DETERMINATION OF THE BIOCIDAL EFFECT OF SODIUM HYPOCHLORITE IN SMEAR MICROSCOPY FOR DIAGNOSIS OF TUBERCULOSIS IN ABSENCE OF SAFETY CABINET

BY

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Infectious Diseases Diagnosis, of Kenyatta University.
Declaration

I, Sophia Wanja Matu, duly declare that this thesis is my original work and has not been presented for a degree in any other university or any other award.

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Dedication

To my husband Matu and my son Roy with all my love.
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Pulmonary tuberculosis (PTB) is the most common presentation of tuberculosis (TB) worldwide. To diagnose PTB, direct sputum smear microscopy is usually used because it is technically simple, non-invasive and cheap. The reliability of this technique for the diagnosis of TB has frequently been questioned due to its low sensitivity. liquefaction and concentration of sputum using sodium hypochlorite (NaOCl-Jik-bleach) before preparing a smear has been used to increase sensitivity in many settings. Results from these studies have, however, been conflicting. Nevertheless, the protective value of NaOCl in processing of sputum smears for diagnosis of TB particularly in places where adequate safety measures are lacking has not been ascertained. This study was therefore designed to determine the biocidal effect of NaOCl in terms of appropriate concentration and time of exposure required to completely sterilize the specimens and making recommendations for use in absence of a safety cabinet. Sputum specimens of over 10 ml from newly diagnosed patients were processed for microscopy and culture. Two direct smears were prepared from each specimen, one stained with fluorescence microscopy (FM) and the other using Ziehl Neelsen (ZN) method. The remaining sputum was divided into five equal portions after homogenization. One portion was processed for culture. The remaining four portions were treated with NaOCl at 3.5% (household bleach jik™) and 5% (commercial bleach) concentrations. Two specimens, of each concentration were centrifuged and the other two sedimented. Smears were prepared from all sputum specimens that tested smear negative by direct FM and ZN, and examined by FM and ZN methods. Sedimented and centrifuged sputum specimens were cultured at various intervals ranging from ½ hrs to 24 hours, to determine their viability upon which the biocidal effect of NaOCl was evaluated. The sensitivity of direct ZN was 66% and that of direct FM 77.5%. ZN sensitivity was 42.9% and 27.1%, respectively, after centrifugation and sedimentation with 3.5% NaOCl, while it was 37.1% and 10%, respectively, after centrifugation and sedimentation with 5%. The number of specimens that were positive with ZN after treatment with 3.5% was significantly higher than those treated with 5% after centrifugation and sedimentation (p = 0.0014; p = 0.001), respectively. There was also significant reduction in debris in sputum giving clear field definition under the microscope. The 3.5% NaOCl showed total immobilization of MTB, between 1 hr and 3 hr of exposure whereas 5% NaOCl showed total sterilization after 15hrs. Use of appropriate concentration of NaOCl and appropriate time of exposure, may be recommended as a safety measure during smear microscopy for AFB in setups where a safety cabinet is not available.
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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Tuberculosis (TB) is a disease of public health importance globally. One-third of the world’s population is estimated to have been infected with *Mycobacterium tuberculosis* (MTB) and nine million new cases of TB are reported each year (Murray, 1991; Snider et al., 1994; WHO, 1998a). The TB crisis is likely to escalate since the human immunodeficiency (HIV) epidemic has triggered an even greater increase in the number of TB cases. The majority of TB patients are 15 to 45 years of age, persons in their most productive years of life. The disease kills over two million people worldwide each year, more than all other infectious diseases combined, including AIDS and malaria (WHO, 1998b).

Transmission of TB is virtually entirely by droplet infection, created through coughing by untreated persons suffering from pulmonary tuberculosis (PTB) in a confined environment. PTB is the most common form of TB. Infected droplets remain airborne for a considerable time, and may be inhaled by susceptible persons. PTB usually occurs in the apex of the lungs, which develop cavities containing large populations of tubercle bacilli that can be detected in a sputum specimen. PTB is suspected if there is persistent productive cough for three weeks or longer, weight loss, night sweats and chest pains (Collins et al., 1997). The diagnosis can only be made reliably by demonstrating the presence of tubercle bacilli in the sputum by means of microscopy and/or culture in the laboratory (Mitchson, 1966).
Examination by bacteriological culture provides the definitive diagnosis of TB (WHO, 1998c). Compared to other bacteria, which typically reproduce within minutes, MTB proliferate extremely slowly (generation time 18-24 hours). The only media, which allow growth of MTB, are egg-enriched media containing glycerol, asparagine, and agar or liquid medium supplemented with serum or bovine albumin. Culture increases the number of TB cases found, often by 30-50%, and detects cases earlier, often before they become infectious (WHO, 1998c). Culture also provides the necessary isolates for drug susceptibility testing. Culture procedure is however, much more costly than microscopy and requires facilities for media preparation as well as skilled staff (WHO, 1998c).

Direct microscopic examination of appropriately stained sputum specimens usually by Ziehl-Neelsen (ZN) technique is the cornerstone of the diagnosis of TB (WHO, 1998a). The technique is simple, inexpensive and detects those cases of TB that are infectious. Its specificity is at least 99%, although it only identifies acid-fast bacilli (AFB) in the vast majority cases in most developing countries when AFB are observed in sputum, they correspond to MTB complex (IUATLD, 2000).

Fluorescent microscopy is also used for diagnosis of TB. The technique is both more rapid and sensitive than ZN method (Githui et al., 1993) but because of the cost component of the equipment needed, it is rarely used in most developing countries. Between 5 000 and 10 000 tubercle bacilli per milliliter of sputum are required for direct smear microscopy to be positive (David, 1996). Sputum specimens from patients with PTB particularly those with cavitary disease – often contain sufficiently large numbers of acid-fast bacilli (AFB) to be readily detected by direct microscopy (WHO, 1998b). The sensitivity can further be improved by examination of more than one smear from a
patient. Many studies have shown that examination of two smears will on average detect more than 90% of infectious TB cases (WHO, 1998b). The incremental yield of AFB has been shown to range between 80 and 83%, 10 and 14%, and 5 to 8% from the first, second and third specimen respectively (WHO, 1998b). Therefore, three sputum specimens are recommended from each suspect of PTB. Nevertheless, a negative smear result does not exclude the presence of TB since some patients’ harbor fewer tubercle bacilli than can be detected by microscopy. A poor quality specimen may also produce negative result (WHO, 1998b) further complicating TB diagnosis.

Sputum examination by microscopy must be performed on all cases suspected of having PTB. Most patients with infectious TB have respiratory symptoms and the use of smear microscopy in those presenting to health services with suggestive symptoms constitutes the most efficient means of case detection (NACO, 2004). Sputum microscopic examination is usually done in designated TB microscopy center. These centers have a skilled laboratory technician, trained intensively for a sputum examination and are well equipped with microscopes and reagents (NACO, 2004).

Smear sensitivity is poor in extra-pulmonary TB and in diseases caused by mycobacteria other than tubercle bacilli (MOTT) (WHO, 1998b). It is also virtually impossible to distinguish between Mycobacterium species by smear microscopy. Additional problems that undermine the importance of this technique include; (i) lack of sensitivity, particularly in patients with TB and HIV co-infection due to lack of cavities, and (ii) risk of laboratory acquired infections when the technique is performed outside a safety cabinet.
1.2 Etiology

Tuberculosis is an infectious and communicable disease, caused by *Mycobacterium tuberculosis*, and occasionally by *M. bovis* and by *M. africanum*, which are the main pathogenic species within MTB complex (Dubos and Dubos, 1952). Mycobacteria can be divided into two major groups: those belonging to MTB complex (*M. tuberculosis, M. bovis, M. microti, M. cannetti and M. africanum*) and those referred to as nontuberculous mycobacteria (NTM) (IUATLD, 2000).

Nontuberculous mycobacteria include a wide range of species and are usually isolated from the environment and rarely cause illness (IUATLD, 2000). However, *M. avium* complex shows some virulence towards humans, in whom it causes infection and illness, especially in immunodepressed persons, such as HIV-AIDS cases (IUATLD, 2000).

1.2.1 Types of TB cases

TB patients are classified as sputum positive PTB, sputum negative PTB or extrapulmonary TB by site and bacteriological status.

**Pulmonary Tuberculosis, smear positive:**

- Two or three sputum smears positive for AFB.
- One sputum smear positive for AFB, with radiographic abnormalities consistent with active TB as determined by the medical officer.
- One sputum smear positive for AFB, with culture positive for AFB (NACO, 2004).
Pulmonary Tuberculosis, smear negative:

- Three sputum smear negative for AFB, but showing radiographic abnormalities consistent with active PTB after 2 weeks of antibiotic treatment
- Three sputum smears negative for AFB, but positive on culture (NACO, 2004).

Extra-pulmonary tuberculosis

- TB of any organ other than lungs such as pleura, lymph nodes, intestines, meninges, skin, joints and bones, genital system etc.
- Three sputum smears negative for AFB, but positive on culture (NACO, 2004).

1.2.2 Sputum for microscopy in diagnosis of tuberculosis

Sputum is a pathological material expectorated from lungs and bronchi, in the strict sense, and it consists of saliva and secretion from nasal, laryngeal and mouth cavities in different proportions when evacuated from mouth.

Application and value

- Sputum microscopy is a non-invasive procedure, which is generally used as a first-line diagnostic investigation or a screening tool for the detection of AFB.

Sputum Collection

For satisfactory sampling, fresh, deep-cough specimen is required. The best specimen should be taken early in the morning when the patient wakes. The specimen should be sent to the laboratory immediately to prevent deterioration of the cells. Sputum
collected should show the presence of alveolar macrophages, as macrophages have not been reported in the secretion of upper respiratory tract. Its presence is especially important for specimen adequacy for diagnosis of TB. Other cells include epitheloid cells, pus cells and giant cells.

1.3 Epidemiology

Tuberculosis, when introduced into a susceptible population, takes the form of an epidemic wave. Unlike other infections the wave runs its course in about 300 years. The decline of TB in the 1980s, led to its neglect as a major health priority, particularly by the wealthy nations. The problem had become so small that there was discussion of possible elimination of the disease in the twenty-first centuries (Gordon and Saunders, 1996). In an article by Horne in 1983 titled “Eradication of TB in Europe: so near yet so far” the following statement was made: “forecasts that TB would disappear within a generation have been made nearly a century, but it seems likely that several more generations will come and go before eradication is achieved.” This proved prophetic since two years later USA observed for the first time a deviation from the expected logarithmic decline in TB (Gordon and Saunders, 1996). This is attributed to HIV co-infection and patients harboring resistant-often multi-drug resistant-tubercle bacilli (Gordon and Saunders, 1996).

The WHO estimates that one-third of the world population is infected with tubercle bacilli. Furthermore, in 1990, eight million new cases of TB occurred, 95% of them in developing world and 5% in industrialized countries. Close to 3 million people die of this disease every year (Grzybowski, 1991). In early 1992 WHO estimated that
approximately 4 million people had been infected with both TB and HIV since the beginning of the pandemic. (IUATLD, 2000). Whereas the largest number is in Asia, the highest incidence rate is seen in sub-Saharan Africa (Gordon and Saunders, 1996).

1.4 Transmission, Pathogenesis and clinical symptoms of Tuberculosis

Transmission of bacilli (infection) occurs almost exclusively from person to person by aerogenic route. The source of infection is a patient with PTB. When such a person coughs, tiny droplets are produced which evaporate rapidly to the size of infectious droplet nuclei.

Fig 1: Diagrammatic illustration of Transmission of TB

TB is spread from person to person through the air. The dots in the air represent droplet nuclei containing tubercle bacilli. (Adopted from CDC, 2004)

A single cough may produce up to 3 000 such droplets. The nuclei contain one or more tubercle bacilli which being very small (less than 5μm in diameter), remain suspended in air, and when inhaled may reach pulmonary alveoli. These infectious droplets are the principle source of transmission of MTB (IUATLD, 2000).

The probability of becoming infected by MTB depends on the number of infectious droplet nuclei per volume of air (infectious particle density) and the duration of exposure of a susceptible individual to that particle density. The risk is reduced by air renewal, exterior ventilation and exposure to ultra violet light (IUATLD, 2000).
Once inside the body, the tubercle bacilli can remain in latent state in the body tissues for years. The tubercle bacilli are phagocytized by alveolar macrophages, taken to the nearest lymph node where they continue to multiply, thereby destroying the cells and ensuing bacilli are taken up by other cells. The bacteria multiply and reach critical mass that spills out of the destroyed macrophages through the lymphatics into the blood stream and the bacilli are carried to many parts of the body. At this stage, some individuals with intact immune system mob out most of the bacilli but a few remain at the apical portion of the lung where they are walled off. These may be reactivated by insult on the immune system and cause secondary TB. In some immunodeficient hosts the initial bacteremia seeds bacteria throughout the host, which is unable to control them, leading to disseminate or milliary TB. Growth of bacteria in host macrophages and histiocytes in the lungs causes influx of lymphocytes, neutrophils and histiocytes leading to a granuloma, tissue destruction and cavity formation (CDC, 2004).

**Fig 2: Diagrammatic illustration of Pathogenesis of TB infection and disease**

Droplet nuclei containing tubercle bacilli are inhaled, enter the lungs, and travel to the alveoli.
Tubercle bacilli multiply in the alveoli.

A small number of tubercle bacilli enter the bloodstream and spread throughout the body. The bacilli may reach any part of the body, including areas where TB disease is more likely to develop (such as the lungs, kidneys, brain, or bone).

Within 2-10 weeks, the immune system produces special immune cells called macrophages that surround the tubercle bacilli. The cells form a hard shell that keeps the bacilli contained and under control (TB infection).

If the immune system cannot keep the bacilli under control, the bacilli begin to multiply rapidly (TB disease). This process can occur in different places in the body, such as the lungs, kidneys, brain, or bone (see diagram in box 3).

(Adopted from CDC, 2004)

Clinical manifestations of patients with MTB complex may range from asymptomatic to acutely symptomatic. Patients who are symptomatic can have systemic symptoms, pulmonary signs and symptoms, signs and symptoms related to other organ involvement, or a combination of these features. Common presenting symptoms include
low-grade fevers, night sweats, fatigue, anorexia, and weight loss (Betty et al., 1998). If a patient presents with PTB, a productive cough is usually present, along with fevers, chills, aches and sweating. Individuals infected with HIV are particularly susceptible to develop active tuberculosis (Betty et al., 1998).

1.5 Resurgence of TB

In recent years, there have been dramatic increases in the numbers of new cases worldwide. Although a cure for TB was developed more than 50 years ago, TB continues to kill between 2 and 3 million people every year (Fact sheet, 2003). The World Health Organization (WHO) estimates that 36 million people will die of TB by 2020 if it is not controlled. Nine million people develop active TB every year, nearly 98 percent of who live in the developing world (WHO, 1998). In Kenya, the number of TB cases (all forms) notified each year continues to increase steadily (WHO, 2003). However, the number of new smear-positive cases does not correspond to the increase. There are several possible explanations for this and includes: cases in HIV-positive patients are less likely to be smear-positive and there may be over-diagnosis of extra pulmonary or smear-negative pulmonary TB; or the quality of diagnosis may be falling as the work-load increases (WHO, 2003).

The association between TB and HIV presents an immediate and grave public health and socio-economic threat particularly in the developing world (Itoh et al., 1986). The association between TB and HIV is evident from the high incidence of TB, estimated at 5 – 8% per year among HIV infected persons, the HIV sero-prevalence among patients with TB, the high recurrence of TB among AIDS patients and the coincidence of
increased TB notifications with the spread of the epidemic in several African countries (Gordon and Saunders, 1996). Tuberculosis is the principal ‘‘opportunistic’’ infection and cause of death among HIV patients. It differs from other opportunistic infections in that it is transmitted aerogenically to uninfected persons, thus becoming a problem for the whole community.

HIV infection influences the natural history of TB in several ways: i) Active TB occurs within 6 months of acquiring MTB infection in 37% of persons with HIV-induced immunosupression (Pablo et al., 2003), (ii) it occurs in 2% of immunocopetent adults during the first year of acquiring MTB infection (Pablo et al., 2003), (iii) HIV infected persons with TB also have increased frequency of disseminated and meningeal diseases, other extra pulmonary diseases, drug related adverse effects, and negative sputum smears for AFB (Murray, 1997). TB appears at any point in the progression of HIV infection, which is the strongest known factor in the increasing risk of the disease (IUATLD, 2000).

1.5.1 Treatment and Drug Resistance

The chemotherapy of PTB demands not only a detailed knowledge of individual drugs but also of the regimens that are essential to a successful outcome of treatment. The drugs used for treatment of TB fall into two groups viz.; those commonly used in previously untreated patients that include Isoniazid (H), Rifampicin (R), Ethambutanol (E), Pyrazinamide (Z), Streptomycin (S) and Thiacetazone (T) and secondly those used if primary treatment has failed mainly Cycloserine (CYC), Prothionamide (PRO), Capreomycin (CAP) and Viomycin (VIO) (Gordon and Saunders, 1996)
Drug resistance is a state when MTB organisms are resistant to antimicrobial agents at the levels attainable in blood and tissue (Mitchison, 1998). Resistance can develop either spontaneously (primary) or under selective pressure (acquired) (Jacobs, 1994). Multi-drug resistant TB (MDR-TB) is defined as TB caused by MTB that is resistant to at least isoniazide and rifampicin (WHO, 1994; WHO/IUATLD, 1997).

The MDR-TB has become an increasing concern. Factors that promote the emergence of MDR-TB include inappropriate regimens, non-adherence to therapy, failure to identify drug resistance, adding a single drug to a failing regimen, Laboratory delays in growth, and identification and reporting of these MDR-TB cases (Iseman, 1993; Tenover, 1993; Mitchson, 1998). MDR therefore threatens global TB control and MDR has been associated with inadequate treatment regimens, poor adherence to treatment; poorly managed TB programmes and unenforced hospital control programs as well as HIV infection (Gordin et al., 1996).

1.5.2 Case definition by previous treatment

- **A new case** is a patient who has never had treatment for TB for more than four weeks.

- **A relapsed case** is a patient who has been declared cured of any form of TB in the past, by a physician, after one full course of chemotherapy, and has become sputum smear positive.

- **A case treatment failure** is a patient who, while on treatment, remained smear positive; OR became again smear positive at the 5th month or later during the course of treatment.
• **Treatment after interruption (Defaulter)** is a patient who completed at least one month of treatment, interrupted this treatment for two months or more, and returned to the health service with smear-positive sputum, or as smear-negative but still with active TB as judged on clinical and radiological assessment.

### 1.6 Current Diagnostic techniques for TB

The sputum acid-fast stain remains the cornerstone for the diagnosis of active respiratory TB around the world; however, the technique has repeatedly lacked sensitivity and, to some extent, specificity (Lipsky, 1984). Since the mid 1990s, a few commercially available molecular amplification assays have been introduced in an attempt to improve the accuracy and speed of detecting MTB in clinical specimens. Despite the development of quicker and more sensitive novel diagnostic techniques, their complexity and high cost has limited their use in many resource-poor countries (Githui, 2002).

#### 1.6.1 ZN staining

The Ziehl Neelsen carbolfuchsin or Kinyoun stains have been used in TB diagnosis for nearly 100 years. Although less sensitive than culture, the acid-fast smear is a rapid and inexpensive test that can be performed with a minimum of equipment and is very specific for mycobacteria (WHO, 1998b). Depending on the bacteria load, a single sputum smear has sensitivity between 22% and 80%, but the yield is improved when multiple sputum specimens are examined (Nolte and Metchock, 1995; Van *et al.*, 2003).
1.6.2 Fluorochrome staining

This technique uses fluorochrome stains such as Auramine-rhodamine stain. With this technique, mycobacteria fluoresce with bright orange color and can easily be seen on low-power microscopy, increasing the sensitivity of smears (Lempert, 1944).

1.6.3 Culture technique

Culture of MTB using the conventional Lowenstein-Jensen (L-J) media is the “gold standard” in the diagnosis of TB (Aber, 1980). Culturing the specimen is necessary to determine whether the specimen contains MTB and therefore confirm a diagnosis of the disease. One of the newest and fastest technique for growing MTB is the mycobacterial Bactec - based Growth Indicator Tube. The advantage of this system is the rapidity with which it detects and isolates mycobacteria (Hanna, 1996).

1.6.4 Drug susceptibility testing

Drug susceptibility tests, the final part of the bacteriologic examination, are done to determine *in vitro* which drugs will kill the tubercle bacilli that are causing disease in a particular patient. Tubercle bacilli that are killed by a particular drug are said to be susceptible to that drug, whereas those that can grow even in the presence of a particular drug are said to be resistant to that drug (CDC, 2004).

Drug susceptibility tests are done when a patient is first found to have a positive culture for MTB (that is, the first isolate of MTB). In addition, drug susceptibility tests is repeated if a patient has a positive culture for MTB after 2 months of treatment or if a patient does not seem to be getting better. That way, the clinician can find out whether
the patient's strain of TB has become resistant to certain drugs; if necessary, the clinician may change the drugs used for treating the patient (CDC, 2004).

1.6.5 Sensitivity, Specificity and Predictive value of diagnostic tests

The actual value of a diagnostic test, is its reliability for distinguishing between persons who have disease and those who have not, depends on mainly two characteristics: The first is the Sensitivity which is the capacity to correctly identify diseased individuals in a population, or “true positives”. The greater the sensitivity, the smaller the number of unidentified case (false negatives) (IUATLD, 2000).

The second value is the Specificity, which is the capacity to correctly exclude individuals who are free of the disease, or” true negatives”. The greater the specificity, fewer false positives will be included. Sensitivity and specificity are attributes proper to each diagnostic method. However, when these methods are used in the field, the certainty of results is affected by the frequency of the phenomenon being measured or prevalence. Other diagnostic values include:

Positive predictive value (PPV): This is the probability of the disease being present, among those with positive diagnostic test results. The PPV of smear microscopy will decrease as prevalence drops.

Negative predictive value (NPV): is the probability that the disease was absent, among those whose diagnostic test results were negative.
1.6.6 Limitations of diagnosis by smear microscopy and culture

Although microscopy is of intermediate complexity and gives results within a few hours, it fails to detect low numbers of mycobacteria present in a specimen and is not specific to MTB complex (Githui, 2000). On the other hand, culturing which allows confirmation of cases and facilitates species identification requires skilled personnel and additional equipment. It also takes 2 to 8 weeks before results are available (Githui, 2000).

1.6.7 Commonly used disinfectants and antiseptics for mycobacteria

Antiseptics are frequently used to prevent mycobacterial infections. However, the reported activities of antiseptics against mycobacteria are not always consistent due to several basic methodological problems as well as the high intrinsic resistance to antiseptics due to mycobacterial membrane structure (Petit and Lederer, 1978). In Europe and United States much effort has been made to ensure uniformity of methods for the evaluation of antibacterial activities of antiseptics (Rikimaru et al., 2000).

Standardization of methods for the evaluation of antimycobacterial activities of antiseptics has been attempted (Cole et al., 1990). Nevertheless, it is unclear whether the results of tests conducted according to these guidelines accurately reflect antiseptic activity against mycobacteria (Best et al., 1990). This uncertainty exist because i) currently available antiseptics include wide variety of agents, such as aldehydes, alcohols, surfactants and halogens, and therefore individualized study designs should be established to evaluate each of these chemicals taking into account their individual properties, and ii) evaluation of antiseptic activity requires consideration of the
relationship between time of exposure and potency, as antiseptics may affect mycobacteria after prolonged exposure (Rikimaru et al., 2000). In this respect, antiseptics need to be neutralized and inactivated after exposure to bacteria.

The Center for Disease Control and Prevention (CDC) defines three levels of disinfection in the “Guidelines for the prevention of Transmission of HIV and Hepatitis B Virus to healthcare and public—safety workers.” High-level disinfection for example formaldehyde 6-8% can be expected to destroy all microorganisms, with the exceptional of high numbers of bacterial spores (Miller, 2004).

Intermediate—level disinfection for example 70% alcohol inactivates MTB, vegetative bacteria, most viruses, most fungi, but it does not kill bacterial spore (Miller, 2004).

Low-level disinfection for example Quaternary ammonium mixtures can kill most bacteria, some viruses, and some fungi, but it cannot be relied on to kill resistant microorganisms such as tubercle bacilli or bacterial spores (Miller, 2004).

It is a remarkable fact that no one knows precisely how disinfecting chemicals kill microorganisms (Biosafety Guide, 2004). There are plenty of theories and correlations but proof is lacking and recommendations are based on purely empirical findings (Biosafety Guide, 2004).

Many factors influence the effectiveness of germicides, including the:

- Amount of microbiologicals on the germicide.
- Resistance of the microbiologicals to germicide.
• Amount of additional organic buildup on the object, including blood, mucous or tissue.

• Chemical composition of the germicide.

• Time of exposure to the germicide.

• Temperature of the germicide (Facts, 2004).

Some of the commonly used disinfectants and antiseptics for mycobacteria include iodophor, glutaldehyde, phenolic compounds and chlorine compounds.

Iodophor

Iodophor is effective against vegetative bacteria and viruses and has a rapid biocidal action. It is effective against Gram-negative and Gram-positive organisms, some viruses, tubercle bacilli, and is most effective in acid solutions. For tubercle bacilli it should be used at concentrations of 3%-5% and contact time should be 15-30 minutes depending on the type and volume of material to be disinfected. Iodophors are useful for mopping spills and for hand washing. It is still active if the solution is brown or yellow (WHO, 1998a; Miller, 2004). Examples of iodophor include Vanodine, FAM 30 and Biocid.

Glutaraldehyde

Glutaraldehyde is useful for decontaminating bench surfaces and glassware. It does not require dilution but an activator. It is usually supplied as 2% solution, while the activator is a bicarbonate compound. The activated solution should be used within two weeks and discarded if turbid (WHO, 1998a).
Phenolic compounds

Phenolic compounds are effective against vegetative bacteria, fungi, and lipid-containing viruses and tubercle bacilli. For tubercle they should be used at concentrations 2-5% and contact time 15-30 minutes, depending on the type and volume of material to be disinfected. They are useful in soaked paper towels to cover working surfaces. This minimizes spatter and aerosol formation in the event of spilling (WHO, 1998a) and include Trilafon and Flavons 500.

Chlorine compounds

Chlorine compounds are good disinfectants for the clean up of blood or body-fluid spills. They have biocidal effect on MTB, S.aureus, other vegetative bacteria, and HIV after 10-20 minutes, and is used in 1:5 dilution (250 ppm) for bacterial spores and mycobacteria (Miller, 2004). An example is oxine, which is a chlorine dioxide disinfectant.

1.7 Sodium hypochlorite

Sodium hypochlorite is a greenish-yellow liquid commonly referred to as "bleach". It is formed by reacting chlorine with sodium hydroxide as indicated in the following formular:

\[ \text{Cl}_2 + 2\text{NaOH} \rightarrow \text{NaOCl} + \text{NaCl} + \text{H}_2\text{O} \]

The chemical formula for sodium hypochlorite is NaOCl.

The use of hypochlorite salts for disinfection dates back to the mid-18\textsuperscript{th} century. Hypochlorite is routinely used as a sanitizer for domestic uses, as well as in food-
processing plants to remove surface contaminants, which can alter food quality or lead to food-borne diseases. Hypochlorite is known to be very effective killer of bacteria; even micromolar concentrations are enough to reduce bacterial populations significantly (Nakagawara et al., 1998). However little is known about the exact mechanisms of bacterial killing by this sanitizer. Hypochlorite solutions (5%) are useful for disinfection of material containing organic debris because of their digesting action (WHO, 1998a).

When diluted in water, the hypochlorite salts used (NaOCl, Ca(OCl)₂, LiOCl, and KOCl) lead to formation of hypochlorous acid (HOCl), whose concentration is correlated with bactericidal activity (Nakagawara et al., 1998). Bacterial killing by HOCl may be due at least in part to lethal DNA damage (Dukan et al., 1999). However, HOCl itself is so reactive that it is unlikely to penetrate cells and reach the DNA; rather, it seems that the bactericidal activity is due to formation of secondary products, as hypochlorous acid reacts avidly with a wide variety of sub cellular compounds (membranes, proteins). In particular, HOCl reacts with NH₄⁺ and organic amines to form highly toxic chloramines, which are also strong oxidizing and chlorinating compounds and could be the actual killing agents (Lucie and Jacques, 2001).

1.7.1 Domestic and Industrial General Use of Sodium Hypochlorite

Sodium hypochlorite is used as bleach disinfectant/sterilizer at desirable concentrations for general domestic and industrial use. For example, it is used as a bottle sterilizer for baby feeding, wine bottles, fabric bleach, bath cleaner, water sterilizer, clothes/fabric prewash product, drain cleaner, scourer, dishwashing liquid/powder for machine washing, septic tank cleaner among others.
1.7.2 Scientific use of sodium hypochlorite

Sodium Hypochlorite has proven to be an excellent solution for the chemical-biomechanical preparation of root canals. One of its most important characteristics is the capability to liquefy/dissolution of tissue (Spano et al., 2001). Studies have shown that HIV is inactivated rapidly after being exposed to commonly used chemical germicides at concentrations that are much lower than used in general practice (Spire et al., 1985). A solution of NaOCl (household bleach) prepared daily is inexpensive and effective germicide. Concentrations ranging from approximately 500 ppm (1:100 dilution of household bleach) NaOCl to 5000 ppm (1:10 dilution of household bleach) are effective depending on the amount of organic material present on the surface to be cleaned and disinfected (CDC, 1995).

For the last decade various investigators have used NaOCl for liquefaction and concentration by either centrifugation or sedimentation to increase the sensitivity of direct smear microscopy for diagnosis of PTB (Gebre et al., 1995; Miomer et al., 1996; Habeenzu et al., 1999). In studies performed in Ethiopia and India, the use of NaOCl method increased the number of positive for AFB by more than 100% (Gebre et al., 1995). A study carried out on HIV positive patients showed that sensitivity increased from 38.5% to 50% after concentration with NaOCl (Bruchfeld et al., 2000). Most investigators used 5% NaOCl (household bleach) (Miomer et al., 1996; Gebre – selassie, 2003), one used 1% NaOCl (Rattan et al., 1994) and others never indicated the sodium hypochlorite content (Farnia et al., 2002). Other studies have reported over 70% increase in sensitivity and specificity of 100% in both ZN and FM methods (Habeenzu et al., 1999; Saxena et al., 2001; Gebre-selassie, 2003). However, although most investigators
refer to NaOCl as a potent disinfectant and suggest that it will reduce laboratory-acquired infections, it has not been established what concentration and duration of exposure to NaOCl is protective in the absence of safety cabinet (Rattan *et al.*, 1994; Gebre *et al.*, 1995; Miomer *et al.*, 1996; Saxena *et al.*, 2001). The main objective of this study was to ascertain at what concentration and time of exposure NaOCl has biocidal effect and thus is protective to the user when used in smear microscopy for diagnosis of TB in absence of appropriate safety measures and particularly a safety cabinet.

In Kenya, diagnosis of TB is done in centralized laboratories, which are equipped with safety cabinets. Establishment of the biocidal effect of NaOCl will provide insight of the protective value to the user and may assist the decentralization of TB diagnostic centers thus increasing efficiency of diagnosis by reducing the workload and subsequently facilitating early detection and reduction of TB transmission.

1.8 Problem Statement and Justification

In developing countries, sputum smear microscopy forms the cornerstone of diagnosis of TB. However, its reliability has been frequently questioned due to its relatively low sensitivity, which has further been influenced by the increasing number of HIV-related smear-negative cases. Although liquefaction and concentration of sputum before ZN staining, using NaOCl, improves yield by up to 70%, the protective value of this technique to the user in absence of appropriate precautions especially a safety cabinet, has not been ascertained.

In spite of its disinfecting properties, killing or immobilization effect of NaOCl on Mycobacteria has not been proved. There is no data on the appropriate concentrations and
appropriate exposure time required to kill or immobilize MTB (Angeby et al., 2004). Use of NaOCl may therefore pose a health hazard to workers in settings where appropriate safety measures are not observed. This may be as a result of the assumption that NaOCl is a potent "disinfectant" as it has been used against HIV (Flynn et al., 1994), and its use would therefore contribute to the risk of laboratory-acquired infections. In addition, in Kenya TB diagnosis is still centralized to microscopy centers, which are equipped with appropriate safety measures. These laboratories are usually situated distances away from most populations who need their services. This leads to delays in diagnosis of TB since patients or specimens are usually transported long distances to these microscopy centers. These laboratories are also overwhelmed with increasing workload. Therefore, there is need to ascertain the biocidal effect against which the protective value of NaOCl would be recommended for use in settings without appropriate safety measures. This will lead to early diagnosis of infectious cases and decentralization of TB diagnosis.

1.8.1 Research question

How protective is NaOCl when used as a disinfectant for direct sputum smear microscopy for diagnosis of pulmonary TB outside a safety cabinet?

1.8.2 Hypothesis

Sodium hypochlorite has a biocidal effect when used in appropriate concentrations and adequate time of exposure in the preparation of sputum smears for diagnosis of pulmonary tuberculosis in the absence of a safety cabinet.
1.9 Objectives

The general objective of the study was to evaluate the biocidal effect of NaOCl when used as a disinfectant in processing of sputum smears for diagnosis of PTB.

1.9.1 Specific objectives

1. To compare the specificity and sensitivity of ZN with FM methods using concentrated sputum treated with NaOCl and smear from untreated sputum.

2. To determine the liquefying effect of NaOCl on ZN staining.

3. To determine the effect of different concentrations of NaOCl on the viability of MTB using culture as a gold standard at different time intervals of exposure.

1.9.2 Significance and Anticipated Output

Data from this study will be useful in improving diagnosis of TB especially in the areas where appropriate safety procedures are difficult to establish. Its application will also increase the efficiency of TB control programmes
CHAPTER TWO

MATERIALS AND METHODS

2.1 Study site and design

This prospective study was carried out at Kenya Medical Research institute (KEMRI)-Center for Respiratory Diseases Research (CRDR). A total of 370 sputum samples were collected from patients attending Mbagathi District Hospital (MDH) Nairobi to examine for AFB. MDH is a public hospital that serves as the TB referral center for Nairobi province. The hospital is located in Nairobi City, a walking distance from Kenyatta National Hospital (KNH) complex where CRDR is situated.

2.2 Sample size

A sensitivity level of 60% was anticipated with a significance level of 5%, 95% confidence interval and 5% error margin (Habeenzu et al., 1999).

The minimum sample size was estimated using Lwanga and Lememshaws (1991) formula as follows:

\[ n = \frac{((Z\alpha/2)^2 \cdot pq)}{d^2} \]

Where: \((Z\alpha/2)^2\) is the corresponding value to the 95% confidence interval

\[ P \text{ is the level of sensitivity} \]

\[ q = 1 - p \]

\[ d \text{ is the allowable error margin} \]
Therefore: \[ n = (1.96)^2 \times (0.6)(0.4) = 369 \approx 370 \text{ samples} \]
\[ (0.05)^2 \]

2.3 Inclusion and Exclusion criteria

Sputum specimens from all suspected new cases of PTB were included in sample collection while those from defaulters and patients under medication were excluded.

2.4 Ethical consideration

The specimens used in this study were already used for routine smear diagnosis of TB using routine procedure at MDH. Therefore, patients from whom the specimens were derived were not affected by results from this study.

2.5 Transport of sputum specimens and Preparation of smears for microscopy

Fresh sputa of 10ml volume or more were collected in clean well-labeled polypots daily and where necessary was pooled. The polypots were packed securely in cool boxes and transported to CRDR by car where the sputa were transferred aseptically to sterile 50ml centrifuge tubes.

In the laboratory, smears for direct microscopy were prepared under a type IIA-safety cabinet. A small portion of purulent part of sputum was picked with a sterile loop in accordance with methods previously described (WHO, 1998) as follows:

A new, clean, unscratched slide was labeled at one end with the specimen number. An appropriate portion of the specimen was transferred to the slide using a bacteriological loop. Blood-specked, opaque, grayish or yellowish cheesy mucus for smear
preparation was used when present. The specimen was smeared on the slide over an area approximately 2.0 by 1.0 centimeter and made thin enough to be able to read through it. The smear was allowed to air dry for 15 minutes, and then fixed by passing the slide through a flame three or four times with the smear uppermost. It was allowed to cool before staining.

Two smears were prepared; one was stained using the ZN method and the other one using FM technique.

2.6 Diagnosis Methods

2.6.1 Ziehl Neelsen method

A standard staining procedure previously described by IUATLD (1998) was used for Ziehl Neelsen method. Briefly, heat fixed smears were stained with carbol fuchsin, heated to steamrise and then rinsed with tap water, after which they were decolorized with acid-alcohol and counterstained with malachite green. The smears were examined under a light microscope. The AFB, which appeared as red rods against a green background, were counted and recorded.

2.6.2 Fluorescence microscopy method

A standard staining procedure previously described by Lempert (1944) was used for fluorescent microscopy method. Briefly, heat fixed smears was stained with auramine phenol, rinsed, decolourized with acid alcohol, and counterstained with potassium permanganate in bulk lots of 64 using an automatic (Shandon- Elliot) staining machine.
(Clancey, 1976). The smears were examined using fluorescent microscope. The number of AFB, which fluoresced with bright orange color, were counted and recorded.

2.6.3 Liquefaction and concentration

The remaining sputum specimen was vortexed and divided into five equal parts in 50-ml conical screw-cap tubes. One tube was treated with sodium hydroxide for culture; two tubes were treated with equal volume of 3.5% NaOCl and the remaining two with equal volume of 5% NaOCl. The NaOCl concentration was determined using a HTH kit (Appendix 4). The tubes treated with NaOCl were left to stand for 30 minutes at room temperature in a safety cabinet for liquefaction to take place (Yassin et al., 2003). Two tubes one containing 3.5% and the other 5% NaOCl were centrifuged at 3000 RCF for 15 minutes and the other two tubes sedimented at room temperature and analyzed after 12-15 hours. The supernatant of each tube was carefully decanted off, the sediment was vortexed, and 1-2 drops were transferred with a sterile pipette on to a pre-labeled slide. Two sets of smears were made from each concentrate. One set of slides were air dried, heat fixed and stained for ZN technique.

The stained slides were examined using a bright field microscope under oil immersion (X 1000), and they were reported as negative when no AFB were seen in at least 100 microscopic fields. Smears were recorded as positive for any of the following observation: (i) When 1-9 bacilli were seen in 100 microscopic fields (Few bacilli), (ii) 10-99 AFB were seen in 100 microscopic fields (1+), (iii) 1-10 AFB were seen in at least 50 fields (2+), and (iv) more than 10 AFB were seen per field in at least 20 fields (3+).
The other set of slides were air dried, heat fixed and stained using FM technique. The stained slides were examined using a Leitz SM binocular fluorescence microscope. Smears were recorded as positive for any of the following observation: (i) when 1-9 bacilli were seen in 100 microscopic fields (Few bacilli), (ii) 10-99 AFB were seen in 100 microscopic fields (1+), (iii) 1-10 AFB were seen in at least 50 fields (2+), and (iv) more than 10 AFB were seen per field in at least 20 fields (3+). The sensitivity and specificity of direct and NaOCl sediment smear method were determined by using the culture as the “Gold standard” (Flow chart on page 33).

2.6.4 Quality control

Known positive and negative sputum specimens were included in every batch of specimens processed. All smears prepared from sputum specimens (both bleach digested and direct) were retained. An arbitrary 10% of the positive smears and 5% of the negative smears were selected at random (Yassin et al., 2003) and re-examined by an experienced microscopist from KEMRI-CRDR TB laboratory. The microscopist was blinded to the initial results. All reagents and media were prepared in accordance with SOPs used at CRDR-KEMRI. NaOCl was reconstituted on a weekly basis.

2.7 Determination of the liquefying effect of NaOCl on ZN staining

2.7.1 Sample by Estimation

Assuming that one third of the specimen will have the characteristics of interest i.e. type and number of cells found in sputum including pus cells, epithelial cells, debris,
and morphology of tubercle bacilli (Saluja et al., 1999). The minimum sample size was estimated using WHO (1986) formula on sample size estimation as follows:

\[ n \geq \frac{((Z\alpha/2)^2 \times \delta^2)}{d^2} \]

Where: \((Z\alpha/2)^2\) is the corresponding value to the 95% confidence interval
\(d^2\) is the allowable error taken to be 1/3 of previous other studies
\(\delta^2\) is the variance

Therefore, \(d = \frac{1}{3} \delta\)

\[ n \geq \frac{((Z\alpha/2)^2 \times \delta^2)}{((1/3) \delta)^2} \]

\[ n = \frac{(1.96)^2}{1/3^2} = 34.56 \text{ cases} (\approx 35 \text{ cases}) \]

Sampling was done by sequential random sampling method.

\[ \frac{370}{35} = 10 \]

One number between 1 and 10 was randomly chosen and the number was number 6.

Therefore the serial number was 6.

\[ N^{th} \text{ term} = 6 + (n-1) + 10 \]

The type, number and morphology of cells found in sputum including pus cells, epithelial cells, and debris were counted and recorded in stained smear following treatment with NaOCl and were compared with those found in direct smears.
2.8 Effect of sodium hypochlorite on viability of *Mycobacterium tuberculosis* using culture as a gold standard at different time intervals of exposure

To ascertain the biocidal effect of NaOCl, all smear-positive sputum samples were aliquoted into five portions. One portion was directly processed for culture on Lowenstein-Jensen (L-J) medium; the other portions were treated with different concentrations of NaOCl reagent, and the sediment were cultured for MTB at different time intervals, starting at, \( \frac{1}{2} \) hr, 1 hr, 3hrs, 15hrs, and after 24 hrs.

The portion that was directly processed for culture used the Petroffs modified sodium hydroxide technique (Aber, 1980; Githui *et al.*, 1993, 2000). Briefly, sputum was homogenized in a vortex mixer. An equal volume of 4% NaOH was added and the mixture was vortexed. To effect decontamination, the tubes were kept at room temperature in the safety cabinet for 15 minutes. The mixture was centrifuged at 3000 RCF for 15 minutes. The supernatant was poured off, and the resulting sediment was neutralized with 20ml of sterile distilled water. Samples were again centrifuged and 1-2 drops of the sediment was inoculated into two slopes of L-J media using a sterile pipette and incubated for up to 8 weeks at 37°C checked weekly before they were declared negative if no growth was observed.

Two of the other 4 portions were treated with equal volumes of 3.5% NaOCl homogenized by a vortex and then 1-2 drops inoculated immediately on L-J media using a sterile pipette. The remaining sample was left to stand for 30 minutes at room temperature for liquefaction to take place. After liquefaction, one portion was centrifuged and 1-2 drops of the sediment inoculated in two slopes of L-J medium using a sterile pipette after one and three hrs respectively. The other portion was left to stand at room
temperature in a safety cabinet for 15 hrs after which the supernatant was poured off and 1-2 drops of the sediment inoculated in two slopes of L-J medium using a sterile pipette at 15hrs and 24 hrs respectively. All the cultures were incubated for 8 weeks at 37°C and checked weekly for 8 weeks after, which were declared negative if no growth was obtained.

The remaining two portions were treated with 5% NaOCl, and the same procedure followed as described above as for 3.5% NaOCl (Flow chart on page 33).

Growth was graded according to the method previously described by Githui et al. (1993). Briefly, the numbers of viable colonies from 1-20 were recorded as actual, 21-100 colonies were recorded as moderate growth, more than 100 distinct colonies as innumerable colonies (IC) and more than 100 indistinguishable colonies as confluent growth (CG). The treated samples which showed growth after treatments with NaOCl were subjected to strain identification and Drug Susceptibility Testing (DST) was done.

### 2.8.1 Drug Susceptibility Testing and strain identification

Drug Susceptibility testing analysis was done using the conventional resistance ratio (RR) method (WHO/IUATLD, 1997; Githui et al., 2004). Doubling dilutions of each drug were added to L-J medium, and the final drug concentrations used were in the following ranges: 0.025 – 1.0 μg/ml for Isoniazid (INH), 4 – 64 μg/ml for rifampicin (RMP), 2 – 32 μg/ml for streptomycin (SM), and 1- 8μg/ml for ethambutal (EMB). Control strains of known susceptibility patterns, including a sensitive H37Rv, as well as resistant strains to respective drugs were included in each batch tested. The slopes were incubated at 37°C for 21 days. Growth of less than 20 colonies on the slope containing
the lowest concentration of the drug was taken at the end point. The results were recorded as a RR, defined as the minimum inhibitory concentration (MIC) of the test organism divided by MIC of the H₃₇Rᵥ control strain. A RR of 2 or less indicated susceptibility, while a ratio of 4 and above indicated resistance. Strain identification was done in accordance to procedures previously described by WHO/IUATLD (1997).
Figure 3: Flow chart illustration of specimen processing

10ml sputum specimen (N = 370)
(vortexed)

Direct smear microscopy

4 equal proportions
Treated with equal volumes of NaOCl

ZN FM 3.5% NaOCl
Centrifuged
Sedimented

3.5% NaOCl
Centrifuged

5% NaOCl
Centrifuged
Sedimented

5% NaOCl

Smear negative
NaOCl treated smears
N = 232 ZN
N = 205 FM

Smear positive
NaOCl treated
(Cultured)(n = 156)

½ h 1h 3h 15h 24h

KEY: Smear negative → Smear positive
2.9 Data Analysis

Culture results were used as gold standard in the assessment for sensitivity and specificity for microscopy. The results were also used to determine the biocidal effect and duration at which NaOCl treated specimens can safely be handled outside a safety cabinet. The level of significance was determined using the normal t distribution test at 5% level of significance. Chi- square test was used for comparisons of means of different treatment, morphology, type of cells and the viability. Data were entered and processed using Microsoft Excel and were analyzed by using SPSS (Version 11.5).
CHAPTER THREE

RESULTS

3.1 Specificity and Sensitivity of Ziehl-Neelsen and Fluorescence Microscopy staining methods using direct smears

Three hundred and seventy sputum specimens from tuberculosis suspects were processed for direct microscopy using ZN and FM staining methods with culture as the 'Gold Standard'. Table 1 indicates the number of positive specimens identified by direct smear microscopy and culture. Of these, 158 (42.7%) were smear positive for AFB and 200 (54%) were culture positive. However using ZN 138 (37.3%) smears were positive for AFB while 232 (62.7%) were negative. Similarly using FM 155 (41.9%) smears were positive for AFB while 205 (55.4%) were negative. The higher number of positive specimens using culture was statistically significant ($\chi^2 = 5.991; \text{df} = 2; P = 0.05$). Of the smear positive specimens 129 (34.8%) were positive by both FM and ZN staining methods, 26 (7%) and 3 (0.8%) by FM and ZN methods respectively. A total of 42 (11.4%) culture positive specimens were negative with both FM and ZN stained smears. Of the 170 (46%) specimens, which were negative by culture, 159 (42.7%) were also negative by both ZN and FM staining methods. Of the 11 (3.3%) smear positive, but culture negative, 4 (1.1%) were positive by both FM and ZN methods, while 6 (1.9%) and 1 (0.3%) were positive by FM and ZN methods respectively (Table 1).
Table 1: Number of positive and negative specimens identified by direct Ziehl Neelsen, Fluorescence Microscopy and culture

<table>
<thead>
<tr>
<th>Culture</th>
<th>Smear</th>
<th>Ziehl-Neelsen</th>
<th>Specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fluorescence</td>
<td>Positive</td>
<td>Number</td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>Negative</td>
<td>42</td>
</tr>
<tr>
<td><strong>Sub-Total</strong></td>
<td></td>
<td></td>
<td><strong>200</strong></td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>Negative</td>
<td>159</td>
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<tr>
<td><strong>Sub-Total</strong></td>
<td></td>
<td></td>
<td><strong>170</strong></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>370</strong></td>
</tr>
</tbody>
</table>

3.2 Frequency distribution of acid-fast bacilli identified using Ziehl-Neelsen and Fluorescence Microscopy

Out of the 200 sputa that showed growth on routine culture, 72 (36 %) had confluent growth while 47 (23.5 %), 42 (21 %) and 39 (19.5 %) had innumerable colonies (IC), 20-100 colonies and 1-20 colonies respectively. The number of culture(s) with confluent growth was significantly higher than that with IC, 20-100 colonies and 1-20 colonies ($\chi^2 = 164.838; df = 3; P = 0.0001$).

Table 2 indicates the frequency distribution of acid-fast bacilli identified by ZN and FM staining methods. Of the 138 AFB positive smears on ZN, 7 (5.1 %) were identified as heavy while 54 (39.1 %) and 77 (55.8 %) had moderate and scanty AFB respectively (Table 2). Of the 165 positive smears for AFB identified using FM, 26 (15.7 %) had heavy while 80 (48.5 %) and 59 (35.8 %) had moderate and scanty AFB.
respectively (Table 2). The frequency and distribution of AFB was significantly higher using the FM ($\chi^2 = 198.454; df = 2; P = 0.0001$) compared to ZN (Table 2).

Table 2: Frequency distribution of acid-fast bacilli identified by Ziehl-Neelsen and Fluorescence Microscopy

<table>
<thead>
<tr>
<th>Technique</th>
<th>Grading</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>ZN</td>
<td>5.1 %</td>
<td>39.1 %</td>
</tr>
<tr>
<td>FM</td>
<td>15.8 %</td>
<td>48.5 %</td>
</tr>
</tbody>
</table>

Key: 3+ = Heavy, 2+ = Moderate, 1+ = Scanty

3.3 Comparative detection of positive specimens using Ziehl-Neelsen, Fluorescence Microscopy and culture

A total of 370 sputa were analyzed for the presence of AFB and MTB using direct ZN and FM staining methods and culture. Of these, 232 (62.7%) sputum specimens were negative for AFB using ZN. However 68 (29.6 %) sputum specimens, which were negative for AFB using ZN method were culture positive. Similarly 205 (55.4%) sputum specimens were negative for AFB using FM method. However, 45 (22.5 %) direct FM smear negative were culture positive. There was significant difference in the numbers identified by ZN and FM staining methods ($\chi^2 = 225.693; df = 1; P = 0.05$). There was also significantly higher number of positive samples detected by culture method than by FM and ZN (p<0.05). The order of positive detection from highest to lowest was: Culture >FM >ZN ($\chi^2: 21.079; df: 2; p = 0.0001$) (Table 3)
Table 3: Proportion of positive specimens identified by Ziehl-Neelsen, Fluorescence Microscopy and culture

<table>
<thead>
<tr>
<th>Method</th>
<th>Total (N)</th>
<th>Positive</th>
<th>Negative</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>370</td>
<td>200</td>
<td>170</td>
<td>54.05</td>
</tr>
<tr>
<td>FM</td>
<td>370</td>
<td>165</td>
<td>205</td>
<td>44.59</td>
</tr>
<tr>
<td>ZN</td>
<td>370</td>
<td>138</td>
<td>232</td>
<td>37.30</td>
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</tbody>
</table>

3.4 Sensitivity and specificity of direct Ziehl-Neelsen and Fluorescence Microscopy staining methods

Table 4 indicates sensitivity, specificity, positive and negative predictive values of both FM and ZN staining methods. Culture was used as the gold standard. The sensitivity for FM (77.5%) was significantly higher than that of ZN (66%) (p<0.001) while the specificity of FM and ZN methods were 94.1% and 96.5% respectively (Table 4).

Table 4: Sensitivity, specificity, positive and negative predictive values of both Ziehl-Neelsen and Fluorescence Microscopy staining methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZN</td>
<td>66.0</td>
<td>96.5</td>
<td>95.7</td>
<td>70.7</td>
</tr>
<tr>
<td>FM</td>
<td>77.5</td>
<td>94.1</td>
<td>93.9</td>
<td>78.0</td>
</tr>
</tbody>
</table>

Key: PPV- Predictive value of positive smear, NPV- Predictive value of Negative smears
3.5 Sensitivity and specificity of Ziehl-Neelsen and Fluorescence Microscopy staining methods after treatment with sodium hypochlorite

All smear negative samples by either direct ZN or FM were treated with 3.5% and 5% NaOCl and then subjected to centrifugation and sedimentation. Table 5 indicates sensitivity, specificity, negative and positive predictive values of ZN staining method after treatment with 3.5% and 5% NaOCl. Culture was used as the gold standard. Using 3.5% NaOCl the sensitivity was 42.9% and 27.1% after centrifugation and sedimentation, respectively. With 5% NaOCl the sensitivity was 37.1% and 10% after centrifugation and sedimentation, respectively (Table 5).

Table 5: Sensitivity, specificity, negative and positive predictive values of Ziehl-Neelsen staining method using two concentrations of sodium hypochlorite followed by either centrifugation or sedimentation

<table>
<thead>
<tr>
<th>Method</th>
<th>% NaOCl Concentration</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>NPV (%)</th>
<th>PPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifugation</td>
<td>3.5</td>
<td>42.86</td>
<td>98.13</td>
<td>79.70</td>
<td>90.91</td>
</tr>
<tr>
<td>Sedimentation</td>
<td>3.5</td>
<td>27.14</td>
<td>99.38</td>
<td>75.71</td>
<td>95.00</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>5.0</td>
<td>37.14</td>
<td>100.00</td>
<td>78.43</td>
<td>100.00</td>
</tr>
<tr>
<td>Sedimentation</td>
<td>5.0</td>
<td>10.00</td>
<td>98.75</td>
<td>71.49</td>
<td>77.78</td>
</tr>
</tbody>
</table>

Key: PPV- Predictive value of positive smear, NPV- Predictive value of Negative smear

Table 6 indicates the sensitivity, specificity, negative and positive predictive values of FM staining method after treatment with 3.5% and 5% NaOCl. Culture was used as the gold standard. Using 3.5% NaOCl the sensitivity was 28.9% and 22.2% after centrifugation and sedimentation, respectively. While with 5% NaOCl the sensitivity was 22.4% and 17.8% after centrifugation and sedimentation, respectively.
Table 6: Sensitivity, specificity, negative and positive predictive values of Fluorescence Microscopy staining method using two concentrations of sodium hypochlorite followed by either centrifugation or sedimentation

<table>
<thead>
<tr>
<th>Method</th>
<th>NaOCl Concentration (%)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>NPV (%)</th>
<th>PPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifugation</td>
<td>3.5</td>
<td>28.9</td>
<td>98.7</td>
<td>83.1</td>
<td>86.7</td>
</tr>
<tr>
<td>Sedimentation</td>
<td>3.5</td>
<td>22.2</td>
<td>100.0</td>
<td>82.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>5.0</td>
<td>24.4</td>
<td>100.0</td>
<td>82.4</td>
<td>100.0</td>
</tr>
<tr>
<td>Sedimentation</td>
<td>5.0</td>
<td>17.8</td>
<td>100.0</td>
<td>81.1</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Key: PPV- Predictive value of positive smear, NPV- Predictive value of negative smear

3.6 Effect of sodium hypochlorite on Ziehl-Neelsen smear negative specimens

An equal portion from each of the two hundred and thirty two specimens which were negative by direct ZN microscopy was treated with either 3.5 % or 5% NaOCl and concentrated by centrifugation. Of these, 33 (14.2%) showed presence of AFB while 199 (85.8 %) remained smear negative after treatment with 3.5% NaOCl. However, on treatment with 5% NaOCl, only 26 (11.2%) showed presence of AFB (Fig 4). There was significant difference on the effect of 3.5 % and 5 % NaOCl treatment after centrifugation ($\chi^2 = 999.98; df = 1; P = 0.05$).

Another portion of each of the 232 ZN direct smear negative specimens was treated with 3.5% and 5% NaOCl and concentrated by sedimentation. Of these, 20(8.6%) showed presence of AFB while 212 (91.4 %) remained smear negative after treatment with 3.5% NaOCl. On treatment with 5% NaOCl, only 9 (3.9%) showed presence of AFB (Fig 4). The number of positive specimens identified after treatment with 3.5 % and
5% NaOCl were significantly different after sedimentation. ($\chi^2 = 2107.46; \text{df} = 1; P = 0.05$). There was a significant difference between the number of specimens that were positive after treatment with 3.5% and 5% NaOCl after centrifugation ($\chi^2 = 15.581; \text{df} = 3; P = 0.0014$). The order of positive detection from highest to the lowest was: 3.5% centrifuged > 5% centrifuged > 3.5% sedimented > 5% sedimented. Centrifugation thus had a higher detection power than Sedimentation (Fig 4).

![Bar chart showing positive detection](image)

**N = 232**

**Figure 4: Proportion of positive detection after treatment with 3.5% and 5% sodium hypochlorite followed by either centrifugation or sedimentation using Ziehl-Neelsen**

**3.7 Effect of sodium hypochlorite on Fluorescence Microscopy smear negative specimens**

An equal portion of each of the two hundred and five FM direct smear negative sputum specimens were treated with 3.5% and 5% NaOCl and concentrated with centrifugation. Of these, 15(7.3%) were smear positive after treatment with 3.5% NaOCl. On treatment with 5% NaOCl, 10(4.9%) were smear positive (Fig 5). There was
significant difference in the number of positive specimens following centrifugation between the 3.5 % and 5 % NaOCl treated specimens ($\chi^2 = 2233.364; \text{df} = 1; P = 0.05$).

Another portion of each of the 205 FM direct smear negative specimens was treated with 3.5% and 5% NaOCl and concentrated by sedimentation. Of these, 10 (4.9%) were smear positive after treatment with 3.5% NaOCl, and 9 (4.4%) on treatment with 5% NaOCl (Fig 5). The number of positive specimens identified after treatment with 3.5 % and 5 % NaOCl was significantly different after sedimentation ($\chi^2 = 3580.512; \text{df} = 1; P = 0.05$). There was no significant difference in the number of positive samples detected using 3.5% and 5% NaOCl followed by both centrifugation and sedimentation ($\chi^2 = 2.498; \text{df} = 3; P = 0.4756$) (Fig.5).

\[\text{Figure 5: Proportion of positive detection after treatment with 3.5\% and 5\% sodium hypochlorite followed by either centrifugation or sedimentation using Fluorescence Microscopy}\]

Table 7 indicates the proportion of positive specimens using ZN and versus FM staining methods with the two NaOCl concentrations. The proportion of positive ZN prepared sputum smears following treatment with 3.5% and 5% NaOCl after
prepared sputum smears following treatment with 3.5% and 5% NaOCl after centrifugation were significantly higher than the similarly treated FM smears \((p>0.05)\). The proportion of positive specimens following sedimentation was not significantly different for both FM and ZN \((p<0.05)\).

Table 7: Comparison of Ziehl-Neelsen versus Fluorescent Microscopy staining methods at same sodium hypochlorite concentrations

<table>
<thead>
<tr>
<th>Method</th>
<th>NaOCl Concentration (%)</th>
<th>ZN Positive (%)</th>
<th>FM Positive (%)</th>
<th>(p^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifugation</td>
<td>3.5</td>
<td>14.4</td>
<td>7.4</td>
<td>0.0303</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>5.0</td>
<td>11.3</td>
<td>5.4</td>
<td>0.0424</td>
</tr>
<tr>
<td>Sedimentation</td>
<td>3.5</td>
<td>8.7</td>
<td>2.4</td>
<td>0.1721</td>
</tr>
<tr>
<td>Sedimentation</td>
<td>5.0</td>
<td>3.91</td>
<td>3.9</td>
<td>0.9964</td>
</tr>
</tbody>
</table>

Key: \(p^a\) value for Chi square test for association

3.8: Overall sensitivity for both direct and treated smears by Ziehl-Neelsen and Fluorescence Microscopy

Table 8 indicates overall sensitivity, specificity, positive and negative predictive values of both FM and ZN staining methods. Use of NaOCl increased the sensitivity of ZN. The sensitivity of the NaOCl treated smears followed by centrifugation combined with direct smears was 80%, with a specificity of 96% (95-97%), a PPV of 96% (95-97%), and a NPV of 80.5% (80-81%). Similarly, the sensitivity of the NaOCl –treated smears followed by sedimentation was 73% (70-76%), with a specificity of 96%, a PPV of 95.5% (95-96%), and a NPV of 75% (73-77%). Overall, there was a significant
difference in the sensitivity between NaOCl treated smears and direct smears (p>0.05), but there was no significance difference in the specificity.

Table 8: Overall sensitivity, specificity, positive and negative predictive values of 370 specimens using Ziehl-Neelsen after treatment of negative specimens with sodium hypochlorite

<table>
<thead>
<tr>
<th>Total Samples</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.5% NaOCl + Centrifuged</td>
<td>81.1</td>
<td>95</td>
<td>95</td>
<td>81</td>
</tr>
<tr>
<td>3.5% NaOCl + Sedimentation</td>
<td>76</td>
<td>96</td>
<td>96</td>
<td>77</td>
</tr>
<tr>
<td>5% NaOCl + Centrifuged</td>
<td>79</td>
<td>97</td>
<td>97</td>
<td>80</td>
</tr>
<tr>
<td>5% NaOCl + Sedimentation</td>
<td>70</td>
<td>96</td>
<td>95</td>
<td>73</td>
</tr>
<tr>
<td>Direct Microcopy ZN</td>
<td>66</td>
<td>97.1</td>
<td>96.4</td>
<td>78</td>
</tr>
</tbody>
</table>

Key: PPV- Predictive value of positive smear, NPV- Predictive value of Negative smear

Table 9 indicates overall sensitivity, specificity, positive and negative predictive values of FM staining methods. Use of NaOCl increased the sensitivity of FM. The sensitivity of NaOCl-treated smears followed by centrifugation combined with direct smears was 83%, with specificity of 94%, a PPV of 94%, and a NPV of 82%. The sensitivity of the NaOCl treated smears followed by sedimentation was 83%, with specificity of 94%, a PPV of 94%, and a NPV of 82%. There was no significant difference in the sensitivity and specificity between NaOCl treated smears and directs smears.
Table 9: Overall sensitivity, specificity, positive and negative predictive values of 370 specimens using Fluorescent Microscopy after treatment of negative specimens with sodium hypochlorite

<table>
<thead>
<tr>
<th>Total Samples</th>
<th>Method</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>370</td>
<td>3.5% NaOCl + Centrifuge</td>
<td>83</td>
<td>93</td>
<td>93</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>3.5% NaOCl + Sedimentation</td>
<td>83</td>
<td>94</td>
<td>94</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>5% NaOCl + Centrifuged</td>
<td>83</td>
<td>94</td>
<td>96</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>5% NaOCl + Sedimentation</td>
<td>82</td>
<td>94</td>
<td>94</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Direct Microscopy FM</td>
<td>77.5</td>
<td>94.1</td>
<td>96.4</td>
<td>70.8</td>
</tr>
</tbody>
</table>

Key: PPV- Predictive value of positive smear, NPV- Predictive value of Negative smears

3.9 Liquefying effect of sodium hypochlorite on sputum specimens using Ziehl-Neelsen smear microscopy

To determine the liquefying effect of NaOCl a total of 35 sputum specimens were examined and the prevalence of epithelial, pus cells, macrophages and debris compared with smears prepared after treatment of sputum specimens with different concentrations of NaOCl.

(a)
Plate 1: The different cell types in smears (a) A slide with smear for direct microscopy (b) A smear prepared from NaOCl treated specimen

Table 10 indicates the frequencies of macrophages, epithelial, pus cells and debris on the sputum specimens before and after treatment with 3.5% and 5% NaOCl. Of the untreated sputum specimens, 10 (28.6%) had no macrophages while 1 (2.9%), 1 (2.9%), and 23 (65.7%) had scanty, moderate and few respectively. Fifteen (42.9%) smears had no epithelial cells while 4 (11.4%) and 16 (54.7%) had scanty and few respectively. Of the 3.5% NaOCl treated sputum specimens, 23 (65.7%) had no macrophages while 2 (5.7%) and 10 (28.6%) had scanty and few respectively. The number of macrophages was significantly reduced after treatment with 3.5 % NaOCl (t = 3.633; df = 34; P = 0.01). Similarly, 31 (88.6%) smears had no epithelial cells while 2 (5.7%) and 2 (5.7%) had scanty and few epithelial cells respectively. There was a significant reduction in the number of epithelial cells (t = 4.148; df = 34; P = 0.0001). Of the 5% NaOCl treated sputum specimens, 25 (71.4%) had no macrophages while 10 (28.6%) had few
macrophages. The number of macrophages was significantly reduced after treatment with 5% NaOCl (t = 3.927; df = 34; P = 0.0001). Similarly, 32 (91.4%) smears had no epithelial while 3 (8.6%) had few and the number of epithelial cells was significantly different (t = 4.442; df = 34; P = 0.0001).

Table 10: Frequencies of macrophages and epithelial on untreated and sodium hypochlorite-treated sputum specimens

<table>
<thead>
<tr>
<th>Grading</th>
<th>Macrophages (%)</th>
<th></th>
<th></th>
<th>Epithelial cells (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated 3.5% NaOCl</td>
<td>5% NaOCl</td>
<td>Untreated 3.5% NaOCl</td>
<td>5% NaOCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>28.6</td>
<td>65.7</td>
<td>71.4</td>
<td>42.9</td>
<td>88.6</td>
<td>91.4</td>
</tr>
<tr>
<td>1+</td>
<td>68.6</td>
<td>34.3</td>
<td>28.6</td>
<td>47.1</td>
<td>11.4</td>
<td>8.6</td>
</tr>
<tr>
<td>2+</td>
<td>2.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

N = 35
Key: 1+ = scanty, 2+ = Moderate, 3+ = heavy, Negative = no cells

Similarly, 13 (37.1%) untreated smears had scanty pus cells while 4 (11.4%), 2 (5.7%) and 16 (45.7%) had moderate, heavy and few respectively. All the 35 (100%) untreated smears showed numerous debris. Of the 3.5% NaOCl treated sputum specimens, 18 (51.4%) treated smears had no pus cells while 1 (2.9%), 2 (5.7%) and 14 (40%) had scanty, moderate and few respectively and the frequency distribution of pus cells were significantly reduced (t = 2.253; df = 34; P = 0.031). However, 34 (79.1%) treated smears had reduced debris while only 1 (2.9%) smear had many number of debris. Of the 5% NaOCl treated sputum specimens, 15 (42.9%) treated smears had no pus cells and 2 (5.7%), 1 (2.9%) and 17 (48.6%) had scanty, and heavy respectively.
The frequency distribution of pus cells was not significantly different ($t = 1.342; \ df = 34; \ P = 0.188$). However 34 (79.1 %) treated smears had reduced debris while only 1 (2.9 %) smear had many number of debris.

Table 11: Frequencies of pus cells and debris on untreated and sodium hypochlorite-treated sputum specimens

<table>
<thead>
<tr>
<th>Grading</th>
<th>Pus cells (%)</th>
<th>Debris (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated 3.5%NaOCl 5% NaOCl</td>
<td>Untreated 3.5%NaOCl 5% NaOCl</td>
</tr>
<tr>
<td>Negative</td>
<td>0 0 42.9</td>
<td>0 0 0</td>
</tr>
<tr>
<td>1+</td>
<td>82.8 91.4 54.3</td>
<td>0 97.1 97.1</td>
</tr>
<tr>
<td>2+</td>
<td>11.4 2.9 2.9</td>
<td>0 0 0</td>
</tr>
<tr>
<td>3+</td>
<td>5.7 5.7 0</td>
<td>100 2.9 2.9</td>
</tr>
</tbody>
</table>

$N = 35$

Key: 1+ = scanty, 2+ = Moderate, 3+ = heavy, Negative = no cells

3.10 Effects of different concentrations of sodium hypochlorite on the viability of *Mycobacterium tuberculosis*

Table 12 indicates the number of viable colonies of one hundred and fifty six smear positive sputum specimens treated with different concentrations of NaOCl at different times of exposure and subsequently cultured on LJ media. Only 18 showed growth at different time intervals; specimens treated with 3.5% NaOCl did not show any growth after 1 to 3 hrs of exposure but there was growth at 15 and 24 hrs respectively. Those treated with 5% NaOCl showed no growth of MTB after 15 to 24 hrs of exposure. The number of specimens with viable MTB decreased significantly over 0-24 hrs period after treatment with either 3.5% or 5% NaOCl ($p<0.05$)
Table 12: Effect of different concentrations of sodium hypochlorite on the viability of *Mycobacterium tuberculosis*

<table>
<thead>
<tr>
<th>SL No</th>
<th>3.5% NaOCl</th>
<th>5% NaOCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>½ hr 1 hr 3 hrs 15 hrs 24 hrs</td>
<td>½ hr 1 hr 3 hrs 15 hrs 24 hrs</td>
</tr>
<tr>
<td>1</td>
<td>0 0 0 0 0 0</td>
<td>7 col 1 col 0 0 0</td>
</tr>
<tr>
<td>2</td>
<td>0 0 0 0 0 0</td>
<td>IC 20-100 col 20-100 col 0 0</td>
</tr>
<tr>
<td>3</td>
<td>0 0 0 4 col 0</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>4</td>
<td>0 0 0 0 0 0</td>
<td>10 col 4 col 0 0 0</td>
</tr>
<tr>
<td>5</td>
<td>0 0 0 0 0 0</td>
<td>20-100 col 4 col 0 0 0</td>
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<tr>
<td>6</td>
<td>0 0 0 0 0 0</td>
<td>20-100 col 0 0 0 0</td>
</tr>
<tr>
<td>7</td>
<td>0 0 0 0 0 0</td>
<td>20-100 col 0 0 0 0</td>
</tr>
<tr>
<td>8</td>
<td>0 0 0 0 0 0</td>
<td>IC 10 col 0 0 0</td>
</tr>
<tr>
<td>9</td>
<td>0 0 0 0 0 0</td>
<td>IC 0 0 0 0</td>
</tr>
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<td>0 0 0 2 col 0</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>11</td>
<td>0 0 0 IC 20-100 col</td>
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<td>0 0 0 20-100 col 4 col</td>
<td>0 0 0 0 0</td>
</tr>
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<td>13</td>
<td>1 col 0 0 0 0</td>
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</tr>
<tr>
<td>14</td>
<td>20-100 col 0 0 0 0</td>
<td>IC 20-100 col 2 col 0 0</td>
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<tr>
<td>15</td>
<td>0 0 0 0 0</td>
<td>CG IC IC 0 0</td>
</tr>
<tr>
<td>16</td>
<td>0 0 0 0 0 0</td>
<td>5 col 1 col 0 0 0</td>
</tr>
<tr>
<td>17</td>
<td>0 0 0 20-100 col 0</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>18</td>
<td>0 0 0 20-100 col 5 col</td>
<td>0 0 0 0 0</td>
</tr>
</tbody>
</table>

Key: CG = Confluent Growth, IC = Innumerable colonies

Table 13 indicates smear, culture and drug susceptibility results of specimens, which showed growth after treatment with NaOCl. Of the 18 specimens, which showed growth after treatment with NaOCl, all had confluent growth on culture, while the smears had scanty, moderate or heavy AFB. However, 2 (1.3%) specimens showed resistance to anti-TB drugs: One showed resistance to Isoniazid and Streptomycin and the other to Isoniazid only. Drug Susceptibility testing analysis was done using the conventional resistance ratio (RR) method (WHO/IUATLD, 1997; Githui et al., 2004).
Table 13: Routine smear, culture and drug susceptibility results of specimens, which showed growth after treatment with sodium hypochlorite

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Culture</th>
<th>Smear</th>
<th>Susceptibility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FM</td>
<td>ZN</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>CG</td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>2</td>
<td>CG</td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>3</td>
<td>CG</td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td>4</td>
<td>CG</td>
<td>3+</td>
<td>2+</td>
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<tr>
<td>5</td>
<td>CG</td>
<td>1+</td>
<td>1+</td>
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<tr>
<td>6</td>
<td>CG</td>
<td>2+</td>
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<td>CG</td>
<td>2+</td>
<td>3+</td>
</tr>
<tr>
<td>9</td>
<td>CG</td>
<td>2+</td>
<td>3+</td>
</tr>
<tr>
<td>10</td>
<td>CG</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>11</td>
<td>CG</td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td>12</td>
<td>CG</td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td>13</td>
<td>CG</td>
<td>1+</td>
<td>2+</td>
</tr>
<tr>
<td>14</td>
<td>CG</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>15</td>
<td>CG</td>
<td>1+</td>
<td>2+</td>
</tr>
<tr>
<td>16</td>
<td>CG</td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td>17</td>
<td>CG</td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>18</td>
<td>CG</td>
<td>3+</td>
<td>2+</td>
</tr>
</tbody>
</table>

Key: CG = Confluent Growth, 3+ = Heavy, 2+ = Moderate, 1+ = Scanty, H = Isoniazid, S = Streptomycin, R = rifampicin, E = Ethambutanol

Figure 6 shows the proportion of the 156 initially smear positive samples which showed growth after treatment with 3.5% NaOCl. Of these, 2 showed growth at 0 hrs but there was no growth at 1 hr and 3 hrs while there was growth after 15 hrs and 24 hrs in 6 and 3 cultures respectively. Treatments of the samples with 3.5% NaOCl significantly reduced the growth of MTB after 24 hrs ($\chi^2 = 156.124; df = 3; p = 0.05$). There was generally a logarithmic decrease of growth with increase in exposure time (Fig 6).
Figure 7: Proportion of the smear positive specimens which showed growth after Exposure to 5.0% NaOCl

Table 14 indicates the results of 11 sputum specimens that showed growth after treatment with 3.5% NaOCl. Of these, 5 had less than 20 colonies, while 5 yielded 20-100 colonies and 1 had innumerable colonies. At ½ hrs only two specimens showed growth, one yielded only 1 colony and the other one yielded 20-100 colonies. All specimens that showed growth after treatment with 3.5% NaOCl had confluent growth in untreated cultures. The sample, which had 20-100 colonies, was also resistant to H but sensitive to S, R and E. At 15 hrs and 24 hrs, growth was evident with 4 specimens showing countable colonies, 4 yielded innumerable colonies, and 1 had 20-100 colonies. The growth decreased with time, with 6 specimens showing growth at 15 hrs and only 3 (50%) at 24 hrs.
Figure 6: Proportion of smear positive samples which showed growth after Exposure to 3.5% NaOCl

Figure 7 shows the proportion of the initially smear positive samples which showed growth after treatment with 5% NaOCl. Of these, 13 specimens showed growth at 0 hrs, 10 at 1 hr and 5 at 3hrs respectively but there was no growth at 15 hrs and 24 hrs respectively. Treatment of the specimens with 5% NaOCl significantly reduced the growth of the MTB over 24 hrs ($\chi^2 = 148.594; \text{df} = 9; p = 0.011$). There was generally a logarithmic decrease of growth with increase in exposure time. There was no significant difference on the biocidal effect of the two concentrations between 0 and 24 hr period ($\chi^2 = 13.805; \text{df} = 9; p = 0.061$) (Fig 7).
Table 14: Sputum specimens showing growth after treatment with 3.5% sodium hypochlorite and exposed at various time intervals and either Centrifuged or Sedimented

<table>
<thead>
<tr>
<th>Scores</th>
<th>Time</th>
<th>Centrifuged</th>
<th>Sedimented</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hrs</td>
<td>1 hr</td>
<td>3 hrs</td>
<td>15 hrs</td>
</tr>
<tr>
<td>CG</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IC</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>20-100 colonies</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>1-20 Colonies</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

Key: CG = Confluent Growth, IC = Innumerable colonies

Table 15 indicates the results of 28 sputum specimens that showed growth after treatment with 5% NaOCl. Of these, 10 yielded less than 20 colonies, 8 had 20-100 colonies, 9 had innumerable colonies and 1 had confluent growth. It was evident that growth decreased with time. The specimens that showed growth at 1/4 hrs were 13 and in the same specimens 10 showed growth after 1 hr and 5 after 3 hrs respectively. However there was no growth after 15 hrs of exposure.
Table 15: Sputum specimens showing growth after treatment with 5 % sodium hypochlorite and exposed at various time intervals and either centrifuged or sedimented

<table>
<thead>
<tr>
<th>Scores</th>
<th>Time</th>
<th>Centrifuged</th>
<th>Sedimented</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hrs</td>
<td>1 hr</td>
<td>3 hrs</td>
<td>15 hrs</td>
</tr>
<tr>
<td>CG</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IC</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>20-100 colonies</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1-20 Colonies</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>10</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

Key: CG = Confluent Growth, IC = Innumerable colonies
A core element of any effective TB control programme is to diagnose cases with active disease promptly and initiate effective therapy. The main aim is to reduce individual morbidity and mortality that can result from delays in diagnosis and also break the cycle of disease transmission. It is therefore important to minimize both TB diagnosis and treatment delays to improve the impact of any TB control programme. Direct smear microscopy for AFB, is still the cornerstone of TB diagnosis especially in resource-poor settings. It is fairly rapid, inexpensive, and highly specific method for detection of AFB in sputum specimens. The major disadvantage of this technique is its low sensitivity especially when used in overburdened control programs. The low sensitivity has often been reported to be due to high rates of HIV-related PTB cases (Garay, 2000), among other factors. Therefore, more sensitive techniques need to be developed if the patients with active TB are to be accurately and appropriately diagnosed in resource – poor settings. One such technique that potentially provides the opportunity to improve TB diagnosis is the use of NaOCl that has been seen to improve the sensitivity of smear microscopy (Gebre et al., 1995; Habeenzu et al., 1999). However, the safety to the user of NaOCl has not yet been determined.

This study was the first to determine biocidal effect of NaOCl, and to compare the effect of different concentrations of NaOCl on MTB. This study also determined the
liquefying effect of NaOCl on ZN staining, and to compare the sensitivity and specificity for ZN and FM staining methods.

The sensitivity obtained in this study (ZN 66% and FM 77%) is consistent with results from other studies. For instance, two studies in Kenya, one which showed a sensitivity of 65% and 80% using ZN and FM respectively (Githui et al., 1993) and the other a sensitivity of 60% and 78% using ZN and FM respectively (Kivihya et al., 2003). However, a study in Senegal showed less pronounced differences between ZN and FM, which were attributed to specimens with scanty numbers of AFB, but results obtained in follow-up patients in the same study were similar with those from this study. The observation in this study that the majority of specimens identified as positive by FM only, were also positive by culture supports earlier findings by Holst et al. (1959) that FM does not yield more false positives than ZN. However, FM showed more smear positives, which were negative by culture than did the ZN method. Since culture is the gold standard these results could be interpreted as false positives (Collins et al., 1981). Nevertheless, an additional possibility of these findings is that the observed bacilli were from patients that could have been already on treatment (Githui et al., 1993). The apparent advantage of FM is however compromised by the fact that skill is essential to distinguish between genuine AFB from fluorescent artifacts, the failure of which could lead to high false positivity rate. It is, therefore, advisable to have all doubtful smears counter checked by a more experienced senior technician and/or restain by the ZN method (Githui et al., 1993). This is an additional advantage of using FM method, as the same technique of restaining cannot be applicable to the ZN stained smears.
Previous studies aimed at increasing sensitivity of smear microscopy have used NaOCl to liquefy sputum specimen and then concentrate the bacilli by either centrifugation or sedimentation. Most studies used 5% NaOCl to liquefy the sputum, while in others the concentration was not indicated, followed by either centrifugation or sedimentation. In a study in New Delhi, 1% NaOCl was used and the bacilli concentrated by floatation on a layer of xylene before staining (Rattan et al., 1994). In most of these studies the household NaOCl indicated as 5% NaOCl, was used (Miorner et al., 1996). However, in Kenya NaOCl is presented as jik brand, which is usually 3.5%, while other available bleaches do not indicate the actual concentration.

In this study therefore, household bleach Jik™ (3.5%) and commercial bleach (5%) NaOCl were used to liquefy sputum specimens followed by either centrifugation or overnight sedimentation to concentrate the bacilli. The 3.5% NaOCl was used to concentrate sputum for AFB since it is the most common presentation of NaOCl for household use and the NaOCl concentration specified. The 5% concentration was selected for comparison, since it has been used in a number of studies. There was a significant increase in the sensitivity of ZN smear microscopy using both concentrations of NaOCl unlike previous studies, which have indicated contradicting results. For instance, a study in South Africa using 4-5% NaOCl showed no increase at all (Wilkinson and Sturm, 1997). Another study by Miorner et al. (1996) using 5% NaOCl showed sensitivity close to 70% while Gebre et al. (1995) using 5% NaOCl reported that NaOCl increased the number of samples positive with AFB by more than 100%. A study in Ethiopia by Bruchfeld et al. (2000) using 5% NaOCl however showed that sensitivity increased from 54.2% to 63.1% and 38.5% to 50% in HIV-positive patients. In these
studies, however, the bacilli were concentrated by centrifugation only. The limitation with this approach is that it requires access to a centrifuge, which is lacking in many peripheral laboratories in resource-poor countries. Studies have shown that simple tabletop centrifuges that are available in many laboratories for serum and urine analysis can obtain enough centrifugal force (Gebre et al., 1995). This may, however, not be adequate for sedimentation of mycobacteria since they require high centrifugal force of 3000 RCF since lower centrifugal force leaves mycobacteria suspended in the supernatant (WHO/IUA/TLID, 1997).

Results showed that 3.5% NaOCl treated specimens had a higher sensitivity than the 5% NaOCl treated specimens using ZN method. The higher sensitivity observed with 3.5% over 5% NaOCl treated sputum specimens in ZN method might be due to the fact that with the 5% NaOCl treated smears being washed off during staining due to lack of stickiness on the slides. These findings are consistent with those of a study by Collins et al. (1985). It has been suggested that this problem can be prevented by use of ammonium sulphate to enhance adhesion of the smears on the slide. Ammonium Sulphate acts as a homogenizer and fixative, making the deposit of smears thick and adherent to the slides (Garay, 2000). This approach was not used in this study.

There was significant increase in sensitivity by ZN using both centrifugation and sedimentation. However, there were a higher number of positive specimens following centrifugation than sedimentation. This is because centrifugation makes better sediment than sedimentation that is not affected during decanting of supernatant. These findings are consistent with a study by Miorner et al (1996) that observed a higher number of positive specimens after centrifugation than sedimentation. However, concentration of
sputum by centrifugation did not significantly increase the sensitivity of the fluorochrome stain similar to observations by Gail et al (1995). Lempert (1944) described two reasons for superiority of FM over ZN method with respect to weakly positive smear specimens: (i) an increased area of smear per field and, (ii) an increased contrast between the stained bacilli and the background.

It is thought that NaOCl increases the sensitivity by digesting the sputum which when followed by concentration of bacilli by either centrifugation or sedimentation greatly increases the number of bacilli per microscopic field (Ratnam and March, 1986). Improved recovery of bacilli after treatment by NaOCl might also be attributed to changes in surface properties of the mycobacteria (i.e., charge and hydrophobicity), and for denaturing of sputum constituents leading to flocculation and subsequent increased sedimentation rate of mycobacteria (Gebre et al., 1995). The duration of exposure of sputum specimen to NaOCl is therefore important when using NaOCl method for optimal liquefaction to occur. A study by Yassin et al. (2003) showed that the optimal liquefaction was achieved within 30-45 minutes of exposure to 5% NaOCl. However, other investigators exposed the sputum specimens to 5% NaOCl for 10-15 minutes (Aung et al., 2001; Gebre-Selassie, 2003) which could be partly the cause of the varied results.

The presence of specific cells in sputum is diagnostic of a good specimen for TB smear microscopy. The presence of alveolar macrophages, which are usually absent in the secretion of upper respiratory tract (Saluja et al., 2003), is especially important for appropriate specimen for diagnosis of TB. Sputum contains different cell types that include epitheloid cells, pus cells and giant cells. In this study the liquefying effect of NaOCl on different cell types using ZN was determined. Results showed that
liquefaction does not affect the morphology of bacilli but affected other cells. This finding contradicts findings by Yassin et al (2003) who indicated that prolonged exposure to NaOCl (≥ 60min) gradually reduced the possibility of detecting the bacilli. In this study the sputum was exposed to NaOCl for over 18hrs and the bacilli were still visibly clear. There was a significant decrease in the number of macrophages and epithelial cells after treatment with 3.5% and 5% NaOCl. The amount of debris was also reduced significantly leaving a clear microscopic field and thus it was easy to identify the bacilli (Refer to plate (b)). The reduction on the amount of debris significantly leaves a clear microscopic field which makes slides reading simpler and hopefully less time consuming than the 5 min recommended for reading the direct smear (WHO, 1998), and possibly increases the concentration of bacilli thereby increasing the sensitivity.

Although NaOCl is probably one of the most commonly used mycobacterial disinfectant, the data on the concentrations and exposure times is insufficient (Angeby et al., 2004). According to Kent and Kubica. (1985), 0.1-0.5% is enough to kill MTB, but the time needed was not stated while Best et al. (1990) also showed that 1% concentration of NaOCl was enough to kill MTB, but the time required was not stated. Yassin et al. (2003) in a study on the efficacy and safety of NaOCl in Ethiopia concluded that NaOCl kills mycobacteria but the concentration and the time of exposure was not indicated.

In this study, 3.5% and 5% NaOCl showed gradual biocidal activity on MTB since all the specimens that under routine cultures showed confluent growth, and had high number of bacilli in direct smears yielded between 20-100 colonies to less than 20 colonies after exposure to NaOCl. Of the specimens that indicated growth with NaOCl
treatment, 1.3% showed antimicrobial resistance. This may suggest that there could be a relationship between NaOCl resistance and antimicrobial resistance. However, this observation requires further investigations, as the sample size in this study was small.

The observation of growth of MTB at 15 and 24 hrs would mean that the 3.5% NaOCl does not kill but rather immobilizes the MTB, which remain viable. Consequently, it may be safe to use 3.5 % NaOCl between 1hr and 3 hrs of exposure when there is total immobilization of MTB followed by centrifugation since sedimentation requires 15-18hrs. On the other hand, the 5% NaOCl was an effective sterilizing agent since no growth was observed after 15 hours of exposure suggesting that this concentration effectively kill the MTB.

The use of NaOCl-method is advantageous in that: (i) The treated samples lose resemblance to sputum samples, thus they are less aesthetically offensive to the laboratory technicians (ii) It is safe since the sputum is rendered sterile after 15hrs of exposure using 5% concentration. As a result, NaOCl treated samples can easily be disposed together with other laboratory wastes. This increases the biosafety in laboratories and would be more important in settings with high incidence of HIV, where a high proportion of laboratory staff could be HIV-infected and thus more susceptible to TB. (iii) NaOCl is inexpensive, stable at room temperature and locally available. (iv) It increases the sensitivity of smear microscopy significantly. However, the NaOCl-treated specimens cannot be used for mycobacterial culture, as the NaOCl kills MTB and if culture is required, a different sample must be obtained.

Although this study did not address the influence of HIV infection on smear positivity, FM may be more appropriate in the diagnosis of HIV – associated TB where
sputum smears are repeatedly reported negative due to low bacillary content. The FM may also be used in detecting a significant number of early PTB cases who may be excreting small number of bacilli with negative smears by ZN method but who will later cavitate and become infectious.

4.2 CONCLUSIONS

- Findings from this study indicated that NaOCl has a biocidal effect on MTB and maybe useful for diagnosis of TB in absence of a safety cabinet.
- The 3.5% NaOCl showed immobilizing effect to the MTB between 1-3 hrs of exposure whereas 5% showed killing effect after 15 hrs of exposure.
- There was a significant reduction of debris and other cells giving a clear microscopic field, and concentration of bacilli.
- The results also confirm previous findings that liquefaction of sputum with NaOCl followed by concentration of bacilli through centrifugation or overnight sedimentation significantly increases the sensitivity of direct microscopy.
- Centrifugation increased the yield of AFB more than sedimentation which was attributed to both the use of a centrifuge with high centrifugal force for centrifugation and the interference of sediment that often occurs during the removal of supernatant after overnight sedimentation.
- The use of NaOCl technology is appropriate for TB control programs, since NaOCl is readily available at low costs as household bleach when used at appropriate concentrations and time.
4.3 RECOMMENDATIONS

- Using appropriate concentrations of NaOCl and appropriate time of exposure may be adequate safety measure in smear microscopy for AFB in instances where a safety cabinet is not available.
  - 3.5% NaOCl should be used to concentrate the bacilli between 1hr and 3hr of exposure, followed by centrifugation but may not be safe after overnight sedimentation.
  - 5% NaOCl should be used to concentrate the bacilli after sedimentation for 15 hrs. However, this concentration has a disadvantage of smears being washed off during the staining process. This can be prevented by use of ammonium sulphate to enhance adhesion of smears on the slide (Garay, 2000).

- Centrifugation could be used in settings with centrifuges since it is more efficient for concentration of bacilli than sedimentation.

- It would be of great importance to establish the activity of NaOCl against multi-drug resistant tuberculosis (MDR-TB) since it is increasingly becoming a significant clinical problem.

- Use of NaOCl method should be adopted in routine laboratory work particularly national TB control programs in developing countries after further studies have been done on its application in such settings.

- A standard protocol should be established to enable uniformity in different settings.
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ICT Buyers Guide.


APPENDICES

APPENDIX 1: Protocol for various diagnostic techniques

1. Ziehl-Neelsen stain

Reagents

Carbol Fuchsin

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic fuchsin powder</td>
<td>1 g.</td>
</tr>
<tr>
<td>Phenol crystals</td>
<td>4.5 g.</td>
</tr>
<tr>
<td>Ethyl alcohol, absolute</td>
<td>10 ml.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml.</td>
</tr>
</tbody>
</table>

Weigh the basic fuchsin and phenol crystals into an appropriate calibrated flask/bottle, dissolve by heating over boiling water bath. Shake contents occasionally until solution is dissolved. Cool and add alcohol, mix thoroughly, add distilled water to required volume. Stand overnight at room temperature. Filter using whatman filter paper.

Acid Alcohol

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrochloric acid (concentrated)</td>
<td>3 ml.</td>
</tr>
<tr>
<td>Ethyl alcohol, absolute</td>
<td>97 ml.</td>
</tr>
</tbody>
</table>

Slowly add acid to alcohol with occasional mixing.

Malachite Green

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malachite Green</td>
<td>0.5 g.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml.</td>
</tr>
</tbody>
</table>

Dissolve the malachite green in distilled water by gentle heating.

Method

- In the safety cabinet make a smear on a properly labelled slide.
- Fix smear by heat - either using heating plate or passing over bunsen burner flame.
On a staining rack over a water sink, flood slide with carbol fuchsin, heat and stain for 10 minutes

- Wash in tap water until all free stain is washed away
- Decolourise with acid alcohol for a maximum of 3 minutes
- Wash in tap water
- Counter-stain with methylene blue or malachite green for 60 seconds.
- Wash in tap water
- Allow drying on a slide rack – Do not blot.

For every new batch of stains, include known positive and negative slides, for quality control purposes.

- The following semi quantitative reporting method is used for the Ziehl Neelsen (ZN) technique.

If no AFB are found in 100 fields, report:

No acid-fast bacilli observed.

If 1-9 are found in 100 fields:

Record the exact figure.

If 10-99 AFB are found in 100 fields:

Report as 1+

If 1-10 are found per field,

Report as 2+

If greater than 10 AFB per field,

Report as 3+

- Include the following information in the report:
  - Evaluation of the specimen
  - Staining method used
  - Smear results
  - Date of examination
  - Signature of microscopist
2. Auramine – Phenol stain

Reagents

Auramine Phenol

Auramine O  
Phenol crystals  
Distilled water  

0.3 g.  
3g.  
to 100 ml.  
6 g.  
60g.  
to 2000 ml.  

Dissolve phenol crystals to distilled water by gentle heating, add auramine O gradually and shake vigorously. Stand overnight in a 37 degrees celcius incubator, filter and store in a dark bottle at room temperature.

Acid Alcohol

Sodium Chloride  
Distilled water  
Hydrochloric acid (conc.)  
Ethyl alcohol (absolute) or Methylated spirit  

0.5 g.  
20 ml.  
0.75 ml.  
to 100 ml.  
10 g.  
200 ml.  
15 ml.  
to 2000 ml.  

Dissolve the sodium chloride in distilled water. Gradually add the acid and make up to the required volume with ethyl alcohol (absolute) or Methylated spirit.

Potassium Permanganate

Potassium permanganate  
Distilled water  

0.1 g.  
100 ml.  
1 g.  
1,000 ml.  

Dissolve the potassium permanganate in the distilled water.

Method

- In the safety cabinet, make smear on a properly labeled slide.
- Fix smear by heat - either using heating plate or passing over bunsen burner flame
- Stain with auramine/phenol for 10 minutes
- Wash with tap water
- Decolourise with acid alcohol for 5 minutes
- Wash with tap water
- Counter stain with potassium permanganate for 30 seconds
- Wash with tap water
Reporting smear results

It is vital that results of all sputum examinations performed be entered correctly into the tuberculosis laboratory register as follows:

- Enter positive results in red ink for quick reference.

If acid-fast bacilli are seen, report the observation as **"Smear Positive for AFB"**.

The WHO and IUATLD recommended method for reporting results is followed as follows:

**Negative Report:** “Negative for acid-fast bacilli” where no organisms have been observed in 100 fields.

**Positive Report:** “Positive for acid-fast bacilli”

- Include the following information in the report:
  - Evaluation of the specimen
  - Staining method used
  - Smear results
  - Date of examination
  - Signature of microscopist

3. Culture procedures

- Egg-based media, the Lowenstein-Jensen (L-J), is the most commonly used in our laboratory.

- Procedures which involve digestion and decontamination of specimens before culture process include modified Petroff’s NaOH technique and N-acetyl-L-cysteine (NALC)/ sodium hydroxide (NaOH) technique.

Accordingly, the use of modified Petroff’s NaOH decontamination technique and egg-based L-J medium for culture is described.

**Lowenstein-Jensen egg medium**

**Complete Medium base**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume 1</th>
<th>Volume 2</th>
<th>Volume 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eggs (less than a week old)</td>
<td>1,000 ml</td>
<td>1,500 ml</td>
<td>1,625 ml</td>
</tr>
<tr>
<td>Mineral salt solution (sterile)</td>
<td>600 ml</td>
<td>900 ml</td>
<td>1,000 ml</td>
</tr>
</tbody>
</table>
Malachite green solution (sterile) 20 ml. 30 ml. 33 ml.

**Method of Preparation**

- Gently scrub the eggs with a soft brush in soap water.
- Wipe the clean and dry eggs with cotton wool soaked in methylated spirit.
- Aseptically break the eggs into a sterile 500 ml measuring jug. Transfer to a sterile 5-litre flask containing sterile glass beads (5 mm diameter), until a required volume is achieved.
- Shake to break up the eggs to a homogeneous solution.
- Aseptically add appropriate amount of mineral salt solution and malachite green solution.
- Shake well to mix.
- Add penicillin (100,000 units/ml.) to the media.
  i.e. 2.4 ml for 1,500 ml of eggs and 1.6 ml for 1,000 ml of eggs.
- Filter mixture using a sterile stainless steel funnel and gauze.
- Aliquot as required in appropriate sterile glass containers.
- Inoculate at 85°C (80% humidity) for maximum of 60 minutes.

**Penicillin Solution 100,000 units/ml.**

To a lyophilised vial of 1,000,000 units of penicillin add aseptically 2 ml. of sterile distilled water shake to dissolve. Transfer to a 10 ml. sterile cylinder and make up to 10 ml. Store in a sterile universal container for up to 1 month at 4°C. For use add 0.1ml to 100 ml of L-J media.

**Mineral Salt Solution (with Glycerol)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium dihydrogen Phosphate</td>
<td>4.0 g.</td>
</tr>
<tr>
<td>Magnesium Sulphate</td>
<td>0.4 g.</td>
</tr>
<tr>
<td>Magnesium tricitrate</td>
<td>1.0 g.</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>6.0 g.</td>
</tr>
<tr>
<td>Glycerol</td>
<td>20.0 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>to 1,000.0 ml.</td>
</tr>
<tr>
<td></td>
<td>6,000.0 ml.</td>
</tr>
</tbody>
</table>

**Method of Preparation**

Usually a volume of 6,000 ml is made up at a time.
• Add all the reagents and glycerol to 2,000 ml distilled water, dissolve by heating in a boiling water bath.
• Bring the volume up to 6,000 ml.
• Distribute in 600 ml and 400 ml amounts.
• Autoclave for 15 minutes at 121°C.
• Store in the dark at 4°C.

For a batch of Pyruvate containing media – add 2 ml pyruvate solution in every 100 ml of L-J media.

Malachite Green Solution 2%
Malachite Green 20 g.
Distilled water to 1,000 ml.

Dissolve by heating, filter and distribute in 10 ml amounts and autoclave for 15 minutes at 121°C.

Modified petroff's decontamination method for culture of mycobacterial species

• All specimen manipulations were done in a safety cabinet.
• Add an equal volume of 4% sodium Hydroxide (NaOH) to a volume of sputum in an appropriate container.
• Homogenize using either mechanical shaker or vortex mixture, at intervals, for maximum of 15 minutes.
• Centrifuge at 3000 rpm for 15 minutes. Preferably use centrifuge with capped buckets.
• Decant/pipette off the supernatant into a container with 3% Lysol.
• Dilute the deposit with 10-15 ml sterile distilled water (in separate universal containers for each specimen) and vortex to mix.
• Centrifuge at 3000 rpm for 15 minutes.

Note - Centrifugal time critical
- Ensure that the total period of time of contact with the decontaminating solution (NaOH) does not exceed 30 minutes.
• Decant/pipette off the supernatant as in step 4 and vortex mix. Inoculate L-J slopes with either sterile pipette (1-2 dops) or sterile disposable loop (3mm).
Incubate slopes at 37°c for up to 8 weeks. Initially incubate slopes in a horizontal position overnight at 37°c to prevent inoculum from sliding to the bottom of the tube. The subsequent incubation is done in an upright position. Ensure continuous recording of the temperature in the incubator. Use a thermometer kept inside the incubator at all times to confirm that the recordings are accurate. Temperature charts are pinned on incubator doors.

- Growth is checked weekly.

**Species identification**

Identification of mycobacterial species was based on:

- On L-J media, colonies of MTB appear rough dry with buff colour.
- For each isolate, a film was prepared and stained with ZN method to confirm the presence of AFB and cord formation (strong indicator for stained smears of MTB).

**Growth/culture**

- Rate of growth: rapid (1-5 days), checked daily
- Preference for growth on L-J slopes containing:
  - 5mg/l thiopene carboxylic acid hydrazide (TCH)
  - 500mg/l p-nitro benzoate (PNB)

**Biochemical characteristics**

Niacin production

**Interpretation of results**

Classical MTB strains grow in TCH containing medium, produce niacin, do not grow at 25°c and 42°c, do not produce pigment, are inhibited by PNB and visible growth is not apparent within three days of subculture.

4. **Determination of the concentration of NaOCl**

Commercially available Bleach ("JIK") is used, but the amount of available chlororine in the JIK solution must be verified weekly and/or when a new Bottle is opened.

**Requirements:**

1. Plastic bottle
2. 3 glass tubes
3. 25ml Distilled Water
4. "JIK" (3.5% sodium hypochlorite when packed).

Method:
1. Dilute the bleach from the main bottle at $d = 2.10^4$ (20 000 times) as follow:
2. Take 3 glass tubes
3. Label the first once with “2” the second one with “100” and the last one with “100”
4. Add 5 ml of distilled water into the last two tubes
5. With the same pipette take 3 m of bleach and add it into the first tube
6. Mix very well and discard the pipette
7. Take 100ul with the precision pipette of the solution from the 1st tube and add it into the 2nd tube
8. Mix very well and discard the tip
9. Take 100ul of the solution from the 2nd tube and add it into the 3rd tube
10. Mix very well and discard the tip
11. Put the obtained solution from the 3rd tube into the pool tester on the side of chlorine testing
12. Add one tablet of HTH and replace the cap on the test cell.
13. Shake gently to dissolve tablet and match the color immediately with the standard chlorine colors on the right hand side of your test kit.

Ideal Range

1.5 to 2 ppm of chlorine for a stabilized pool and 1.0 to 1.5 ppm for Non-stabilized pools.

14. Transfer 200ml in a plastic bottle of the reagent.
15. Write the date on the bottle and the expiry date.

Shelf life: One week.
If the reading is acceptable:
16. Transfer 200 ml in a plastic bottle of the reagent.
17. Write the date on the bottle and the expiration date:

**Shelf life**: One week. Do a fresh solution every week. Discard the remaining solution and start again from step 1.

Follow the instructions about the expiration date according the supplier.
Batch number of the reagent and the expiration date must be if possible the same for the duration of the study.
nontuberculous mycobacteria – mycobacteria that do not cause TB disease and are not usually spread from person to person; one example is *M. avium* complex

pathogenesis – how an infection or disease develops in the body

pulmonary TB – TB disease that occurs in the lungs (about 85% of all U.S. cases), typically causing a cough and an abnormal chest x-ray; pulmonary TB is usually infectious if untreated

transmission – the spread of an organism, such as *M. tuberculosis*, from one person to another; depends on the contagiousness of the patient, the type of environment, and the

tubercle bacilli – another name for *Mycobacterium tuberculosis* organisms, which cause TB disease

tuberculous mycobacteria – mycobacteria that can cause TB disease or other diseases very similar to TB; the tuberculous mycobacteria are *M. tuberculosis*, *M. bovis*, and *M. africanum*. 