

PREVALENCE OF BACTERIAL INFECTION
IN ACUTE HEPATITIS IN NEPAL

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ABSTRACT

A clinical and lab-based study was conducted at CDM, TU; taking blood samples from 118 consecutive patients with clinically suspected acute hepatitis attending Liver Foundation Nepal, Liver Unit of Bir Hospital, Nepal Ayurveda Hospital and Sukraraj Tropical & Infectious Disease Hospital during Aug 2001—Aug 2002; with an objective to find the association of bacteria with acute hepatitis.

The venous blood samples collected aseptically from the included patients were subjected to blood culture & sensitivity, serology for viral markers & biochemistry and haematology for blood picture.

Prevalence of aerobic bacterial infection among acute hepatitis cases was found to be 38%. Among these, 49% were due to gram-negative rods, 44% due to gram-positive cocci and 7% due to gram-positive rods. *E. coli* (9) was the most prevalent bacteria, followed by *Klebsiella spp.* (6), *Pseudomonas aeruginosa* (3), *Salmonella spp.* (2), *Enterobacter spp.* (1) & *Acinetobacter spp.* (1) among GNB. *Staphylococcus aureus* (11), CONS (5), *Group D Streptococcus* (1) were also isolated among GPC.

Prevalence of bacterial infection was found to be more in male than in female & in the age group 20-29.

Incidence of bacterial infection was found to be significantly higher during rainy season in the month of August.

Prevalence of bacterial and viral co-infection in acute hepatitis was found to be 17%. Co-infection was most common in patients infected with gram-negative bacteria and hepatitis E virus.

All kinds of signs and symptoms were more common among cases with bacterial infection or co-infection than among acute hepatitis cases without bacterial infection, including acute viral hepatitis cases. More leukocytosis was observed in bacterial infection &/or co-infection cases while more leukocytopenia in acute viral hepatitis cases.

In-vitro antibiotic susceptibility test showed that most bacterial isolates were susceptible to ciprofloxacin & chloramphenicol (93%); however, gram-negative rods were most susceptible towards amikacin (100%).

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ABBREVIATIONS

<u>Abbreviation</u>	:	<u>Referent</u>
HAV	:	Hepatitis A Virus
HBV	:	Hepatitis B Virus
HCV	:	Hepatitis C Virus
HDV	:	Hepatitis D Virus
HEV	:	Hepatitis E Virus
HBsAg	:	Hepatitis B surface antigen
GNB	:	Gram-negative bacilli
GPC	:	Gram-positive cocci
GPB	:	Gram-positive bacilli
CONS	:	Coagulase-negative Staphylococci
ALT	:	Alanine aminotransferase
BHI	:	Brain Heart Infusion
BA	:	Blood Agar
CA	:	Chocolate Agar
MA	:	MacConkey Agar
MHA	:	Mueller Hinton Agar
NA	:	Nutrient Agar
NB	:	Nutrient Broth
SIM	:	Sulphide Indole Motility
TSI	:	Triple Sugar Iron Agar
AK	:	Amikacin
A	:	Ampicillin
CX	:	Cefotaxime
C	:	Chloramphenicol
CI	:	Ciprofloxacin
Q	:	Co-trimoxazole
E	:	Erythromycin
J	:	Gentamicin
M	:	Methicillin
P	:	Penicillin-G
T	:	Tetracycline
VA	:	Vancomycin

CHAPTER – ONE

INTRODUCTION

Hepatitis, originally a Latin word, refers to the inflammation of the liver. Liver is an essential organ responsible for performing a number of vital activities; including metabolism, excretion, detoxification, synthesis and filtration in the human body. Dysfunction of the liver due to infection or other disturbances affects the normal physiology rendering a number of disabilities and ailments (Pedersen & Ove, 2000).

Acute hepatitis is defined as a clinical syndrome resulting from massive necrosis of hepatocytes resulting in sudden and severe impairment of liver function. In case of acute viral hepatitis, the syndrome should have developed within eight weeks of the onset of symptoms (Sherlock, 1987). Acute hepatitis is characterized by the elevation of serum alanine transaminase level by 2.5 times its normal value (30-40 U/L); and the acute stage can last from several weeks to several months (Shrestha, 2000).

The most common cause of acute hepatitis in Nepal is found to be hepatitis E, a water-borne viral infection. Acute hepatitis due to hepatitis E virus is found to be very severe and fatal. Lack of quality drinking water and proper sanitation has often proved to be the cause of several outbreaks of water-borne infections in Nepal. (Shrestha, 2000)

The incidence of acute hepatitis B is 6-9% in Nepal. Hepatitis A virus is also transmitted faeco-orally but rarely causes water-borne outbreaks (Shrestha, 2000). Hepatitis C virus is associated more with chronic hepatitis and very rarely with acute hepatitis. (Shrestha, 2000)

The role of bacteria as aetiological agent of hepatitis, however, has not yet been understood as such although the documented data of last few years at several hospitals reveal the frequent incidence of acute bacterial hepatitis (Shrestha, 2001; Bir Hospital, 2001-2002). Some incidences of Salmonella hepatitis have been reported (El-Newihi *et al.*, 1996).

The liver being a bacterial filter, hepatic venous blood is normally sterile. But due to the linkage of portal and systemic venous systems via the hepatic veins, infection of liver is usually probable if the significant number of pathogens or opportunistic normal microflora of the digestive system could enter the liver and establish the disease. Also, when the liver is infected and inflamed, release of these agents into the blood stream and their spreading to various other parts through inferior vena cava is quite likely. Further, the portal-hepatic venous anastomoses developing in cirrhosis may allow intestinal organisms to cause septicaemia (Sherlock, 1987).

Bacteria may enter the blood stream giving rise to bacteraemia from a focus of infection within the body, from a surface site with a normal flora or by the introduction into the vascular system of contaminated material. However, if the immune system is overwhelmed, invaded organisms persist in the blood and the symptoms and signs of septicaemia may supervene either briefly until the causal organisms are eliminated or as a more constant or recurrent event. (Phillips *et al.*, 1990)

Bacterial infection should always be considered as a possible cause of deterioration in the clinical picture; it may be a complication of pre-existing acute/chronic liver/biliary tract disease/cause of hepatobiliary disease; prompt administration of appropriate antibiotics may save the patient's life. (McIntyre, 1991)

Presence of bacteria in the blood can be detected by using different standard microbiological techniques. Isolation and identification of bacteria from blood helps in diagnosis of disease caused by bacteria and the study of antibiotic susceptibility pattern of these isolates undoubtedly helps the physicians to decide the suitable therapy for the patients suffering from bacterial infection. (Stokes *et al.*, 1993)

It has been reported that drinking water in the Kathmandu valley is usually polluted and the extent of water pollution is greater during the rainy season (Sharma, 1978; Vaidya *et al.*, 1986). Most bacterial infections like diarrhoeal disease and typhoid fever occur in the months of July and August in Nepal (Shrestha, 2003). Gram-negative bacilli such as *Salmonella* are known to cause hepatitis, the clinical picture of which is frequently indistinguishable from acute viral hepatitis (El-Newihi *et al.*, 1996). Therefore, the involvement of bacterial infection needs to be taken into consideration in patients with acute hepatitis of unknown etiology. (Shrestha, 2003)

The prevalence of acute bacterial hepatitis, which might be associated with inferior vena cava obstruction (Budd-Chiari syndrome), has not been well documented in Nepal. Feeling the need to find out the prevalence of bacterial infection in acute hepatitis in Nepal, the study was planned and carried out during August 2001- August 2002 in Central Department of Microbiology, T.U.; in collaboration & cooperation with Liver Foundation Nepal, Tripureswor; The Liver Unit, Bir Hospital; Sukraraj Tropical & Infectious Disease Hospital, Teku; and Nepal Ayurveda Hospital, Naradevi.

OBJECTIVES OF THE STUDY

GENERAL OBJECTIVE: -

To study the prevalence of bacterial infection in acute hepatitis in Nepal

SPECIFIC OBJECTIVES: -

- Isolation and identification of the bacterial agent(s) from the blood samples collected from acute hepatitis cases
- Determination of antibiotic susceptibility pattern of the bacterial isolates against commonly used antibiotics
- Study of distribution of bacterial infection in acute hepatitis cases
- Study of co-infection of bacteria with hepatitis viruses

CHAPTER – TWO

LITERATURE REVIEW

2.1. HEPATITIS

Inflammation of the liver is termed “hepatitis,” which is characterized by the destruction of a number of hepatocytes and the presence of inflammatory cells in the liver tissue. Hepatitis can be caused by diseases which primarily attack the liver cells (Pedersen & Ove, 2000). The first reference of epidemic jaundice has been ascribed to Hippocrates (Sherlock, 1987).

2.2. THE LIVER

The liver is one of the heaviest organs in the body (1.2-1.5 kg). It comprises one-fiftieth of the total adult body weight (Filayson *et al.*, 1999). It is located below the diaphragm in the right upper quadrant of the abdominal cavity. It extends approximately from the right fifth rib to the lower border of the rib cage. (Wilson, 1990)

The liver is separated into a right and left lobe by the falciform ligament. The right lobe is much larger than the left lobe. The functional unit of liver is the hepatic acinus and the working cells of the liver are known as hepatocytes that have a unique capacity to reproduce in response to liver injury. The liver has important synthetic and metabolic functions. It detoxifies and excretes end products of metabolism. (Wilson, 1990)

2.2.1. Blood supply in the liver: -

The liver possesses a double blood supply. Portal circulation allows the passage of venous blood from the capillary bed of the abdominal part of the digestive system, the spleen and pancreas to the liver. Blood passes through a second capillary bed into the liver before entering the general circulation via the inferior vena cava. Hepatic veins, varying in number, leave the posterior surface and immediately enter the inferior vena cava just below the diaphragm (Wilson, 1990).

This linkage of portal and systemic venous systems by hepatic veins ease the spread of the pathogens from liver to the systemic circulation (Sherlock, 1987). The hepatic artery, coming from the coeliac axis, supplies the liver with arterial blood (Wilson, 1990).

Rows of hepatocytes radiate from the central hepatic vein and are separated by sinusoidal spaces along the wall of which are interspersed hepatic macrophages, the Kupffer cells, which as a part of the reticuloendothelial system, are phagocytic. At the corners of each lobule are the portal tracts, which contain branches of the hepatic artery, the portal vein and branches of the bile duct. Blood flows from the corners of the lobules towards the central hepatic vein (Filayson *et al.*, 1999). All nutrients from the gastrointestinal tract, except fat, pass through the sinusoidal spaces before entering the systemic circulation. (James *et al.*, 1989)

Distortion of this architectural arrangement, by diseases like cirrhosis of the liver, may allow the blood to pass directly from the portal to the hepatic vein, so bypassing the hepatocytes and significantly reducing the detoxifying capacity. This causes many features of liver disease. (Kuo *et al.*, 1991)

Therefore, in patients with poor hepato-cellular function, such as acute hepatitis, the damaged cells are unable to metabolize the contents of portal venous blood completely so that they pass unaltered into the hepatic veins and then through systemic circulation into various vital organs like brain, kidneys, lungs etc causing serious damage to them. (Sherlock, 1987)

2.3. FUNCTIONS OF LIVER:

2.3.1. General metabolic functions of the liver: -

Postprandially, when the glucose concentration in the portal vein is high, glycogen is synthesized and stored, and glucose is converted to fatty acids that are transported to the adipose tissue. During fasting, the systemic plasma glucose concentration is maintained by the breakdown of stored glycogen or by the synthesis of glucose from such substances as glycerol, lactate and amino acids by gluconeogenesis. Fatty acids reaching the liver from fat stores may be metabolized in the tricarboxylic acid cycle, converted to ketones, or incorporated into triglycerides. (Filayson *et al.*, 1999)

2.3.2. Synthetic functions of the liver: -

The hepatocytes synthesize plasma proteins except immunoglobulins and complement; most coagulation factors, including fibrinogen, and factors 2 (prothrombin), 5, 6, 7, 9, 10, 11, 12 and 13. Of these, prothrombin (2) and factors 7, 9, 10 cannot be synthesized without vitamin K; the lipoproteins, VLDL (very low density lipoprotein) and HDL (high density lipoprotein) and primary bile acids. (Sherlock, 1987)

2.3.3. Excretion and detoxification by the liver: -

Besides bilirubin, other substances that are inactivated and excreted by the liver include (a) amino acids which are deaminated in the liver; the amino groups, and any ammonia produced by intestinal bacterial action which is absorbed in the portal vein are converted to urea; (b) cholesterol, which is excreted in the bile either unchanged or after conversion to bile acids; (c) steroid hormones, which are metabolized and inactivated by conjugation with glucuronate and sulphate, and are excreted in the urine in this water soluble form; and (d) many drugs, which are metabolized and inactivated in the endoplasmic reticulum, including the P450 system, are excreted in the bile to a certain extent. (Sherlock, 1987)

The efficient excretion of end products of metabolism and bilirubin depends on the normal functioning of the liver cells, the normal blood flow through the liver and the patent biliary ducts. (Filayson *et al.*, 1999)

2.3.4. Filtering function of the liver: -

The liver is the largest reticuloendothelial organ in the body and its situation is important in removing infecting bacteria and bacterial products, which often enter the body from the gut (Filayson *et al.*, 1999). Liver is the principal organ for reticuloendothelial uptake of the bacteria accounting for 60% of total uptake (Holdsworth *et al.*, 1989). The reticuloendothelial Kupffer cells in the hepatic sinusoids are well placed to extract toxic substances that have been absorbed from the gastrointestinal tract. They act as filters to any substances entering or leaving the organ (Wilson, 1990).

2.3.5. Metabolism and excretion of bilirubin: -

Bilirubin is the main bile pigment that is formed from the breakdown of heme in red blood cells. The broken down heme travels to the liver, where it is secreted into the bile by the liver. Normally, a small amount of bilirubin circulates in the blood. Production and excretion of bilirubin follows a specific pathway. When the reticuloendothelial system breaks down old red blood cells, bilirubin is one of the waste products. This free bilirubin is in a lipid soluble form that must be made water soluble. Once it is made water soluble, bilirubin can be excreted in the urine. An enzyme, glucuronyl transferase, is necessary for the conjugation of bilirubin. (Haslett *et al.*, 1999)

2.4. LIVER FUNCTION TESTS:

Biochemical investigations are commonly used to assess the structural integrity of the liver, its ability to transport material from the blood into bile and its ability to synthesize and secrete substances into the blood. Liver function tests, also referred to as LFTs, are blood tests that assess the state of the liver and biliary system.

Liver function tests can be classified as:

- i. Tests of excretion by the liver
- ii. Evaluation of synthesis in liver
- iii. Evaluation of enzyme activity

Liver function tests are most often employed to determine the presence of liver disease, type of the liver disease and the extent and progression of liver disease. The most commonly available laboratory tests for the diagnosis of liver disease include measurement of plasma levels of:

1. Bilirubin for the evaluation of the excretory function;
2. Transaminases (AST and/or ALT) for evaluation of hepatocellular damage;
3. Alkaline phosphatase for assessing cholestasis;
4. Albumin and/or prothrombin time for evaluation of synthetic function.

2.4.1. The transaminases: -

Liver cells contain enzymes that may be released into the blood in various pathological conditions. The plasma enzymes that are most commonly measured as indicators of liver damage are aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The transaminases are concerned in the synthesis and degradation of amino acids. Normal plasma contains low activities of these enzymes, the source of which is unknown. A rise in plasma transaminase activities is a sensitive indicator of damage to cytoplasmic and/or mitochondrial membranes even if there is no detectable impairment of function. Measurement of their activity in the blood may give evidence of hepatobiliary diseases.

Alanine aminotransferase (ALT): AST is not specific to the liver but ALT occurs in much higher concentration in the liver than elsewhere and consequently increased serum ALT activity reflects hepatitis damage more specifically. ALT is liberated into the blood whenever liver cell is damaged and increased plasma enzyme activity is a very sensitive index of hepatitis damage. The normal serum level for ALT is 10-40 U/L (Haslett *et al.*, 1999).

ALT is confined to the cytoplasm in which its concentration is higher than that of AST. In conditions such as viral hepatitis, the cytoplasmic membrane sustains the main damage; and leakage of cytoplasmic contents leads to a relatively greater increase of plasma ALT than AST.

Acute liver damage, irrespective of the cause, usually increase the activity in serum more than tenfold but these values may be much higher in acute viral hepatitis when there is widespread hepatic necrosis. In viral hepatitis, the amino transferase activity may be raised even in the prodromal phase and maximal levels of 10-100 times the normal values are usually reached in the jaundiced phase. (Haslett *et al.*, 1999)

2.4.2. Bilirubin: -

The major pigment in the body is the haem part of haemoglobin. When red blood cells die, the haemoglobin is liberated and the haem is separated from the globin. The iron atom is removed and the porphyrin ring opened, converting the haem to biliverdin and then to bilirubin in the cells of the reticuloendothelial system, especially those in the spleen and liver.

The bilirubin, although it is transported in the blood loosely bound to plasma albumin, is known as 'free bilirubin' and it is normally present in the plasma or serum in very small amounts. In the liver, free bilirubin is taken up by the hepatic cells and combined chemically (conjugated) with glucuronic acid to form the diglucuronide salt of bilirubin, and it is then referred to as 'conjugated bilirubin.' A small amount of conjugated bilirubin gets into the blood and can be detected in the serum, but most of it is excreted by the liver in the bile to which it gives the normal yellow green colour. Conjugated bilirubin is not bound to protein and it can therefore pass through the glomerular filter and appear in the urine.

Free bilirubin is poorly soluble in water, whereas conjugated bilirubin glucuronide is very soluble. This difference in the solubility of the two forms of bilirubin allows distinguishing between the two in the chemical procedure used to measure bilirubin in the serum. Since the conjugated bilirubin is soluble, it produces an immediate reaction when added to the aqueous reagent. It is thus referred to as 'Direct bilirubin.' The free bilirubin does not react with the reagent unless it is first solubilized usually by the addition of alcohol. Free bilirubin is therefore referred to as 'Indirect bilirubin.'

In a normal adult, approximately 7.5 g of haemoglobin is catabolized producing 250-300 mg of bilirubin daily. Approximately 80% of bilirubin is derived from the destruction of senescent of RBCs, with the remainder being derived from young but defective RBCs, hepatic haem, myoglobin and tissue enzymes. Normal liver has the capacity to generate up to 850 mmol/day of bilirubin, and haemolysis has to be 2 times the normal value before the plasma bilirubin level rises. (Sherlock, 1987)

2.5. ACUTE HEPATITIS

According to its duration, hepatitis is classified into acute hepatitis (lasting less than six months) and chronic hepatitis (lasting longer than six months). Acute hepatitis is defined as a clinical condition resulting from considerable necrosis of hepatocytes, or sudden and severe impairment of liver function. (Pedersen & Ove, 2000).

Absence of preceding liver disease and development of the syndrome within eight weeks of the onset of symptoms characterize acute viral hepatitis (Sherlock, 1987). The acute hepatitis is accompanied by the elevation of serum alanine transaminase level above twice the upper limit of normal value and usually there is the presence of jaundice (Shrestha, 2001). The acute stage can last from several weeks to several months.

2.5.1. Pathophysiology of acute hepatitis: -

Clinical Features: Hepatitis varies from a minor flu-like illness to fulminant, fatal liver failure, depending on the patient's immune response and other poorly understood virus-host factors. The picture of acute hepatitis varies widely, ranging from anicteric with only slight malaise to a severe and fatal disease culminating in hepatic coma. The symptoms of acute hepatitis vary considerably from person to person. Some patients have no symptoms at all, and in most cases, children only show mild symptoms. (El-Newihi *et al.*, 1996)

Generally, acute hepatitis shows following three different stages:

(i) **The early stage or prodromal phase:** It is characterized by tiredness, general malaise, slight fever, nausea, poor appetite, changes in taste perception, pressure or pain below the right ribs caused by an enlarged liver, aching muscles and joints, headache, and skin rash. Urticarial eruptions and arthralgias occasionally occur, especially in HBV infection. After 3 to 10 days, the urine darkens, followed by jaundice or icteric phase. However, the mildest attack is asymptomatic and marked only by a rise in serum transaminase levels.

(ii) **The jaundice/ icteric phase:** It is distinguished by yellowing of sclerae, skin and mucous membranes, dark urine, and light-coloured stools. Around this time, the other symptoms subside except worsening jaundice. The temperature returns to normal and there may be bradycardia. Appetite returns and abdominal discomfort and vomiting cease. Pruritus may appear transiently for a few days. Physical examination shows variable jaundice. The liver is usually enlarged and often tender, but the edge remains soft and smooth. Mild splenomegaly is present in 15 to 20% of patients. Signs of chronic liver disease are not seen in uncomplicated cases. Jaundice usually peaks within 1 to 2 wk, and then fades during a 2- to 4-wk **recovery phase**.

(iii) **The recovery phase:** It is accompanied by tiredness and can last for weeks after an icteric period of about 1-4 weeks. Clinical and biochemical recovery is usual within six months of onset.

However, association of bacteria at any stage may lead to complications resulting in increase in the duration of hospitalization of the patients. Despite long hospitalization, bacterial hepatitis responds to proper antibiotic therapy provided timely diagnosis and application of therapy (El-Newihi *et al.*, 1996).

Also, chronic hepatitis may follow types B and C. (Shrestha, 2000)

2.5.2. Hematology of acute hepatitis: -

Total serum bilirubin levels range widely. An increase in conjugated pigment is early, even when the total bilirubin level is still normal. Serum transaminase estimations are useful in early diagnosis, in detecting the anicteric case. The peak level is found one or two days before or after onset of jaundice. Later in the course the level falls, even if the clinical condition is worsening. The estimation cannot be used prognostically. Values may remain elevated for six months in those who are recovering uneventfully.

The pre-icteric stage of acute viral hepatitis is marked by leucopenia, lymphopenia and neutropenia. These revert towards normal as jaundice appears. The prothrombin time is lengthened in the more severe cases.

2.6. AETIOLOGY OF ACUTE HEPATITIS

Acute hepatitis has a number of possible causes—infectious hepatotropic viral hepatitis such as viruses of hepatitis A, hepatitis B, hepatitis C, hepatitis D and hepatitis E; other viral diseases such as mononucleosis and cytomegalovirus; severe bacterial infections, amoebic infections; medicines, eg paracetamol poisoning and halothane (an anaesthetic); and toxins such as alcohol and fungal toxins, eg toadstool poisoning are also reported as the aetiological agents of acute hepatitis (Pedersen & Ove, 2000).

The commonest cause of acute hepatitis in Nepal is found to be hepatitis E virus infection—80% to 90%. The incidence of acute hepatitis B infection is 6% to 9% in Nepal. Hepatitis A Virus is one of the agents of acute hepatitis but more common among children. Hepatitis C virus has also been found to cause acute hepatitis (Shrestha, 2001).

2.7. BACTERIAL HEPATITIS

The role of bacteria in causing acute hepatitis was not found to have been studied in detail so far and no literatures related to such study could be obtained.

However, a case study at department of Geriatric Medicine, Alexandra Hospital, Singapore (2000) revealed ischaemic hepatitis due to septicaemia. Markedly raised liver transaminases need not always be drug-induced or viral-related. It could be ischaemic in origin and the serious underlying condition needs to be sought and treated urgently. (Lim *et al.*, 1999)

Researchers of Los Angeles County-University of Southern California Medical Center revealed several incidences of Salmonella hepatitis (El-Newihi *et al.*, 1996).

Similarly, incidence of bacterial hepatitis, with/without viral co-infection, has been reported in Nepal at different time. Incidence of 6 cases with bacterial hepatitis in the year 2057 B.S., 48 cases with bacterial hepatitis in the year 2058 B.S., involving 2 cases of bacterial and viral (HEV) co-infection have been documented in the medical records of the patients in Bir Hospital (Bir Hospital, 2001-2002) and at LFN (Shrestha, 2000).

2.7.1. Bacteria involved in hepatitis: -

Different researchers have reported the association of various bacteria with hepatitis accompanied by bacteraemia in various case studies and reports. Some frequently reported organisms are as found and listed below: -

Gram-negative bacilli: *E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Salmonella enteritidis*, *Aeromonas hydrophila*, Non-O1 *Vibrio cholerae*, *Salmonella paratyphi B*, *Salmonella typhi*, *Edwardsiella tarda*, *Yersinia enterocolitica*, etc. [Wyke *et al.*, 1982; Worthington *et al.*, 1984; Sharma *et al.*, 1995]

Gram-positive cocci: *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Streptococcus pyogenes*, coagulase-negative staphylococci, *Streptococcus bovis*, *Streptococcus milleri*, etc. [Viscoli *et al.*, 1997; Flannery *et al.*, 2002; Gonzalez-Quintela *et al.*, 2001; Castroagudin *et al.*, 1997]

Gram-positive bacilli: *Listeria monocytogenes*, *Corynebacterium urealyticum*, *Campylobacter jejuni* etc. [Chadwick *et al.*, 1978; Nguyen *et al.*, 1994; Korman *et al.*, 1997]

2.7.2. Bacteria & hepatitis: -

Virus has always been considered the major aetiological agent for causing infectious hepatitis, and routine diagnostic tests for the acute hepatitis involves LFT's and test for viral markers but barely a blood culture! However, various researchers have depicted the picture of bacterial hepatitis, mostly salmonella hepatitis, to be common and more often confusing with the viral hepatitis due to similarity in the clinical picture of the patient (El-Newihi *et al.*, 1996 & Shrestha, 2000).

Bacterial infection should always be considered as a possible cause of deterioration in the clinical picture of hepatobiliary disease; it may be a complication of pre-existing acute or chronic liver or biliary tract disease or a cause of hepatobiliary disease; prompt administration of appropriate antibiotics may save the patient's life (McIntyre, 1984). Liver failure, hepatoma, and cirrhosis may be the outcome when liver function is reduced to such an extent that other body activities are impaired (Wilson, 1990).

Inflammation of the liver may result from infection with bacteria (Peter, 1999). In addition to isolation of bacteria in culture followed by their identification, the evidence of bacterial association may be implied by exclusion of other infective and non-infective causes of acute hepatitis and improvement of patient's symptoms and liver function values after antimicrobial therapy. (Korman *et al.*, 1997)

2.7.3. Bacterial infection in other liver diseases: -

Positive blood cultures were found in 6.4% of cirrhotic patients—both biliary and non-biliary (Jones *et al.*, 1967). The mortality of those with hepato-cellular disease was 59% and the organisms were frequently Gram-positive. This compared with 29% of those with predominantly biliary disease in which infection was often with Gram-negative organisms. Septicaemia is frequent in terminal hepato-cellular failure. Clinical features may be atypical with inconspicuous fever, no rigors and only slight leukocytosis. Spontaneous bacterial peritonitis may be a complication and also bacterial endocarditis (Snyder *et al.*, 1977). These patients have often been receiving previous corticosteroids or neomycin therapy.

E. coli has been found in two-thirds of pyogenic liver abscess. *Streptococcus faecalis*, and *Proteus vulgaris* have also been reported as frequent causative agents. *Salmonella typhi* has been found to cause recurrent pyogenic liver infection. It has also been ascribed as the agent causing bacterial hepatitis (El-Newihi *et al.*, 1996). *E. coli* has also been reported to cause secondary infection following infection due to liver flukes. (Lefkowitz *et al.*, 1982)

Small intestinal flora have been reported to increase in patients with liver diseases. Intoxicant produced by these bacteria may reach the brain without being metabolized by the liver because the damaged cells are unable to metabolize the contents of the portal venous blood completely so that they pass unaltered into the hepatic veins. Urea-splitting bacteria and the small intestinal flora generally are increased in patients with liver disease (Martini *et al.*, 1957).

Anaerobic organisms such as *Bacteroides*, aerobacteria, actinomyces and anaerobic and microaerophilic streptococci have also been found to be significant agents causing pyogenic liver abscess. *Streptococcus milleri* Lancefield group F, which is neither a true anaerobic nor a microaerobe, is reported as a very common causative organisms (Gleeson *et al.*, 1983). Staphylococci were found in nearly a half, especially in those who have received chemotherapy. *Pseudomonas* and *Clostridium welchii* have also been reported to be isolated from pyogenic liver abscess. (Moore-Gillon *et al.*, 1981)

The typhoid hepatitis has been ignored as a clinical entity. The importance of typhoid hepatitis and its clinical significance for the diagnosis of enteric fever is highlighted, particularly at places where viral hepatitis and hepatic amoebiasis are usually common and enteric fever is endemic. (Sharma *et al.*, 1995)

2.7.4. Fever, septicaemia and hepatitis: -

About a third of patients with active, advanced cirrhosis show a continuous low-grade fever which rarely exceeds 38°C (Tisdale *et al.*, 1960). The pyrexia is unaffected by antibiotics or by altering the protein content of the diet. It seems to be attributable to the liver disease alone. It is frequent in alcoholics. (Sherlock, 1987)

2.7.5. Possible portals of entry of bacteria into the liver: -

The possible sources of bacterial infection of the liver are as listed below: (i) Biliary obstruction (cholangitis); (ii) Haematogenous—Portal vein (mesenteric infections) & Hepatic artery (bacteraemia); (iii) Direct extension; (iv) Trauma—Penetrating or non-penetrating; (v) Infection of liver tumor or cyst. (Finlayson, 1999)

The human liver is bacteriologically sterile and the portal venous blood only rarely contains organisms (From *et al.*, 1956). Liver is the bacterial filter and the blood coming from the liver is normally bacteriologically sterile (Sherlock, 1987). Portal circulation carries blood from the gastrointestinal tract into the liver. Blood from liver enters the general circulation via inferior vena cava. Portal system can hence be considered the possible portal of entry for dissemination of bacteria from gastrointestinal organs into the liver and from liver to general circulation.

Such organisms could, however, reach the general circulation either by passing through a faulty hepatic filter or through portal—systemic collaterals (Caroli *et al.*, 1958). Therefore, intercurrent infections, often with coliform organisms, are frequent.

2.7.6. Blood and defense mechanism: -

Blood contains powerful antimicrobial systems, including leukocytes, immunoglobulins and complement. The chemical systems act in the moving blood streams. For eg., antibodies and complement can attach to circulating bacteria. On the other hand cellular defenses such as neutrophils act efficiently only after the blood has delivered them to a suitable site in another tissue (Schaechter *et al.*, 1989). Once evaders penetrate the subepithelial layer, they become prey to the host's second line of defense which includes cellular element of the blood, the lymphatic system and other non-specific resistance factor (Boyd *et al.*, 1980).

2.7.7. Bacterial and viral co-infection: -

Any authorized and published article regarding bacterial and viral co-infection could not be obtained. However, the record of discharge patients in the Liver Unit of Bir Hospital in the year 2058 B.S. shows documentation of two cases having co-infection of HEV and bacteria (Bir Hospital, 2002). There is a probability that aetiological agents of water borne hepatitis—viruses and bacteria may co-infect the vulnerable patients due to their common mode of transmission. (Dr SM Shrestha, LFN—personal communication).

Super-infection with opportunistic pathogens among normal flora is also probable. (Pedersen & Ove, 2000)

2.7.8. Laboratory diagnosis and treatment of bacterial hepatitis: -

The bacterial agents present in the circulating blood of the patients can be detected by applying the standard microbiological techniques. When blood is cultured for the diagnosis of bacteraemia, the number of viable organisms which may be circulating in it is usually small, often about one or two per millilitre, sometimes less (Stokes *et al.*, 1993).

Blood culture system comprise septic collection, culture of blood in broth medium to allow organisms present in small numbers to multiply, a system of combination of system to detect them, and a final phase of subcultures so that the bacteria can be identified and their antibiotic susceptibility determined (Phillips *et al.*, 1990).

Acute infectious viral hepatitis usually improves at its own; less than 1 in 300 patients develop liver failures and risk dying (Shrestha, 2000). However, bacterial infection may lead to various complications in the patient's state and may delay recovery. In the absence of proper diagnosis and treatment, however, the patient's death may occur if infection is severe. Administration of appropriate antibiotic in appropriate order may save the patient's life (McIntyre, 1984).

2.8. ACUTE VIRAL HEPATITIS

Viral hepatitis caused by virus is a systemic disease with primary inflammation in the liver. Viral hepatitis may be also clinically defined as an acute infectious illness in which hepatic cell necrosis is responsible for the most frequent, prominent and characteristic symptoms. This common, important group of worldwide diseases shares clinical, biochemical, and morphological features but has different viral causes. At least six specific viruses appear to be responsible for causing viral hepatitis namely—Hepatitis A Virus (HAV), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Hepatitis D Virus (HDV), Hepatitis E Virus (HEV), and Hepatitis G Virus (HGV).

It is also known that many other viruses may be implicated in hepatitis such as Cytomegalovirus, Epstein-Barr Virus, Yellow Fever Virus and Rubella Virus. Viruses of Herpes Simplex, Varicella and Adenoviruses can also cause severe hepatitis in immunocompromised individuals but are rare (Park, 2000). Liver infections caused by these viruses however are considered separate disorders and generally are not termed acute viral hepatitis. (Pedersen & Ove, 2000)

2.8.1. Hepatitis A Virus (HAV): -

Hepatitis A virus is a single-stranded RNA picornavirus. Viral antigen is found in serum, stool, and liver only during acute infection. IgM antibody appears early in the disease and persists for over 90 days, but diminishes within several weeks, followed by the slow development of protective IgG antibody (anti-HA), which persists usually for many years.

HAV spreads primarily by fecal-oral route. Parenteral transmission is extremely rare, but can follow transfusion of blood from a donor who is in the incubation stage of the disease (Hollinger, Khan & Oefinger, 1983). It may occur as a sexually transmitted infection among homosexual men because of oral-anal contact.

Sero-epidemiological studies carried out in India, Bangladesh, Bhutan and Nepal demonstrated that 85-95% of children have been infected and are immune to HAV infection by 10 years of age. However, only a small number of infected children develop symptoms of disease. In case of Nepal, 55% of the population contract hepatitis A by the age of 5 (Shrestha, 2000). The exact incidence of the disease is difficult to estimate because of the high proportion of asymptomatic cases. However, according to WHO about 10-50 persons per 100,000 are affected annually. Most infections are subclinical or unrecognized. The disease has, in general, a low mortality (0.1%)

Hepatitis A infection never leads to ongoing chronic hepatitis or cirrhosis. Both the sexes are equally susceptible. Immunity acquired after attack probably lasts for life; second attacks have been reported in about 5% of patients.

2.8.2. Hepatitis B Virus (HBV): -

Hepatitis B virus is the most thoroughly characterized and complex etiologic agent. The infective Dane particle consists of a viral core plus an outer surface coat. The core contains circular double-stranded DNA and DNA polymerase, and it replicates within the nuclei of infected hepatocytes. Surface coat is added in the cytoplasm and, for unknown reasons, is produced in great excess; it can be detected in serum by immunologic means as hepatitis B surface antigen (HBsAg), formerly Australia antigen.

At least three distinct antigen-antibody systems are intimately related to HBV:

1. HBsAg is associated with the viral surface coat; its presence in serum is usually the first evidence of acute HBV infection and implies infectivity of the blood. HBsAg characteristically appears during the incubation period, usually 1 to 6 wk before clinical or biochemical illness develops, and disappears during convalescence. The corresponding protective antibody (anti-HBs) appears weeks or months later, after clinical recovery, and usually persists for life; thus, its detection indicates past HBV infection and relative immunity. In up to 10% of patients, HBsAg persists after acute infection, and anti-HBs does not develop; these patients usually develop chronic hepatitis or become asymptomatic carriers of the virus.
2. Core antigen Core antigen (HBcAg) is associated with the viral core. It can be found in infected liver cells but is not detectable in serum except by special techniques that disrupt the Dane particle. Antibody to HBcAg (anti-HBc) generally appears at the onset of clinical illness; thereafter, titers gradually diminish, usually for years or life. Its presence with anti-HBs is not significant beyond indicating previous HBV infection. It is also regularly found in chronic HBsAg carriers, who do not mount an anti-HBs response. In chronic infection, anti-HBc is mainly of the IgG class, whereas in acute infection, IgM anti-HBc predominates. Occasionally, IgM anti-HBc is the only marker of recent HBV infection, reflecting a "window" between disappearance of HBsAg and appearance of anti-HBs.
3. The e antigen (HBeAg) appears to be a peptide derived from the viral core. Found only in HBsAg-positive serum, HBeAg tends to parallel the production of viral DNA polymerase. Its presence, therefore, reflects more active viral replication and is

generally associated with greater infectivity of the blood and a greater likelihood of progression to chronic liver disease. In contrast, presence of the corresponding antibody (anti-HBe) points to relatively lower infectivity and usually portends a benign outcome.

HBV is often transmitted parenterally, typically by contaminated blood or blood products. Hepatitis B virus causes at least 80% of posttransfusion hepatitis cases and a substantial proportion of sporadic acute hepatitis cases. Routine screening of donor blood for HBsAg has dramatically diminished posttransfusion HBV infection, but transmission via needles shared by drug abusers remains an important problem. Up to 50% of cases are related to intravenous drug users who share needles. The risk is increased for patients in renal dialysis and oncology units and for hospital personnel in contact with blood.

Nonparenteral spread occurs between both heterosexual and male homosexual partners and in closed institutions (eg, those for the mentally retarded and prisons). The risk of sexual and maternal-neonatal transmission is small (Park, 2000). The role of transmission by insect bites is unclear. A low rate of secondary transmission to household contacts has been recognized. Many cases of acute hepatitis B occur sporadically without a known source.

For healthcare workers it is an occupational hazard requiring adherence to universal precautions. Traditional practices such as circumcision, tattooing and scarification with contaminated instruments can spread HBV infection.

Chronic HBV carriers provide a worldwide reservoir of infection. Prevalence varies widely according to several factors, including geography and other epidemiologic factors, including previous use of illicit drugs. Vertical transmission from mother to infant is partly responsible, especially where prevalence is high.

HBV is associated with a wide spectrum of liver diseases, from a subclinical carrier state to acute hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma. It is clinically characterized by a tendency to a long incubation period (6 weeks to 6 months) and a protracted illness with a variety of outcomes. Usually, it is an acute self-limiting infection,

which may be either subclinical or symptomatic. In approximately 5-15% of cases, HBV infection fails to resolve and the affected individuals then become persistent carriers of the virus. Persistent HBV infection may cause progressive liver disease including chronic active hepatitis (a high rate of >50%) which may lead to cirrhosis of liver or hepatocellular carcinoma and is more likely to do so in men than in women, and in alcohol consumers. There is also evidence of a close association between hepatitis B and primary liver cancer (Spira *et al.*, 1980). Hepatitis B virus can form a dangerous alliance with delta virus and produce a new form of virulent hepatitis which is considered to be a widespread threat for much of the world.

Human is the only reservoir of infection which can be spread either from carriers or from cases. The risk of an adult becoming a carrier following acute infection is 5-10%; in infants, it may exceed 50%. Contaminated blood is the main source of infection, although the virus has been found in body secretions like saliva, vaginal secretions and semen of infected persons.

The virus is quite stable and capable of surviving for days on environmental surfaces. It can be readily destroyed by sodium hypochlorites, as is by heat sterilization in an autoclave for 30 to 60 minutes.

In countries in which infection with HBV is relatively common, the highest prevalence of the surface antigen is found in the 20-40 year age group. In countries, where infection with HBV is common, much infection occurs perinatally or during early childhood. Certain groups carry higher risks. For example, in USA, the annual incidence of HBV infection in surgeons is estimated to be 50 times greater than in the general population, and is more than twice that of other physicians. Other high risk groups comprise recipients of blood transfusions, health care and laboratory personnel, HBV carrier mothers and patients who are immunocompromised (Alter *et al.*, 1994).

Antibodies form in a week or two after onset of jaundice—the order being, first core antibody, then “e” antibody and much later surface antibody. The appearance of surface antibody signals recovery from HBV infection and the development of immunity.

The HBV infection is a global problem, with 66% of all the world's population living in areas where there are high levels of infection. It is endemic throughout the world, especially in tropical and developing countries and also in some regions of Europe. Its prevalence varies from country to country and depends upon a complex mix of behavioural, environmental and host factors. In general, it is lowest in countries or areas with high standard of living. More than 2 billion people world wide have evidence of past or current HBV infection and 350 million are chronic carriers of the virus, which is harboured in the liver, the virus causes 60-80% of all primary liver cancer, which is one of the three top causes of cancer death in East and SEAR, the Pacific Basin and Sub-Saharan Africa (Masley, 1970).

Infection with HBV is a major cause of morbidity and mortality in the SEAR. More than one-third of the population has been infected with HBV, and it is estimated that there are 80 million HBV carriers (about 6% of total population). Many are life long carriers, although not all are infectious, and some will clear the virus after intervals varying from many months to years. Between 5-10% of adults and up to 80% of infants, infected with HBV will become carriers. Among these, 25% will, in the long term, develop serious liver disease. (Park, 2000)

Approximately 14-16 million people are infected with HBV each year in the region. It is estimated that approximately 2 lakh deaths associated with acute HBV infection and related disease occur in the Region annually. Transmission of HBV infection by blood transfusion and other medical interventions in both modern and traditional health practices is also common in the region.

The national five-yearly average data shows that the incidence of acute hepatitis B was 9% in 1982-1986; it rose to 15% in 1987-1991 and 1992-1996 and 36% in 1997. It suggests that we may be witnessing an emergence of epidemic of hepatitis B in Nepal. At present, about 200 new cases of active hepatitis B are recorded annually, and hepatitis is responsible for 34% of liver cancer in Nepal (Shrestha, 2000).

2.8.3. Hepatitis C virus (HCV): -

Hepatitis C virus (HCV), identified in the year 1989, has been shown to be the major cause of parenterally transmitted non-A, non-B (PT-NANB) hepatitis (Spira, 1980). This single-stranded, flavivirus-like RNA agent causes the vast majority of posttransfusion and sporadic NANB hepatitis. Most cases of hepatitis C are subclinical, even in the acute stage. The infection has a much higher rate of chronicity (about 75%) than hepatitis B. Thus, hepatitis C is often uncovered by the serendipitous detection of anti-HCV in apparently healthy persons.

WHO estimates that 3% of the world population is infected with HCV and around 170 million individuals are chronic carriers at risk of developing liver cirrhosis and liver cancer. In many countries, particular population subgroups such as voluntary blood donors have a very high prevalence of HCV infection especially 4 million people have contracted the disease, 4 times more than HIV infection. Approximately 30,000 new acute infections and 8000-10,000 deaths occur each year. It has also become a leading reason for liver transplantation ((Masley, 1970). Incubation period of HCV infection is about 3 to 16 wk.

The annual incidence of HCV infection in SEAR countries is largely unknown, primarily because over 50% of infectious cases are asymptomatic. In addition, many symptomatic acute HCV cases are not laboratory-confirmed since testing of patients for HCV markers is not commonly done.

2.8.4. Hepatitis D virus (HDV): -

Hepatitis D virus or delta agent, is a unique, defective RNA virus that can replicate only in the presence of HBV, never alone. It occurs as a co-infection with acute hepatitis B or as a superinfection in established chronic hepatitis B. Infected hepatocytes contain delta particles coated by HBsAg. Delta hepatitis has not been reported as significant in Nepal. Shrestha, 2000)

2.8.5. Hepatitis E virus (HEV): -

Hepatitis E virus, formerly termed enterically transmitted hepatitis non-A, non-B (HNANB), is a 29-nm to 32-nm non-enveloped, single-strand RNA virus. It belongs to the family Calciviridae and genus Calcivirus. The infection caused by the hepatitis E virus (HEV) which was discovered in 1990, is essentially a waterborne disease. HEV Outbreaks have occurred exclusively in developing countries. The infection may be severe, especially in pregnant women, but chronicity does not occur and there is no known chronic carrier state.

HEV replicates in the hepatocytes and is excreted in stool. Transmission is predominantly by the fecal-oral route, usually through contaminated water. Although person-to-person transmission is rare, maternal-neonatal transmission has been documented. The course of HEV is usually more severe than other forms of epidemic jaundice. The fatality rate is generally 1% to 2%. It is, however, a very concerning illness in pregnant women as it may cause liver failure with a mortality rate of 20%. Prolonged viraemia or fecal shedding are unusual and chronic infection does not occur.

The incubation period ranges from 15 to 60 days. The virus is detected in stools as early as 1 week prior to onset of clinical illness and persists for 1 to 2 weeks afterwards, during which stools are highly infectious.

Anti HBE antibody (IgM) appears soon after the onset of the clinical infection, immunoglobulin G (IgG) soon after that and IgG remains detectable for as long as 20 months.

Water or food supplies contaminated by faeces in which the virus is excreted have been implicated in major outbreaks reported in all parts of the world that have a hot climate. After an incubation period of 2-9 weeks, a self-limiting acute viral hepatitis appears, lasting for a period of several weeks, which is followed by recovery. No case of chronic disease has been reported. Mainly young adults, aged 15-40 years, have been affected by acute hepatitis E (Shrivastava, 1990).

In addition, HEV has a propensity to induce a fulminating form of acute disease (the mortality ranges between 0.5% to 4%), particularly in pregnant women, up to 20% of whom may develop fulminant hepatitis E, with a mortality that may reach about 80% of such cases.

2.8.6. Prevalence of HEV infection & water quality and sanitation: -

Hepatitis E appears to be widespread problem in developing countries where there are problems in providing safe drinking water and adequate sewage disposal (Park, 2000).

It is estimated that three quarters of the people in the developing world do not have reasonably safe supply of drinking water. In a 1975 estimate, only 22% (313 million) out of 1,422.72 million rural people in 75 developing countries (excluding China) had reasonable access to safe water. The remaining 78% have to rely on various sources of water supply, mostly contaminated and unsafe (Obeng, 1982). Sidwick, 1985, has written that over 1800 M people in developing countries lack a reasonable supply of water and at least 2400 M people have no sanitation facilities.

Millions die each year in the developing countries from diseases spread by contaminated water. 80% of all sickness in the world is attributable to inadequate water or sanitation, according to WHO estimates. Over 1.5 billion people in the Third World countries of Asia, Africa, and Latin America-75% of the population lack a supply of safe water for drinking and washing and/or adequate sanitation (Morrison, 1984).

Worldwide, the lack of sanitary waste disposal and of clean water for drinking, cooking, and washing is to blame for over 12 million deaths a year and 2.3 billions suffer water related diseases. Millions of people have little access to sanitary waste disposal or to clean water for personal hygiene. An estimated 3 billion people lack a sanitary toilet, for example. Over 1.2 billion people are at risk because they lack access to safe freshwater (Popline, 1998).

In Nepal only 3% of the urban population have access to a sewerage system with another 10% having access to some sort of excreta-disposal system, often a totally inadequate one; the remaining 87% of the urban dwellers have nothing; and in urban core-areas there are no fields. (Maharjan, 1998)

The infection caused by the hepatitis E virus (HEV) which was discovered in 1990, is essentially a waterborne disease. HEV Outbreaks have occurred exclusively in developing countries. Water or food supplies contaminated by faeces in which the virus is excreted have been implicated in major outbreaks reported in all parts of the world that have a hot climate.

HEV is distributed in endemic and epidemic forms in Southeast and central Asia, the Middle East, Africa and Mexico. These epidemics appear epidemiologically similar to HAV epidemics. Sporadic cases also occur, probably via enteric transmission.

The first major epidemic was reported in New Delhi in the winter of 1955-56. After the flooding of Yamuna river, 30,000 cases of jaundice were described, and retrospectively attributed to hepatitis E. China reported 100,000 cases of jaundice between 1986 and 1988. Since then, additional outbreaks have been reported from Borneo, India, Indonesia, Mexico, Nepal, Pakistan etc. However, Hepatitis E outbreaks or even sporadic cases are rare in temperate climates.

Hepatitis E infection occurs in Kathmandu valley as epidemic, sporadic, or focal outbreak. In 1973 A.D., hepatitis E epidemic led to the death of about 10,000 people. The second epidemic was reported in 1981 and 1982 with more than 12,000 cases and the third epidemic occurred in 1987 with 7405 cases with similar epidemiological features (Shrestha, 2000). Similarly, focal outbreak of Hepatitis E has been common in police training center, central jail or military barracks from time to time. HEV hepatitis causes substantial morbidity due to its acute presentation and prolonged convalescent period. Hepatitis E disease rates in the Kathmandu valley were estimated to be 20/1000 person-years. Acute hepatitis due to E virus is found to be very severe and fatal. It transmits by faecal-oral route, and there is no vaccine against it (Shrestha, 2000).

2.8.7. Hepatitis G virus (HGV): -

Hepatitis G virus is a new flavivirus-like agent that has been detected in a few cases of non-A-E hepatitis. HGV can apparently be transmitted by blood and may be responsible for some cases of chronic hepatitis. The role of HGV and other unidentified agents in cases of unexplained hepatitis remains unclear.

2.8.8. Laboratory findings: -

Striking aminotransferase elevations are the hallmark of the disease. High values appear early in the prodromal phase, peak before jaundice is maximal, and fall slowly during the recovery phase. AST and ALT are typically 500 to 2000 U/L, although correlation with clinical severity is poor. The ALT is typically higher than the AST, but this is of only limited value in differentiation from alcoholic hepatitis, in which the reverse is usual. Urinary bile usually precedes jaundice; its early detection is valuable for diagnosis. The degree of hyperbilirubinemia is variable, and fractionation is of no clinical value. Alkaline phosphatase is only moderately raised unless cholestasis is severe. Major prolongation of the prothrombin time is uncommon and, if present, portends a severe illness. The WBC count is usually low-normal, and a blood smear often shows a few atypical lymphocytes.

2.8.9. Diagnosis of acute viral hepatitis: -

First steps in the diagnosis of the jaundiced patient involve clinical history and examination; urine and stool examination; serum biochemical tests, including bilirubin, transaminases, albumin, quantitative immunoglobulin, alkaline phosphatase, total cholesterol; haematology, including haemoglobin, WBC, platelets; blood film, and prothrombin time (Sherlock, 1987).

Tests most useful in the diagnosis of hepatitis are the serum alkaline phosphatase level, serum transaminase (Alanine aminotransferase, SGPT and aspartate aminotransferase, SGOT) values, and serum bilirubin (total and conjugated) level (O'Connor, Snodgrass, 1983).

The serum bilirubin level confirms jaundice. Jaundice might arise in acute and chronic hepatitis. Rapid onset of pruritus followed by slow fall with recovery, slight weight loss and hepatic tenderness accompany the jaundice in acute virus hepatitis (Sherlock, 1987).

Increased transaminase level, Leucopenia with lymphocytosis, bilirubinaemia, yellow urine, yellow discolouration of sclera and nails, dark urine and pale to dark coloured stool often accompany hepatitis.

The HAV can be identified in the stool of sufferers from about two weeks before until one week or less after the onset of jaundice. A serum antibody (anti-HAV) appears as the stool becomes negative for virus, reaches a maximum in several months and is detectable for many years. Anti-HAV probably gives immunity from further infection with hepatitis A. The appearance of serum IgM anti-HAV is more helpful diagnostically and implies a recent infection. This antibody persists for only 2-6 months.

Hence, a specific laboratory diagnosis of hepatitis A can be obtained by demonstration of HAV particles or specific viral antigens in the faeces, demonstration of a rise in anti-HAV titre, detection of IgM antibody to HAV in the patient's serum; this antibody appears early in the illness, and persists for a limited time, usually 3 to 4 months after onset; and IgG antibody indicates past infection and immunity. Hepatitis A is diagnosed by detecting IgM antibody; as noted above, IgG anti-HA is merely a marker of remote exposure and does not indicate current HAV infection.

Hepatitis B is specifically diagnosed by identifying HBsAg in serum, with or without concomitant anti-HBc. In cases in which antigenemia may be transient, the isolated presence of IgM anti-HBc may establish the diagnosis.

Diagnosis of hepatitis C is based on the presence of serum antibody (anti-HCV), which is not protective and implies active infection. First-generation serologic tests were often falsely positive, but newer second- and third-generation tests are more reliable. Anti-HCV often appears several weeks after acute infection, so a negative test does not exclude recent infection.

For the diagnosis of HCV infection, the diagnosis of acute NANB hepatitis must first be established in persons with signs and symptoms consistent with acute hepatitis by ruling out acute HAV and HBV infections. Currently, only immunoassays for antibodies to part of the non-structural region of HCV (anti-HCV) are available, as well as supplemental recombinant immunoblot assay (RIBA) tests used to confirm anti-HCV positive results.

The disease due to HEV is frequently cholestatic, with bilirubin and alkaline phosphatase elevations. The presence of anti-HEV antibody (IgM) in the serum confirms the disease and persists for at least 6 weeks after the peak of the illness. IgG antibody peaks soon after that and remains detectable for as long as 20 months.

2.8.10. Treatment: -

In most cases, no special treatment is required. Appetite usually returns after the first several days, and patients need not be confined to bed. Undue restrictions on diet or activity have no scientific basis. Vitamin supplements are not required. Corticosteroids are contraindicated in ordinary cases. Most patients may safely return to work after jaundice resolves, even if AST or ALT is not entirely normal.

2.8.11. Prophylaxis: -

Personal hygiene helps to prevent spread of HAV. Careful handling of blood of acute hepatitis patients and stool of patients with hepatitis A is essential. Posttransfusion infection can be minimized by avoiding unnecessary transfusions, using volunteers rather than paid donors, and screening all donors for HBsAg and anti-HCV.

Vaccination is advisable for persons at increased risk of contacting hepatitis B, eg, patients and staff in hemodialysis units, medical and other health care personnel exposed to blood, dentists and dental hygienists, residents and staff of institutions for the mentally retarded, and male homosexuals. Vaccine against HAV has recently become available; it is safe and effective and provides more prolonged protection than prophylactic immune globulin (probably for several years). Travelers to highly endemic areas should therefore receive the vaccine. There is no vaccine against HCV.

CHAPTER – THREE

MATERIALS & METHODS

Study population:

The study population included randomly selected clinically suspected acute hepatitis cases of all age group; attending Liver Foundation Nepal, Liver Unit of Bir Hospital, Nepal Ayurveda Hospital and Sukraraj Tropical & Infectious Disease Hospital during 2001 August-2002 August. Clinically suspected acute hepatitis cases with a rise in SGPT/ALT twice the normal limit were involved in the study. Clinical signs & symptoms including anorexia, nausea, malaise, abdominal pain, dark urine, and jaundice were considered as acute hepatitis cases. Patients with a history of chronic hepatitis, exposure to hepatotoxic drugs/chemicals or chronic alcohol use were excluded.

Data collection:

The relevant data of all the patients, including personal informations (name, address, age, sex, socioeconomic status), short clinical history and presenting signs and symptoms were recorded in the schedule either from direct interview or from the medical records in the respective collaborative health institutions as applicable; and the laboratory findings were documented in the laboratory record sheet. (Appendix I)

Specimen collection and handling:

Ten millilitre of blood was collected (Appendix II) and 5 mL of it was dispensed to a culture bottle containing 50 mL BHI broth. Bed-site & on-site inoculation was practiced for admitted patients and out patients respectively. The culture bottles were then subjected to bacterial culture and sensitivity testing following standard protocol (Baron *et al.*, 1990).



Photograph 1: History taking.



Photograph 2: Data collection from patient's profile.



Photograph 3: Blood collection in BHI bottle.

Three and 2 mL of blood sample were collected in separate plain vial and EDTA vial respectively. Serum separated (Collee *et al.*, 1989) was assayed for detection of HBsAg (Appendix VI), anti-HEV IgM (Appendix VII), anti-HCV antibody (Appendix VIII), ALT (Appendix IX), total bilirubin & conjugated bilirubin (Appendix X); and leukocyte count (Cheesbrough, 1984) was performed using anticoagulated blood from EDTA vial.

The samples were processed at laboratory of the Central Department of Microbiology, Tribhuvan University and Liver Foundation Nepal.

Sample processing:

A. BACTERIOLOGICAL EXAMINATION

3.1. Processing of sample for isolation/identification of bacteria:

Five millilitre of collected blood was aseptically inoculated at bed site into 50 ml of sterile BHI broth in blood culture bottles that were processed for culture by incubating at 37°C for up to 7 days as per standard protocol (Collee *et al.*, 1989).

Macroscopic examination of broth culture bottle: The culture bottles were examined daily for any visual evidence of microbial growth; such as turbidity, haemolysis of red cells, gas production, clot formation or formation of discrete colony. This helped in making presumptive diagnosis of positive broth cultures as shown in Table below:

Table 3.1: Macroscopic examination of broth culture bottles. (Tibrewal, 1999)

Observation	Presumptive Diagnosis
1. Uniform turbidity with gas bubble formation.	Enteric Gram-negative bacilli
2. Greenish discolouration.	<i>Pseudomonas spp.</i>
3. Partial haemolysis of blood with greenish tinge.	<i>Streptococcus spp.</i>
4. Haemolysis with cotton ball colony.	<i>Streptococcus spp.</i>
5. Jelly like coagulum throughout the broth, discrete colonies on the surface of red cell layer and in the broth.	<i>Staphylococcus spp.</i>

Microscopic examination: The microscopic examination was performed after preparing Gram-stain smear for the macroscopically positive cultures in order to make the presumptive diagnosis of bacterial infection. (Appendix II)

Subculture from broth culture: The broth cultures were subcultured on Blood Agar, MacConkey Agar, Nutrient Agar and Chocolate Agar plates. Repeated subcultures of the culture bottles were made at 24, 48, 72, 96 hours up to 7 days, till growth was negative. (Collee *et al.*, 1989)

All the subculture plates were incubated aerobically at 37°C in an incubator. The subculture plates were then examined after 18-72 hours of incubation. Morphological, biochemical & metabolic characteristics of the bacterial colony were studied and noted as in result section.

Identification of isolated organisms: Grown colonies were identified on the basis of morphological, biochemical & metabolic reactions as per the standard protocols (Cheesbrough, 1984; Barrow *et al.*, 1993; Reeler, 1982).

3.3. Antibiotic susceptibility tests of isolated organisms:

Antibiotic susceptibility pattern of the isolates was studied using Kirby Bauer method (Bauer *et al.*, 1966). Mueller Hinton agar (Hi-Media) was used for antibiotic susceptibility testing. (Appendix IV & V)

3.4. Quality control for the test (Cheesbrough, 1984):

Quality control of media, sample collection, processing, antibiotic susceptibility testing were done as per standard protocol (Cheesbrough, 1984).

B. SEROLOGY FOR TESTING VIRAL MARKERS

3.5. Detection of hepatitis B surface antigen (HBsAg) in patients' sera:

The quantitative detection of Hepatitis B surface antigen (HBsAg) based on reversed passive haemagglutination (RPHA) was done as per manufacturer's instruction. (Appendix VI)

3.6. Detection of anti-hepatitis E virus antibody:

Anti-HEV IgM was assayed using greiner ELISA-F kits produced by the Institute of Hepatology, Tokyo. (Appendix VII)

3.7. Detection of anti-hepatitis C virus antibody:

Anti-Hepatitis C Virus antibody (anti-HCV) was detected using Ortho HCV 3.0 ELISA as per manufacturer's instruction. (Appendix VIII)

C. LIVER FUNCTION TESTS

Serum alanine aminotransferase, ALT (Appendix IX); and total bilirubin were analyzed using Human kit. (Appendix X)

D. HAEMATOLOGY

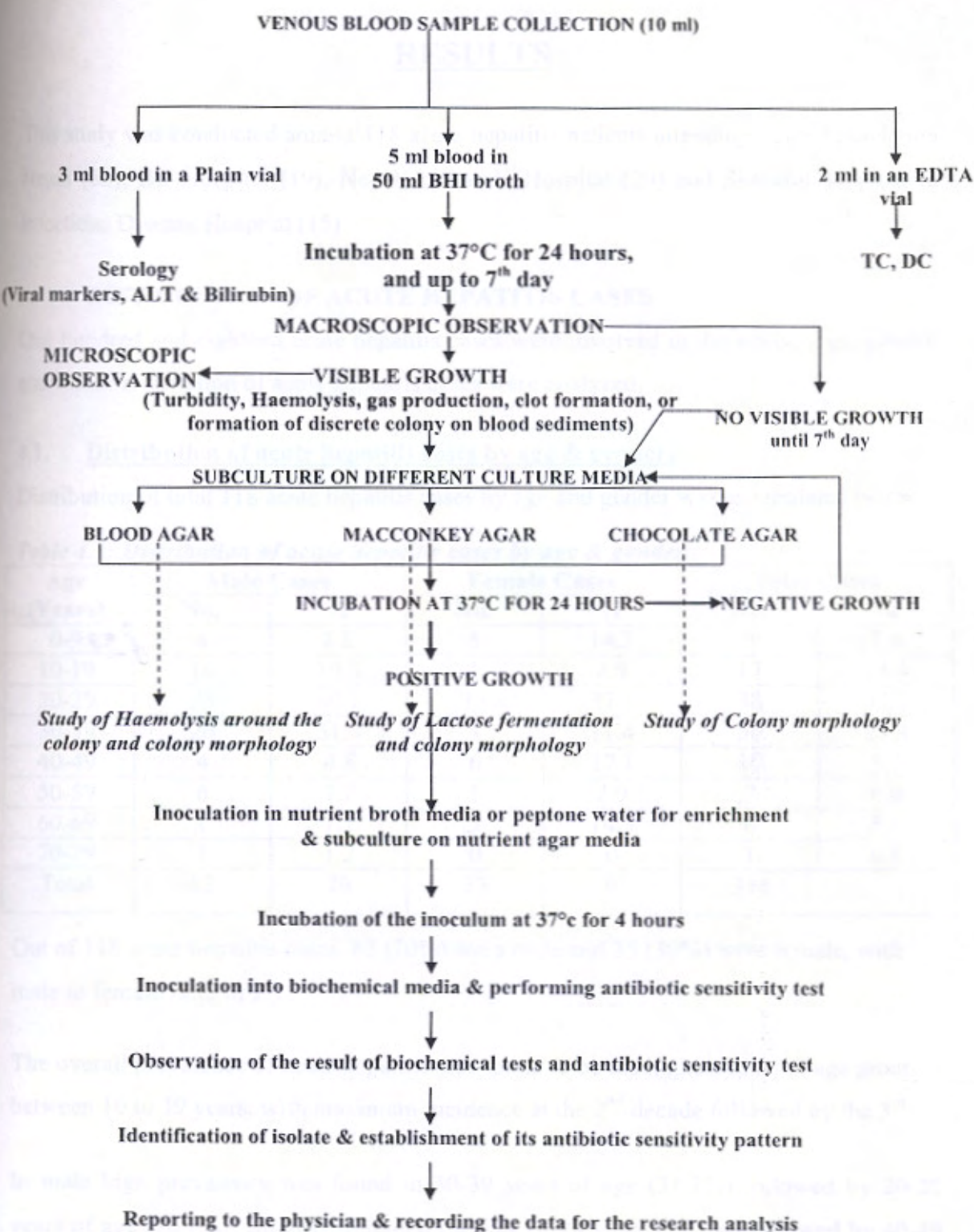
Total and differential leukocyte count of the suspected patients were performed using standard methodology. (Cheesbrough, 1984)



Photograph 4: Sample processing for bacterial identification.



Photograph 5: Microscopic investigation.

SAMPLE PROCESSING FLOW CHART

CHAPTER – FOUR

RESULTS

This study was conducted among 118 acute hepatitis patients attending Liver Foundation Nepal (55), Bir Hospital (19), Nepal Ayurveda Hospital (29) and Sukraraj Tropical & Infectious Disease Hospital (15).

A. DISTRIBUTION OF ACUTE HEPATITIS CASES

One hundred and eighteen acute hepatitis cases were involved in the study. Age, gender, and seasonal variation of acute hepatitis cases were analyzed.

4.1. Distribution of acute hepatitis cases by age & gender:

Distribution of total 118 acute hepatitis cases by age and gender was as tabulated below:

Table 4.1: Distribution of acute hepatitis cases by age & gender.

Age (Years)	Male Cases		Female Cases		Total Cases	
	No.	%	No.	%	No.	%
0-9	4	4.8	5	14.3	9	7.6
10-19	16	19.3	1	2.9	17	14.4
20-29	25	30.1	13	37.1	38	32.2
30-39	26	31.3	4	11.4	30	25.4
40-49	4	4.8	6	17.1	10	8.5
50-59	6	7.2	1	2.9	7	6.0
60-69	1	1.2	5	14.3	6	5.1
70-79	1	1.2	0	0	1	0.8
Total	83	70	35	30	118	

Out of 118 acute hepatitis cases, 83 (70%) were male and 35 (30%) were female, with male to female ratio of 2:1.

The overall prevalence of acute hepatitis was found to be the highest in adult age group between 10 to 39 years, with maximum incidence at the 2nd decade followed by the 3rd.

In male high prevalence was found in 30-39 years of age (31.3%) followed by 20-29 years of age (30.1%); whereas in females it was 20-29 years (37.1%) followed by 40-49 years (17%).

Figure 4.1: Distribution of acute hepatitis cases by age & gender.

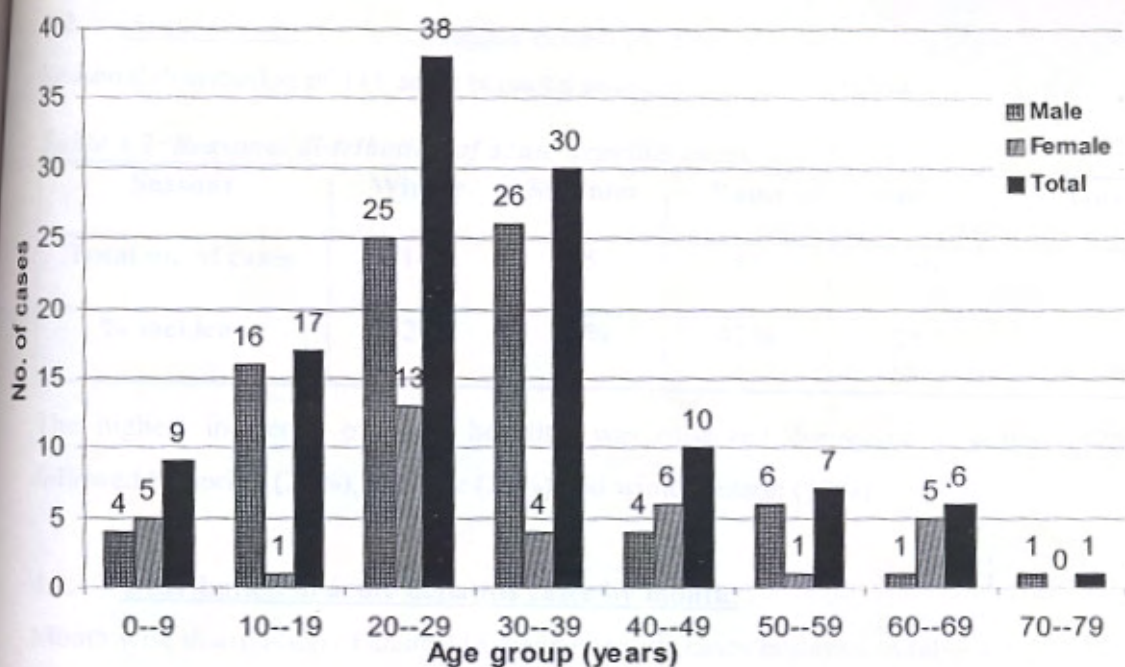
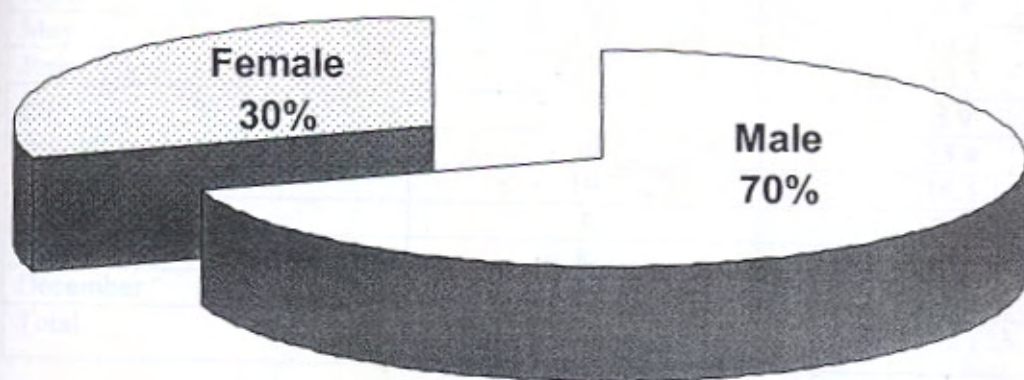


Figure 4.2: Proportional distribution of acute hepatitis cases by gender.



4.2. Seasonal distribution of acute hepatitis cases:

Seasonal distribution of 118 acute hepatitis cases is as tabulated below:

Table 4.2: Seasonal distribution of acute hepatitis cases.

Seasons	Winter	Summer	Rainy	Spring	Total
Total no. of cases	14	25	49	30	118
% incidence	12%	21%	42%	25%	

The highest incidence of acute hepatitis was observed during rainy season (42%), followed by spring (25%), summer (21%) and winter season (12%).

4.3. Distribution of acute hepatitis cases by month:

Month wise distribution of acute 118 acute hepatitis cases is shown in table 4.3.

Table 4.3: Distribution of acute hepatitis cases by month.

Month	No. of cases	Percentage (%)
January	1	0.8
February	5	4.2
March	5	4.2
April	8	6.8
May	12	10.2
June	12	10.2
July	7	5.9
August	30	25.4
September	19	16.1
October	3	2.5
November	8	6.8
December	8	6.8
Total	118	

Maximum samples from acute hepatitis cases (25.4%; 30/118) could be collected in the month of August, followed respectively by September (16.1%; 19/118); May & June (each 10.2%, 12/118); July (5.9%, 7/118), April, November and December (each 6.8%, 3/118); February and March (each 4.2%, 5/118); October (2.5%, 3/118) and only 1 (0.8%) sample could be collected in the month of January.

Figure 4.3: Seasonal distribution of acute hepatitis cases.

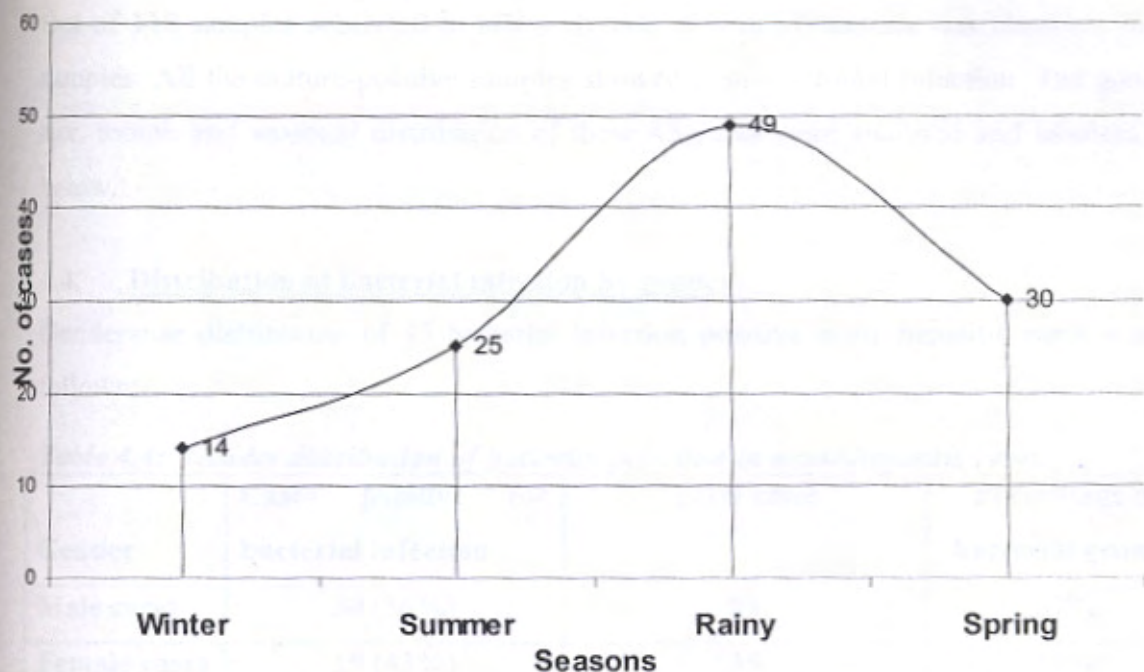
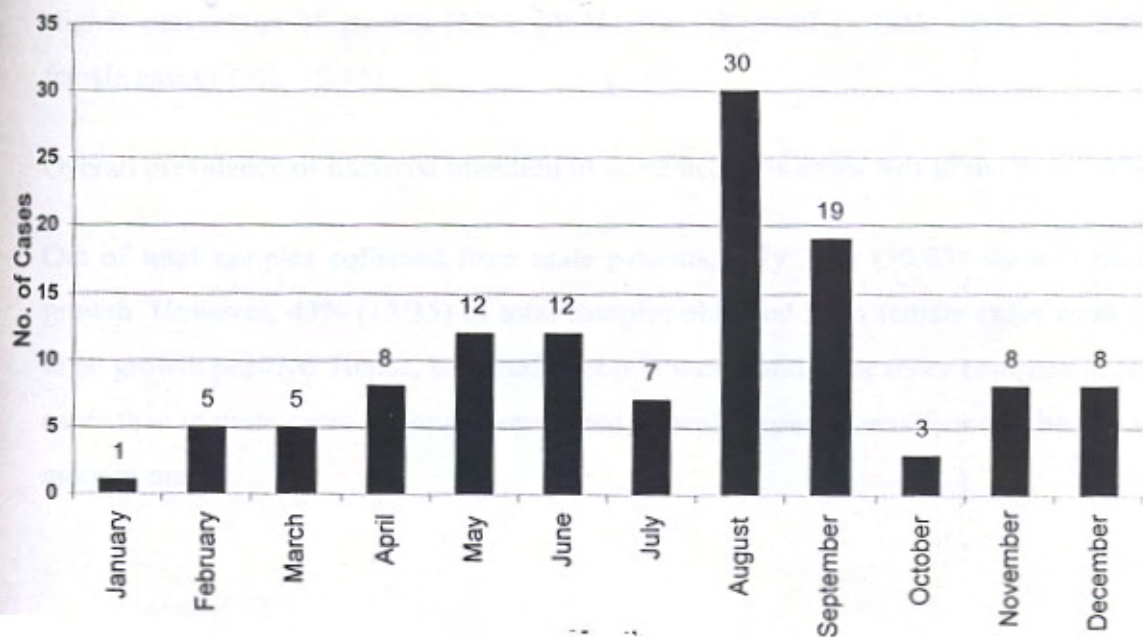


Figure 4.4: Distribution of acute hepatitis cases by month.



B. PREVALENCE OF BACTERIAL INFECTION IN ACUTE HEPATITIS

Out of 118 samples subjected to blood culture, growth of bacteria was observed in 45 samples. All the culture-positive samples showed monomicrobial infection. The gender, age, month and seasonal distribution of these 45 cases were analysed and tabulated as below.

4.4. Distribution of bacterial infection by gender:

Genderwise distribution of 45 bacterial infection positive acute hepatitis cases was as follows:

Table 4.4: Gender distribution of bacterial infection in acute hepatitis cases.

Gender	Cases positive for bacterial infection	Total cases	Percentage of bacterial growth
Male cases	30 (36%)	83	67%
Female cases	15 (43%)	35	33%
Total cases	45 (38%)	118	

Male : Female ratio = 2:1

Higher percentage of growth (67%, 30/45) was observed in male cases compared to female cases (33%, 15/45).

Overall prevalence of bacterial infection in acute hepatitis cases was found to be 38%.

Out of total samples collected from male patients, only 36% (30/83) showed bacterial growth. However, 43% (15/35) of total samples obtained from female cases were found to be growth positive. Hence, bacterial infection was found to be more common in female cases than in male cases although prevalence of acute hepatitis was found to be two times more in male.

4.5. Distribution of bacterial infection by age:

Age-wise distribution of 118 acute hepatitis cases is tabulated as follows:

Table 4.5: Age group distribution of bacterial infection in acute hepatitis cases.

Age-group (Years)	Bacterial infection cases	No. of total cases
0-9	4 (9%)	9
10-19	8 (18%)	17
20-29	15 (33%)	38
30-39	10 (22%)	30
40-49	5 (11%)	10
50-59	1 (2%)	7
60-69	1 (2%)	6
70-79	1 (2%)	1
Total	45 (38%)	118

High prevalence of bacterial infection was observed in the age group 20-29 years (33%) followed by 30-39 years (22%), 10-19 years (18%), 40-49 years (11%) and 0-9 years (9%). Only 1 case (2%) each belonging to age groups 50-59, 60-69, and 70-79 revealed bacterial infection.

Figure 4.5: Proportional distribution of bacterial infection by gender.

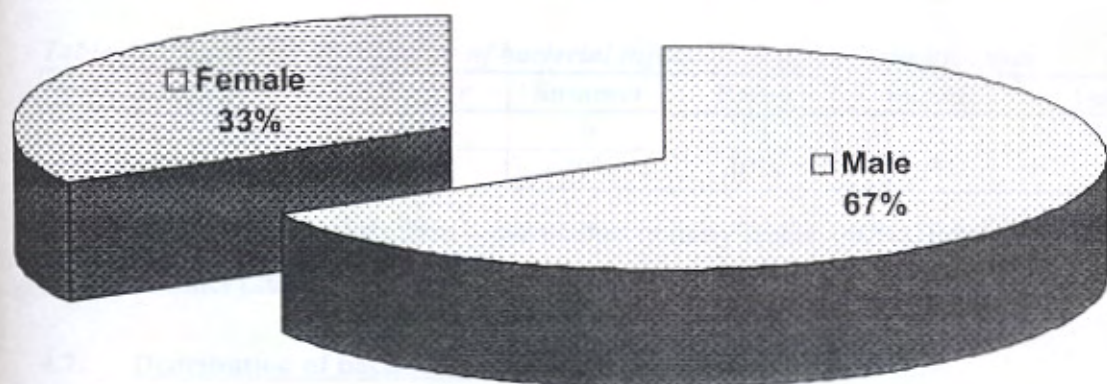
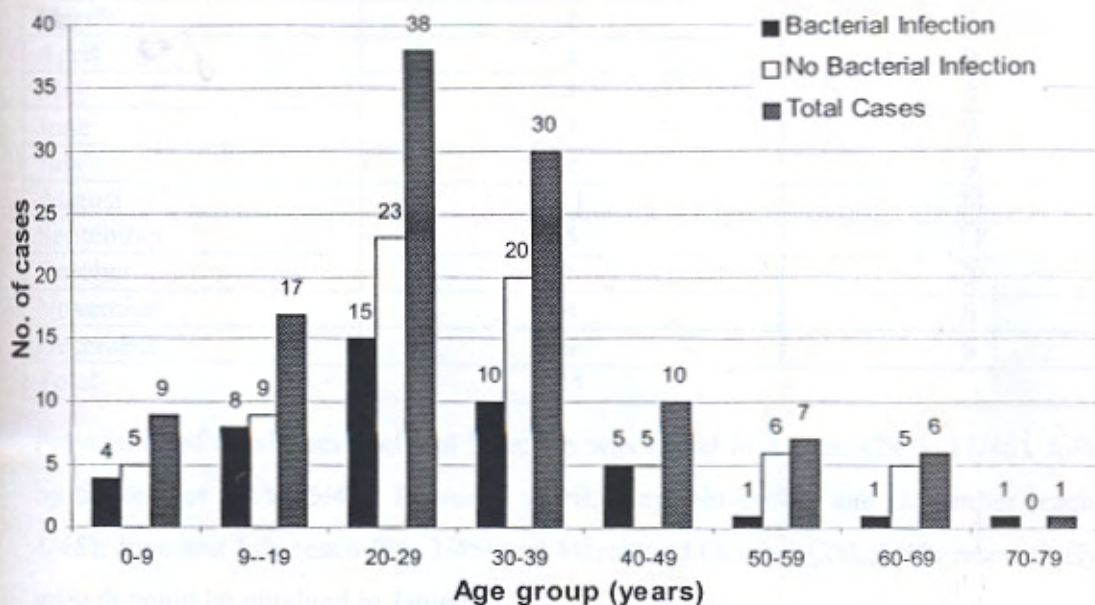


Figure 4.6: Age distribution of bacterial infection.



4.6. Seasonal distribution of bacterial infection:

Seasonal distribution of 45 bacterial infection cases was as shown below.

Table 4.6: Seasonal distribution of bacterial infection in acute hepatitis cases.

Seasons	Winter	Summer	Rainy	Spring	Total
Total no. of cases	8	9	18	10	45
% of cases	18%	20%	40%	22%	

The maximum bacterial infection occurred during rainy season (40%), followed by spring (22%), summer (20%) and winter season (18%).

4.7. Distribution of bacterial infection by month:

Month wise distribution of bacterial infection in acute hepatitis cases was found as follows:

Table 4.7: Distribution of bacterial infection in acute hepatitis cases by month.

Month	No. of Cases	%
January	0	0
February	4	9
March	1	2
April	4	9
May	4	9
June	3	7
July	4	9
August	11	24
September	5	11
October	1	2
November	4	9
December	4	9
Total	45	

Prevalence of maximum bacterial infection was found in August (24%, 11/45), followed by September (11%, 5/45); February, April, May, November and December (each 9%, 4/45); June and July (each 7%, 3/45) and March and October (2%, 1/45) respectively. No growth could be obtained in January.

Figure 4.7: Seasonal distribution of bacterial infection.

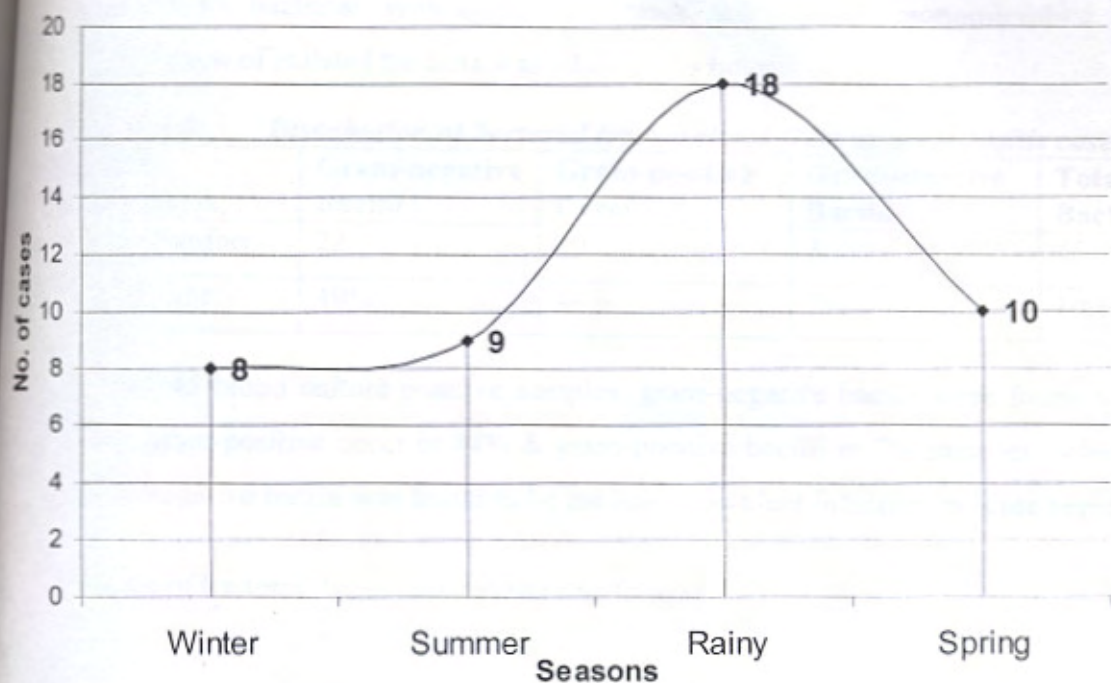
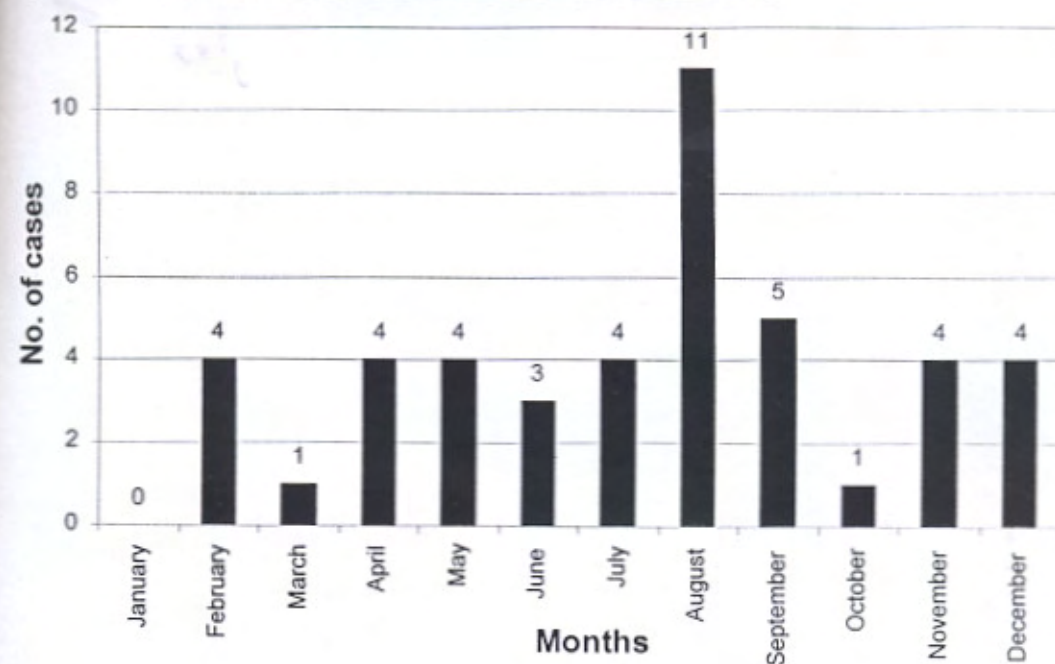


Figure 4.8: Distribution of bacterial infection by month.



C. DISTRIBUTION OF BACTERIAL PATHOGENS

Out of 45 bacterial growth positive cases, all showed monomicrobial infection.

Distribution of isolated bacteria was observed as below.

Table 4.8: Distribution of bacterial types isolated from acute hepatitis cases.

Types of bacteria	Gram-negative Bacilli	Gram-positive Cocci	Gram-positive Bacilli	Total Bacteria
Total Number	22	20	3	45
Percentage	49%	44%	7%	100%

Out of 45 blood culture positive samples, gram-negative bacilli were found to grow in 49%, gram-positive cocci in 44% & gram-positive bacilli in 7% samples. Infection with gram-negative bacilli was found to be the most prevalent infection in acute hepatitis.

Details of bacterial types isolated were as follows:

Table 4.9: Bacterial organisms isolated from blood culture.

Bacterial organisms	Total number	%
<i>Escherichia coli</i>	9	20
<i>Klebsiella spp.</i>	6	13
<i>Pseudomonas aeruginosa</i>	3	7
<i>Salmonella spp.</i>	2	4
<i>Enterobacter spp.</i>	1	2
<i>Acinetobacter spp.</i>	1	2
Total gram-negative bacilli	22	49
<i>Staphylococcus aureus</i>	11	24
Coagulase-negative Staphylococci	5	11
Gr. D Streptococcus	1	2
Other GPC	3	7
Total gram-positive cocci	20	44
Beta-haemolytic <i>Bacillus spp.</i>	2	4
Other GPR	1	2
Total gram-positive bacilli	3	7
Total Number of bacterial isolates	45	

Six different types of gram-negative bacteria and 3 different types of gram-positive cocci were isolated and identified. *Staphylococcus aureus* was the most common isolate (24%, 11/45) followed by *E. coli* (20%, 9/45).

Among gram-negative bacilli, *E. coli* (20%, 9/45), *Klebsiella spp* (13%, 6/45), *Pseudomonas aeruginosa* (7%, 3/45), and *Salmonella spp.* (4%, 2/45), *Enterobacter spp.* (2%, 1/45) and *Acinetobacter spp.* (2%, 1/45) were isolated.

Among gram-positive cocci, *Staphylococcus aureus* (24%, 11/45), coagulase-negative staphylococci (11%, 5/45), group-D streptococcus (2%, 1/45) were isolated.

Beta-haemolytic *Bacillus spp* (4%, 2/45) were also isolated.

Figure 4.9: Proportional distribution of bacterial types isolated from acute hepatitis cases.

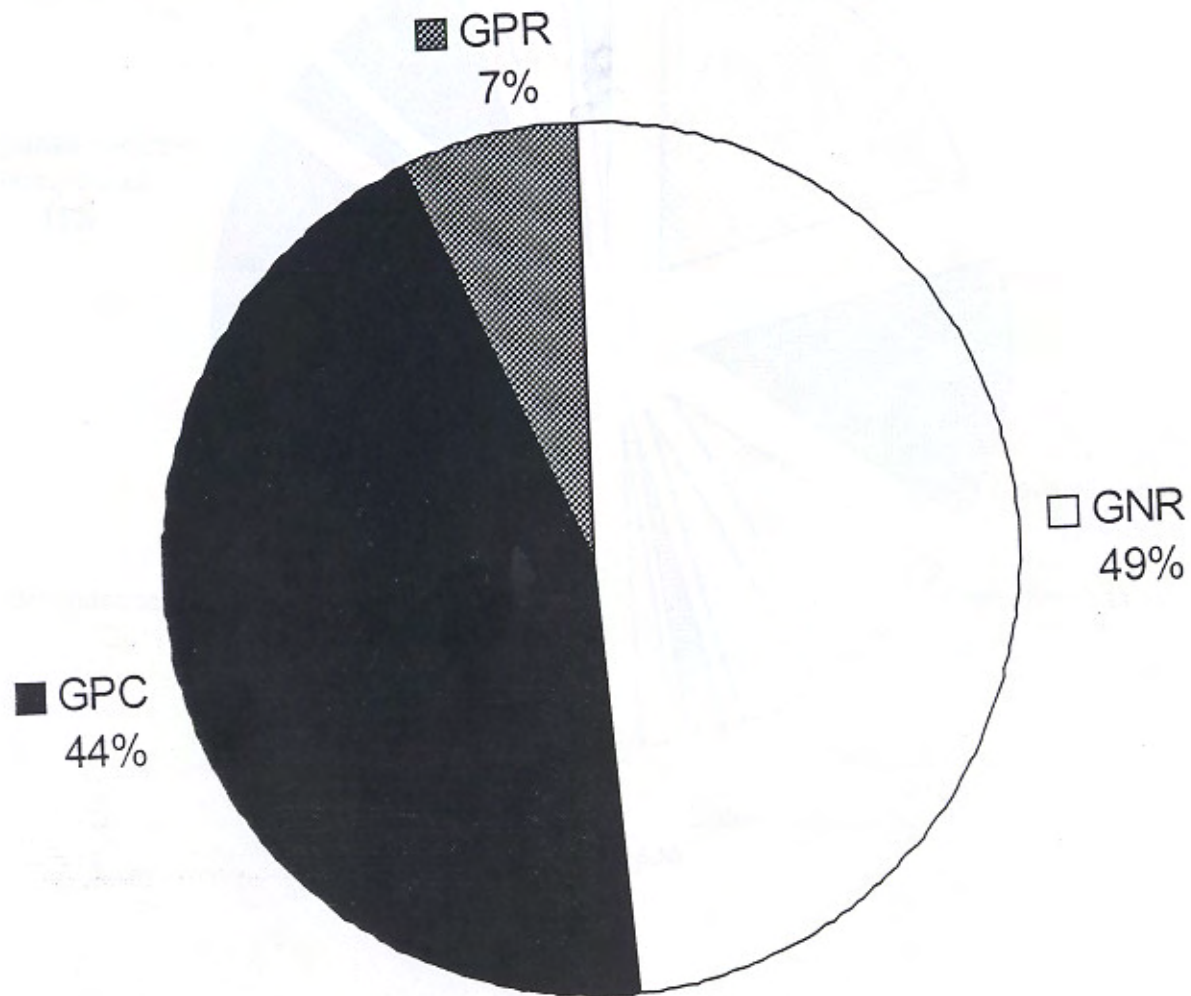
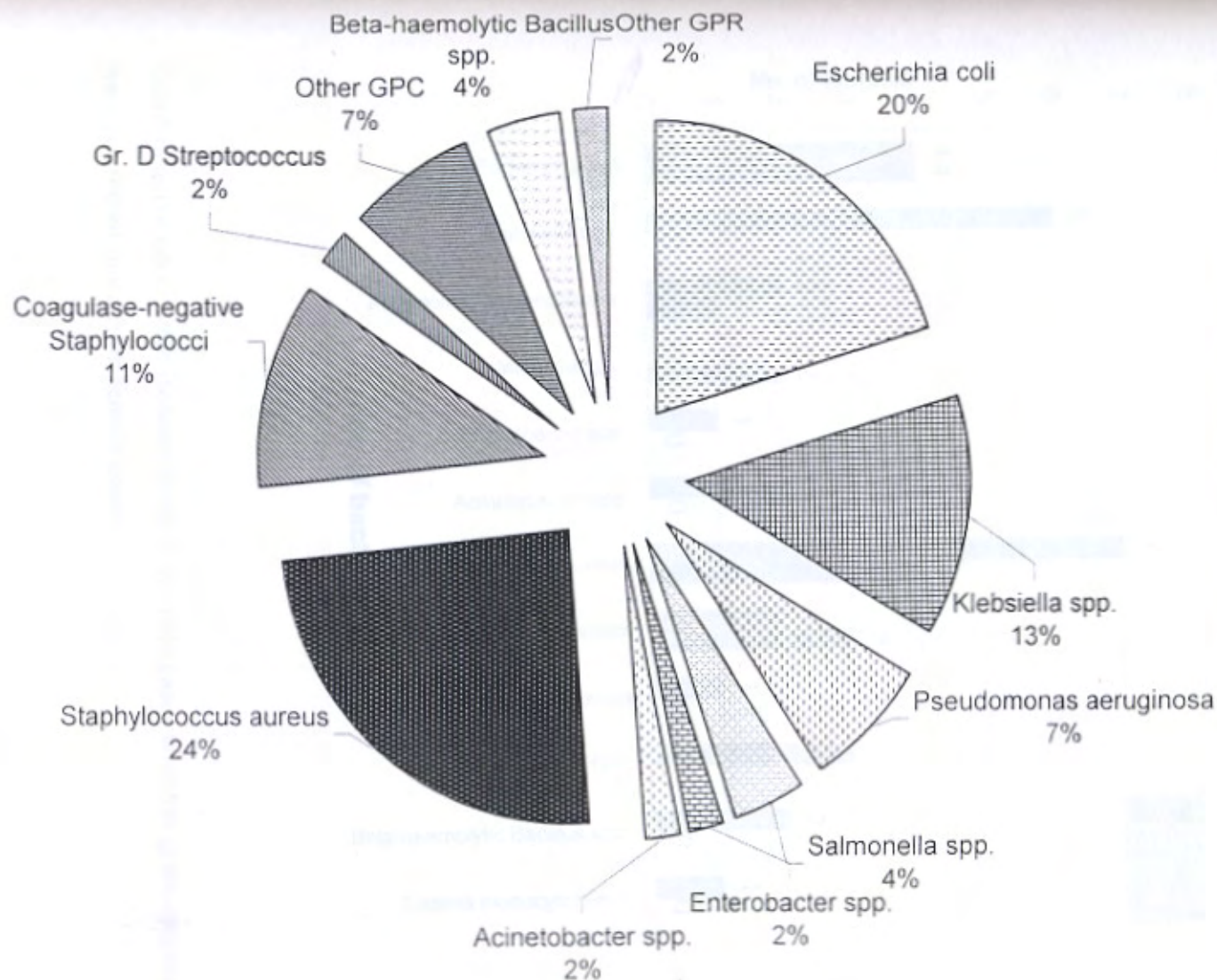


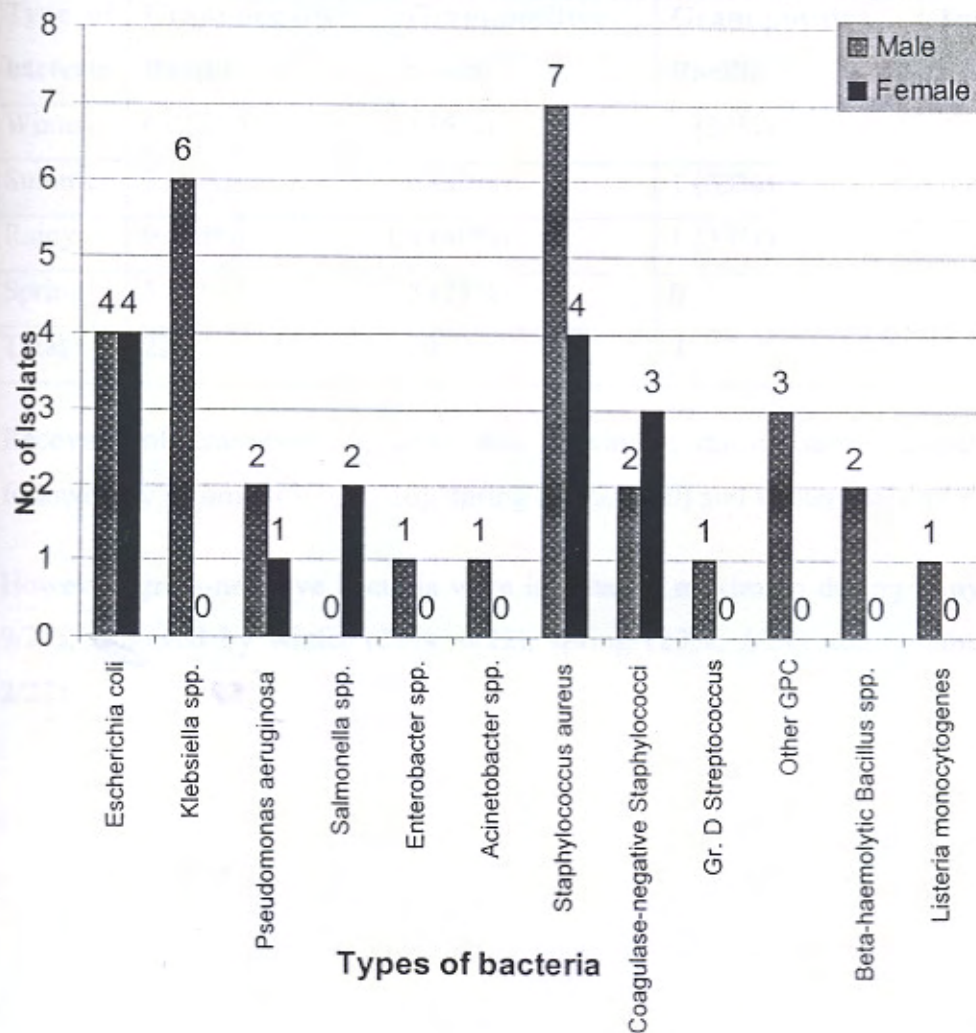
Figure 4.10: Proportional distribution of different bacterial organisms isolated from blood culture



4.8. Distribution of bacterial pathogens by gender:

Distribution of isolated bacterial pathogens was observed as below.

Figure 4.11: Gender distribution of different bacterial pathogens.



Gram-positive cocci were isolated more from male cases whereas gram-negative bacteria were recovered more from female cases.

4.9. Seasonal distribution of bacterial isolates:

Seasonal distribution of gram-positive and gram-negative bacteria was found as below.

Table 4.10: Seasonal distribution of bacterial isolates.

Type of bacteria	Gram-negative Bacilli	Gram-positive Cocci	Gram-positive Bacilli	Total number of bacterial isolates
Winter	6 (27%)	1 (5%)	1 (33%)	8
Summer	2 (9%)	6 (30%)	1 (33%)	9
Rainy	9 (40%)	8 (40%)	1 (33%)	18
Spring	5 (23%)	5 (25%)	0	10
Total	22	20	3	45

Recovery of gram-positive cocci was maximum during rainy season (40%, 8/20), followed by summer (30%, 6/20), spring (25%, 5/20) and winter season (5%, 1/20).

However, gram-negative bacteria were isolated in maximum during rainy season (40%, 9/22), followed by winter (27%, 6/22), spring (23%, 5/22) and summer season (9%, 2/22).

4.10. Seasonal distribution of bacterial pathogens:

Distribution of different bacterial pathogens in different seasons was as shown below:

Table 4.11: Seasonal distribution of bacterial pathogens.

Bacterial organisms	Seasonal distribution of bacteria No. (%)				Total
	Winter	Summer	Rainy	Spring	
<i>Escherichia coli</i>	3 (38%)	-	6 (33%)	-	9 (20%)
<i>Klebsiella spp.</i>	-	1 (11%)	-	5 (50%)	6 (13%)
<i>Pseudomonas aeruginosa</i>	1 (13%)	-	2 (11%)	-	3 (7%)
<i>Salmonella spp.</i>	-	1 (11%)	1 (6%)	-	2 (4%)
<i>Enterobacter spp.</i>	1 (13%)	-	-	-	1 (2%)
<i>Acinetobacter spp.</i>	1 (13%)	-	-	-	1 (2%)
Total GNR	6 (75%)	2 (22%)	9 (50%)	5 (50%)	22 (49%)
<i>Staphylococcus aureus</i>	1 (13%)	5 (56%)	2 (11%)	3 (30%)	11 (24%)
Coagulase-negative Staphylococci	-	1 (11%)	3 (17%)	1 (10%)	5 (11%)
Gr. D Streptococcus	-	-	1 (6%)	-	1 (2%)
Other GPC	-	-	2 (11%)	1 (10%)	3 (7%)
Total GPC	1 (13%)	6 (67%)	8 (44%)	5 (50%)	20 (44%)
Beta-haemolytic <i>Bacillus spp.</i>	1 (13%)	1 (11%)	-	-	2 (4%)
Other GPR	-	-	1 (6%)	-	1 (2%)
Total GPR	1 (13%)	1 (11%)	1 (11%)	-	3 (7%)
Total number of bacteria	8 (18%)	9 (20%)	18 (40%)	10 (22%)	45
Number of types of bacterial isolates	6	5	8	4	12

Out of total 45 bacterial isolates of 12 different types, 8 different types of bacterial isolates were isolated during rainy season: *E. coli* (6), CONS (3), *Pseudomonas aeruginosa* (2), *Staphylococcus aureus* (2), *Salmonella spp* (1), Gr.-D *Streptococcus* (1), other GPC (2) and other GPB (1).

Similarly, 6 different types of bacteria including *E. coli* (3), *Pseudomonas aeruginosa* (1), *Enterobacter spp* (1), *Acinetobacter spp.* (1), *Staphylococcus aureus* (1) and β -hemolytic *Bacillus spp* (1) were isolated in winter.

In summer, 5 different types of bacteria namely, *Staph. aureus* (5), *Klebsiella spp* (1), *Salmonella spp* (1), CONS (1), and β -hemolytic *Bacillus spp* (1) could be isolated.

In spring 4 types of bacteria *Klebsiella spp* (5), *Staphylococcus aureus* (3), CONS (1), other GPC (1) were isolated.

D. ANTIBIOTIC SUSCEPTIBILITY PATTERN OF THE ISOLATED BACTERIAL PATHOGENS

Antibiotic susceptibility pattern of the bacterial isolates was studied as follows.

Table 4.12: Antibiotic susceptibility pattern of isolated bacteria.

Antibiotics	Gram-positive bacteria (#23)			Gram-negative bacteria (#22)			Total isolated bacteria (#45)		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Ciprofloxacin	89.5	-	10.5	90.9	9.1	-	93.3	6.7	-
Chloramphenicol	94.7	-	5.3	90.9	-	9.1	93.3	-	6.7
Tetracycline	68.4	-	31.6	50	18.2	31.8	62.2	8.8	28.8
Ampicillin	31.6	10.5	57.9	40.9	13.6	45.5	42.2	11.1	46.7
Amikacin	-	-	-	100	-	-	-	-	-
Cefotaxime	-	-	-	86.4	-	13.6	-	-	-
Cotrimoxazole	-	-	-	68.2	9.1	22.7	-	-	-
Gentamicin	45.4	36.4	18.2	77.3	4.5	18.2	-	-	-
Erythromycin	68.4	15.8	15.8	-	-	-	-	-	-
Penicillin	31.6	-	68.4	-	-	-	-	-	-
Methicillin	81.2	-	18.8	-	-	-	-	-	-
Vancomycin	100	-	-	-	-	-	-	-	-

S = Susceptible, I = Intermediate, R = Resistant.

Antibiotic susceptibility pattern of 45 bacterial isolates including 22 gram-negative bacilli, 20 gram-positive cocci and 3 gram-positive bacilli was found as shown.

The isolates were most susceptible to Ciprofloxacin & chloramphenicol (both 93.3%), followed by tetracycline (62.2%) and ampicillin (42.2%).

Gram-negative bacilli were highly susceptible to amikacin (100%), followed by chloramphenicol & ciprofloxacin (both 90.9%), cefotaxime (86.4%), cotrimoxazole (68.2%), gentamicin (77.3%), tetracycline (50%) & ampicillin (40.9%).

Gram-positive bacteria were most susceptible to chloramphenicol (94.7%), followed by ciprofloxacin (89.5%), tetracycline & erythromycin (both 68.4%) and ampicillin & penicillin (both 31.6%). Susceptibility of Methicillin 16 bacterial isolates (*Staphylococcus aureus* & CONS) was 81.2% cases. The isolates resistant to methicillin were further tested against vancomycin and were found to be 100% susceptible to it.

4.11. Antibiotic susceptibility of gram-negative bacteria:

Antibiotic susceptibility pattern of gram-negative bacilli were as follows:

Table 4.13: Antibiotic susceptibility pattern of gram-negative bacilli.

Isolated gram-negative bacilli (no.)	Antibiotic Susceptibility (%)							
	AK	CI	CX	C	J	Q	T	A
<i>E. coli</i> (#9)	100	100	100	88.9	88.9	77.8	44.5	44.5
<i>Klebsiella pneumoniae</i> (#6)	100	83.3	83.3	100	83.3	83.3	83.3	33.3
<i>Pseudomonas aeruginosa</i> (#3)	100	66.7	67.7	100	33.3	0	0	0
<i>Salmonella spp.</i> (#2)	100	100	100	50	100	100	0	50
<i>Enterobacter spp.</i> (#1)	S	S	R	S	R	I	S	S
<i>Acinetobacter spp.</i> (#1)	S	S	S	S	S	S	S	S

adjacent number in parenthesis represents the total number of isolates.

S = Susceptible, I = Intermediate, R = Resistant.

Isolated *E. coli* was found to be 100% susceptible to amikacin, ciprofloxacin and cefotaxime.

Klebsiella pneumoniae were highly susceptible to chloramphenicol and amikacin (both 100%).

All 3 isolated *Pseudomonas aeruginosa* were susceptible to chloramphenicol and amikacin (100%).

Both of the isolated *Salmonella spp.* were susceptible to amikacin, cotrimoxazole, ciprofloxacin, cefotaxime and gentamicin.

The isolated *Enterobacter spp.* was susceptible to chloramphenicol, ciprofloxacin, amikacin, ampicillin and tetracycline; and the *Acinetobacter spp.* isolated from the blood culture of acute hepatitis cases was susceptible to all used antibiotics.

4.12. Antibiotic susceptibility of gram-positive cocci:

Antibiotic susceptibility pattern of gram-positive cocci were as follows:

Table 4.14: *Antibiotic susceptibility pattern of gram-positive cocci.*

Isolated positive (no.)	gram-bacteria	Antibiotic Susceptibility (%)							
		C	Cl	M	T	E	J	P	VA
<i>Staphylococcus aureus</i> (#11)		90.9	81.8	72.7	63.6	63.6	45.5	36.4	100
<i>Coagulase-negative staphylococcus</i> (#6)		100	100	100	100	100	-	20	-
<i>Group D Streptococcus</i> (#1)		S	S	-	S	R	-	S	-

adjacent number in parenthesis represents the total number of isolates.

S = Susceptible, I = Intermediate, R = Resistant.

Staphylococcus aureus was most susceptible to chloramphenicol (90.9%), followed by ciprofloxacin (81.8%). Susceptibility towards vancomycin was tested for 3 methicillin-resistant *Staphylococcus aureus* and were found to be 100% susceptible.

Coagulase-negative staphylococci were 100% susceptible to chloramphenicol, ciprofloxacin, methicillin, tetracycline and erythromycin.

Isolated *Group-D Streptococcus* was susceptible to chloramphenicol, ciprofloxacin, cotrimoxazole, ampicillin, tetracycline and penicillin.

E. BACTERIAL & VIRAL CO-INFECTION IN ACUTE HEPATITIS

Sera of 118 patients were tested for the presence of HBsAg, anti-HEV IgM & anti-HCV IgM/IgG.

None of these markers were found in 57.6% (68/118) of cases while markers of at least one of hepatitis viruses (HBV/ HCV/HEV) could be detected in 42.4% (50/118) of cases. Pattern of bacterial infection &/or viral co-infection was as shown below:

Table 4.15: *Bacterial & viral infection pattern in acute hepatitis cases.*

Bacterial Infection	Cases positive for viral markers				Cases with none of the viral markers	Total
	HBV	HCV	HEV	Total		
Present	10 ^{*1}	-	13 ^{*1}	20	25	45
Not present	14	5 ^{*2}	16 ^{*2}	30	43	73
Total	24	5	29	50	68	118

^{*1}Three cases with co-infection of HBV & HEV; ^{*2}Five cases with co-infection of HCV & HEV

The overall prevalence of bacterial and viral co-infection was found to be 17% (20/118).

4.13. Bacterial & viral co-infection pattern:

Distribution of viral infection in 45 different bacterial infection positive cases was analyzed as below:

Table 4.16: Bacterial co-infection with different hepatitis viruses.

Type of bacteria	Cases positive for viral markers					None of the viral markers detected	Total
	HBV	HEV	HBV & HEV	Total Co-infection			
				No.	%		
<i>Escherichia coli</i>	1	2	1	4	20	5	9
<i>Klebsiella spp.</i>	1	0	1	2	10	4	6
<i>Pseudomonas aeruginosa</i>	0	2	0	2	10	1	3
<i>Salmonella spp.</i>	0	1	0	1	5	1	2
<i>Enterobacter spp.</i>	1	0	0	1	5	0	1
<i>Acinetobacter spp.</i>	1	0	0	1	5	0	1
Total GNR	4	5	2	11	55	11	22
<i>Staphylococcus aureus</i>	1	2	0	3	15	8	11
Coagulase-negative Staphylococci	0	0	0	0	0	5	5
Gr. D Streptococcus	0	1	0	1	5	0	1
Other GPC	1	1	0	2	10	1	3
Total GPC	2	4	0	6	30	14	20
Beta-haemolytic <i>Bacillus spp.</i>	1	1	0	2	10	0	2
Other non-sporing GPR	0	0	1	1	5	0	1
Total GPR	1	1	1	3	15	0	3
Total Number	7	10	3	20		25	45
Percentage	35%	50%	15%	17%			

Greater percentage of bacterial co-infection was observed in cases infected with Hepatitis E Virus (50%, 10/20), followed by Hepatitis B Virus (35%, 7/20) & both HEV & HBV (15%, 3/20).

Co-infection was seen to occur more with gram-negative bacteria (55%, 11/20) than with gram-positive cocci (30%, 6/20) and gram-positive bacilli (15%, 3/20).

Out of 22 cases infected with gram-negative bacilli, 11 (50%) showed co-infection with hepatitis viruses while 11 (50%) revealed sole bacterial infection. However, only 6 out of 20 (30%) cases infected with gram-positive cocci revealed co-infection while 13 (70%) revealed sole bacterial infection. All 3 gram-positive bacilli were found in co-infection cases.

Prevalence of co-infection therefore was found more in gram-negative bacilli than gram-positive cocci (50% and 30%) while prevalence of bacterial hepatitis was greater in gram-positive cocci (70% and 50%).

Highest percentage of co-infection was seen to occur in cases infected with *E. coli* (20%, 4/20), followed by *Staphylococcus aureus* (15%, 3/20); *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and β -hemolytic *Bacillus spp* (each 10%, 2/20); *Salmonella spp*, *Enterobacter spp.*, *Acinetobacter spp.*, Coagulase-negative Staphylococci, and Gr. D Streptococcus (each 5%, 1/20).

Figure 4.12: Bacterial & viral infection pattern in acute hepatitis cases.

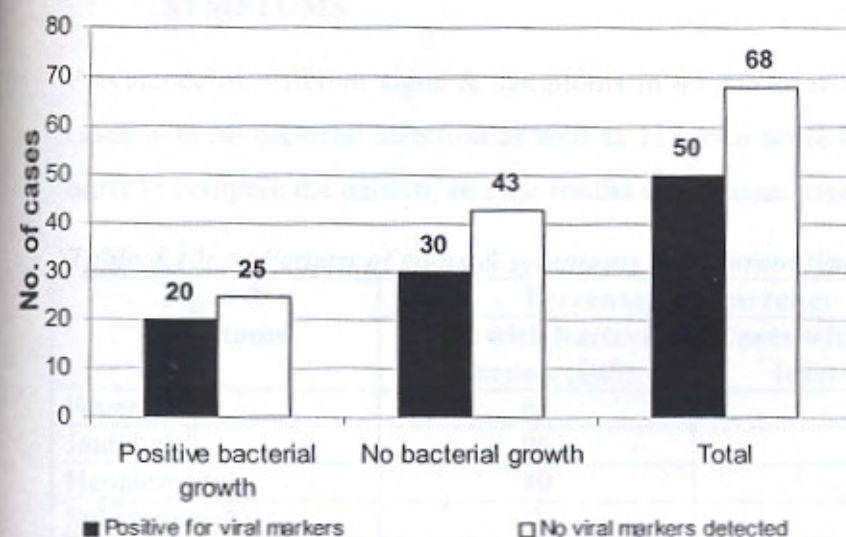
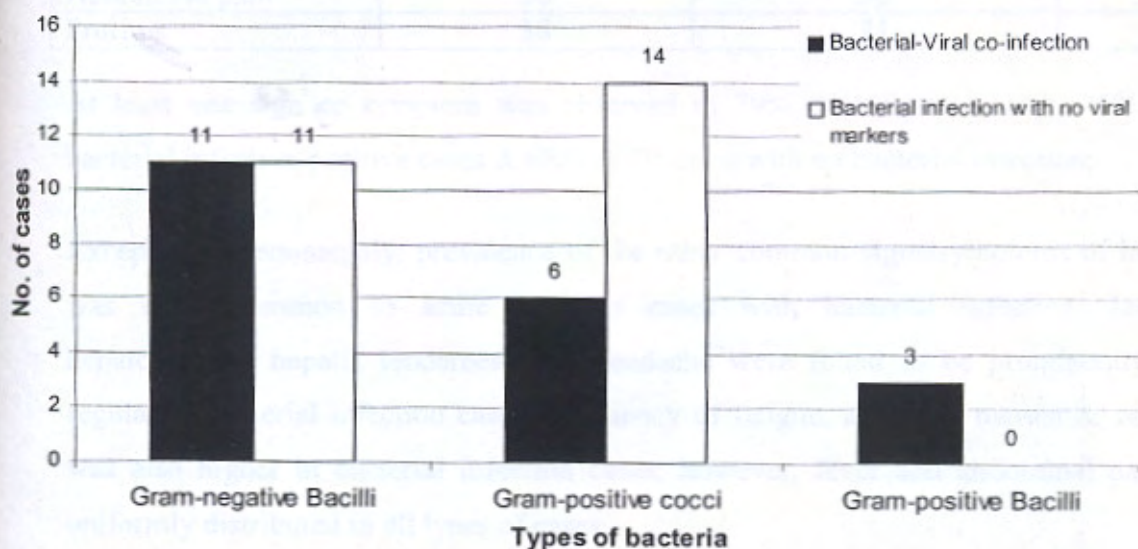


Figure 4.13: Prevalence of bacterial & viral co-infection in bacterial growth-positive cases.



A. CORRELATION OF BACTERIAL INFECTION AND SIGNS AND SYMPTOMS

Prevalence of different signs & symptoms in 45 bacterial growth-positive cases and 73 cases with no bacterial infection as well as 118 total acute hepatitis cases was studied in order to compare the pattern; and the results were summarized as below:

Table 4.17: Pattern of signs & symptoms in different types of acute hepatitis cases.

Signs & Symptoms	Percentage occurrence of signs/symptoms		
	Cases with bacterial infection (#45)	Cases with no bacterial infection (#73)	Total cases (#118)
Fever	67	64	65
Jaundice	96	68	79
Hepatomegaly	40	21	28
Splenomegaly	2	4	3
Hepatic tenderness	20	7	12
Headache	11	-	4
Fatigue	44	22	31
Anorexia	67	42	52
Nausea &/or vomiting	47	27	35
Abdominal pain	36	30	32
Pruritus	38	21	

At least one sign or symptom was observed in 79% of 118 total cases, 96% of 45 bacterial infection positive cases & 68% of 79 cases with no bacterial infection.

Except for splenomegaly, prevalence of the other common signs/symptoms of hepatitis was more common in acute hepatitis cases with bacterial infection. Jaundice, hepatomegaly, hepatic tenderness and headache were found to be prominently more regular in bacterial infection cases. Frequency of fatigue, anorexia, nausea & vomiting was also higher in bacterial infection cases; however, fever and abdominal pain was uniformly distributed in all types of cases.

4.14. Correlation of ALT level & bacterial infection in acute hepatitis cases:

Serum ALT level in acute hepatitis cases were studied in order to correlate the ALT level with infection (viral/bacterial).

Table 4.18: *ALT level in different types of acute hepatitis cases.*

ALT level (U/L)	Cases with bacterial infection		Cases without bacterial infection		Total acute hepatitis cases (#118)	
	Viral markers detected (#20)	Viral markers not detected (#25)	Viral markers detected (#30)	Viral markers not detected (#43)	Number	Percentage
100-199	7 (35%)	10 (40%)	21 (70%)	25 (58%)	63	53%
200-299	8 (40%)	9 (35%)	4 (13%)	5 (12%)	26	22%
300-399	2 (10%)	4 (16%)	5 (17%)	6 (14%)	17	14%
400-499	2 (10%)	-	-	6 (14%)	8	7%
500-599	-	1 (4%)	-	-	1	0.8%
Up to or >600	1 (5%)	1 (4%)	-	1 (2.3%)	3	2.5%

The percentage distribution of ALT level in different types of acute hepatitis cases was found as shown.

53% of acute hepatitis cases presented with their ALT value of 100-199 U./L, followed by 200-299 U./L (22%), 300-399 U./L (14%), 400-499 U./L (7%), 500-599 U./L (0.8%) and up to or >600 U./L (2.5%).

4.15. Total leukocyte count in different types of acute hepatitis cases:

Total leukocyte count in 45 bacterial infection cases and 30 acute viral hepatitis cases with no bacterial infection was found as depicted in table 4.28.

Table 4.19: *Total leukocyte count in different types of acute hepatitis cases.*

Total leukocyte count (per mm ³)	Bacterial infection cases (#25)	Bacterial-viral co-infection cases (#20)	Acute viral hepatitis cases (#30)
<6,000	1 (4%)	1 (5%)	18 (60%)
6,000-10,000	10 (40%)	9 (45%)	8 (27%)
>10,000	14 (56%)	10 (50%)	4 (13%)

Leukocytosis (WBC>10,000 per mm³) was observed more in bacterial infection (56%) followed by bacterial-viral co-infection (50%) while leucopenia (WBC<6,000 per mm³) occurred more in acute viral hepatitis cases with no bacterial infection (60%).

Figure 4.14: Distribution of co-infection cases by bacterial pathogen.

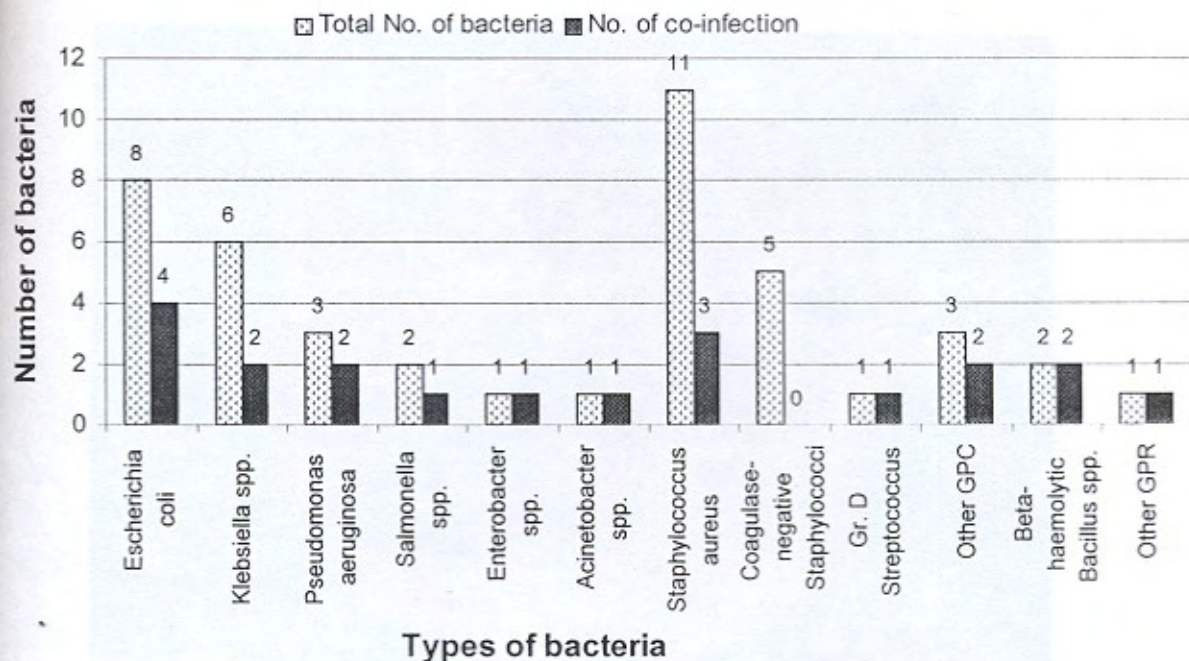
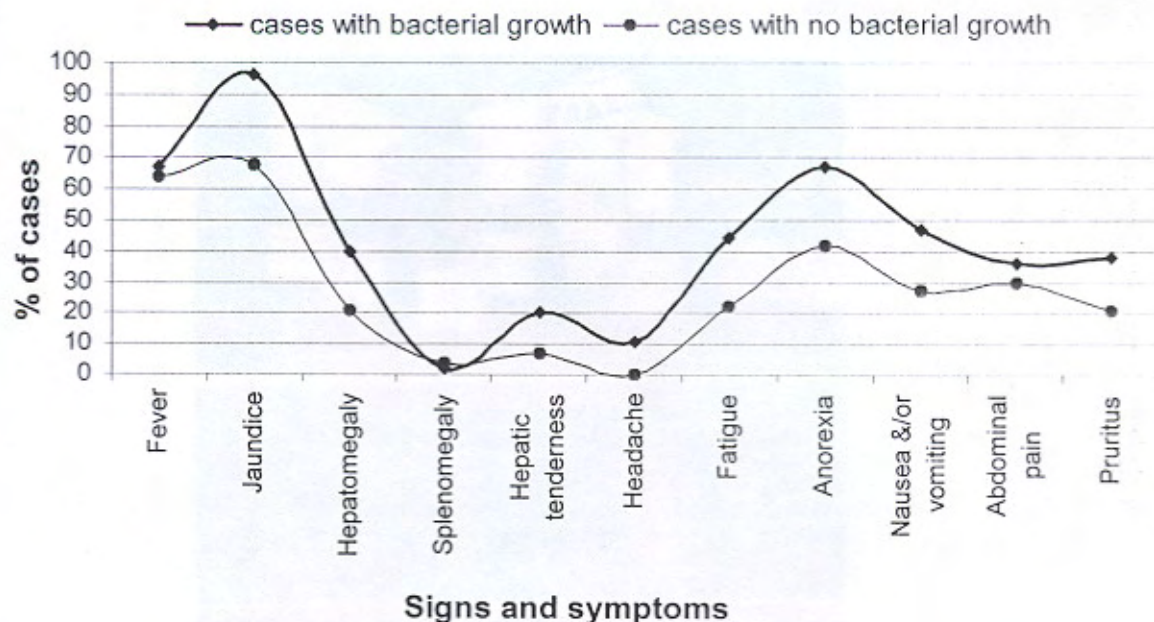
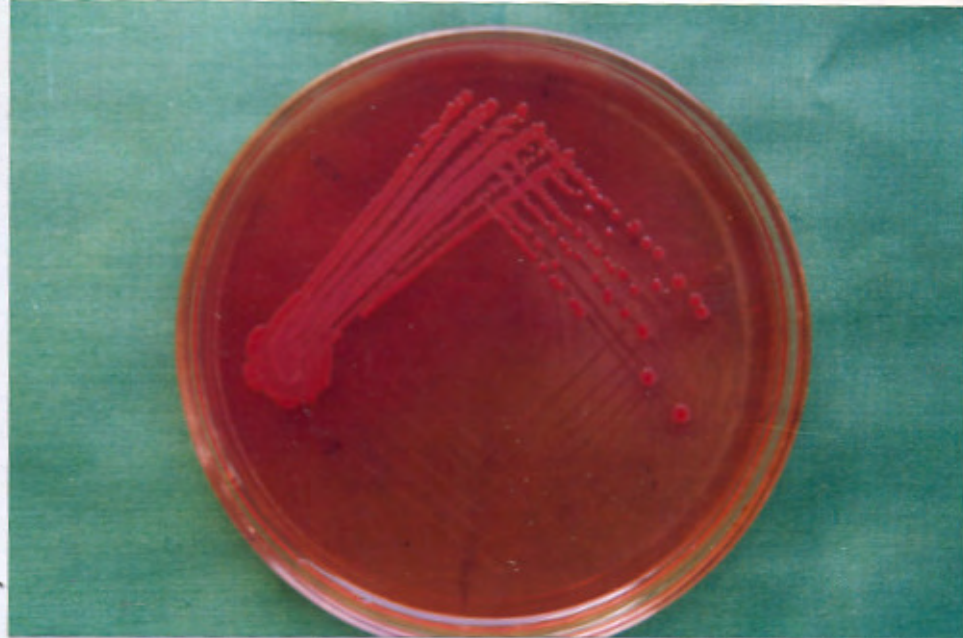


Figure 4.15: Pattern of signs & symptoms in different types of acute hepatitis cases.





Photograph 6: Twenty-four hour culture of *E. coli* on MacConkey agar.



Photograph 7: Results of biochemical tests for *E. coli*.

(H_2S -, Indole +, Motile; MR +; VP -; Citrate utilization -; Urease -; A/A, G++; Fermentative)

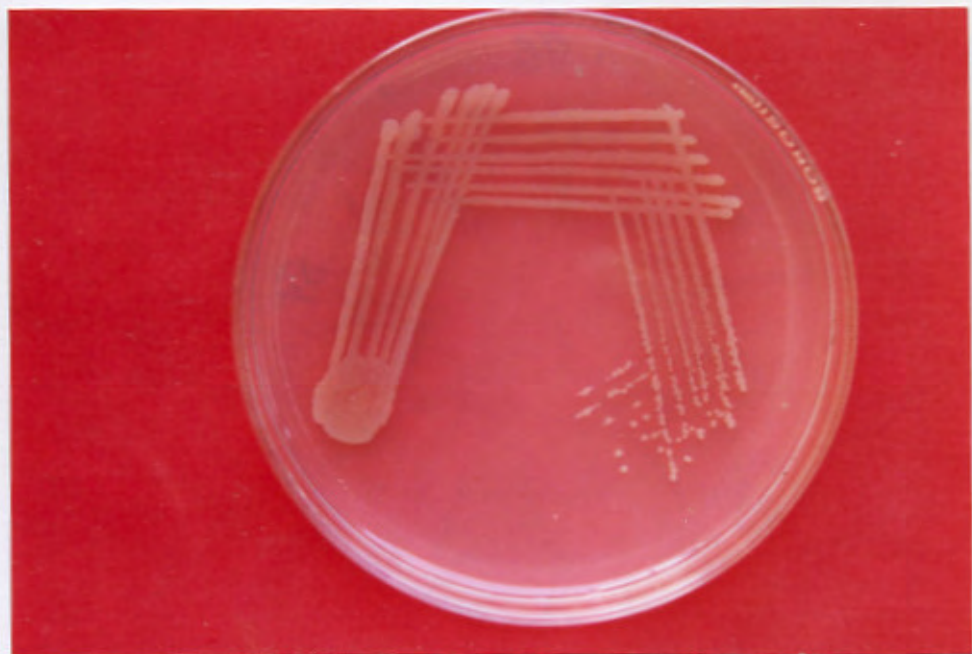


Photograph 8: Twenty-four hour culture of *Klebsiella* spp. on MacConkey agar.

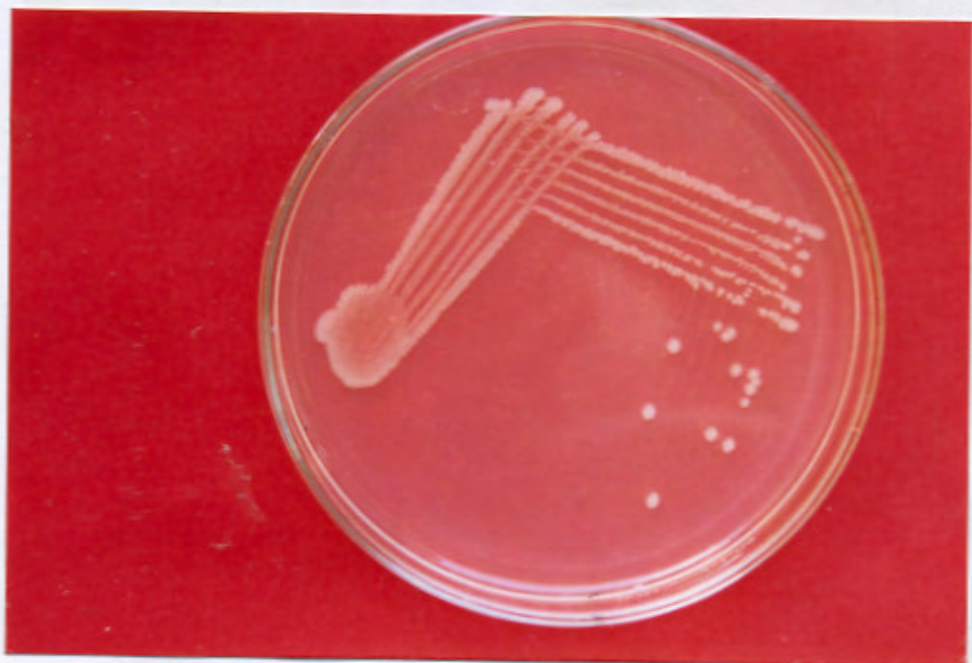


Photograph 9: Results of biochemical tests for *Klebsiella* spp.

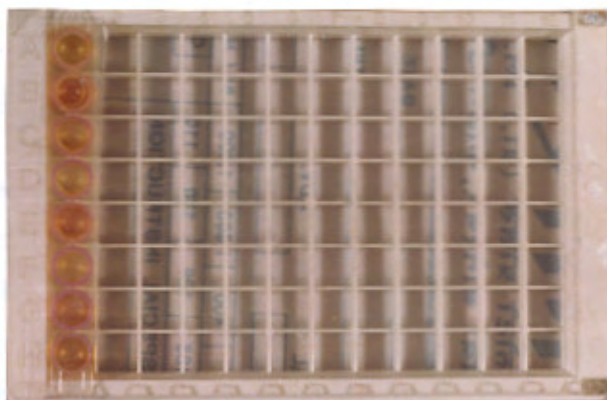
(H₂S -, Indole -, Nonmotile; MR -; VP +; Citrate utilization +; Urease +; A/A, G+; Fermentative)



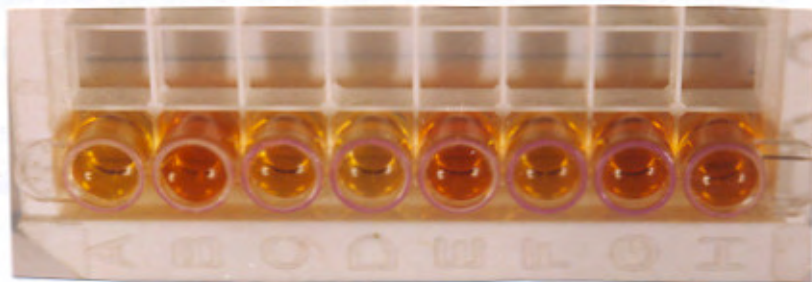
Photograph 10: Twenty-four hour culture of *Pseudomonas aeruginosa*.



Photograph 11: Twenty-four hour culture of *Staphylococcus aureus*.



Photograph 14: Microplate loaded with samples & controls.



Photograph 15: Result of ELISA for detection of anti-HCV antibody.

A = Negative Control, B: Positive Control;

C, D, F & H = Negative test samples; E & G = Positive test samples

CHAPTER – FIVE

DISCUSSION

In the present study, prevalence of bacterial infection in acute hepatitis was found to be 38% and that of bacterial-viral co-infection was found to be 17%.

Numerous facts aroused the probability for involvement of bacteria in acute hepatitis as an aetiological agent: Several epidemic, focal & sporadic outbreaks of water-borne enteric non-A-non-B hepatitis have been reported; prevalence of viral hepatitis E, a water-borne infection, was found to be 80% in Kathmandu valley and exact etiology of nearly one-third of acute hepatitis cases remained unknown in the study done by Shrestha in 1997; and poor quality drinking water and lack of hygiene have been noticed (Sharma, 1978; Sharma, 1986; and Maharjan, 1998).

Several incidences of bacterial hepatitis and bacterial-viral co-infection cases have been experienced by concerned health personnel in Nepal in the past few years. Involvement of gram-negative bacilli such as *Salmonella* in causing hepatitis, the clinical picture of which is indistinguishable from acute viral hepatitis, has been reported (El-Newihi *et al.*, 1996).

Increasing frequency of inferior vena-cava obstruction cases & spontaneous bacterial peritonitis in Nepal also pushed up the suspicion for bacteria being involved in acute hepatitis (Shrestha, 2001). Because the linkage of portal and systemic venous systems by hepatic veins ease the spread of the pathogens from systemic circulation to the liver and vice versa (Sherlock, 1987; Haslett *et al.*, 1999).

These facts directed the researchers to undertake the study in order to find the prevalence of bacterial hepatitis among acute hepatitis cases in Nepal.

Altogether 118 clinically suspected patients having their ALT level more than 2.5 times its normal upper limit were included in the study. After filling up a schedule (Appendix

1) and taking their consent, blood samples were collected from the patients for performing culture and sensitivity; and separated sera were analyzed for viral markers (antigens or antibodies of HBV, HCV & HEV) and hematological parameters in order to study their possible correlation.

The other potent causes of acute hepatitis were ruled out by careful questioning and taking details of the patients' social and medical history.

Overall incidence of bacterial infection in acute hepatitis cases in the present study was found to be 38%, which is relatively higher in comparison to the studies made on bacteraemia among the patients attending Tribhuvan University Teaching Hospital, 2001 (15% of all types of cases); Bir Hospital, 1999 (17% of all types of cases); Zhao *et al.*, 2001 (17.1% of severe acute hepatitis patients in China) and Wyke *et al.*, 1978 (22.3% of fulminant hepatitis cases). This difference may be ascribed to the difference in the type of study population, inclusion & exclusion criteria, and geographic variation.

A relatively higher incidence of bacterial infection observed among acute hepatitis cases could be due to relatively greater tendency of acute hepatitis cases to contact bacterial infection. The greater tendency of bacterial infection in acute hepatitis might be attributed to the following facts: liver is a bacterial filter (Filayson *et al.*, 1999); liver is the principal organ for reticuloendothelial uptake of the bacteria accounting for 60% of total uptake (Holdsworth *et al.*, 1989); bacteria can infect the liver, and the liver is almost inevitably involved to some extent in all blood-borne infections (Betel *et al.*, 1997); Left shift of white blood cells occurs in acute hepatitis cases (El-Newihi *et al.*, 1996). However, the impairment and dysfunction of the liver in hepatitis may also facilitate the pathogenesis of bacteria.

In the present study, acute hepatitis was found to be more common among males, in the age group 20-29 years followed by 30-39 years, which is consistent with the findings of Shrestha, 2000 and Betel *et al.*, 1997. The greater prevalence of acute hepatitis in males and in adult age group might be ascribed to probability of their maximum exposure to the outside environment; adult age group being the most active age group & hence exposed to outside world more than the others. Various other sociocultural & biological factors,

however, might attribute to males being more susceptible than the females. Further advanced researches may provide exact correlation.

Similarly, prevalence of bacterial infection was also relatively greater in males (43%) than in females (36%) and in the age group 20-29 years. Of total infection, 67% was observed among males & only 33% among female cases. Hence, male cases were found to be 2 times more prone to contract bacterial infection compared to females. This result was corroborating with the findings of Ghimire, 1995. Higher prevalence of bacterial infection in age group 20-29 (33%) was similar to findings of Banjade, 1999.

Maximum incidence of both acute hepatitis (42%) and bacterial hepatitis (40%) were observed during rainy season (June-August). This result complies with the findings of Shrestha, 1991; Kane, 1984 and Clayson, 1997. The humid atmosphere, optimum environmental conditions and increased water pollution might have favoured the increased incidence of acute hepatitis during rainy season. Similarly, ravenous environmental condition during winter season might be the cause for its least incidence during winter season. Higher prevalence of bacterial isolation during June-August, i.e., rainy season is also corroborating with findings of higher bacterial load in the water by EDCD, 2000; ENPHO/DISVI, 1995; and Maharjan, 1998. This indicates towards the possibility that bacterial infection in acute hepatitis may be a water-borne infection.

In total bacterial infection, 49% was due to gram-negative bacilli (GNB), 44% due to gram-positive cocci (GPC) and 7% due to gram-positive bacilli (GPB). This complies with the findings of Chakraborty, 1995 (60-70% GNB); Ghimire, 1995 (40% GPC & 53% GNB); Tibrewal, 1999 (81.6% GNB & 18.3% GPC); and Shakya, 2001 (61.68% GNB, 33.7% GPC & 4.54% GPB).

Among gram-negative bacilli, *E. coli* was the most common isolate (41% of total GNR, 20% of total bacteria). This is found to be higher in comparison to the findings of Wyke, 1982 (26%); Ispahani *et al.*, 1987 (27.4%); Shakya, 2001 (15.78%) & Ghimire, 1998(17%). Chakraborty, 1995, stated that *E. coli* was the commonest isolate from blood culture in U.K.

Klebsiella spp was the second most common gram-negative isolate (27% of GNR) accounting for 13% of total infection. This finding shows relatively higher incidence of *Klebsiella* infection in the present study in comparison to those of Bir Hospital, 2002 (8.3%); Shakya, 2001 (5.26%) and Banjade, 1999 (0.8%).

Isolation of *Pseudomonas aeruginosa* (7%) in the present study is little higher than in the findings of Shakya, 2001 (3.15%), Banjade, 1999 (1.7%) and Tibrewal, 1999 (3%). Result is however consistent with the finding of Planes, 1992.

Salmonella spp. accounted for 4% of total bacterial pathogens isolated from acute hepatitis cases. The finding is consistent with those of El-Newihi *et al.*, 1996; Sharma *et al.*, 1995 (Hepatic involvement in 23-60% of typhoid cases); and Sirmatel *et al.*, 2001.

Enterobacter spp. accounted for 2% of total bacterial infection in the present study which corroborates with the findings of Shakya, 2001 (0.64%). Isolation of *Acinetobacter spp.* (2%) was consistent with the finding of Shakya, 2001 (2.59%) & Tibrewal, 1999 (15%).

In the present study, 24% of total isolates were *Staphylococcus aureus*. This finding is consistent with those of Wyke, 1982 (61%); Ispahani, 1987 (11.2%); Banjade, 1999 (42.1%) and Ghimire, 1995 (17%). The result, however, are not consistent with the findings of Shakya, 2001 (73%) & Tibrewal, 1999 (57%). Isolation of *Staphylococcus aureus* from blood cultures of patients with liver abscess during 2058 has also been reported in Bir Hospital in 2002.

Isolation of coagulase-negative Staphylococci, CONS, (11%) was consistent with the finding of Sanchis *et al.*, 1999. Group-D *Streptococcus* (2%) and β -hemolytic *Bacillus spp.* (4%) were also isolated.

It was observed that gram-positive cocci are more frequently isolated from male cases whereas gram-negative bacteria are recovered comparatively more from female cases. Although the precise reason for this difference is unknown, this may however partly be due to the limited number of cases in the present study.

The maximum number of *E. coli* was isolated during the rainy season, which indicates that *E. coli* infection might be water-borne. Further epidemiological research might elaborate on this.

All the isolated & identified bacterial pathogens were tested for their susceptibility against the commonly used antibiotics. Microbial susceptibility was highest (both 93%) to ciprofloxacin and chloramphenicol, followed by tetracycline (69%) and ampicillin (42%).

The present study showed that all 22 gram-negative isolates were susceptible to amikacin (100%), followed by chloramphenicol & ciprofloxacin (both 91%), cefotaxime (86%), cotrimoxazole (82%), gentamicin (77%), tetracycline (64%) and ampicillin (41%).

All isolated *E. coli* were highly susceptible (100%) to amikacin, ciprofloxacin and cefotaxime. *Klebsiella spp.* was most susceptible to chloramphenicol and amikacin (100%). All isolated *Pseudomonas aeruginosa* were found to be susceptible (100%) to chloramphenicol and amikacin. Both *Salmonella spp.* were susceptible (100%) to ciprofloxacin, amikacin, cotrimoxazole, cefotaxime and gentamicin. *Enterobacter spp.* isolated in the present study was susceptible to all but cotrimoxazole, gentamicin and cefotaxime. The present findings are corroborating with findings of Trenholme *et al.*, 1989; Mahmood, 2000; Shakya, 2001; Arstila *et al.*, 1994; Cohen *et al.*, 1987; Walder, 1994; and Mandal *et al.*, 2002. The findings however are not complying with the findings of Hanel, 2000. This variation might be due to different strain of the infecting organism as well as different patient type.

All types of gram-positive bacteria were found susceptible to chloramphenicol (95%), followed by ciprofloxacin (90%), tetracycline & erythromycin (both 68%); and ampicillin & penicillin (32%). Most *Staphylococcus aureus* & CONS were susceptible to methicillin (81%) while all of them were susceptible to Vancomycin (100%).

Staphylococcus aureus were most susceptible to chloramphenicol (91%), the findings corroborating with those of Shakya, 2001 (95%). Coagulase-negative staphylococci were susceptible (all 100%) to chloramphenicol, ciprofloxacin, methicillin, tetracycline and erythromycin. Findings are consistent with those of Huovinen, 1966. The isolated *Group D Streptococcus* was susceptible to all used antibiotics except cefotaxime and erythromycin.

Out of 118 acute hepatitis cases studied, 20 (17%) cases revealed bacterial and viral co-infection. A greater percentage, 50% (10/20), of co-infection was observed in the cases infected with Hepatitis E Virus, 35% (7/20) with Hepatitis B Virus & only 15% (3/20) with HEV & HBV together.

Out of 45 bacterial infection cases, co-infection was observed in 44% (20/45) and sole bacterial hepatitis was observed in 56% (25/45). Most of 20 co-infection cases had bacterial infection due to gram-negative bacteria (55%, 11/20), followed by gram-positive cocci (30%, 6/20) and gram-positive bacilli (15%, 3/20).

Out of 22 cases infected with gram-negative bacilli, 50% (11) showed co-infection with hepatitis viruses; however, only 30% (6/20) cases infected with gram-positive cocci revealed co-infection and all 3 gram-positive bacilli were isolated from co-infection cases. Hence in the present study, prevalence of co-infection was found mostly in gram-negative bacteria while sole bacterial infection was found mostly in gram-positive cocci.

E. coli showed the highest percentage of co-infection (20%, 4/20), followed by *Staphylococcus aureus* (15%, 3/20).

Ninety-six percent of bacterial infection cases (43/45) but only 68% of acute hepatitis cases with no bacterial infection (50/73) showed at least one of the characteristic signs & symptoms of acute hepatitis. Prevalence of almost all different signs and symptoms was greater in bacterial infection cases. Prevalence of jaundice was relatively much higher among bacterial infection cases than that among cases without bacterial infection (96% vs. 68%) while headache was present only in former. The findings are agreeing with those of Franson *et al.*, 1985 & Patrick *et al.*, 1988. This reflects the association of bacteria and abnormal liver function. Cases with systemic infections, particularly when

caused by gram-negative organisms such as *E. coli*, are often jaundiced, possibly because of circulating bacterial endotoxins (Patrick *et al.*, 1988).

There was no significant difference in the prevalence of fever in bacterial infection cases and in acute hepatitis cases without bacterial infection, including those with acute viral hepatitis. However, high fever was found to be associated with most of the bacterial infection while low-grade fever characterized acute viral hepatitis. This finding is consistent with that of El-Newihi *et al.*, 1996, who reported that high fever could raise the possibility of *Salmonella* hepatitis.

No significant difference was observed for ALT level in different types of acute hepatitis cases. All types of infection showed similar distribution pattern of ALT level. Hence, ALT level is found insignificant in characterization purpose. This is not corroborating with the finding of El-Newihi *et al.*, 1996; who reported lower peak serum alanine transaminase (ALT) to be associated with *Salmonella* hepatitis. They also found that ALT/LDH (lactate dehydrogenase) ratio, when levels of both enzymes were expressed as multiples of upper limit of normal value for each, was significantly lower in *Salmonella* hepatitis. This variation might be because their study was concerning only on *Salmonella* hepatitis while the present study included all types of bacterial infection.

Leukocytosis was found to be associated with bacterial infection corroborating with the statement of Snyder *et al.*, 1977; Robson *et al.*, 1963 & Finlayson *et al.*, 1999; however, El-Newihi *et al.* in 1996 found left shift of WBC in salmonella hepatitis. This is due to difference in type of bacterial infection (Godkar, 1992). In contrast, leukocytopenia was found more in acute viral infection without bacterial infection. This is corroborating with statement of Chan *et al.*, 1975 & Godkar, 1992. Although this finding might be of some help for the suspicion of bacterial infection in acute hepatitis cases, the bacterial infection, viral infection or co-infection cases could not be distinguished on the basis of signs and/or symptoms and the respective tests are very essential and useful for the diagnosis purpose. Detailed study focusing on this aspect, taking greater number of cases, may be more informative before generalization of the findings.

CHAPTER – SIX

SUMMARY AND RECOMMENDATIONS

6.1. SUMMARY

Bacterial infection in acute hepatitis is found to be significant in Nepal with the prevalence of 38%. Blood culture of 45 acute hepatitis cases showed bacterial growth.

Gram-negative bacilli accounted for 49% (22/45) of total bacterial infection, followed by gram-positive cocci (44%, 20/45) and gram-positive rods (7%, 3/45).

The most prevalent gram-negative rod was *E. coli* (9/22), followed by *Klebsiella spp.* (6/22), *Pseudomonas aeruginosa* (3/22), *Salmonella spp.* (2/22), *Enterobacter spp.* & *Acinetobacter spp.* (both, 1/22).

Among gram-positive cocci, *Staphylococcus aureus* was the most common isolate (11/20), followed by coagulase-negative staphylococci (5/20) and *Group D streptococcus* (1/20).

The prevalence of bacterial infection in acute hepatitis was found to be significantly higher among male patients with male to female ratio nearly 2:1. The incidence of bacterial infection was higher among patients between the age of 20-29 (33%) followed by 30-39 (22%).

Prevalence of bacterial infection and acute hepatitis was highest during rainy season (40% and 42%), followed by spring (22% and 25%), summer (20% and 21%) and least during winter season (18% and 12%) indicating water might be the source of infection.

Most of *E. coli* were isolated during rainy season, *Klebsiella spp.* during spring season & *Staphylococcus aureus* during summer season.

Maximum bacteria could be grown in the month of August (24%), followed by September (11%).

In-vitro antibiotic susceptibility test of bacterial isolates showed that the most bacteria were susceptible to ciprofloxacin & chloramphenicol (both 93%), followed by tetracycline (69%) and ampicillin (42%). In addition, most gram-negative bacilli were susceptible to amikacin (100%) while all 19% of methicillin-resistant *Staphylococcus aureus* (MRSA) were found to be susceptible to vancomycin.

Prevalence of bacterial and viral co-infection was found to be 17% (20/118).

Out of 45 bacterial isolates, 20 (44%) co-infected with hepatitis viruses while 25 (56%) were found solely to be bacterial infection. In all, 50% of co-infection was seen to occur with HEV, 35% with HBV and 15% with both HBV and HEV.

GNB infection was most common in co-infected cases (50%) whereas GPC infection was most common in sole bacterial infection in acute hepatitis cases.

✓ More acute hepatitis cases with bacterial infection were symptomatic (96%) than acute hepatitis cases without bacterial infection.

Leukocytosis was higher (58%) in acute hepatitis cases with bacterial infection while leukocytopenia was higher (60%) in acute viral hepatitis cases.

6.2. RECOMMENDATIONS

Following recommendations are put forward for further researches in this area:

1. Bacterial infection in acute hepatitis was found to be one of the common problems in Nepal. Therefore, it is suggested that bacteria should be considered a potential aetiological agent for acute hepatitis and blood culture should be made mandatory for the diagnosis of acute hepatitis in patients presenting with the suggestive symptoms.
2. Study & analysis with higher sample numbers representing the country may be useful to have country profile.
3. Further research considering involvement of anaerobic bacteria may be more informative.
4. As bacterial infection in acute hepatitis was common during rainy season, better sanitary & drinking water policy may be useful for lowering infection rate.

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APPENDIX – I

I. MICROBIOLOGICAL AND CLINICAL PROFILE OF THE PATIENTS

A. Clinical Profile:

Name Age Sex

Addressa.....

Patient I.D. No. O.P.D./I.P.D. No.

Brief History of the patient:

Provisional Diagnosis:

Type of Specimen:

Date & Time of collection:

B. Microbiological Profile:

I. Direct Macroscopic Examination (Gram-Stain):

Culture: Broth Culture Medium	Subculture Media	Growth on subculture medium after incubation of			
		24 hours	48 hours	72 hours	days
Brain Heart Infusion (BHI) Broth	Blood Agar MacConkey Agar Chocolate Agar				

II. Culture Colony Characteristics:

Media	Shape	Size	Colour	Consist-ency	Configu-ration	Margin	Opacity	Other characters

III. Microscopic Examination:

Shape	GPC	GPR	GNR	GNC
Size				
Arrangement				

IV. Biochemical Tests:

Tests	Results	Tests	Results	Tests	Results	Other Tests	Results
•Catalase •Oxidase •Coagulase • Slide • Tube		• Indole • MR/VP • Citrate • TSI • Urease		• O/F • Motility • Optochin Sensitivity			

V. Identification of organism:

VI. Result of Antibiotic Susceptibility Test:

Antibiotics	Stren- gth	Zone of Inhibition	Inference	Antibiotics	Stre- ngth	Zone of Inhibition	Inference

VI. Laboratory Findings:

2. SCHEDULE

Reg. No. _____

Health Institution: _____

1. Name:
2. Address: _____
a) Local: _____
b) Permanent: _____
In Kathmandu from: _____
3. I.D. No. _____ OPD No./IPD No. _____
4. Age in years:
5. Gender: M ☐ F ☐
6. Date of Registration: _____
7. Date of first symptom: _____
8. Features suggestive of alcoholic hepatitis: Y ☐ N ☐
9. Definite and sufficient recent exposure to hepatotoxic agents: Y ☐ N ☐
10. Blood transfusion/infusion, injection/tattooing/acupuncture during 12 months: Y ☐ N ☐
11. History of undergoing surgical procedures during last 12 months: Y ☐ N ☐
12. History of recent contact with patients of acute hepatitis: Y ☐ N ☐
13. History of immunoglobulin injection: Y ☐ N ☐
14. Past history suggestive of hepatitis: Y ☐ N ☐

SYMPTOMS:-

15. Fatigue ☐
16. Anorexia ☐
17. Vomiting, Nausea ☐
18. Loose motions ☐
19. Yellow urine ☐
20. Yellow eyes ☐
21. Abdominal pain ☐
22. Fever ☐
23. Flu like symptoms ☐
24. Pruritus ☐
25. Arthralgia ☐
26. Others ☐
Specify: _____

SIGNS:-

29. Fever ☐
If present specify temperature _____ °C
30. Jaundice: ☐
31. Hepatomegaly ☐
If present specify size _____ cm
32. Hepatic tenderness ☐
33. Splenomegaly ☐
34. Lymphadenopathy ☐
35. Skin rash ☐
36. Ascites ☐
38. Signs of Liver failure ☐
If present specify _____

LABORATORY INVESTIGATIONS:

Liver Function tests: -

39. SGPT/ALT level: _____
40. SGOT/AST level: _____
41. Serum Bilirubin: _____
Total: _____
Conjugated: _____
42. Alkaline phosphatase: _____
43. HBsAg: _____
46. Assessment: - _____

44. Final category of Acute Hepatitis

- | | | | |
|---------------|--------------------------|---------------------|--------------------------|
| (a) HAV | ☐ | (f) Acute icteric | ☐ |
| (b) HBV | ☐ | (g) Acute anicteric | ☐ |
| (c) NANB | ☐ | (h) Asymptomatic | ☐ |
| (d) HEV | ☐ | | |
| (e) Bacterial | ☐ | | |

45. Bacteria: _____

APPENDIX – II

A. Blood Collection Procedure (Baron, 1990, Cheesbrough, 1984)

Blood for culture must be collected aseptically as possible the following technique is recommended.

1. Using a pressure cuff, a suitable vein is located in the arm.
2. The skin over the vein is cleansed in a circle approximately 5 cm in diameter with 70% alcohol, rubbing vigorously.
3. Starting in the centre of the circle, 2% tincture iodine is applied in everwidening circles until the entire circle has been saturated with iodine. The iodine is allowed to remain on the skin for at least 1 minute. The timing is critical, a watch or timer should be used.
3. Using a sterile syringe and size 21 gauze needle, appropriate volume (10 ml) of blood is withdrawn by inserting the needle into the vein.
4. After the needle has been removed, the site should be cleansed with 70% alcohol again, as many patients are sensitive to iodine.

B. Gram Staining Procedure (Baron, 1990)

Gram staining is very easy and reliable procedure for differentiating gram-positive and gram-negative organisms. It was first devised by Hans Christian Gram during the late 19th century. The following steps are involved in gram stain.

1. A thin film of a material to be examined is prepared and dried.
2. The material on the slide is heat fixed and allowed to cool before staining.
3. The slide is flooded with crystal violet stain and allowed to remain without drying for 10-30 seconds.
4. The slide is rinsed with tap water, shaking off all excess.
5. The slide is flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.
6. The slide is rinsed with tap water, shaking off all excess.
7. The slide is flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further colour flows from the slide with the decolorizer thicker smear requires more aggressive decolorizing.
8. The slide is flooded with counterstain (Safranin) for 30 seconds and washed off with tap water.
9. The slide is blotted between clean sheets to bibulous paper and examined microscopically under oil immersion at 100 x.

APPENDIX – III

A. Composition and Preparation of Different Culture Media. (Hi-Media Manual)

1. *Brain-Heart Infusion Broth (Hi-Media)*

Composition	gm/Lt
Calf Brain Infusion	200
Beef heart infusion	250
Protease peptone	10
Dextrose	2
Sodium chloride	5
Disodium phosphate	2.5
Final pH at 25°C	7.4 ± 0.2

Preparation: As directed by the manufacturing company, gm of the medium was dissolved in 1000 ml of distilled water. The medium was then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min.

2. *Blood Agar (Hi-Media)*

Blood agar base (infusion agar) – 5% blood.

Composition	gm/Lt
Beef heart infusion	500
Tryptose	10
Sodium Chloride	5
Agar	15
Final pH at 25°C	7.3 ± 0.2

Preparation: As directed by the manufacturing company, 40 gm of blood agar base was dissolved in 1000 ml distilled water. The medium is then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Thus prepared medium was cooled to about 40-50°C, to which 50 ml of sterile defibrinated blood was added aseptically.

3. *Chocolate Agar*

When the blood agar is heated at 75°C the red cells are lysed and the medium becomes brown in colour. It is referred to as chocolate agar.

4. *MacConkey Agar (Hi-Media)*

Composition	gm/Lt
Peptone	20
Lactose	10
Sodium turocholate	5
Agar	20
Neutral red	0.04
Final pH at 25°C	7.4 ± 0.2

Preparation: As directed by the manufacturing company, 55 gm of the medium was dissolved in 1000 ml distilled water. And it was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

5. **Mueller Hinton Agar (Hi-Media)**

Composition	gm/Lt
Beef heart infusion	300
Peptone	17.5
Starch	1.5
Agar	17
Final pH at 25°C	7.4±0.2

Preparation: As directed by the manufacturing company, 38 gm of the medium was dissolved in 1000 ml distilled water. The medium was then sterilized by autoclaving at 15 lbs pressure 9121°C) for 15 minutes.

6. **Nutrient Agar (Hi-Media)**

Composition	gm/Lt
Beef extract	10
Peptone	10
Sodium Chloride	5
Agar	12
Final pH at 25°C	7.4±0.2

Preparation: As directed by the manufacturing company, 39 gm of the medium was dissolved in 1000 ml distilled water and was sterilized by autoclaving at 15 lbs pressure 9121°C) for 15 minutes.

7. **Nutrient Broth**

Composition	gm/Lt
Lab-lemco powder	1.0
Yeast Extract	2.0
Peptone	5.0
Sodium Chloride	5.0

Preparation: As directed by the manufacturing company, 28 gm of the medium was dissolved in 1000 ml distilled water and distributed in test tubes. The medium was sterilized by autoclaving at 15 lbs pressure 9121°C) for 15 minutes.

B. **Composition and Preparation of Different Types of Biochemical Media. (Hi-Media Manual)**

1. **Christensen's Urea Broth (Hi-Media)**
Urea broth base + 40% (w/v) sterile urea solution

Composition	gm/Lt
Peptone	1
Dextrose	1
Sodium Chloride	5
Disodium Phosphate	1.2
Monopotassium phosphate	0.8
Phenol red	0.012
Final pH at 25°C	6.8±0.2

Preparation: As directed by the manufacturing company, 24 gm of the medium was dissolved in 950 ml distilled water and sterilized by autoclaving at 15 lbs pressure (121°C) for 20 minutes. The medium was cooled to about 45°C to which 50 ml of sterile 40% urea solution was mixed aseptically and distributed into sterile test tubes. (The medium was allowed to set in a slant position.

2. *Hugh-Leifson Medium (O/F basal medium) (Hi-Media)*

Composition	gm/Lt
Bacto peptone (Tryptone)	2
Sodium Chloride	5
Dipotassium Phosphate	0.3
Bacto bromothymol blue	0.08
Bacto agar	2

Preparation: As directed by the manufacturing company, 0.94 gm of the basal medium was dissolved in 100 ml distilled water and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Then 10 ml of sterile 10% dextrose solution was added to the medium and mixed thoroughly. Finally, the medium was dispensed in 5 ml amounts into sterile test tubes.

3. *Simmon's citrate Medium (Hi-Media)*

Composition	gm/Lt
Magnesium Sulphate	0.2
Monoammonium phosphate	1
Dipotassium phosphate	1
Sodium citrate	2
Sodium chloride	5
Agar	15
Bromothymol blue	0.08
Final pH at 25°C	6.8±0.2

Preparation: As directed by the manufacturing company, 24.2 gm of the medium was dissolved in 1000 ml distilled water. The medium was then dispensed in test tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The sterilized medium in the tubes was allowed to set in slopes.

8. *Sulphate Indole Motility Medium (SIM) (Hi-Media)*

Composition	gm/Lt
Beef extract	3
Peptone	30
Peptonized iron	0.2
Sodium thiosulphate	0.025
Agar	3
Final pH at 25°C	7.3±0.2

Preparation: As directed by the manufacturing company, 36 gm of the medium was dissolved in 1000 ml distilled water and distributed in test tubes. The medium was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

9. *Triple sugar Iron Agar (TSI) Hi-Media*

Composition	gm/Lt
Peptone	10
Tryptone	10
Yeast extract	3
Beef extract	3
Lactose	10
Sucrose	10
Dextrose	1
Ferrous Sulphate	0.2
Sodium chloride	5
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12
Final pH at 25°C	7.4±0.2

Preparation: As directed by the manufacturing company, 65 gm of the medium was dissolved in 1000 ml distilled water and then distributed in test tubes. The medium was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes then the medium was allowed to set in a sloped with a butt of about 1 inch.

C. *Composition and Preparation of Staining Reagents. (Cheesbrough, 1984)*

I. *Gram Stain Reagents.*

I. *Crystal Violet Stain*

Composition	gm/lit
Crystal violet	20 gm
Ammonium oxalate	9 gm
Ethanol (absolute)	9.5 ml
Distilled water (D/S) to make	1 lit.

Preparation: In a piece of clean paper, 20 gm of crystal violet weighed and transferred to a clean brown bottle. Then, 95 ml of ethanol was added and mixed until the dye was completely dissolved. To the mixture, 9 gm of ammonium oxalate dissolved in 200 ml of D/W was added. Finally, the volume was made 1 Lt by adding D/W.

II. *Lugol's Iodine Solution*

Composition	gm
Potassium iodide	20
Iodine	10
Distilled water (D/W) to make	1 lit.

Preparation: To 250 ml of distilled water, 20 gm of potassium iodide was dissolved. Then 10 gm of iodine was mixed to it until it is dissolved completely. Finally, the volume was made 1 Lt by adding D/W.

III. Acetone – Alcohol Decolorizer.

Composition

Acetone	500 ml
Ethanol (absolute)	475 ml
Distilled water	25 ml

Preparation: To 25 ml distilled water, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then immediately, 500 ml acetone is added to the bottle and mixed well.

IV. Counterstain Solution

Composition

Safranin, 99% dye content	10 g.
Distilled water to make	1 Lt.

Preparation: In a piece of clean paper, 10 gm of safranin is weighed and transferred to a clean bottle. Then 1 Lt distilled water is added to the bottle and mixed well until safranin dissolves completely.

D. Composition and Preparation of Test Reagents. (Cheesbrough, 1984)

1. Catalase Reagent

To make 100 ml

Hydrogen peroxide solution	3 ml
Distilled water	97 ml

Preparation: To 97 ml distilled water, 3 ml of hydrogen peroxide was added and mixed well.

2. Oxidase Reagent

To make 10 ml

Tetramethyl-p-phenylenediamine dihydrochloride (TPD)	0.1 gm
Distilled Water	10 ml

Preparation: This reagent solution was made by dissolving 0.1 gm TPD in 10 ml distilled water. (To that solution strips of Whatman's No. 1 filter paper were soaked and drained for about 30 seconds. Then these strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap)

3. Kovac's Indole Reagent

To make 40 ml

4-dimethylaminobenzaldehyde	2 gm
Isoamyl alcohol	30 mg
Hydrochloric acid, concentrated	10 ml

Preparation: In 30 ml of isoamyl alcohol, 2 gm of 4-dimethyl aminobenzaldehyde was dissolved and transferred to a clean brown bottle. Then to it, 10 ml of Conc. HCl was added and mixed well.

3. ***Methyl Red Solution***

To make 50 ml

Methyl red	0.5 gm
Ethanol, absolute	28 ml
Distilled water	22 ml

Preparation: To 28 ml ethanol, 0.05 gm of methyl red was dissolved and transferred to a clean brown bottle. Then 22 ml distilled water is added to that bottle and mixed well.

4. ***Voges-Proskauer Reagent (Barritt's Reagent)***

I. VP Reagent A:

To make 100 ml

α -naphthol	5 gm
Ethanol, absolute	100 ml

Preparation: To 25 ml distilled water, 5 gm α -naphthol was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

II. VP reagent B:

To make 100 ml

Potassium hydroxide	40 gm
Distilled water	100 ml

Preparation: To 25 ml distilled water, 40 gm of KOH was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml adding distilled water.

5. ***Hydrochloric acid (1 ml 1N HCl) Solution***

To make 100 ml

Hydrochloric Acid, concentrated	8.6 ml
Distilled water to make	100 ml

Preparation: 8.6 ml of concentrated hydrochloric acid was added to 25 ml distilled water into a clean bottle. Then the final volume was made 100 ml by adding distilled water.

APPENDIX-IV

A. Equipments Used.

1. Autoclave
2. Centrifuge
3. Incubator
4. Oven
5. Refrigerator
6. Precision Microplate Reader, Molecular devices, Emax.
7. Automated spectrometric analyzer: Biochemical System, Screen Master.

B. Antibiotic Discs Used

Different antibiotic discs manufactured by "Span Diagnostics Ltd., Surat, India & HiMedia Laboratories Ltd., Mumbai, India" were used for susceptibility tests following Kirby Bauer Method.

The various types of antibiotic discs used according to the nature of organisms are as follows.

1. Gram Positive Organism: **Ampicillin, Ciprofloxacin, Amikacin, Chloramphenicol, Tetracycline, Cephalexin, Methicillin, Penicillin, Erythromycin, Vancomycin.**
2. Gram Negative Organism: **Ampicillin, Cotrimoxazole, Ciprofloxacin, Cefotaxime, Cephalexin, Amikacin, Chloramphenicol, Tetracycline, Gentamicin.**

**The zone size interpretative chart of these antibiotic discs is given in appendix V.*

C. Kits used for identifying viral markers:

- I. HBsAg : - Mycell II HBsAg, Mar., 1996; RPHA. (Appendix VII)
- II. Anti-HEV: - greiner ELISA-F; Institute of Hepatology, Tokyo. (Appendix VIII)
- III. Anti-HCV: - Ortho HCV 3.0 ELISA Test System with Enhanced SAVE. (Appendix IX)

D. Kits used for biochemistry:

- I. ALT: - Human Gasellschaft for Biochemica und Diagnostica mbH. (Appendix X)
- II. Serum bilirubin (total & conjugated):- Human Gasellschaft for Biochemica und Diagnostica mbH. (Appendix XI)



MICROBIAL SENSITIVITY DISCS

Code Nos. 13301 to 13359

DESCRIPTION

Sensitivity Discs are prepared by accurately impregnating special quality of absorbent paper with standard amounts of Antibiotic/Chemotherapeutic agent. Discs are clearly marked with codes and numbers designating the active ingredient and its amount respectively. SPAN's Sensitivity Discs are suitable for a wide range of Antibiotics and Chemotherapeutic Agents in levels that closely approximate those attainable *in vivo*.

PRINCIPLE

Microbial susceptibility testing with Discs is a simple and rapid method and provides a reproducible means of testing bacterial sensitivity to various Antibiotics and Chemotherapeutic Agents. The test is based on the fact that for a given Antibiotic, the size of the zone of inhibition is inversely related to the MIC (determined by the dilution method) of the strain being tested when the test conditions are held constant.

REAGENTS

Trypticase Soy Broth, 5 ml (or any suitable broth)

Mueller Hinton Agar, pH 7.3 $\pm$ 0.1

Barium Sulphate Standard

Sterile Saline or Broth

DRAGE & STABILITY

The sensitivity discs are stable at 2-8°C till the expiry date mentioned on the individual label.

PRECAUTIONS

1. Allow the vial (s) to come to room temperature before opening, to avoid condensation of moisture.
2. As soon as the application is over, tightly stopper and promptly transfer the vial(s) to the refrigerator.
3. Use the oldest discs first and discard the expired ones.

PROCEDURE

Preparation of Plates

Sterile Mueller Hinton Agar (pH 7.3 $\pm$ 0.1), is poured into plates (Petri dishes) kept on a level surface. The depth of the medium should be approx. 4 mm. After the medium has solidified, dry the plates for 30 minutes in an incubator (35-37°C) to remove excess moisture from the surface. While pouring into the plates, 5% defibrinated sterile sheep blood should be aseptically added to MHA for testing Streptococci and other fastidious organisms.

Preparation of Inoculum

Use only pure culture for sensitivity testing. Perform gram staining before preparing an inoculum.

- a. Select 4 to 5 similar colonies and transfer them into a tube containing 5 ml of Trypticase Soy Broth (or a similar broth) with the help of wire loop.
- a. Incubate the broth culture at 35-37°C for 2 to 5 hours to obtain moderate turbidity.
- a. Dilute the broth culture of actively growing organisms with sterile broth or saline to obtain a turbidity equivalent to that of Barium Sulphate standard, which is prepared by adding 0.5 ml of 1.175% BaCl₂·2H₂O solution to 99.5 ml of 0.36 N H₂SO₄ (equivalent to half the density of McFarland Standard No. 1). Overnight cultures of aerobes/non-fastidious organisms generally have too much growth to be used undiluted. When tube does not permit for the development of a turbid broth culture, colonies can be suspended directly into a small volume of saline or broth which is then further diluted. In order to prevent further growth the diluted and standardized inoculum should not be allowed to stand longer than 15 to 20 minutes before the plates are inoculated.

Inoculation

- a. Dip a sterile cotton swab into the diluted culture in a test tube and rotate it along the inner surface of the upper margin of the plate, all over the fluid level to remove excess inoculum.
- a. Streak the agar surface of the plate in three directions, turning the plate by 60° between each streaking.
- a. Replace the lid of the petri dish and keep it at room temperature for 5 to 10 minutes, but no longer than 15 minutes to dry the inoculum. Confluent growth is desirable for accurate results.

Application of Discs

Remove sensitivity discs from their respective vials with the help of a flamed forceps and carefully place them in the plate (s), atleast 15 mm away from the edge, at equal distance and sufficiently separated from each other to avoid the overlapping of the zones of inhibition. Finally, press them lightly with the forceps to make complete contact with the surface of the medium. Allow the plates to stand at room temperature for 30 minutes (Pre-incubation time).

Incubation

Incubate the plates 35-37°C for 16 to 18 hours.

Reading of Zones

Measure the diameter of the zone of inhibition at the end of the incubation period. If plates are showing proper growth, they may be read after 6 to 8 hours in clinically urgent situations. Measure only those zones that are showing complete inhibition and record the zone diameter to the nearest millimeter. If only isolated colonies grow instead of confluent growth, the inoculum is too light and the test should be repeated.

INTERPRETATION

Though there is some correlation between the size of the zone of inhibition and the susceptibility of the organism to the drug, the former is a function of many variable e.g. density of the inoculum, depth of the medium, diffusibility of the drug etc. The size of the inhibition zone at which the organism is considered Resistant, Intermediate or Sensitive is given in the zone size interpretative chart at the end of this literature.

PACKAGING

ITEM	PACKING	CODE NOS.
Sensitivity Discs	50 discs	13301 to 13359

REFERENCES

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- Journal of Clinical Microbiology, 4th Edition (1985), P. 978-987. American Society for Microbiology, Washington DC.
- Performance Standards for Antimicrobial Disk Susceptibility Tests Fifth Edition, National Committee for Clinical Laboratory Standards Vol. 13, 24, 1993.
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ZONE SIZE INTERPRETATIVE CHART

Antibiotic or Chemotherapeutic Agent	Symbol	Strength	Diameter of Zone of Inhibition		
			Resistant: mm or less	Intermediate mm	Sensitive mm or more
Amikacin	AK	30mcg	14	15-16	17
Ampicillin	AM	10mcg	13	14-17	18
Staphylococci			13		20
Other organisms			13		18
Ampicillin		10mcg	13	14-16	17
Gram-negative organisms			13		20
Staphylococci			13		20
Enterococci			13		20
Streptococci (Not S. pneumoniae)			13		20
Listeria monocytogenes			13		20
Bacitracin	B	10 Units	8	9-12	13
Carbenicillin	CB	100mcg	13	14-16	17
Pseudomonas aeruginosa			13		20
Other gram-negative organisms			13		20
Cefaclor	CG	30mcg	14	15-17	18
Cefadroxil	CD	30mcg	14	15-17	18
Cefazolin	CF	30mcg	14	15-17	18
Cefotaxime	CX	30mcg	14	15-17	18
Ceftazidime	CZ	30mcg	14	15-17	18
Ceftiofur	CO	30mcg	13	14-20	21
Cefuroxime sodium	CR	30mcg	14	15-17	18
Cephalexin (Mainly for Gm+ve)	CP	30mcg	14	15-17	18
Cephadrine	H	30mcg	11	12-15	16
Chloramphenicol (Haemophilus influenzae + salmonella)	C	30mcg	12	13-17	18
Chlortetracycline (infection)	A	30mcg	14	15-18	19
Ciprofloxacin (Staph aureus (Not given for child))	CI	5mcg	15	16-20	21
Clindamycin	CM	2mcg	14	15-20	21
Cloxacillin	V	5mcg	11	12-13	14
Colistin	W	10mcg	8	9-10	11
Co-trimazine	CT	25mcg	10	11-15	16
Co-trimoxazole	Q	25mcg	10	11-15	16
Damocycline	L	30mcg	14	15-18	19
Doxycycline	VB	30mcg	12	13-15	16
Erythromycin	E	15mcg	13	14-22	23
Framycetin	SF	50mcg	15	16-20	21
Furazolidone	FZ	100mcg	14	15-16	17
Gentamicin	J	10mcg	12	13-14	15
Kanamycin	K	30mcg	13	14-17	18
Lincomycin	LN	15mcg	13	14-17	18
Methenamine Mandelate	M	2mg	11	12-16	17
Nalidixic Acid	NA	30mcg	13	14-18	19
Neomycin	N	30mcg	12	13-16	17
Netilmicin	NT	30mcg	12	13-14	15
Nitrofurantoin (UTI for both Gm+ve & +ve)	F	300mcg	14	15-16	17
Norfloxacin (But disp. for Gm+ve)	NF	10mcg	12	13-16	17
Ofloxacin	OF	5mcg	12	13-15	16
Oxytetracycline	O	30mcg	14	15-18	19
Penicillin G	P	10 Units	28		29
Staphylococci			28		29
Enterococci			14		15
Listeria monocytogenes			13		20
Streptococci (Not S. pneumoniae)			13		20
Polymyxin B	X	300 Units	8	9-11	12
Sisomicin	SS	10mcg	12	13-15	16
Streptomycin	S	10mcg	11	12-14	15
Sulphadiazine	Z	300mcg	12	13-16	17
Sulphadiazine	D	300mcg	12	13-16	17
Sulphafurazole	G	300mcg	12	13-16	17
Sulphamethazole	U	300mcg	12	13-16	17
Sulphaphenazole	Y	300mcg	12	13-16	17
Tetracycline	T	30mcg	14	15-18	19
Tobramycin	TB	10mcg	12	13-14	15
Trimethoprim	TR	5mcg	10	11-15	16
Triple Sulphas	TS	300mcg	12	13-16	17
Pefloxacin	PF	5mcg	12	13-16	17
Cefoperazone	PZ	75mcg	15	16-20	21
Ceftiofur	ZN	30mcg	14	15-19	20
Azithromycin	AZ	15mcg	13	14-17	18
Loneloxacin	LF	10mcg	18	19-21	22
Spiramycin	R	25mcg	16	17-20	21

Span Diagnostics Ltd.

PAN Plot No. 336-340, G.I.D.C., Sachin - 392 230 (Surat) INDIA

300mcg Pseudomonas 29 → All (R)

15-16
10-11

23
22

Antimicrobial Susceptibility Test Discs

Susceptibility Test Procedure :

1. Prepare plates with Mueller Hinton Agar (M173) for use in the Bauer-Kirby Method for rapidly growing aerobic organisms. For fastidious organisms such as *Streptococci*, the Agar (M173) is supplemented with 5% sterile, defibrinated blood. For *Haemophilus* spp, Haemophilus Test Medium (M1259) and for *N. gonorrhoeae*, GC Agar Base (M434) with 1% defined growth supplement are recommended respectively. The medium in the plates should be sterile and have a depth of about 4 mm.
2. Use pure cultures as inoculum. Select 3-4 similar colonies and transfer them into about 5 ml of suitable broth such as Tryptone Soya Broth (M011). Incubate at 35°C for 2-8 hours till light to moderate turbidity develops. If the turbidity in the broth is sufficient, further incubation is not necessary.
3. Dip a sterile non-toxic cotton swab on a wooden applicator into the standardized inoculum (turbidity so adjusted, as to obtain confluent growth on the petri plate) and rotate the soaked swab firmly against the upper inside wall of the tube to express excess fluid. Streak the entire agar surface of the plate with the swab three times, turning the plate at 60° angle between each streaking. Allow the inoculum to dry for 5 - 15 minutes with lid in place.
4. Apply the discs using aseptic technique. Deposit the discs with centres at least 24 mm apart. For fastidious organisms and for Penicillins and Cephalosporins, the discs should preferably be deposited with centres 30 mm apart. Deposit OctoDiscs in the centre of the plate.
5. Incubate immediately at 37°C and examine after 14-19 hours or later if necessary. Measure the zones showing complete inhibition and record the diameters of the zones to the nearest millimeter.

Precautions :

1. Accuracy of the test depends on the disc potency, proper inoculum, functional pretested medium plates (nature of medium and its depth), inoculation technique, incubation temperature and time, etc.
2. To maintain the potency of discs, store the stock containers of discs in the freezer at -20°C. The discs when required for use within a week, may be kept in refrigerator (below 8°C) and the remainder should be kept with desiccant and tightly closed container caps in the freezer.
3. Remove the antimicrobial discs from refrigerator to room temperature 1 - 2 hours before use to avoid moisture condensation. Return unused discs to refrigerator immediately after applications.
4. While reading results :
 - (i) Ignore swarming of *Proteus* species if zones of inhibition are clearly defined.
 - (ii) Measure the Sulphonamide zones at the margin of heavy growth since Sulphonamide may not inhibit organisms for several generations and slight growth may appear within zones of inhibition.
 - (iii) Subculture, reidentify and retest any large colony growing within a clear zone of inhibition.
5. Control tests using known cultures should be included each time a sensitivity test is performed.
6. Antibacterial agents other than those listed in Interpretative Chart are in current use. Susceptibility tests employing these agents should be interpreted on the basis of presence or absence of a definite zone of inhibition and should be considered as only qualitative until the time interpretative zones have been established.

Note :

1. Ampicillin disc is used for testing susceptibility to Amoxycillin as well.
2. Cephalothin disc is used for testing susceptibility to Cephapirin, Cephaloridine, Cephalexin, Cefactor, Cephoxitin, Cefazolin, Cephadrine and Cephadroxil as well.
3. The Sulfisoxazole (Sulphafurazole) disc can be used to represent any of the currently available sulfonamide preparations.
4. The category "Intermediate" should be reported. Infections with bacteria of intermediate susceptibility may be considered moderately susceptible and may respond to antimicrobial agents with a wide safe dosage range.
5. While using OctoDiscs, known positive (sensitive) control cultures must be used. Results can be interpreted as:

Sensitive	: a zone within 3 mm radius of that of the positive control.
Resistant	: a zone of not more than 2 mm radius.
Intermediate	: a zone falling between the above limits.
6. Since certain strains of *Providencia* spp. have been reported to give false susceptible results with Cefprozil discs, strains of this genus should not be tested & reported with this disc.
7. All *Staphylococcal* isolates with zone diameters of 14 mm or less should be tested by an MIC method.
8. When testing Vancomycin against *Enterococci*, plates should be held a full 24 hrs & examined using transmitted light; the presence of a haze or any growth within the zone of inhibition indicates resistance.
9. Ofloxacin susceptible *S. pneumoniae* will also be susceptible to Levofloxacin.
10. Susceptibility & resistance to Azithromycin, Clarithromycin & Dirithromycin can be predicted by using Erythromycin for *Streptococci*.

ONE SIZE INTERPRETATIVE CHART

based on Results obtained using Mueller Hinton Agar

Product	Antimicrobial Agent	Symbol	Disc content	Resistant mm or less	Intermediate mm	Sensitive mm or more	Diameter of zone of inhibition in mm					
							E. coli ATCC 25922	S. aureus ATCC 25923	P. aeruginosa ATCC 27853	H. influenzae ATCC 49247	S. pneumoniae ATCC 49619	N. gonorrhoeae ATCC 49226
SD035	Amlkacin	Ak	30 mcg	14	15-16	17	19-26	20-26	18-26	—	—	—
SD063	Amoxycillin / Clavulanic acid	Ac	20/10 mcg (30 mcg)	13	14-17	18	18-24	28-36	—	15-23	—	—
When testing Haemophilus spp. and Staphylococci												
When testing gram-negative enteric organisms												
SD062	Ampicillin	A	10 mcg	13	14-16	17	16-22	—	—	—	—	—
When testing gram-negative enteric organisms												
When testing Staphylococci												
When testing Haemophilus spp.												
When testing S. pneumoniae												
When testing Streptococcus other than S. pneumoniae												
SD112	Ampicillin / Sulbactam	As	10/10 mcg	11	12-14	15	19-24	29-37	—	14-22	—	—
When testing gram-negative enteric organisms and Staphylococci												
When testing Haemophilus spp.												
SD204	Azithromycin	At	15 mcg	13	14-17	18	—	21-26	—	—	19-25	—
When testing Haemophilus spp.												
SD084	Azlocillin	Az	75 mcg	17	—	18	—	—	24-30	—	—	—
SD212	Aztreonam	Ao	30 mcg	15	16-21	22	28-36	—	23-29	—	—	—
When testing gram-negative enteric organisms and P. aeruginosa												
When testing Haemophilus spp.												
SD003	Bacitracin	B	10 units	8	9-12	13	—	12-22	—	—	—	—
SD004	Carbenicillin	Cb	100 mcg	13	14-16	17	23-29	—	18-24	—	—	—
When testing gram-negative enteric organisms												
When testing P. aeruginosa												
SD157	Cefaclor	Cj	30 mcg	14	15-17	18	23-27	27-31	—	—	24-32	—
When testing Streptococci												
SD200	Cefamandole	Cef	30 mcg	14	15-17	18	26-32	26-34	—	—	—	—
SD047	Cefazolin	Cz	30 mcg	14	15-17	18	21-27	29-35	—	—	—	—
SD218	Cefdinir	Cdn	5 mcg	16	17-19	20	24-28	25-32	—	—	—	—
When testing gram-negative enteric organisms and Staphylococci												
When testing N. gonorrhoeae												
When testing S. pneumoniae												
SD219	Cefepime	Cepm	30 mcg	14	15-17	18	31-37	23-29	24-30	—	—	—
When testing gram-negative enteric organisms, P. aeruginosa and Staphylococci												
When testing Haemophilus spp.												
When testing N. gonorrhoeae												
When testing Streptococcus other than S. pneumoniae												
When testing S. pneumoniae												
SD211	Cefixime	Cfx	5 mcg	15	16-18	19	23-27	—	—	25-33	—	—
When testing gram-negative enteric organisms												
When testing Haemophilus spp.												
When testing N. gonorrhoeae												
When testing S. pneumoniae												
SD072	Cefoperazone	Cs	75 mcg	15	16-20	21	28-34	24-33	23-29	—	—	—
SD725	Cefpodoxime	Cep	10 mcg	17	18-20	21	23-28	19-25	—	25-31	—	—
When testing gram-negative enteric organisms and Staphylococci												
When testing Haemophilus spp.												
When testing N. gonorrhoeae												
When testing S. pneumoniae												
SD208	Cefprozil	Cfpz	30 mcg	14	15-17	18	21-27	27-33	—	—	25-32	—
When testing gram-negative enteric organisms and Staphylococci												
When testing S. pneumoniae												
SD062	Ceftazidime	Ce	30 mcg	14	15-17	18	25-32	16-20	27-29	—	—	—
When testing Haemophilus spp.												
When testing N. gonorrhoeae												
SD110	Ceftiozone	Ck	30 mcg	14	15-19	20	30-36	27-35	12-17	—	28-34	—
When testing N. gonorrhoeae												
When testing Haemophilus spp.												
SD065	Ceftriaxone	Ci	30 mcg	13	14-20	21	29-35	22-28	17-23	—	—	—
When testing Haemophilus spp.												
When testing S. pneumoniae												
When testing Streptococcus other than S. pneumoniae												
When testing N. gonorrhoeae												

✓ : In accordance to Performance Standards for Antimicrobial Disk Susceptibility Tests, NCCLS.

* : Not included in NCCLS chart; FDA approved performance standards for Antimicrobial Disks obtained from drug manufacturers.

Δ : For E. coli, S. aureus, P. aeruginosa : Mueller Hinton Agar (MHA). For Haemophilus spp. : Haemophilus Test Medium; For S. pneumoniae : Mueller Hinton Agar with 5% sheep blood. For N. gonorrhoeae : GC Agar Base With 1% defined growth supplement.

References: 1. Bauer, Kirby, Sherris and Tenckhoff, 1966, Am. J. Clin. Path., 45 : 483.

For more details refer to this volume.

DISK SIZE INTERPRETATIVE CHART

Based on Results obtained using Mueller Hinton Agar) Δ

Product Code	Antimicrobial Agent	Symbol	Disc content	Resistant mm or less	Intermediate mm	Sensitive mm or more	Diameter of zone of inhibition in mm					
							Quality Control Limits					
							<i>E. coli</i> ATCC 25922	<i>S. aureus</i> ATCC 25923	<i>Ps. aeruginosa</i> ATCC 27853	<i>H. influenzae</i> ATCC 49247	<i>S. pneumoniae</i> ATCC 49619	<i>N. gonorrhoeae</i> ATCC 49226
10061	Cefuroxime Sodium (Parenteral) When testing <i>N. gonorrhoeae</i> When testing <i>Haemophilus</i> spp.	Cu	30 mcg	14 25 16	15-17 26-30 17-19	18 31 20	20-26 — —	27-35 — —	— — —	— — —	— — —	— 33-41 —
10050	Cephalexin	Ch	30 mcg	14	15-17	18	15-21	29-37	—	—	28-32	—
10040	Cephalexime When testing <i>Haemophilus</i> spp. When testing <i>S. pneumoniae</i> When testing <i>Streptococcus</i> other than <i>S. pneumoniae</i> When testing <i>N. gonorrhoeae</i>	Ce	30 mcg	14 — — —	15-22 — — —	23 25 — —	29-35 — — —	25-31 — — —	18-22 — — —	— 31-39 — —	— 31-39 — —	— — — 38-48
10041	Cephalexin When testing <i>N. gonorrhoeae</i>	Cn	30 mcg	14 23	15-17 24-27	18 28	23-29 —	23-29 —	— —	— —	— —	33-41 —
10006	Chloramphenicol When testing <i>Haemophilus</i> spp. When testing <i>S. pneumoniae</i> When testing <i>Streptococcus</i> other than <i>S. pneumoniae</i>	C	30 mcg	12 25 20 17	13-17 26-28 — 18-20	18 29 21 21	21-27 — — —	19-26 — — —	— — — —	— 31-40 — —	— — 23-27 —	— — — —
10060	Ciprofloxacin When testing <i>Haemophilus</i> spp. When testing <i>N. gonorrhoeae</i>	Cf	5 mcg	15 — 27	16-20 — 28-40	21 21 41	30-40 — —	22-30 — —	25-33 — —	— 34-42 —	— — —	— — 48-56
10192	Clarithromycin When testing <i>Streptococci</i> When testing <i>Staphylococci</i> When testing <i>Haemophilus</i> spp.	Cw	15 mcg	16 13 10	17-20 14-17 11-12	21 18 13	— — —	— 26-32 —	— — —	— — 11-17	25-31 — —	— — —
10051	Clindamycin When testing <i>Staphylococci</i> When testing <i>Streptococci</i>	Cd	2 mcg	14 15	15-20 16-18	21 19	— —	24-30 —	— —	— —	— 19-25	— —
10009	Colistin	Cl	10 mcg	8	9-10	11	11-15	—	—	—	—	—
10010	Co-Trimoxazole (Trimethoprim/ Sulphamethoxazole) When testing <i>S. pneumoniae</i>	Co	1.25/ 23.75 mcg	10 15	11-15 16-18	16 19	23-29 —	24-32 —	— —	24-32 —	— 20-28	— —
10012	Doxycycline Hydrochloride	Do	30 mcg	12	13-15	16	18-24	23-29	—	—	—	—
10013	Erythromycin When testing <i>Staphylococci</i> When testing <i>Streptococci</i>	E	15 mcg	13 15	14-22 16-20	23 21	— —	22-30 —	— —	— —	25-30 —	— —
10205	Fosfomycin When testing gram-negative enteric organisms When testing <i>Staphylococci</i>	Fo	200mcg	— 12	— 13-15	— 16	— 22-30	— 25-33	— —	— —	— —	— —
10016	Gentamicin	G	10 mcg	12	13-14	15	19-28	19-27	16-21	—	—	—
10137	Gatifloxacin	Gl	5 mcg	—	—	—	30-37	27-33	20-28	33-41	24-31	45-56
10073	Imipenem When testing <i>Haemophilus</i> spp.	I	10 mcg	13 —	14-15 —	16 15	26-32 —	— —	20-28 —	21-29 —	— —	— —
10017	Kanamycin	K	30 mcg	13	14-17	18	17-25	19-26	—	—	—	—
10218	Levofloxacin When testing gram-negative enteric organisms, <i>Streptococci</i> and <i>Staphylococci</i> When testing <i>Haemophilus</i> spp.	Le	5 mcg	— 13	— 14-16	— 17	— 29-37	— 25-30	— 19-26	— 32-40	— 20-25	— —
10215	Linezolid	Lz	30 mcg	—	—	—	—	27-31	—	—	28-34	—
10206	Lomefloxacin When testing <i>N. gonorrhoeae</i> When testing <i>Haemophilus</i> spp.	Lo	10 mcg	18 26	19-21 27-37	22 38 22	27-33 — —	23-29 — —	22-28 — —	— 33-41 —	— — —	— 45-54 —
10127	Meropenem When testing gram-negative enteric organisms, <i>Staphylococci</i> and <i>Ps. aeruginosa</i> When testing <i>Haemophilus</i> spp. When testing <i>S. pneumoniae</i>	Mr	10 mcg	— 13 —	— 14-15 —	— 16 20	— 28-34 —	— 29-37 —	— 27-33 —	— — 20-28	— — 28-35	— — —
10019	Methicillin	M	5 mcg	9	10-13	14	—	17-22	—	—	—	—
10158	Minocycline	Mi	30 mcg	14	15-18	19	19-25	25-30	—	—	—	—
10220	Moxalactam	Mx	30 mcg	14	15-22	23	28-35	18-24	17-25	—	—	—
10217	Moxifloxacin	Mo	5 mcg	—	—	—	28-35	28-35	17-25	31-39	25-31	—
10021	Nalidixic Acid	Na	30 mcg	13	14-18	19	22-28	—	—	—	—	—
10022	Neomycin	N	30 mcg	12	13-16	17	17-23	18-26	—	—	—	—
10046	Netilmicin (Netilmicin Sulphate)	Nt	30 mcg	12	13-14	15	22-30	22-31	17-23	—	—	—
10023	Nitrofurantoin	Nf	300 mcg	14	15-16	17	20-25	18-22	—	—	23-29	—
10057	Norfloxacin When testing <i>S. pneumoniae</i>	Nx	10 mcg	12 —	13-16 —	17 —	28-35 —	17-28 —	22-29 —	— —	15-21 —	— —
10053	Novobiocin	Nv	30 mcg	17	18-21	22	—	22-31	—	—	—	—
10087	Ofloxacin When testing <i>Haemophilus</i> spp. When testing <i>Streptococci</i> When testing <i>N. gonorrhoeae</i>	Of	5 mcg	12 — 12 24	13-15 — 13-15 25-30	16 — 16 31	28-33 — — —	24-28 — — —	17-21 — — —	— 31-40 — —	— — 16-21 —	— — — 43-51

Y : In accordance to Performance Standards for Antimicrobial Disk Susceptibility Tests, NCCLS

Δ : Not included in NCCLS chart; FDA approved performance standards for Antimicrobial Discs obtained from drug manufacturers.

1 : For *E. coli*, *S. aureus*, *Ps. aeruginosa* : Mueller Hinton Agar (MHA). For *Haemophilus* spp. : *Haemophilus* Test Medium; For *S. pneumoniae* : Mueller Hinton Agar with 5% sheep blood; For *N. gonorrhoeae* : GC Agar Base with 1% defibrinated growth supplement.

References: 1. Bauer, Kirby, Sherris and Tenck, 1966, Am. J. Clin. Path., 45 : 493.

2. Performance Standards for Antimicrobial Disk Susceptibility Tests, NCCLS Vol. 22 No. 1, Jan. 2002. For more details refer to this volume.

ZONE SIZE INTERPRETATIVE CHART

(Based on Results obtained using Mueller Hinton Agar) Δ

Product Code	Antimicrobial Agent	Symbol	Disc content	Resistant mm or less	Intermediate mm	Sensitive mm or more	Diameter of zone of inhibition in mm					
							Quality Control Limits					
							<i>E. coli</i> ATCC 25922	<i>S. aureus</i> ATCC 25923	<i>Ps. aeruginosa</i> ATCC 27853	<i>H. influenzae</i> ATCC 49241	<i>S. pneumoniae</i> ATCC 49619	<i>H. parvus</i> ATCC 49206
SD088	Oxacillin When testing <i>Staphylococci</i> When testing coagulase negative <i>Staphylococci</i> When testing <i>S. pneumoniae</i>	Ox	1 mcg	10	11-12	13	—	18-24	—	—	8-12	—
SD186	Oxolinic acid	Oa	2 mcg	10	—	11	20-24	10-13	—	—	—	—
SD028	Penicillin G When testing <i>Staphylococci</i> When testing <i>S. pneumoniae</i> When testing <i>Streptococcus</i> other than <i>S. pneumoniae</i> When testing <i>N. gonorrhoeae</i>	P	10 units	28	—	29	—	26-37	—	—	24-30	—
SD066	Piperacillin When testing <i>Ps. aeruginosa</i> When testing gram negative enteric organisms	Pc	100 mcg	17	—	18	—	—	25-33	—	—	—
SD210	Piperacillin / Tazobactam When testing gram negative enteric organisms When testing <i>Staphylococci</i> and <i>Ps. aeruginosa</i> When testing <i>Haemophilus spp.</i>	Pt	100/10mcg	17	18-20	21	24-30	—	—	31-38	—	—
SD029	Polymyxin B	Pb	300 units	8	9-11	12	12-16	—	—	—	—	—
SD178	Pristinamycin (Quinupristin/ Dalfopristin) When testing <i>Staphylococci</i> and <i>Streptococci</i> When testing <i>Haemophilus spp.</i>	Pm	15 mcg	15	16-18	19	—	23-29	—	15-21	19-24	—
SD030	Rifampicin When testing <i>E. coli</i> When testing <i>Staphylococci</i> and <i>Haemophilus spp.</i> When testing <i>S. pneumoniae</i>	R	5 mcg	—	—	—	8-10	—	—	—	—	—
SD162	Sparfloxacin When testing <i>E. coli</i> When testing <i>N. gonorrhoeae</i> When testing <i>Haemophilus spp.</i>	Sc	5 mcg	15	16-18	19	—	27-33	21-29	—	21-27	—
SD181	Spectinomycin	Se	100 mcg	14	15-17	18	—	—	—	32-40	—	—
SD031	Streptomycin When testing <i>Staphylococci</i>	S	10 mcg	11	12-14	15	12-20	—	—	—	—	—
SD032	Sulphafurazole (Sulfisoxazole)	Sf	300 mcg	12	13-16	17	15-23	24-34	—	—	—	—
SD213	Telocyclan	Tc	30 mcg	10	11-13	14	—	15-21	—	—	—	—
SD037	Tetracycline When testing <i>Haemophilus spp.</i> When testing <i>Streptococci</i> When testing <i>N. gonorrhoeae</i>	T	30 mcg	14	15-18	19	18-25	24-30	—	14-22	27-31	10-17
SD074	Ticarcillin When testing <i>Ps. aeruginosa</i> When testing gram-negative enteric organisms	Ti	75 mcg	14	—	15	—	—	21-27	—	—	—
SD201	Ticarcillin / Clavulanic acid When testing gram-negative enteric organisms When testing <i>Ps. aeruginosa</i> When testing <i>Staphylococci</i>	Tc	75/10 mcg	14	15-19	20	24-30	—	—	20-28	—	—
SD044	Tobramycin	Tb	10 mcg	12	13-14	15	18-26	19-29	19-25	—	—	—
SD039	Trimethoprim	Tr	5 mcg	10	11-15	16	21-28	19-26	—	—	—	—
SD045	Vancomycin When testing <i>Staphylococci</i> When testing <i>Streptococci</i>	Va	30 mcg	—	—	15	—	17-21	—	—	20-27	—

▼ : In accordance to Performance Standards for Antimicrobial Disk Susceptibility Tests, NCCLS.

* : Not included in NCCLS chart; FDA approved performance standards for Antimicrobial Discs obtained from drug manufacturers.

Δ : For *E. coli*, *S. aureus*, *Ps. aeruginosa* : Mueller Hinton Agar (MHA). For *Haemophilus spp.* : *Haemophilus* Test Medium; For *S. pneumoniae* : Mueller Hinton Agar with 5% sheep blood; For *N. gonorrhoeae* : GC Agar Base with 1% defined growth supplement.

References: 1. Bauer, Kirby, Sherris and Turck, 1986, Am. J. Clin. Path., 45 : 493.

2. Performance Standards for Antimicrobial Disk Susceptibility Tests, NCCLS Vol. 22 No. 1, Jan. 2002. For more details refer to this volume.

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APPENDIX-V

B. Zone size interpretative chart: (Span Diagnostics Ltd., Surat, India & HiMedia Laboratories Ltd., Mumbai, Nepal)

Antibiotic	Symbol	Strength	Diameter of zone of inhibition		
			Resistant (mm or less)	Intermediate (mm)	Sensitive (mm or more)
Amikacin	AK	30 mcg	14	15-16	17
Ampicillin	A	10 mcg			
Gram -ve enteric organism			13	14-16	17
Staphylococci			23	-	29
Enterococci			16	-	17
Cefotaxime	CX	30 mcg	14	15-22	23
Chloramphenicol	C	30 mcg	12	13-17	18
Ciprofloxacin	CI	5 mcg	15	16-20	21
Cotrimoxazole	Q	25 mcg	10	11-15	16
Erythromycin	E	15 mcg	10	14-22	23
Gentamicin	J	10 mcg	12	13-14	15
Methicillin	M	5 mcg	16	17-21	22
Penicillin G	P	10 units	14	-	15
Tetracycline	T	30 mcg	14	15-18	19
Vancomycin	VA	30 mcg	14	15-18	19

APPENDIX - VI

In Vitro Diagnostic Reagent

Instruction Manual

of

Mycell II HBsAg

Diagnostic Reagent for Determination of HBsAg (for 200 or 1000 tests)

Read this instruction manual thoroughly before use of this kit

[Background of the development and features of the kit]

Hepatitis B surface antigen (HBsAg) is envelop protein of hepatitis B virus and as an infection marker, its determination is essential for diagnosis of infection with hepatitis B.

This kit is a reagent for determination of HBsAg based on reversed passive hemagglutination (R-PHA) using duck red blood cells as carrier sensitized with anti-horse anti-HBs. By means of control red blood cells, samples can be easily tested positive or negative in the screening test.

Features:

- Only one drop each of the sensitized and control cells is required for testing a sample allowing, by simple operation, screening of a large number of samples.
- Determination can be made in one hour.
- HBsAg of low concentration can be determined at high sensitivity.
- Confirmatory test available with this kit assures highly reliable results.
- Quantitative determination is also available with this kit.

[Kit Components]

This kit consists of the following components.

1. Red blood cells.
Red blood cells sensitized with anti-horse anti-HBs.
2. Control red blood cells.
Red blood cells sensitized with horse serum.
3. Positive control.
HBsAg positive control (hemagglutination titer: 32 ~ 128 folds).
4. Inhibition solution.
Phosphate buffer solution containing purified anti-HBs used for the confirmatory test of samples of which determination is suspended in the screening test.
5. Suspension solution.
Phosphate buffer solution containing rabbit serum used for suspension of the sensitized and control cells.
6. Sample dilution buffer.
Phosphate buffer solution containing purified horse serum used for dilution of samples.

	200-test Kit	1000-test Kit
Sensitized cells	0.6 ml x 1 vial	1.4 ml x 2 vials
Control cells	0.6 ml x 1 vial	1.4 ml x 2 vials
Positive control	0.5 ml x 1 vial	0.5 ml x 1 vial
Inhibition solution	2 ml x 1 vial	10 ml x 1 vial
Suspension solution	18 ml x 1 vial	65 ml x 1 vial
Sample dilution buffer	24 ml x 1 vial	55 ml x 2 vials
Dropper*	2 ea.	2 ea.

*Droppers for the sensitized and control cells. Not for use other than dispensing the sensitized and control cells.

[Effect]

Determination of serum or plasma HBsAg.

[Assay modes available]

Screening test.

This test examines hemagglutination of red blood cells sensitized with anti-horse anti-HBs. The sensitized red blood cells hemagglutinate when HBsAg presents in samples.

Confirmatory test.

This test is for confirmation of antigen-antibody specific hemagglutination by means of an inhibition test using the inhibition solution. In case of hemagglutination by HBsAg in samples, when the inhibition solution is added to samples their titer is reduced due to absorption of HBsAg by anti-HBs contained in the solution. This hemagglutination inhibition, however, does not occur in non-specific hemagglutination.

Quantitative test.

This test is for determination of the final hemagglutination titer of samples tested positive by the screening or confirmatory test.

[Assay procedures]

1. Tools and Equipment (not supplied with this kit).

- V-shaped well microplate (with mesh)
- Diluter (25 μ l)
- Dropper (25 μ l)
- Microplate mixer
- Cover (for microplate)
- Pipette (micropipette or measuring pipette)
- Centrifugal tubes

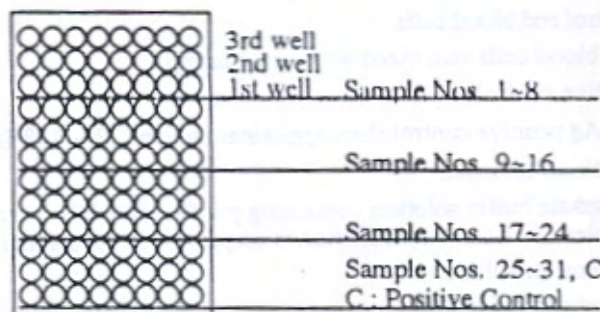
2. Preparation of Reagents

Add 2 ml each of the suspension solution to the sensitized and control cells and dispense in centrifugal tubes. Spin both cell solution at 2000 rpm for 5 min and remove supernatant. Dilute both cells with the suspension solution to make 10-fold diluted solution.

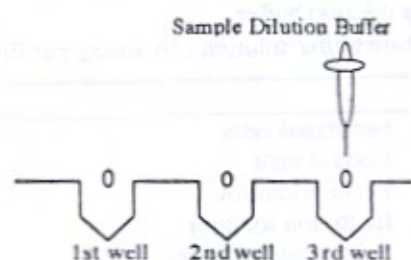
Operation

2) Screening test (refer to Table 1)

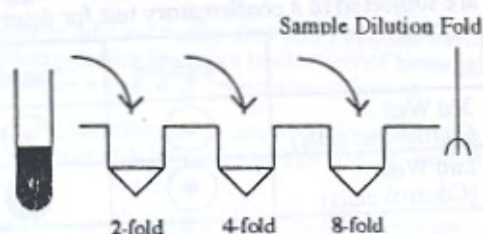
- ① Use a rigid or permanent type microplate with V-shaped wells (with mesh). One sample uses 3 wells.



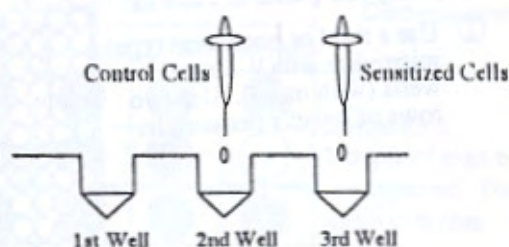
- ② Dispense by a dropper 25 μ l of the sample dilution buffer in the first through to the 3rd well.



- ③ Dispense in the first well by a diluter or micropipette 25 μ l of a sample and serially dilute by two-fold dilution up to the 3rd well.



- ④ Thoroughly suspend the sensitized and control cells prepared in advance, then dispense by a dropper 25 μ l each of the control and sensitized cells in the 2nd well and 3rd well, respectively. Separate droppers must be used for the control and sensitized cells. Do not dispense any cells in the first well.



- ⑤ After thoroughly mixing the microplate contents on a microplate mixer, leave the microplate to stand for one hour on a vibration free table at the room temperature (15~30°C) and determine. It can be left overnight at the room temperature for determination on the following day. After dispensing the sensitized and control cells, make sure to mix them with the sample dilution buffer as quickly as possible. If they are let to precipitate without mixing with the sample dilution buffer, proper hemagglutination patterns may not be obtained.

Table 1: Screening test procedures

Well No.		1	2	3
Sample dilution buffer (μ l)		25	25	25
Sample (μ l)		25	25	25
Sample dilution		1 : 2	1 : 4	1 : 8
Sensitized cells (μ l)		-	-	25
Control cells (μ l)		-	25	-
Thoroughly mix cells and samples and leave to stand for one hour at the room temperature for determination				

Determination of hemagglutination patterns

Determination of hemagglutination patterns is made according to the following criteria.

	Hemagglutination Patterns	Determination (Hemagglutination)
	All cells precipitate at the center of a well.	Hemagglutination (-)
	Most cells precipitate at the center of a well.	
	Cells partly precipitate at the center of a well and partly hemagglutinate around the precipitation like mesh.	Hemagglutination (+)
	Size of precipitation in the center of a well becomes smaller and mesh-like hemagglutination becomes clear.	
	Cells clearly hemagglutinate like mesh across a well.	

Determination by the screening test

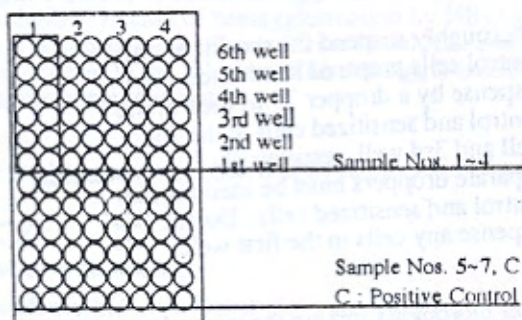
Samples with negative (-) hemagglutination of the sensitized cells are determined HBsAg negative. Samples with negative (-) hemagglutination of the control cells but with positive (+) hemagglutination of the sensitized cells are determined HBsAg

positive. Samples with positive (+) hemagglutination of both sensitized and control cells are subjected to a confirmatory test for determination.

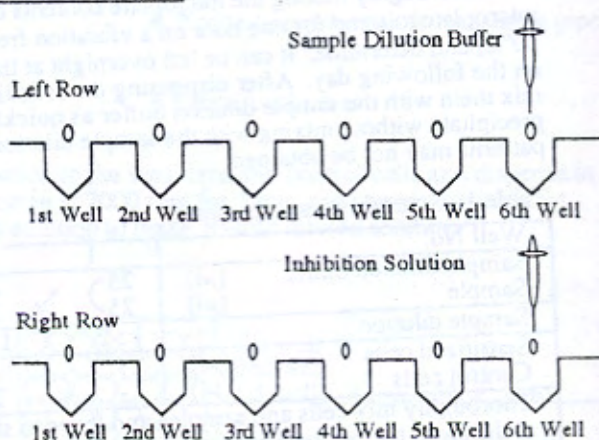
	Negative	Negative	Positive	Suspended (to Confirmatory Test)
3rd Well (Sensitized cells)				
2nd Well (Control cells)				

2) Confirmatory test (Refer to Table 2)

- ① Use a rigid or permanent type microplate with V-shaped wells (with mesh). Use two rows of 6 wells per sample.



- ② Dispense by a dropper 25 μ l each of the sample dilution buffer in 6 wells on the left row and by another dropper 25 μ l each the inhibition solution in 6 wells on the right row.



- ③ Dispense by a micropipett or diluter in the first well in both right and left rows 25 μ l each of a sample and serially two-fold dilute up to the 6th well.
- ④ Dispense by a dropper one drop (25 μ l) of the sensitized cells in all wells.
- ⑤ Thoroughly mix the well contents on a microplate mixer, cover the microplate with a cover and leave it to stand for one hour on a vibration free table at the room temperature (15 ~ 30C) for determination. It can be left overnight at the room temperature for determination on the following day.

Table 2: Confirmatory test procedures

Well No.	1	2	3	4	5	6
Sample dilution buffer (μ l)	25	25	25	25	25	25
Sample (μ l)	25	25	25	25	25	25
Inhibition solution (μ l)	25	25	25	25	25	25
Sample (μ l)	25	25	25	25	25	25
Sample dilution	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64
Sensitized cells (μ l)	25	25	25	25	25	25

Thoroughly mix the well contents and leave to stand for one hour at the room temperature on a vibration free table for determination.

Determination by the confirmatory test

- ① When a sample diluted with the sample dilution buffer (left row) and the inhibition solution shows the difference in hemagglutination titers (reduction of hemagglutination titer by anti-HBs antibody) of 2 wells or more, it is determined positive.
- ② When a sample diluted with the sample dilution buffer and inhibition solution does not show difference in hemagglutination from the 1st well through the 5th well, it is determined negative.
- ③ When hemagglutination difference is other than the above, the following applies.

	Sample Dilution Fold						Determination
	2-fold	4-fold	8-fold	16-fold	32-fold	64-fold	
Sample side							Suspended. Sample of high titer is suspected. Test again at higher dilution.
Inhibition side							
Sample side							
Inhibition side							

Quantitative test

By determining the end titer, this kit can quantitatively determine samples tested positive by the screening or confirmatory test.

- ① Dispense one drop (25 μ l) each of the sample dilution buffer in 10 wells.
- ② Dispense by diluters or micropipettes 25 μ l each of the positive control and a sample in the first well and serially dilute them up to the 10th well by 2-fold dilution.
- ③ Dispense by a dropper 25 μ l of the sensitized cells in all wells.
- ④ After mixing the well contents on a microplate mixer, cover the microplate with a cover and leave it to stand for one hour at the room temperature and determine. (The maximum dilution at which samples hemagglutinate is determined to be their HBsAg titer.)

[Assay precautions]

- 1) Collection of samples and their handling.
 - ① Collect sera or plasma in the normal way and avoid their hemolysis. Inactivation of sera does not practically affect the assay results.
 - ② If samples are not assayed immediately after their collection, store them in a refrigerator (after adding 0.1% final concentration of sodium azide) or in a freezer.
- 2) Interfering substances
 - ① Red blood cells or fibrin adversely affect determination and should be removed.
 - ② Up to 2000 FTU of chylomicron, 20 mg/dl of bilirubin C, 20 mg/dl of bilirubin F and 500 mg/dl of hemoglobin do not adversely affect the determination.

[Specifications]

- 1) Sensitivity
In the qualitative test using the positive control as a sample, this kit shows hemagglutination up to 32 to 128-fold dilution.
- 2) Specificity
This kit tests negative the HBsAg negative panel sera and tests positive the HBsAg positive panel sera.

3) Reproducibility

When the positive control is tested 10 times as a sample, this kit shows hemagglutination within ± 1 well of the mode.

[Correlation]

Correlation of this kit and a kit of the same assay principle manufactured by Company A was studied.

(Result) 70 samples tested showed 100% correlation.

A Company This kit	-	+
-	20	0
+	0	50

[Handling cautions]

This kit must be used according to the instructions and for the purpose described in this manual. No result is guaranteed in any use or for any purpose other than those described in this manual.

1) Cautions in assays

- ① Check accuracy of tools and properly use them according to their instructions.
- ② When using the droppers supplied with this kit, wipe off solution on the tip and slowly dispense at a given speed by holding them upright.
- ③ After dispensing the sensitized and control cells, thoroughly mix the well contents on a microplate mixer with minimum loss of time.
- ④ To prevent the well contents from evaporation, cover the microplate with a plastic plate, etc. while waiting for their reaction.
- ⑤ When frequently used microplates are used, hemagglutination pattern may become poor due to insufficient washing and/or scratches on the well surface.

2) Cautions in the use of this kit

- ① Do not use expired reagents.
- ② Do not use reagents of different production lots.
- ③ Leave to stand the sensitized and control cells for 1 hour at the room temperature (15 ~ 30°C) or overnight at 2 ~ 8°C before use.
- ④ All reagents must be returned to the room temperature (15 ~ 30°C) before use.
- ⑤ All samples should be handled carefully as if they were capable of transmitting HBV, HCV or HIV.
- ⑥ The positive control is not inactivated (heat treatment at 60°C for 10 hours) and should be handled carefully. Although it is tested negative for HBV, HCV antibody and HIV antibody, it should be handled like samples as if it were capable of transmitting these viruses.

3) Cautions after use of this kit

- ① Reagents contains a preservative (0.1% sodium azide) but once they are opened, they should be handled carefully to avoid contamination with microorganisms. Once they are contaminated, it will take a longer time for the sensitized and control cells to precipitate or determination may become difficult. To prevent formation of explosive metal azide by sodium azide, drain them down with a sufficient volume of water.
- ② Microplates used in the assay should be immersed overnight in 1% (W/W) sodium hypochlorite, washed with water using a microplate washer, immersed overnight in detergent, washed again with water, and dried.
- ③ Diluters used in the assay should be sterilized in 2% glutaraldehyde for one hour, rinsed and burnt by gas burner for 5 to 10 seconds.
- ④ It is desirable that other tools are sterilized in the above manners or autoclaved (at

121°C for more than 20 min).

4) Cautions in storage

- ① The sensitized and control cells are stable for one month after preparation if stored at 2 ~ 8°C.
- ② A small quantity of insoluble substance formed in the suspension solution, sample dilution buffer and inhibition solution does not adversely affect the hemagglutination reaction.

5) Others

- ① Vials and tools contained in this kit should not be used for other purposes.
- ② Overall diagnosis should be made not only by the results of this assay but by taking into consideration clinical findings and the results of other tests.

[Storage and shelf life]

Store at 2 ~ 8°C. (Do not freeze).

This kit is stable for one year after the date of manufacture and must be used up before the expiry date.

[Packages]

1 kit: 200 tests or 1000 tests.

[Reference]

Blumberg B.S., Altec H.J., Visnich S.: A new antigen in leukemia sera. JAMA, 191, 541 - 546 (1965).

Institute of Immunology
1-1-10, Koraku, Bunkyo-Ku
Tokyo, Japan

IgM anti-HEV Ab EIA

einer
ISA-FHE(J1)mORF2 5 μ g/mL 50 μ L

RT, >4hrs., standing

No wash

Blocking Solution 100 μ L

RT, >1hr., shaking

wash with Saline x 5times

Sample x100dil. / Sample Diluent 50 μ L

RT, 60', shaking

wash with 0.05% TW20 / Saline x 5times

HRP-Hyb-M49 x1000dil./Conj. Diluent 50 μ L

RT, 60', shaking

wash with 0.05% TW20 / Saline x 5times

TMB Substrate 50 μ L

RT, 10', standing in dark

Stop Solution 50 μ L

Read Abs at 450nm vs 620nm

washing buffer

dil x10

6000 x 10

2 μ L sample + 98 μ L diluent5 μ L 2000 x 1000 diluent

APPENDIX - VIII

Hepatitis C Virus Encoded Antigen

(Recombinant c22-3, c200 and NS5)

ORTHO® HCV 3.0 ELISA Test System
with Enhanced SAVE

(Sample Addition Verification)

Enzyme-Linked Immunosorbent Assay for the Detection of Antibody to
Hepatitis C Virus (Anti-HCV) in Human Serum or Plasma

NAME AND INTENDED USE

ORTHO HCV 3.0 ELISA Test System with Enhanced SAVE is a qualitative, enzyme-linked, immunosorbent assay for the detection of antibody to hepatitis C virus (anti-HCV) in human serum or plasma.

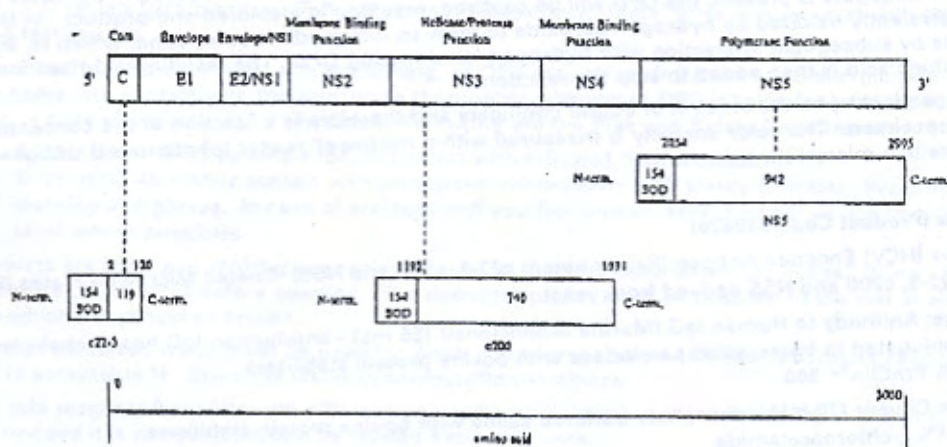
SUMMARY AND EXPLANATION

ORTHO HCV 3.0 ELISA Test System with Enhanced SAVE is an enzyme-linked immunosorbent assay (ELISA) which utilizes microwells coated with recombinant hepatitis C virus encoded antigens as the solid phase. ELISA technology utilizes the principle that antigens or antibodies which become bound to the solid phase can be detected by complementary antibody or antigen which is labeled with an enzyme capable of acting on a chromogenic substrate. When enzyme substrate is applied, the presence of antigen or antibody can be detected by the development of a colored end-product. Immunoassays of this type were first developed in the early 1970s.¹ Since that time, ELISA technology has been extensively used for the detection of antigens and antibodies for a wide range of infectious diseases.

The hepatitis C virus (HCV) is now known to be the causative agent for most, if not all, blood-borne non-A, non-B hepatitis (NANBH).²⁻⁷ Studies throughout the world indicate that HCV is transmitted through contaminated blood and blood products, through blood transfusions or through other close, personal contacts. Studies in the United States show that greater than 90% of transfusion-associated hepatitis infections are considered to be NANBH infections.^{8,9} Worldwide, other forms of NANBH are recognized.

Three recombinant hepatitis C virus encoded antigens are used in ORTHO HCV 3.0 ELISA Test System with Enhanced SAVE. The three recombinant antigens, developed by Chiron Corporation, are c22-3, c200 and NS5. A graphic representation of the putative HCV genome and recombinant proteins appears in Figure 1.

Figure 1
HCV Genome and Recombinant Proteins



HCV recombinant protein c22-3 is encoded by the putative core region of the HCV genome. Amino acid and nucleotide sequence comparisons of flaviviruses and pestiviruses with HCV suggest that c22-3 is derived from a structural region of the genome which encodes the RNA-binding nucleocapsid protein. Nucleocapsid proteins are thought to be involved in forming the viral core structure. Studies have indicated that antibodies which develop following infection with HCV are often reactive with c22-3.¹⁰ Moreover, studies performed using the CHIRON™ RIBATM HCV 2.0 Strip Immunoblot Assay (SIA) for anti-HCV have shown that in many cases antibodies to c22-3 develop sooner following HCV infection than those to c100-3.¹¹

HCV recombinant protein c200 is encoded by the putative NS3 and NS4 regions of the HCV genome. Amino acid and nucleotide sequence comparisons of flaviviruses and pestiviruses with HCV suggest that c200 is derived from nonstructural regions of the genome. The c200 recombinant protein contains the c33c protein sequence genetically linked to the c100-3 protein sequence.

c33c is encoded by the putative NS3 portion of the HCV genome. Amino acid and nucleotide sequence comparisons of flaviviruses and pestiviruses with HCV suggest that the NS3 region encodes the viral helicase, an enzyme involved in the unwinding of RNA during replication of the viral genome by RNA-dependent RNA polymerase.

Studies have indicated that antibodies which develop following infection with HCV are frequently reactive with c33c. Studies performed using the CHIRON RIBA HCV 2.0 SIA for anti-HCV have shown that antibodies reactive with c33c often develop sooner following HCV infection than do those to c100-3.¹¹

HCV recombinant protein c100-3 is encoded by the putative NS4 region of the HCV genome. Amino acid and nucleotide sequence comparisons of flaviviruses and pestiviruses with HCV suggest that c100-3 is derived from a nonstructural region of the genome. At present, the function of this portion of the HCV genome is unknown. Antibodies which develop following infection with HCV are often reactive with c100-3.¹²

HCV recombinant protein NS5 is encoded by the putative NS5 region of the HCV genome. Amino acid and nucleotide sequence comparisons of flaviviruses and pestiviruses with HCV suggest that NS5 is derived from a nonstructural region of the genome that encodes the viral polymerase, an enzyme involved in replication of HCV. Studies have indicated that a significant proportion of persons infected with HCV develop antibodies to NS5.^{12,13}

The use of HCV recombinant proteins derived from the core, NS3, NS4 and NS5 regions of the HCV genome has shown to be effective in identifying a greater number of diagnosed acute and chronic non-A, non-B hepatitis patients than single antigen (c100-3) assays.^{12,13} In addition, the use of these additional proteins allows for earlier detection of seroconversion following HCV infection. Although antibody responses to NS5 region-encoded antigens are not as prevalent in response to HCV infection as those to core and NS3 region-encoded antigens, the addition of NS5 to c22-3 and c200 recombinant proteins in ORTHO HCV 3.0 ELISA Test System with Enhanced SAVA affords antibody detection to a greater number of HCV-encoded epitopes.

The amino acid sequence of the three HCV recombinant proteins is as follows.

Recombinant Protein	Polypeptide Sequence
c22-3	AA # 2-120
c200	AA # 1192-1931
NS5	AA # 2054-2995

The host organism for all three HCV recombinant proteins is *S. cerevisiae* (yeast).

The primary purpose of this assay is to screen blood donations so that units containing HCV antibody can be identified and eliminated from the blood supply. Although the presence of anti-HCV does not constitute a diagnosis of HCV infection, the determination of anti-HCV may be used as an aid in the diagnosis of hepatitis C and in the differential diagnosis of non-A, non-B hepatitis in conjunction with determination of liver enzymes, additional serological markers and clinical evaluation. The Hepatitis C Virus Encoded Antigen (Recombinant c22-3, c200 and NS5) used in the manufacture of ORTHO HCV 3.0 ELISA Test System with Enhanced SAVA is prepared under U.S. License by Chiron Corporation under a shared manufacturing arrangement.

PRINCIPLE OF THE PROCEDURE

The assay procedure is a three-stage test carried out in a microwell coated with a combination of recombinant hepatitis C virus (rHCV) antigen (c22-3, c200 and NS5).

In the first stage, a diluted test specimen is incubated in the test well for a specified length of time. If antibody reactive to any of the three antigens is present in the specimen, antigen-antibody complexes will be formed on the microwell surface. If anti-HCV is not present, complexes will not be formed. In the subsequent washing step, unbound serum or plasma proteins will be removed.

In the second stage, murine monoclonal antibody conjugated to horseradish peroxidase is added to the microwell. The conjugate binds specifically to the human IgG portion of the antigen-antibody complexes. If antigen-antibody complexes are not present, the unbound conjugate will be removed by subsequent washing.

In the third stage, an enzyme detection system composed of o-phenylenediamine (OPD) and hydrogen peroxide is added to the test well. If bound conjugate is present, the OPD will be oxidized, resulting in a colored end-product. In this reaction, peroxidase is divalently oxidized by hydrogen peroxide to form an intermediate compound, which is, in turn, reduced to its initial state by subsequent interaction with hydrogen-ion-donating OPD. The resulting oxidized form of OPD has an orange color. Sulfuric acid is then added to stop the reaction.

The color intensity is dependent upon the amount of bound conjugate and therefore is a function of the concentration of anti-HCV present in the specimen. The color intensity is measured with a microwell reader (photometer) designed to measure light absorbance in a microwell.

REAGENTS

192 Test Kit Components (Product Code 930820)

- HCV** 2 Hepatitis C Virus (HCV) Encoded Antigen (Recombinant c22-3, c200 and NS5) -Coated Microwell Plates (96 wells each) - c22-3, c200 and NS5 derived from yeast
- CON** 1 bottle Conjugate: Antibody to Human IgG (Murine Monoclonal) (55 mL) -anti-human IgG heavy chain (murine monoclonal) conjugated to horseradish peroxidase with bovine protein stabilizers
Preservative: 1% ProClin™ 300
- SD** 1 bottle Specimen Diluent (70 mL) -phosphate-buffered saline with bovine protein stabilizers
Preservative: 0.1% 2-chloroacetamide
- OPD** 1 vial OPD Tablets (30 tablets) - contains o-phenylenediamine - 2HCl
- SB** 1 bottle Substrate Buffer (190 mL) -citrate-phosphate buffer with 0.02% hydrogen peroxide
Preservative: 0.1% 2-chloroacetamide
- PC** 1 vial Positive Control (Human) (1.2 mL)
Source: Inactivated human serum or plasma containing anti-HCV and nonreactive for HBsAg and antibodies to HIV-1 and HIV-2
Preservatives: 0.2% sodium azide and 0.9% EDTA
- NC** 1 vial Negative Control (Human) (1.8 mL)
Source: Human serum or plasma nonreactive for HBsAg and antibodies to HIV-1, HIV-2 and HCV
Preservatives: 0.2% sodium azide and 0.9% EDTA

21 Plate sealers, disposable

- HCV** 5 Hepatitis C Virus (HCV) Encoded Antigen (Recombinant c22-3, c200 and NS5) -Coated Microwell Plates (96 wells each) - c22-3, c200 and NS5 derived from yeast
- CON** 1 bottle Conjugate: Antibody to Human IgG (Murine Monoclonal) (125 mL) - anti-human IgG heavy chain (murine monoclonal) conjugated to horseradish peroxidase with bovine protein stabilizers
Preservative: 1% ProClin™ 300
- SD** 1 bottle Specimen Diluent (190 mL) - phosphate-buffered saline with bovine protein stabilizers
Preservative: 0.1% 2-chloroacetamide
- OPD** 1 vial OPD Tablets (30 tablets) - contains o-phenylenediamine · 2HCl
- SB** 1 bottle Substrate Buffer (190 mL) - citrate-phosphate buffer with 0.02% hydrogen peroxide
Preservative: 0.1% 2-chloroacetamide
- PC** 1 vial Positive Control (Human) (1.2 mL)
Source: Inactivated human serum or plasma containing anti-HCV and nonreactive for HBsAg and antibodies to HIV-1 and HIV-2
Preservatives: 0.2% sodium azide and 0.9% EDTA
- NC** 1 vial Negative Control (Human) (1.8 mL)
Source: Human serum or plasma nonreactive for HBsAg and antibodies to HIV-1, HIV-2 and HCV
Preservatives: 0.2% sodium azide and 0.9% EDTA

21 Plate sealers, disposable

CAUTION: HANDLE AS IF CAPABLE OF TRANSMITTING INFECTIOUS AGENTS.

Store at 2 to 8°C

For in vitro diagnostic use

PRECAUTIONS

1. **CAUTION:** Some components of this kit contain human blood derivatives. No known test method can offer complete assurance that products derived from human blood will not transmit infectious agents. Therefore, all blood derivatives should be considered potentially infectious. It is recommended that these reagents and human specimens be handled using established good laboratory working practices.^{14,15}
2. Wear disposable gloves while handling kit reagents and specimens. Thoroughly wash hands afterward.
3. All specimens should be handled as potentially infectious agents.
4. Sodium azide is included as a preservative in the Positive Control and Negative Control. Sodium azide has been reported to form lead or copper azides in laboratory plumbing. These azides are potentially explosive. To prevent buildup, flush plumbing with a large volume of water while disposing of these solutions in the sink. Following are the Risk and Safety Requirements.¹⁶
R: 22 - Harmful if swallowed.
S: 28 - After contact with skin, wash immediately with plenty of water.
5. Some kit components may be considered as hazardous or potentially infectious waste. Dispose of all materials according to applicable guidelines and regulations.^{17,18}
6. 4N sulfuric acid (H_2SO_4) is a strong acid. Wipe up spills immediately. Flush the area of the spill with water. If the acid contacts the skin or eyes, flush with copious amounts of water and seek medical attention.
7. Handle OPD tablets with plastic or Teflon®-coated forceps only. Metal forceps may react with tablets and interfere with the test results.
8. Avoid contact of OPD with eyes, skin or clothing, as OPD may cause irritation or an allergic skin reaction. If OPD should come into contact with the skin, wash thoroughly with water. OPD is toxic for inhalation, ingestion and skin contact. In case of malaise, call a physician. Following are the Risk and Safety Requirements.¹⁵
R: 23/24/25 - Toxic by inhalation, in contact with skin and if swallowed.
S: 28/36/37-45 - After contact with skin, wash immediately with plenty of water. Wear suitable protective clothing and gloves. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).
9. OPD tablets are light- and moisture-sensitive. Keep vial tightly closed when not in use. Bring vial to room temperature (15 to 30°C) before opening. The desiccant pouch must be retained in the vial at all times. Do not use tablets which are yellow or broken.
10. Distilled or deionized water must be used for Wash Buffer preparation. Clinical laboratory reagent water Type I or Type II is acceptable.¹⁹ Store the water in nonmetallic containers.
11. Do not mix reagents from kits with different lot numbers. Any lot number of 20X Wash Buffer Concentrate may be used provided it is not used beyond its labeled expiration date.
12. All reagents and components must be at room temperature prior to use and kit components returned to 2 to 8°C after use.
13. The microwell strips are sealed in protective pouches with a humidity indicator desiccant. The desiccant, normally blue/purple in color, will turn pink if moisture is present in the pouch. If the desiccant is pink, the microwell strips should not be used.
14. All unopened components are suitable for use through the labeled expiration date. Unused microwell strips are suitable for use for 42 days after opening the foil pouch when stored at 2 to 8°C with desiccant in the foil pouch. All other components are suitable for use for 84 days after opening when stored at 2 to 8°C.
15. Cross-contamination between reagents will invalidate the test results. Permanently labeled, dedicated reservoirs for the appropriate reagents are recommended.
16. Ensure that specimen is added to the microwell. Failure to add specimen may produce an erroneous nonreactive result. Addition of specimens and controls to the microwells should be verified visually or by photometric reading (see Step 5 of Test Procedure for STANDARD Incubation and SHORT Incubation).
17. Grossly hemolyzed specimens may not present a visible color change when added to microwells containing

Specimen Diluent. Hemolyzed specimens may require visual verification that the pipetting device has delivered the specimen.

18. When using a single-channel micropipette for manual sample addition, use a new pipette tip for each specimen to be assayed. When using a multi-channel micropipette, new tips are to be used for each reagent to be added.
19. Strict adherence to the specified wash procedure is crucial to ensure optimum assay performance (see Step 7 of Test Procedure for STANDARD Incubation and SHORT Incubation).
20. Do not allow microwells to become dry once the assay has begun.
21. Do not touch the bottom exterior surface of the microwells. Fingerprints or scratches may interfere with reading the microwell.
22. Ensure that the microwell strips are level in the microwell strip holder during the test procedure. If necessary, wipe the bottom of the microwell strips carefully with a soft, lint-free, absorbent tissue to remove any moisture, dust or debris before reading.
23. Negative or positive control values which are not within the expected range (refer to Quality Control Procedures section) may indicate a technique problem or product deterioration.
24. Do not allow sodium hypochlorite fumes from chlorine bleach or other sources to contact the microwell strips during the assay as the color reaction may be inhibited.
25. All pipetting equipment should be used with care and calibrated regularly, following the equipment manufacturer's instructions.
26. The microwell reader should contain a reference filter with a setting at 620 nm or 630 nm. If an instrument without a reference filter is used, areas in the bottom of the microwells that are opaque, scratched or irregular may cause elevated readings.

PREPARATION OF REAGENTS

1. Preparation of Wash Buffer (1X): Mix 50 mL of 20X Wash Buffer Concentrate with 950 mL of distilled or deionized water. Wash Buffer (1X) is stable for 30 days when stored at room temperature. For longer storage (up to 60 days), store at 2 to 8°C. Record the date the Wash Buffer (1X) is prepared and the expiration date on the container. Discard Wash Buffer (1X) if visibly contaminated.

NOTE: Any lot number of 20X Wash Buffer Concentrate may be used to prepare this reagent provided it is not used beyond its labeled expiration date.

2. Preparation of Substrate Solution: Clean glass or plastic vessels must be used. Prior to the end of the second incubation, transfer a sufficient amount of Substrate Buffer to a container and protect the contents from light. Completely dissolve the appropriate number of OPD tablets in Substrate Buffer prior to use.

Each microwell plate requires at least 20 mL of Substrate Solution. More Substrate Solution may be needed depending upon the reagent dispenser used. See the instrument manufacturer's instructions for additional reagent requirements. Below are guidelines for general use.

Number of Wells	Number of Plates	Number of OPD Tablets	Substrate Buffer (mL)
24	0.25	1	6
48	0.50	2	12
72	0.75	3	18
96	1	4	24
192	2	7	42
288	3	10	60

The Substrate Solution is stable for 60 minutes after the addition of OPD tablets when held at room temperature in the dark and should be colorless to very pale yellow when used. Record the time when the OPD tablets are added to the Substrate Buffer and when it will expire on the container. If it is noticeably yellow in color at the time of preparation, discard and prepare more Substrate Solution as required.

SPECIMEN COLLECTION AND PREPARATION

No special preparation of the patient/donor is required prior to specimen collection. Blood should be collected by approved medical techniques. Plasma collected with an improper ratio of specimen to anticoagulant should not be used. Serum, including serum collected in serum-separator tubes, or plasma collected in EDTA, heparin or citrate based anticoagulants may be used and should be tested as soon as possible following collection. Do not use any heat-treated specimens. Serum or plasma may be stored at 2 to 8°C for up to seven days. If longer storage is necessary, the specimens should be frozen (-20°C or lower) to limit possible contamination. Storage of specimens in self-defrosting freezers is not recommended. Avoid multiple freeze-thaw procedures. Mix specimen thoroughly after thawing and before testing. No effect was observed on reactivity in ORTHO HCV 3.0 ELISA Test System with Enhanced S/CO when 15 weakly reactive and 15 nonreactive specimens were subjected to five freeze-thaw cycles. Clear, nonhemolyzed specimens are preferred. Precipitates in specimens should be removed by centrifugation. When 17 weakly reactive and 17 nonreactive specimens were treated with 0.050-0.200 g/dL hemoglobin, no effect on reactivity was observed. No effect of visual lipemia (289-1035 mg/dL triglyceride) was observed with 15 weakly reactive and 15 nonreactive specimens.

All specimens should be handled as if capable of transmitting infectious agents. If specimens are to be shipped, they must be packed in compliance with International Air Transport Association (IATA) and other applicable guidelines and regulations.²⁰ Studies have demonstrated that specimens may be shipped at ambient temperature (up to 37°C) or refrigerated (2 to 8°C) for up to 7 days, and upon arrival should be stored at 2 to 8°C. For shipments requiring extensive transit times (greater than 7 days), specimens should be kept frozen (-20°C or lower).

PROCEDURE

Materials Provided

192 Test Kit (Product Code 930820)

480 Test Kit (Product Code 930800)

(See REAGENTS for complete listing)

Materials Required But Not Provided

1. Adjustable multichannel micropipette capable of delivering 50 μ L and 200 μ L with at least $\pm 5\%$ accuracy or equivalent reagent dispenser
2. Fixed or adjustable single-channel micropipettes capable of delivering 20 μ L to 30 μ L with at least $\pm 5\%$ accuracy and 200 μ L to 300 μ L with at least $\pm 5\%$ accuracy or equivalent pipetter-dilutor
3. 5 μ L to 300 μ L disposable pipette tips or equivalent
4. Appropriately sized serological pipette or graduated cylinder
5. Multichannel micropipette reservoir or equivalent reagent container
6. Multichannel aspirator-washer device capable of dispensing and aspirating at least 300 μ L per well. (Consult the device's operator's manual for additional technical information.)
7. Dual wavelength microwell reader capable of reading at 490 nm or 492 nm with a reference filter of 620 nm or 630 nm and 610 nm if performing SAVE read. If an instrument without a reference filter is used, areas in the bottom of the microwells that are opaque, scratched or irregular may cause elevated readings. Reportable range of the microwell reader must be at least 0 to 2.5 absorbance units. Consult the instrument manufacturer's specifications.
8. $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ incubator (dry or humidified)
9. Distilled or deionized water, clinical laboratory reagent water Type I or Type II is acceptable (see PRECAUTIONS section)
10. 5.25% sodium hypochlorite (chlorine bleach)
11. 4N sulfuric acid (H_2SO_4) - To determine suitability of acid, prepare Substrate Solution as described under PREPARATION OF REAGENTS. Add 200 μ L of Substrate Solution to three microwells, then add 50 μ L of 4N H_2SO_4 to be tested to each microwell. Read the microwells at a wavelength of 490 nm or 492 nm with a reference filter of 620 nm or 630 nm at "0 time" and "60 minutes." All absorbance values at each time interval must be less than or equal to 0.050.
12. Black microwell strips (Product Code 936985, Ortho-Clinical Diagnostics, Inc.) or equivalent uncoated microwells
13. 20X Wash Buffer Concentrate (Product Code 933730, 6 x 150 mL; Product Code 933780, 4 x 450 mL; Product Code 933790, 12 x 450 mL; Ortho-Clinical Diagnostics, Inc.) — phosphate buffer with sodium chloride and detergent Preservative: 2% 2-chloroacetamide
14. Variable speed microwell plate shaker, optional

Test Procedure - STANDARD Incubation

1. Approximately 30 minutes prior to the beginning of the procedure, bring kit components to room temperature (15 to 30°C). Invert liquid reagents gently several times, but avoid foaming. Check the incubator temperature; maintain at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$.
2. Determine the total number of wells needed for the assay. In addition to specimens, one reagent blank, three negative controls and two positive controls must be included on each plate or partial plate. Unused wells should be stored at 2 to 8°C in the supplied foil pouch with desiccant, tightly sealed and used within 42 days of opening the foil pouch. Record the date the pouch is opened and the expiration date of the unused wells in the space provided on the pouch.

Performing the test on less than a full plate is permitted as long as the following conditions are met.

Microwell strips from different plates can be mixed to assemble full or partial plates as long as they are from the same lot, within the open pouch expiration date and have come from plates that have previously demonstrated proper response to kit controls.

When assembling a plate which contains strips from a newly opened, previously untested plate, the strips should receive the full complement of kit controls.

CAUTION: Handle microwell strips with care. Do not touch the bottom exterior surface of the wells.

3. Assemble the microwell strips in the microwell strip holder, if necessary. Microwell strips must be level in the microwell strip holder. For incomplete plates, add black or uncoated microwell strips.
4. Prepare a record (plate map) identifying the placement of the controls and specimens in the microwells. Arrange the assay control wells so that well 1A is the reagent blank. From well 1A, arrange all controls in a row (horizontal) or column (vertical) configuration as follows. Configuration is dependent upon software.

Well 1A	Reagent Blank
	Negative Control
	Negative Control
	Negative Control
	Positive Control
	Positive Control

5. Verify that any manual dispensing equipment is set to deliver the specified volumes as stated in the procedure, following the equipment manufacturer's instructions. Visually inspect the microwells upon addition of specimens and controls. A color change from green to blue indicates that the specimen or control has been added to the microwell. If a color change does not occur, the control or specimen result must be voided and a retest performed. Add controls and specimens to the microwells as follows:

A. Direct Sample Addition:

1. Add 200 μ L of Specimen Diluent to all wells, including 1A.
2. Add 20 μ L of the controls or specimens to the appropriate wells.

b. Determine the mean of the negative control values (NCx).

Example:

Negative Control	Absorbance
1	0.010
2	0.030
3	0.020
Total Absorbance	= 0.060
$NCx = \frac{\text{Total Absorbance}}{3}$	= 0.020

3. Positive Control Acceptance Criteria

The positive control is used to verify that the test kit components are capable of detecting a reactive specimen provided the test procedure has been strictly adhered to.

A plate is considered valid with respect to the positive control if both positive control values are greater than or equal to 0.800, within the reportable range of the microwell reader and do not differ by more than 0.600.

NOTE: Results beyond the upper limit of the reportable range of the microwell reader may appear as "OVER" or "***" or ">".

4. Calculation of the Cutoff Value

Cutoff Value = $NCx + 0.600$

Example:

Negative Control	Absorbance
1	0.010
2	0.030
3	0.020
Total Absorbance	= 0.060
$NCx = \frac{\text{Total Absorbance}}{3}$	= 0.020
Cutoff Value = $0.020 + 0.600$	= 0.620

INTERPRETATION OF RESULTS

- Specimens with absorbance values less than -0.025 should be retested in a single microwell. The specimen should be considered nonreactive if the retest absorbance value is less than the Cutoff Value, even if the retest absorbance value remains less than -0.025.
- Specimens with absorbance values less than the Cutoff Value and greater than or equal to -0.025 are considered nonreactive. Further testing is not required.
- Specimens with absorbance values greater than or equal to the Cutoff Value are considered initially reactive and should be retested in duplicate before final interpretation.
- Upon retesting an initially reactive specimen, the specimen is considered repeatedly reactive for antibody to HCV if either or both duplicate determination(s) is (are) reactive, i.e., greater than or equal to the Cutoff Value.
- After retesting an initially reactive specimen, the specimen is considered nonreactive for antibody to HCV if both duplicate determinations are nonreactive, i.e., less than the Cutoff Value.

Test Procedure-SHORT Incubation

- Approximately 30 minutes prior to the beginning of the procedure, bring kit components to room temperature (15 to 30°C). Invert liquid reagents gently several times, but avoid foaming. Check the incubator temperature; maintain at 37°C ± 1°C.
- Determine the total number of wells needed for the assay. In addition to specimens, one reagent blank, three negative controls and two positive controls must be included on each plate or partial plate. Unused wells should be stored at 2 to 8°C in the supplied foil pouch with desiccant, tightly sealed and used within 42 days of opening the foil pouch. Record the date the pouch is opened and the expiration date of the unused wells in the space provided on the pouch.

Performing the test on less than a full plate is permitted as long as the following conditions are met.

Microwell strips from different plates can be mixed to assemble full or partial plates as long as they are from the same lot, within the open pouch expiration date and have come from plates that have previously demonstrated proper response to kit controls.

When assembling a plate which contains strips from a newly opened, previously untested plate, the strips should receive the full complement of kit controls.

CAUTION: Handle microwell strips with care. Do not touch the bottom exterior surface of the wells.

- Assemble the microwell strips in the microwell strip holder, if necessary. Microwell strips must be level in the microwell strip holder. For incomplete plates, add black or uncoated microwell strips.
- Prepare a record (plate map) identifying the placement of the controls and specimens in the microwells.

Arrange the assay control wells so that well 1A is the reagent blank. From well 1A arrange all controls in a row (horizontal) or column (vertical) configuration as follows. Configuration is dependent upon software.

Well 1A	Reagent Blank
	Negative Control
	Negative Control
	Negative Control
	Positive Control
	Positive Control

5. Verify that any manual dispensing equipment is set to deliver the specified volumes as stated in the procedure, following the equipment manufacturer's instructions. Visually inspect the microwells upon addition of specimens and controls. A color change from green to blue indicates that the specimen or control has been added to the microwell. If a color change does not occur, the control or specimen result must be voided and a retest performed. Add controls and specimens to the microwells as follows:

A. Direct Sample Addition:

1. Add 200 μ L of Specimen Diluent to all wells, including 1A.
2. Add 20 μ L of the controls or specimens to the appropriate wells.
3. If the controls and specimens have been manually delivered, ensure that the contents of the microwells are thoroughly mixed. Use of a microplate shaker, manual mixing with a pipette or equivalent is acceptable. If a shaker is used, it should be used at a slow to moderate speed to avoid splashing the contents of the test wells.

B. For Previously Diluted Sample Addition:

1. Add 300 μ L of Specimen Diluent to a tube or container.
2. Add 30 μ L of control or specimen to the tube. Mix thoroughly.
3. Transfer 220 μ L of each previously diluted control or specimen to the appropriate well position.
4. Add 200 μ L of Specimen Diluent to well 1A.

C. A color change can also be read photometrically. To document specimen addition, a SAVE diluent read should be performed after the addition of Specimen Diluent, controls and specimens to the microwells as follows:

1. If necessary, wipe moisture from the bottom of the microwell strips carefully with a soft, lint-free, absorbent tissue before reading. If necessary, level the strips in the microwell holder.
2. Read the microwell strip plate at a wavelength of 610 nm. No reference filter is necessary. Blank the reader on well 1A according to the instrument manufacturer's instructions.
3. Each control or specimen should exhibit a value of greater than or equal to 0.315. If a well has a value of less than 0.315, the control or specimen result must be voided and a retest performed.

6. For manual processing of microwell plates, cover the microwell strip holder with a plate sealer. When using an automated microplate processor for incubation, follow the instrument manufacturer's recommendations with regard to microwell plate sealing. Incubate at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 30 minutes $\pm$ 1 minute.

7. Level the strips in the microwell strip holder, if necessary. With an aspirator-washer device, aspirate and wash all wells five times with Wash Buffer (1X).

CAUTION: Strict adherence to the specified wash procedure is crucial to ensure optimum assay performance. Follow the steps specified in order to ensure thorough washing.

- a. Aspirate the sample solutions from the microwells then fill completely with Wash Buffer. Do not allow the wells to overflow. Allow approximately 20 seconds between the addition of Wash Buffer and subsequent aspiration.
- b. Complete the aspirate/fill sequence four additional times.
- c. Completely aspirate wells. Invert the plate and firmly tap on a clean paper towel to remove excess Wash Buffer, if necessary.

8. Add 200 μ L of Conjugate to all wells, including 1A. If it is necessary to document Conjugate addition, a photometric read should be performed after addition of Conjugate to the microwell strips as follows:

- a. If necessary, wipe moisture from the bottom of the microwell strips carefully with a soft, lint-free, absorbent tissue before reading. If necessary, level the strips in the microwell holder.
- b. Read the microwell strip plate at a wavelength of 490 nm or 492 nm. For dual wavelength readers, set the reference wavelength at 620 nm or 630 nm. Do not blank the reader on well 1A.
- c. Each control or specimen should exhibit a value of greater than or equal to 0.700. If a well has a value of less than 0.700, the control or specimen result must be voided and a retest performed.

9. For manual processing of microwell plates, cover the microwell strip holder with a new plate sealer. When using an automated microplate processor for incubation, follow the instrument manufacturer's recommendations with regard to microwell plate sealing. Incubate at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 30 minutes $\pm$ 1 minute.

10. Prepare sufficient Substrate Solution prior to use in Step 12 to allow time for OPD tablets to dissolve completely. Refer to PREPARATION OF REAGENTS. Do not use more than a single preparation of Substrate Solution on a plate.

11. After the second incubation, wash the wells as described in Step 7.

12. Add 200 μ L of Substrate Solution to all wells, including 1A.

13. Incubate at room temperature in the dark for 30 minutes $\pm$ 1 minute.

14. Add 50 μ L of 4N sulfuric acid (H_2SO_4) to all wells, including 1A. To ensure proper mixing, acid should be added forcibly in a steady stream. If necessary, gently tap the plate or use a microwell plate shaker to mix the contents. Care should be taken to avoid splashing of the contents of the microwells. When using an automated microplate processor, follow the instrument manufacturer's instructions with regard to mixing.

15. If necessary, wipe moisture from the bottom of the microwell strips carefully with a soft, lint-free, absorbent tissue before reading. If necessary, level the strips in the microwell strip holder. Read the microwell strip plate at a wavelength of 490 nm or 492 nm. For dual wavelength readers, set the reference wavelength at 620 nm or 630 nm. Blank the reader on well 1A according to the instrument manufacturer's instructions.

The user should ensure that the blank value (well 1A) has been subtracted from all control and specimen well values prior to applying the Quality Control criteria below.

NOTE: Microwell strip plates must be read within 60 minutes following the addition of 4N sulfuric acid. Plates must be stored in the dark until read.

Quality Control Procedures-SHORT Incubation 21.22

1. Reagent Blank Acceptance Criteria

A plate is considered valid with respect to the reagent blank if the absorbance value of the reagent blank well (well 1A) is greater than or equal to -0.020 and less than or equal to 0.050.

2. Negative Control Acceptance Criteria

- Individual negative control values must be less than or equal to 0.120 and greater than or equal to -0.005. If one of the three control values is outside either of these limits, recalculate the negative control mean (NC $\bar{x}$) based upon the two acceptable control values. The plate is invalid and the test must be repeated if two or more of the three control values are outside either of the limits.
- Determine the mean of the negative control values (NC $\bar{x}$).

Example:

Negative Control	Absorbance
1	0.005
2	0.015
3	0.010
Total Absorbance	= 0.030
NC $\bar{x}$ = $\frac{\text{Total Absorbance}}{3}$	= 0.010

3. Positive Control Acceptance Criteria

The positive control is used to verify that the test kit components are capable of detecting a reactive specimen provided the test procedure has been strictly adhered to.

A plate is considered valid with respect to the positive control if both positive control values are greater than or equal to the Cutoff Value, within the reportable range of the microwell reader.

NOTE: Results beyond the upper limit of the reportable range of the microwell reader may appear as "OVER" or "****" or ">".

4. Calculation of the Cutoff Value

Cutoff Value = NC $\bar{x}$ + 0.330

Example:

Negative Control	Absorbance
1	0.005
2	0.015
3	0.010
Total Absorbance	= 0.030
NC $\bar{x}$ = $\frac{\text{Total Absorbance}}{3}$	= 0.010
Cutoff Value = 0.010 + 0.330	= 0.340

INTERPRETATION OF RESULTS

- Specimens with absorbance values less than -0.025 should be retested in a single microwell. The specimen should be considered nonreactive if the retest absorbance value is less than the Cutoff Value, even if the retest absorbance value remains less than -0.025.
- Specimens with absorbance values less than the Cutoff Value and greater than or equal to -0.025 are considered nonreactive. Further testing is not required.
- Specimens with absorbance values greater than or equal to the Cutoff Value are considered initially reactive and should be retested in duplicate before final interpretation.
- Upon retesting an initially reactive specimen, the specimen is considered repeatedly reactive for antibody to HCV if either or both duplicate determination(s) is (are) reactive, i.e., greater than or equal to the Cutoff Value.
- After retesting an initially reactive specimen, the specimen is considered nonreactive for antibody to HCV if both duplicate determinations are nonreactive, i.e., less than the Cutoff Value.

LIMITATIONS OF THE PROCEDURE

ORTHO HCV 3.0 ELISA Test System with Enhanced SAvE is limited to the detection of anti-HCV in human serum or plasma. The presence of anti-HCV does not constitute a diagnosis of hepatitis C but may be indicative of recent and/or past infection by hepatitis C virus. A nonreactive test result does not exclude the possibility of exposure to hepatitis C virus. Levels of anti-HCV may be undetectable in early infection.

Data obtained from testing persons at increased risk or low risk for HCV infection suggest that repeatedly reactive specimens with high absorbance values are more likely to demonstrate the presence of anti-HCV in supplemental testing. Reactivity at or slightly above the Cutoff Value is more frequently nonspecific, especially in specimens obtained from persons at low risk for infection. However, the presence of anti-HCV in some of these specimens can be demonstrated by supplemental testing.

The positive control in the test kit is not to be used to quantitate assay sensitivity. The positive control is used to verify that the test kit components are capable of detecting a reactive specimen provided the test procedure has been strictly adhered to.

When positive control values are beyond the reportable range of the microwell reader, the positive control cannot be used to assess assay precision.

PERFORMANCE CHARACTERISTICS

SENSITIVITY

Seroconversion Sensitivity

The sensitivity of the ORTHO HCV 3.0 ELISA Test System with Enhanced SAvE was determined by testing multiple lots with 20 commercially available anti-HCV seroconversion panels. The ORTHO HCV 3.0 ELISA Test System with Enhanced SAvE detected antibodies to HCV at the same bleed date as the ORTHO HCV 3.0 ELISA Test System with SAvE in 17 of 20 cases. The ORTHO HCV 3.0 ELISA Test System with Enhanced SAvE detected antibodies to HCV prior to ORTHO HCV 3.0 ELISA Test System with SAvE in 3 of 20 cases. There were no significant differences observed between the bleeds detected by the Standard and Short testing procedures of the ORTHO HCV 3.0 ELISA Test System with Enhanced SAvE.

Clinical Sensitivity

Nineteen serum specimens from patients clinically diagnosed with hepatitis C were tested by the ORTHO HCV 3.0 ELISA Test System with Enhanced SAVE by the Standard and Short procedures and by the ORTHO HCV 3.0 ELISA Test System with SAVE. Eighteen of the 19 cases positive by supplemental strip immunoblot assay were detected by both procedures of the ORTHO HCV 3.0 ELISA Test System with Enhanced SAVE. The single exception was a case identified as active hepatitis which was nonreactive on supplemental strip immunoblot assay. Overall, there was 100% concordance between procedures of the ORTHO HCV 3.0 ELISA Test System with Enhanced SAVE, the ORTHO HCV 3.0 ELISA Test System with SAVE and supplemental HCV strip immunoblot assays.

Seroconversion Sensitivity by Bleed Date

Panel	ALT	PCR	ORTH HCV 3.0 ELISA Test System with Enhanced SAVE		ORTH HCV 3.0 ELISA Test System with SAVE		CHIRON RIBA HCV 3.0 SIA Pattern at Seroconversion
			Short Procedure	Standard Procedure	Short Procedure	Standard Procedure	
NABI SC-0050	1567	+	1	1	1	1	c33c
NABI SC-0100	301	+	1	1	1	1	c33c
NABI SC-0010	162	+	3 ^f	3	2	3	c22p
NABI SC-0030	309	+	2	3	3	3	c100p
BBI PHV904	>450	+	4	4	5	5	c33c
BBI PHV 901	158	+	3	3	3	3	c33c/c100p
BBI PHV 905	144	+	6 ^f	6 ^f	6	7	c33c/c22p
BBI PHV 906	11	+	1	1	1	1	c33c
"D"	804	+	7	7	7	7	c33c
BCP 61067	244	NT	11	11	11	11	c33c/c22p
BCP 61083	44	NT	10 ^f	10 ^f	10	10	c33c
BCP 790989	132	+	39 ^f	39 ^f	39	39	c33c
NABI SC-0090	582	+	6	6	6	6	c33c
NABI SC-0070	561	NT	7	7	7	7	c33c/c22p/c100p/NS5
4814	391	+	5	5	5	5	c33c
NABI SC-0040	70	+	3	4	3	4	c33c
552-358002	20	NT	1	1	1	1	c33c/c100p
NABI SC-0060	47	+	3	3	3	3	c22p
NABI SC-0080	40	+	4	4	4	4	c33c/c100p
STD976	141	NT	15	16 ^f	17	17	c22p

NT = Not tested

f = First bleed which was reactive on 3 lots, this specimen was borderline on prior bleed

Analytical Sensitivity

Twenty-one CHIRON™ RIBATM HCV 3.0 SIA positive specimens were diluted to levels of anti-HCV to be considered negative by ORTHO HCV 3.0 ELISA Test System with SAVE. A comparison between the Short and Standard assay procedures among multiple lots of the ORTHO HCV 3.0 ELISA Test System with Enhanced SAVE demonstrated equivalent sensitivity. The ORTHO HCV 3.0 ELISA Test System with Enhanced SAVE proved equivalent sensitivity when compared to the ORTHO HCV 3.0 ELISA Test System with SAVE.

Specificity Studies

The specificity of the ORTHO HCV 3.0 ELISA Test System with Enhanced SAVE in low-risk populations is based on a population of blood donors tested at four different sites.

Site	Number Tested	Procedure	Initial Reactive	Repeat Reactive
Site 1 (United Kingdom)	3517	Short	1 (0.03%)	1 (0.03%)
Site 2 (Germany)	3445	Standard	7 (0.20%)	3 (0.09%)
Site 3 (France)	2374	Standard	0 (0%)	0 (0%)
		Short	0 (0%)	0 (0%)
Site 4 (Germany)	3808	Standard	5 (0.16%)	4 (0.11%)
Total	13,144		13 (0.09%)	8 (0.06%)

Specimens repeatedly reactive by ELISA were tested by supplemental strip immunoblot assay (SIA) and further evaluated by supplemental peptide EIAs. Upon removal of specimens considered positive or indeterminate by SIA and reactive by peptide EIAs, the specificity* in this low-risk population was 99.96% (4 units per 10,000 donations).

* Specificity was calculated as follows:

$$\frac{TN}{TN + FP} \times 100$$

where TN = true negatives, that is the number of specimens nonreactive by ELISA; FP = false positives, that is repeatedly reactive by ELISA and negative or indeterminate by SIA and other supplemental assays.

Reproducibility Studies

The interassay and intra-assay reproducibility of the ORTHO HCV 3.0 ELISA Test System with Enhanced SAVE was assessed for the Standard and Short procedures at four different sites testing a panel of three samples. The samples consisted of a nonreactive sample, a weakly reactive sample and a strong reactive sample. A minimum of two microwell plates per day were tested with an average of 24 to 30 replicates of the same sample per microwell plate.

After statistical analysis, the intra-assay coefficient of variation (CV) varied from 6.6 to 13.2% with a mean of 9.9%. The interassay CV varied from 1.2 to 14.8% and from one day to another, from 1.2 to 15%.

SUMMARY OF REVISIONS (Since Rev. 5/1998)

Revised to reflect transfer of manufacturing from Germany to United States.

Section	Revisions
REAGENTS	Replaced 96 Test Kit with 192 Test Kit. Revised product codes.
PRECAUTIONS	Revised #4 and #8 to add Risk and Safety Requirements. Revised #14 to reflect increased open vial stability.
PREPARATION OF REAGENTS	
2. Preparation of Substrate Solution	Revised last sentence in paragraph under chart.
PROCEDURE	
Materials Required But Not Provided	Revised #12 and #13.
BIBLIOGRAPHY	Added #16 and renumbered as appropriate.

T (ALAT) IFCC mod.

UV-Test

Aminotransferase (EC 2.6.1.2)

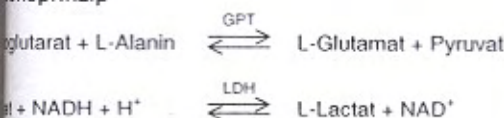
Formen

12212	16 x 5 ml	Kompletter M-Test Kit
12012	10 x 10 ml	Kompletter Testkit
12022	8 x 50 ml	Kompletter Testkit
12032	4 x 250 ml	Kompletter Testkit

de

Die Methode zur Bestimmung der GPT-Aktivität nach den Empfehlungen des Expert Panels der IFCC (International Federation of Clinical Chemistry).

Prinzip



Bestandteile

12212	12012	12022	12032
16 x 4 ml	10 x 8 ml	8 x 40 ml	4 x 200 ml
1 x 16 ml	2 x 10 ml	8 x 10 ml	4 x 50 ml
Puffer / Enzymreagenz			
TRIS-Puffer (pH 7,5)			150 mmol/l
L-Alanin			750 mmol/l
LDH			≥ 8 kU/l
Substrat			
α-Ketoglutarat			90 mmol/l
NADH			0,001 mmol/l

Reagenzienzubereitung und Haltbarkeit

Methode 1 mit Substratstart

Reagenzien sind gebrauchsfertig und bei 2...8°C, lichtgeschützt haltbar, selbst nach Anbruch bis zu dem auf der Packung angegebenen Verfalldatum verwendbar. Kontamination muß unbedingt vermieden werden.

Methode 2 mit Probenstart

12032 und 12022: Den Inhalt einer Flasche [SUB] vollständig in eine Flasche [BUF] geben, gründlich mischen.
12012 und 12012: 1 ml (12212) bzw. 2 ml (12012) aus Flasche in eine Flasche [BUF] pipettieren, gründlich mischen. Die Gebrauchslösung ist 4 Wochen bei 2...8°C und 5 Tage bei 25°C haltbar.

Material

EDTA- oder Heparinplasma
Analyse stört!

Stabilitätsverlust innerhalb von 3 Tagen: bei +4°C: ~ 10 %
bei 20...25°C: ~ 17 %

Messungsansatz

Weglänge: Hg 365 nm, 340 nm oder Hg 334 nm
Wegdicke: 1 cm
Temperatur: 25°C, 30°C, 37°C
Schutz: gegen Luft (Extinktionsabnahme)

Reagenzien und Küvetten vor der Messung auf die gewünschte Temperatur bringen. Temperatur während der Messung konstant halten.

Schema 1 mit Startreagenz

In Küvetten pipettieren	25°C, 30°C	37°C
	200 µl	100 µl
	1000 µl	1000 µl
5 Minuten bei der gewünschten Temperatur inkubieren		
	250 µl	250 µl
5 Minuten, nach 1 Minute Extinktion ablesen und gleichzeitig Stoppuhr starten. Genau nach weiteren 1, 2 und 3 Minuten Ablesewerte wiederholen.		

Pipettierschema 2 mit Probenstart

In Küvetten pipettieren	25°C, 30°C	37°C
Probe	200 µl	100 µl
Gebrauchslösung	1000 µl	1000 µl
Mischen, nach 1 Minute Extinktion ablesen und gleichzeitig Stoppuhr starten. Genau nach weiteren 1, 2 und 3 Minuten Ablesewerte wiederholen.		

Halbmikro-Ansatz; für Makro-Ansatz Volumina verdoppeln

Berechnung

Bei ΔE/min von 0,06-0,08 (Hg 365 nm) bzw. 0,12-0,16 (Hg 334 nm, 340 nm) nur die Messungen der ersten 2 Minuten berücksichtigen (1 Minute inkubieren und 2 Minuten messen).

Methode 1 mit Substratstart

	Hg 334 nm	340 nm	Hg 365 nm
U/l (25°C, 30°C) = ΔE/min x	1173	1151	2132
U/l (37°C) = ΔE/min x	2184	2143	3971

Methode 2 mit Probenstart

	Hg 334 nm	340 nm	Hg 365 nm
U/l (25°C, 30°C) = ΔE/min x	971	952	1765
U/l (37°C) = ΔE/min x	1780	1745	3235

Umrechnungsfaktor von konventionellen Einheiten (U/l) in SI-Einheiten (kat/l):

1 U/l = 16,67 x 10⁻³ µkat/l; 1 µkat/l = 60 U/l

Leistungseigenschaften

Linearität

Die Verdünnungsgrenze liegt bei folgenden Extinktionsdifferenzen bzw. Aktivitäten:

	25°C, 30°C	37°C
Hg 365 nm ΔE/min = 0,080 bzw. 170 U/l		320 U/l
Hg 334/340 nm ΔE/min = 0,160 bzw. 190 U/l		350 U/l

Bei höheren Aktivitäten 0,1 ml Probe mit 0,9 ml Natriumchlorid-Lösung (0,9%) mischen und Bestimmung mit dieser Verdünnung wiederholen (Ergebnis x 10).

Hochaktive Seren können sehr niedrige Anfangsextinktionen zeigen, da das NADH bereits vor der Messung größtenteils verbraucht ist. In diesem Fall wird wie oben angegeben verdünnt.

Typische Leistungsdaten sind im Verification Report zu finden, zugänglich über:

www.human.de/data/gb/vr/EN-GPTLI.pdf

www.human-de.com/data/gb/vr/EN-GPTLI.pdf

Normalwerte

	25°C	30°C	37°C
Männer:	bis 22 U/l	bis 30 U/l	bis 42 U/l
Frauen:	bis 17 U/l	bis 23 U/l	bis 32 U/l

Qualitätskontrolle

Alle Kontrollseren mit GPT Sollwerten für diese Methode können verwendet werden.

Wir empfehlen unsere HUMATROL Kontrollseren, die aus tierischen Seren hergestellt werden, oder unser SERODOS aus Humanserum.

Automation

Vorschläge zur Applikation der Reagenzien auf Automaten stehen auf Anfrage zur Verfügung. Die Validierung der Applikation liegt in der Verantwortung des Labors.

Hinweise

[BUF] und [SUB] enthalten als Konservierungsmittel Natriumazid (0,095 %). Verschlucken, Berührung mit der Haut oder Schleimhäuten vermeiden!

Literatur

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2. Thfeld, W. et al.; Dtsch. med. Wschr. 99, 343 (1974)
3. Thfeld, W. et al.; Dtsch. med. Wschr. 99, 343 (1974)
4. ISO 15223 Medical devices-Symbols to be used with medical device labels, labelling and information to be supplied

EN-GPTLI.DOC
INF 1221201 D
12-2001-12



human

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BILIRUBIN D+T liquidcolor

Photometric Test for Direct (D) and Total (T) Bilirubin

Micro-Biotec Laboratories GmbH Method

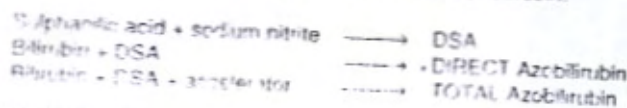
Packaging Size

1000 µl 2 x 100 µl 1000 µl 1000 µl

[FBR]

Principle

Bilirubin reacts with diazotized sulphuric acid (DSA) to form a red azo dye. The absorbance of this dye at 546 nm is directly proportional to the bilirubin concentration in the sample. Water-soluble bilirubin glucuronides react directly with DSA whereas the albumin conjugated indirect bilirubin will only react with DSA in the presence of an accelerator: total bilirubin = direct + indirect bilirubin.



Contents: Reagent Composition in the Test

[FBR]	1 x 100 ml Total bilirubin reagent (white cap)	
	Sulphuric acid	14 mmol/l
	Hydrochloric acid	250 mmol/l
	Caffeine (accelerator)	200 mmol/l
	Sodium benzoate	420 mmol/l
[TNR]	1 x 9 ml T-Nitrite reagent (white cap)	
	for determination of total bilirubin	
	Sodium nitrite	14 mmol/l
	(m: 0.22)	
[DBR]	1 x 100 ml Direct bilirubin reagent (red cap)	
	Sulphuric acid	14 mmol/l
	Hydrochloric acid	250 mmol/l
[DNR]	1 x 9 ml D-Nitrite reagent (red cap)	
	for determination of direct bilirubin	
	Sodium nitrite	0.9 mmol/l

Reagent Preparation and Stability

Both reagents and nitrite solutions are ready for use. They are stable up to the given expiry date if unopened and stored at 15...25°C.

Specimen

Serum or heparinized Plasma

Avoid haemolysis! Samples must be protected from light.

Stability: Bilirubin is stable for 3 days when stored light-protected at 2...8°C.

Assay

Wavelength: 546 nm
Optical path: 1 cm
Temperature: 20...25°C
Measurement: Against sample blank

Procedure

For total bilirubin

Pipette into cuvettes	Sample blank	Sample
[FBR]	1000 µl	1000 µl
[TNR]	---	1 drop ¹
Mix thoroughly, incubate for 5 min.		
Sample	100 µl	100 µl
Mix, incubate at room temperature for 10 to 30 min. Measure the absorbance of sample against sample blank (ΔA_{546})		

1 drop = 40 µl

For direct bilirubin

Pipette into cuvettes	Sample blank	Sample
[FBR]	1000 µl	1000 µl
[DBR]	---	1 drop ¹
Mix thoroughly, incubate for 5 min.		
Sample	100 µl	100 µl
Mix, incubate at room temperature for 10 to 30 min. Measure the absorbance of sample against sample blank (ΔA_{546})		

1 drop = 40 µl

Calculation

Calculate the concentration of total and direct bilirubin by using the factor 13.0.

$$\begin{aligned} \text{Bilirubin concentration [mg/dl]} &= \Delta A_{546} \times 13.0 \\ \text{[mg/dl]} \times 17.1 &= \text{[µmol/l]} \end{aligned}$$

Performance Characteristics

Linearity: The assay is linear up to 25 mg/dl. For bilirubin concentrations exceeding 25 mg/dl dilute the sample 1:4 with physiological saline (0.9%) and repeat the assay. Multiply the result by 5. Typical performance data can be found in the Validation Report, accessible via

www.human.de/data/ghr/SU/CD/OT.rtf or
www.human.de.com/data/ghr/SU/BILDT.pdf

Normal Values

Total bilirubin	mg/dl	µmol/l
At birth up to:		
5 days up to:	5	85.5
1 month up to:	12	205.0
Adults up to:	1.5	25.5
Direct bilirubin	1.1	16.6
Adults up to:	0.25	4.3

Quality Control

All control sera with bilirubin values determined by this method may be employed.

We recommend to use our animal serum based HUMATROL quality control sera or our SERODOS based on human serum.

Notes

- It is important to ensure the bilirubin reagent and the nitrite reagent are thoroughly mixed before adding the sample.
- Bilirubin levels may be reduced if the samples are exposed to light. Haemolysis will also lower the bilirubin value due to the inhibitory effect of haemoglobin on the diazo reaction.

References

- Jendrassik, L., Gróf, P., Biochem. Z. 81, 297 (1938)
- Van der Bergh, A. A., Muller, P., Biochem. Z. 77, 90 (1916)
- ISO 15223 Medical devices - Symbols to be used with medical device labels, labelling and information to be supplied.

SU-BILDT
INF 1074001 GB
05-2002-11

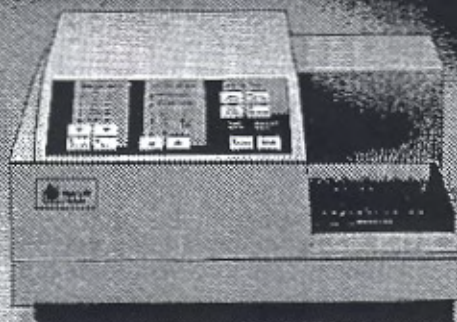


Human

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Untitled

Mode: Kinetics	Microplate Chamber
Run Time: 1:00:00	
Wavelength: —	Instrument Offline
Protocol:	

Data Comment: Protocol Comment: **SOFTmax®**

DR. SANTOSH MAN SHRESTHA, LIVER FOUNDATION NEPAL

For use with instrument: E9626

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