (49-59) years, and 14/362 (3.8%) patients were from (60-70) years as shown in table (4-2).

Regarding the gender 140/362 (38.7%) were males and 222 (61.3%) were females as shown in table (4-3).

Considering hospital admission, 160/362 (38.7%) were inpatients 202 (61.3%) were from outpatients as shown in table (4-4).

Out of 362 specimens 32 (8.8%) showed no growth after cultivation and incubation, 330 (91.2%) specimens showed growth

The prevalence of Enterococcus isolates was (4.1%), while the majority of Enterococcus isolates were *E.faecalis* 14 (3.8%) and only one (0.3%) was *E.faecium* as shown in Figure (4-1).

Considering type of infections 10/15 (66.7%) of Enterococcus isolates were from UTI patients, and 5/15 (33.3%) were from wound infection patients with statistical significance difference between Enterococcus isolate and type of infection (*P*. value = 0.02) as shown in table (4-5).

Of these 15 Enterococcus isolates 12 (80%) were isolated from patients with age group (49-59) years, with statistical significance difference between Enterococcus isolates and age (*P*.value = 0.002) as shown in (Table 4-6).

Eight (53.3%) of Enterococcus isolates were isolated from males and 7 (47.7%) from females with no statistical significance difference between Enterococcus isolates and gender (*P*.value = 0.281) as shown in (Table 4-7).

Considering hospital admission 12/15 (80%) of Enterococcus isolates were isolated from inpatients and 3 (20%) from outpatients with statistical significance difference between Enterococcus isolate and hospital admission (*P*.value = 0.004) as shown in table (4-8).

All Enterococcus spp isolates showed no resistance to Vancomycin (0/15) (0%). *Enterococcus faecalis* showed high resistant rate to antimicrobial used as follow: 12 (85.7%) to both Chloramphenicol and Gentamycin and 10 (71.4%) to Ciprofloxacin.

While the only one *Enteroccous faecium* isolate showed high resistance to Ampicillin and Chloramphenicol 1 (100%) while Gentamycin, Ciprofloxacin and Vancomycin showed no resistance 0 (0%) as shown in table (4-9).

The multidrug resistant Enterococcus isolates in this study were 10/15 (66.7%).

Table (4-1): Distribution of clinical specimens

Specimen	Frequency (%)
Urine	232 (64%)
Wound swab	121(35.5%)
Blood	9 (2.5%)
Total	**362 (100%)**

Table (4-2): Distribution of the study population according to age group

Age group / Year	Frequency (%)
16-26	67 (18.5%)
27-37	20 (5.5%)
38-48	117(32.2%)
49-59	144(39.7%)
60-70	14 (3.9%)
Total	**362(100%)**

Table (4-3): Distribution of the study population according to the gender

Gender	Frequency (%)
Male	140 (38.7%)
Female	222 (61.3%)
Total	**362 (100%)**

Table (4-4): Distribution of the study population according to hospital admission

Hospital admission	Frequency (%)
Inpatients	160 (44.2%)
Outpatients	202 (55.8%)
Total	**362 (100%)**

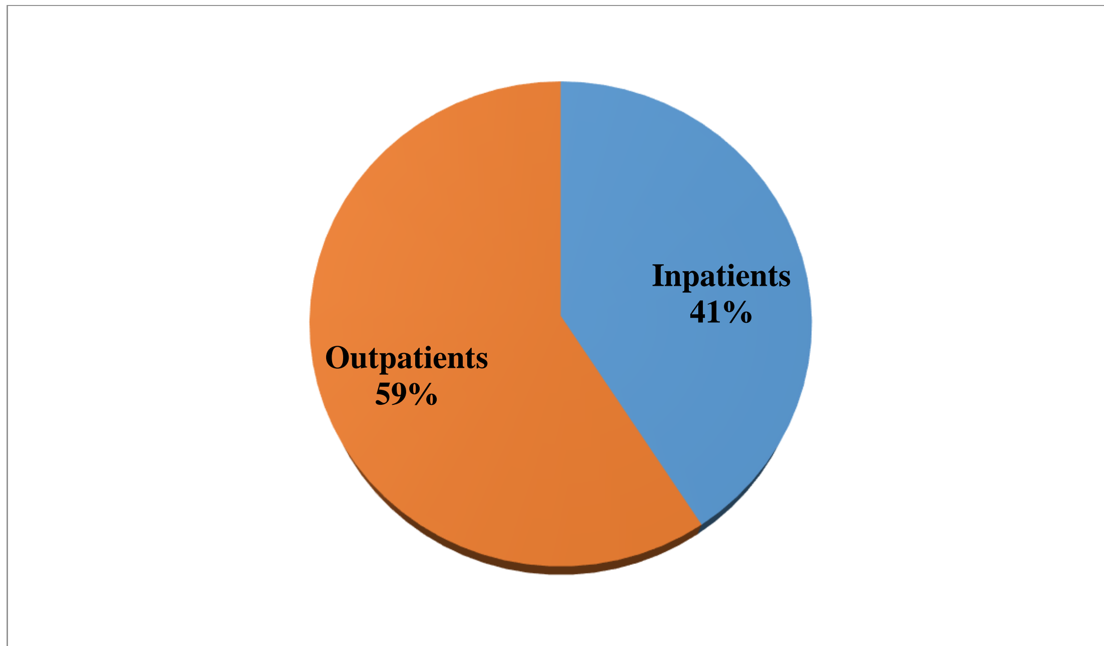

Figure (4-1): Distribution of the study population according to hospital admission

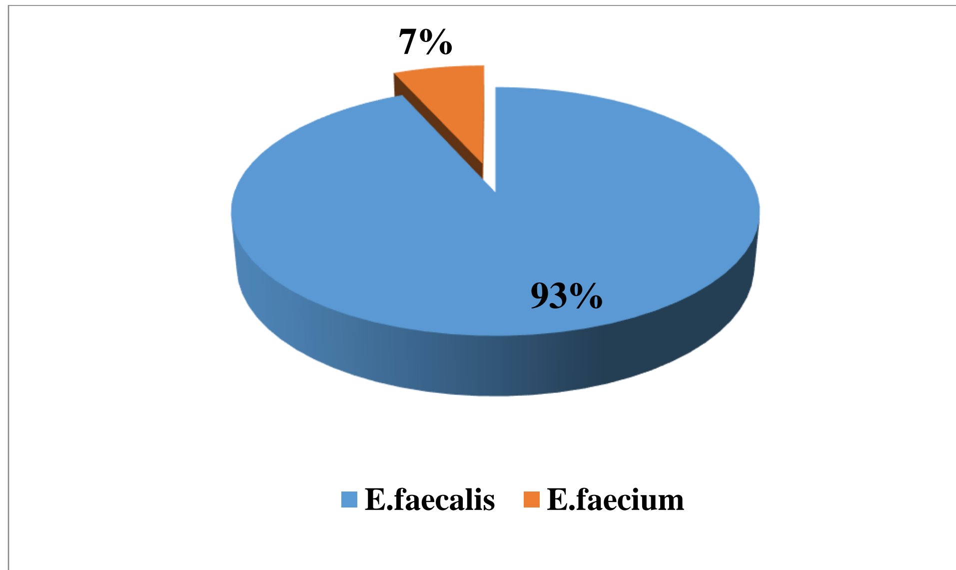

Figure (4-2): Frequency of isolated Enterococcus species

Table (4-5): Association between Enterococcus isolates and type of infections

Type of infection	Enterococcus isolates		*P*. value
	Positive (%)	Negative (%)	
UTI	10 (66.7%)	5 (33.3%)	**0.02**
Wound infection	5 (33.3%)	10 (66.7%)	
Bacteremia	0 (0%)	15 (100%)	

.

Table (4-6): Distribution and association between Enterococcus isolates and age

Age group	Enterococcus isolates		P. value
	Positive (%)	Negative (%)	
16-26	0 (0%)	0 (0%)	**0.002**
27-37	0 (0%)	0 (0%)	
38-48	1 (6.6%)	14 (93.4%)	
49-59	12 (80%)	3 (20%)	
60-70	2 (13.3%)	13 (86.7%)	

Table (4-7): Association between Enterococcus isolates and gender

Gender	Enterococcus isolates		P. value
	Positive (%)	Negative (%)	
Male	8 (53.3%)	7 (46.7%)	**0.281**
Female	7 (46.7%)	8 (53.3%)	

Table (4-8): Association between Enterococcus isolates and hospital admission

Hospital admission	Enterococcus isolates		P. value
	Positive (%)	Negative (%)	
Inpatients	12 (80%)	3 (20%)	**0.004**
Outpatients	3 (20%)	12 (80%)	

Table (4-9): Antimicrobial susceptibility of *E.faecalis* and *E.faecium* isolates

Antimicrobial agents	*E.faecalis* (N=14)		*E.faecium* (N=1)		Total (N=15)	
	S	R	S	R	S	R
Ampicillin	14(100%)	0 (0%)	0(0%)	1(100%)	14(93.3%)	1(6.7%)
Chloramphenicol	2(14.3%)	12(85.7%)	0(0%)	1(100%)	2(13.3%)	13(86.7%)
Gentamycin	2(14.3%)	12(85.7%)	1(100%)	0(0%)	3(20%)	12(80%)
Ciprofloxacin	4(28.6%)	10(71.4%)	1(100%)	0(0%)	5(33.3%)	10(66.7%)
Vancomycin	14(100%)	0(0%)	1(100%)	0(0%)	15(100%)	0(0%)

CHAPTER V

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Intestinal colonization with resistant Enterococcal strains is more common than clinical infection .Colonized patients are a potential source for the spread of organism to the health care workers, the environment and other patients (Jada and Jayakuarm,.2012).

In this study the frequency of Enterococcus species among different clinical isolates was (4.2%), the frequency rate was consistent with the findings of many authors, freed *et al* (2018) whom found the prevalence rate was (3.5%) in Ethiopia, Sreeja *et al* (2012) in India whom found the prevalence rate was (2.3%) and Saha *et al* (2016) whom found the prevalence rate was (4.9%) in Kolkata India.

On the other hand, the prevalence in the present study was higher than the report from Kenya by Kimando *et al* (2010), which was (0.22%) in Kenyatta University.

In this study the frequency of *Enterococcus faecalis and Enterococcus faceium* was (3.8%) and (0.3%) , Respectively which was higher than the findings of the study which was conducted in India by Sreeja *et al*(2012) whom found the prevalence of *Enterococcus faecalis and Enterococcus faceium* was (1.7%) and (0.5%) , Respectively.

The results of this study showed lower prevalence of Enterococci than that reported by Suresh *et al* (2013) in India in which the prevalence of *Enterococcus faecalis and Enterococcus faceium* was (20.8%) and (2.4%) ,Respectively.

The variation in the results might be explained due the different methods used for identification and geographical distribution. The conventional methods for the identification of microorganisms are based on phenotypic and culture characteristics and may not be able to identify the causative organism correctly when strains with unusual phenotype

The majority of samples from which Enterococcus were isolated are urine (10) (66.7%), this findings were similar to the findings of Paul *et al* (2017) in India,

which is Enterococcus were isolated are urine (73) (71.5%). High frequency of Enterococcus isolates was isolated form inpatients 12 (80%) Enterococcus species have emerged as important pathogens in urinary tract infection (UTI), especially in Inpatients.

In the present study most of the Enterococcus isolates were isolated from patients from age group 49-59 years 12 (80%) this is comparable to the study of Paul et al (2017) in India whom found that the most of the patients were from age group (51-60) years the frequency and severity of infectious diseases also increase in elderly patients because of humoral and cellular immunity changes, organ and tissue dysfunctions and underlying chronic diseases in this patient group.

The susceptibility tests showed high resistance to Chloramphenicol 13 (86.7%), Gentamycin, Ciprofloxacin, Ampicillin and Vancomycin was 12 (80%), 10 (67.7%), 1 (6.7%) and 1 (0%), respectively this findings were found to be different from the findings was conducted by Ferede *et al* (2018) whom found that the resistance rate to Ampicillin, Chloramphenicol, Gentamycin, Ciprofloxacin and Vancomycin was 12 (80%), 8 (53.3%), 9 (60%), 8 (53.3%) and 1 (6.7%), respectively.

These findings differ from the results of Salah *et al* (2008) whom found that isolates were 100% susceptible to Ampicillin, Chloramphenicol, Ciprofloxacin, and Vancomycin

The unregulated using of antibiotics in the last decades has helped the bacteria to survive and resist wide range of antibiotic leading to multi drug resistance.

In this study of the isolated Enterococci (10) (66.7%) were multi drug resistances which were resistant to three and above drugs. This result was lower than the results of the study reports in Negeria by Olawale *et al* (2011) in which the prevalence of multi drug resistant Enterococcus was 7 (100%).

5.2 Conclusion

This study concluded that:

The frequency of isolated Enterococci spp among different clinical specimens is moderate and the most predominant species was *Enterococcus faecalis*. This study showed an association between Enterococcus and type of infection, hospital admission and age.

Enterococcus isolates in this study showed high rate of multi drug resistant and they showed high resistance rate to Chloramphenicol, Ciprofloxacin, and Gentamycin, while no Vancomycin Resistant Enterococci (VRE) was isolated.

5.3 Recommendations

- The presence of high percentage multidrug resistant Enterococci in this study should be considered as alarm and further in large scale is needed
- Effort should be made to prevent nosocomial Enterococci infection and spread of Multi Drug Resistant Enterococci
- Uses of advance methods such as molecular methods is recommended for isolation, identification and detection of resistant genus of Enterococci species.

CHAPTER V

DISCUSSION, CONCLUSION AND, RECOMMENDATIONS

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APPENDICES

Appendix I

Questionnaire

Frequency and Antimicrobial Susceptibility Pattern of Isolated Enterococcus species among Different Clinical Specimens at Almak Nemer Hospital, River Nile State Sudan

Age ……………………….years

1. From 16-26( ) 2.From 27-37 ( ) 3.From 38-48( )

4. From 49-59( ) 4.From 60-80 ( )

Sex:-

1. Male ( ) 2.Female ( )

Hospital stay:-

1. Hospitalized ( ) 2.Outpatients ( )

Type of specimen:-

1. Urine ( ) 2.Wound swab ( ) 3.Blood ( )

Result

Positive Bacterial growth:-

1. Positive growth ( ) 2.No growth ( )

Enterococcus isolate:-

1. E.faecalis ( ) 2.E.faecium ( )

Antimicrobial sensitivity:-

1. Vancomycin ( ) 2.Ampicllin ( ) 3.Ciprofloxacin ( )

4. Gentamycin ( ) 5.Chloramphenicol ( )

Appendix II

Culture media:

Different culture media were used for inoculation, isolation, and identification of organisms. These include:

Blood agar

Typical formula g/L:

Contents:

....Peptone0.5 %

Beef extract/yeast extract...................................0.3%

Agar…………………………………………….1.5%

Nacl……………………………………………0.5%

Distilled water

5% Sheep Blood

Preparation

1- Suspend 28 g of nutrient agar powder in 1 litre of distilled water.
2- Heat this mixture while stirring to fully dissolve all components.
3- Autoclave the dissolved mixture at 121 degrees Celsius for 15 minutes.
4- Once the nutrient agar has been autoclaved, allow it to cool but not solidify.
5- When the agar has cooled to 45-50 °C, Add 5% (vol/vol) sterile defibrinated blood that has been warmed to room temperature and mix gently but well.
6- Avoid Air bubbles.
7- Dispense into sterile plates while liquid.

MacConkey Agar

Typical formula g/L:

Contents:

Peptone……………………… 17

Proteose peptone……………...3

Lactose monohydrate…………10

Bile salts…………………….. 1.5

Sodium chloride………………5

Neutral red………………….. 0.03

Crystal Violet……………….. 0.001

Agar…………………………. 13.5

Distilled Water

Preparation

1- Suspend 49.53 grams of dehydrated medium in 1000 ml purified/distilled water.
2- Heat to boiling to dissolve the medium completely.
3- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
4- Cool to 45-50°C.
5- Mix well before pouring into sterile Petri plates.

Bile Esculin agar

Typical formula g/L:

Contents:

Peptic digest of animal tissue………5.00

Beef extract…………………..........3.00

Esculin……………………………...1.00

Bile salts……………………………40.0

Ferric citrate………………………...0.50

Agar……………………………...15.00

Preparation

1. Suspend 64.5 grams in 1000 ml distilled water.
2. Heat to boiling to dissolve the medium completely.
3. Dispense into tubes or flasks.
4. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
5. Allow the tubed medium to solidify in a slanted position with a butt of 2.5cm deep or pour into sterile Petri plates.
6. Allow the BEA medium warm to room temperature before use.
7. Inoculate and streak the medium with one isolated pure colony.
8. Incubate in an aerobic atmosphere at 35ºC for 24-48 hours.
9. Observe for growth and blackening of the medium.

Litmus Milk

Typical formula g/L:

Contents:

Skim milk powder …………….100

Litmus: ………………………...0.5

Sodium sulfite …………………0.5

Preparation

1- Suspend 10.1 grams in 100 ml distilled water.
2- Agitate the mixture continuously.
3- Dispense 10 ml amounts into 15 x 150 mm tubes.
4- Sterilize the medium by autoclaving at 15 pressure (121°C) for 5 minutes.
5- Avoid overheating.

Mueller Hinton agar:

Typical formula g/L:

Contents:

Casein acid hydro lysate……………………………………………17.50

Beef heart infusion……………………………………………...2.00

Starch, soluble……………………………………………………...1.50

Agar………………………………………………………………....17.00 pH (at 25ºc) 7.3±0.1

Preparation:

1- Thirty eight gram of powder were suspended in 1000ml D.W
2- Mixed well and heated until boiling and dissolved completely.
3- Then was sterilized by autoclave at 121ºc for 15mins.

Chemicals and reagents:

1- Mcferland turbidity standard
2- Gram stain

Mcferland turbidity standard:

Contents:

Concentrated sulphric acid…………………………………………..1 ml

Dehydrate barium chloride……………………………………………0.5g

Distilled water………………...……………………………………150 ml

Gram's stain:

Contents:

Crystal violet stain

Lugol's iodine

Acetone

Safranin