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Editorial

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We would like to thank all our readers for their precious inputs & encouragement in making this Journal a successful effort. This issue of the Journal brings forth not just topics to ponder over, but essentials to read & understand.....

Mini Review segment: Foot infections are a common and serious problem in persons with diabetes. Diabetic foot infections (DFIs) typically begin in a wound, most often a neuropathic ulceration. While all wounds are colonized with microorganisms, the presence of infection is defined by ≥ 2 classic findings of inflammation or purulence. Infections are then classified into mild (superficial and limited in size and depth), moderate (deeper or more extensive), or severe (accompanied by systemic signs or metabolic perturbations). Most DFIs are polymicrobial, with aerobic gram-positive cocci (GPC), and especially staphylococci, the most common causative organisms.

Our Current Trends segment: Humans are very susceptible to the Tuberculosis infection but are remarkably resistant to the Tuberculosis disease; which is dependent largely on the state of the hosts immune system. Of all the Mycobacterial species *Mycobacterium tuberculosis* remains the most common cause of pulmonary tuberculosis and remains the most virulent of all the Mycobacterial species.

In Profile segment: Max Theiler was a South African-American virologist and doctor. He was awarded the Nobel Prize in Physiology or Medicine in 1951 for developing a vaccine against yellow fever in 1937. Max Theiler (pronounced Tyler) was a leading scientist in the development of the yellow-fever vaccine. His early research proved that yellow-fever virus could be transmitted to mice. He later extended this research to show that mice that were given serum from humans or animals that had been previously infected with yellow fever developed immunity to this disease. From this research, he developed two different vaccines in the 1930s, which were used to control this incurable tropical disease. For his work on the yellow-fever vaccine, Theiler was awarded the Nobel Prize in medicine or physiology in 1951.

Bug of the month segment: *Bacillus cereus* is a Gram-positive aerobic or facultative anaerobic, motile, spore-forming, rod-shaped bacterium that is widely distributed environmentally. While *B. cereus* is associated mainly with food poisoning, it is being increasingly reported to be a cause of serious and potentially fatal non-gastrointestinal-tract infections. The pathogenicity of *B. cereus*, whether intestinal or non-intestinal, is intimately associated with the production of tissue-destructive exoenzymes. Among these secreted toxins are four hemolysins, three distinct phospholipases, an emesis-inducing toxin, and proteases.

Best Practices Segment: Antiseptics are agents that destroy or inhibit the growth and development of microorganisms in or on living tissue. Unlike antibiotics that act selectively on a specific target, antiseptics have multiple targets and a broader spectrum of activity, which include bacteria, fungi, viruses, protozoa, and even prions. Several antiseptic categories exist, including alcohols (ethanol), anilides (triclocarban), biguanides (chlorhexidine), bisphenols (triclosan), chlorine compounds, iodine compounds, silver compounds, peroxygens, and quaternary ammonium compounds. The most commonly used products in clinical practice today include povidone iodine, chlorhexidine, alcohol, acetate, hydrogen peroxide, boric acid, silver nitrate, silver sulfadiazine, and sodium hypochlorite.

Did You Know segment: To protect drinking water from disease-causing organisms, or pathogens, water suppliers often add a disinfectant, such as chlorine, to drinking water. However, disinfection practices can be complicated because certain microbial pathogens, such as *Cryptosporidium*, are highly resistant to traditional disinfection practices. Also, disinfectants themselves can react with naturally-occurring materials in the water to form byproducts, such as trihalomethanes and haloacetic acids, which may pose health risks.

We are looking forward for your continuous support in making this journal a better effort each time. Feedback & suggestions are always welcomed.

Diabetic Foot Management

Question 1: Definition of diabetic foot infections

1a) What is the definition of diabetic foot infection and what are its clinical presentations?

Infection is defined by invasion of the tissues with proliferation of micro-organisms causing tissue damage with or without an associated inflammatory response by the host. Diabetic foot infections are generally secondary to a skin wound. **The diagnosis of diabetic foot infection is clinical. However, infection must be distinguished from bacterial colonization**, a physiological phenomenon occurring all over the skin and related to minimally virulent aerobic and anaerobic bacteria derived from the skin flora, endogenous flora or the environment. This phenomenon can be modified in diabetes mellitus, with a polymorphic appearance and growth of more virulent bacteria such as *Staphylococcus aureus* or *Streptococcus pyogenes*. The progression to infection occurs as a result of multiple factors related to the characteristics of the wound, the pathogenic bacteria and the host. The diagnosis of infection is based on the presence of at least two of the following signs: swelling, induration, erythema around the lesion, local tenderness or pain, local warmth or presence of pus. The severity of infection is assessed according to the International Consensus on the Diabetic Foot classification system (Table 1A).

Table 1A - International Consensus on the Diabetic Foot classification of foot wound infections

Grade 1	No symptoms, no signs of infection
Grade 2	Lesion only involving the skin (without involvement of deeper tissues nor systemic signs) with at least two of the following signs: • local warmth • erythema > 0.5 - 2 cm around the ulcer • local tenderness or pain • local swelling or induration • purulent discharge (thick, opaque to white or sanguineous secretion) Other causes of inflammation of the skin must be eliminated (for example: trauma, gout, acute Charcot foot, fracture, thrombosis, venous stasis)
Grade 3	Erythema > 2 cm and one of the findings described above or Infection involving structures deeper than skin and subcutaneous tissue, such as deep abscess, osteomyelitis, septic arthritis or fasciitis There must not be any systemic inflammatory response (see grade 4)
Grade 4	Any foot infection, in the presence of a systemic inflammatory response manifested by at least two of the following characteristics: • temperature > 38 °C or < 36 °C • pulse > 90 bpm • respiratory rate > 20 per min • PaCO₂ < 32mmHg • leukocytes > 12,000 or < 4000 per mm³ • 10% of immature (band) forms

Superficial infections involve tissue layers above the superficial fascia and present in the form of acute bacterial cellulitis. Deep infections involve the superficial fascia, muscles or bones and joints. Cellulitis is a bacterial infection of the subdermis. The clinical features are characterized by local signs (erythema, initially around the lesions and then diffuse). Hyperthermia, ascending lymphangitis and regional lymphadenopathy are sometimes absent in diabetic patients. Necrotizing cellulitis is characterized by tissue necrosis of the subdermis and then the dermis. Necrotizing fasciitis corresponds to involvement of the superficial fascia, presenting in the form of sloughing of the skin and a violaceous color of the integument without pus or abscess. Rapid deterioration of the general status, development of renal failure, sudden extension of the lesions, cutaneous sensory loss (difficult to demonstrate in patients with diabetic neuropathy) and

the presence of skin detachment constitute warning signs of necrotizing fasciitis. Wet gangrene is defined by blackish necrotic tissues. It is rapidly progressive with skin detachment and grayish pus with a nauseating odor, and can lead to rapid deterioration of the patient's general status with sepsis, metabolic disorders and renal failure. Purulent collections can present in the form of abscess (collected form) or phlegmon (circumscribed by the tissues) in the soft tissues of the foot, or even the leg that may sometimes be difficult to demonstrate clinically and may require the use of imaging examinations. 1b) What are the pathophysiological mechanisms of diabetic foot infections? Diabetic patients are at greater risk than the general population to infections, particularly foot infections. Fifteen to 25% of diabetic patients develop a foot ulcer at some time during their life and 40–80% of these ulcers become infected. The pathophysiological mechanisms of diabetic foot infections are still a subject of controversy. The various hypotheses proposed include: 1 a deficiency of cell-mediated immune mechanisms accentuated by hyperglycemia that can alter leukocyte functions, 1 the harmful effects of neuropathy and excessive pressure on the wound, 1 the chronic nature of the lesion, 1 hypoxia, due to a poor local perfusion and accentuated by the host's hypermetabolic state and microbial cellular metabolism. Hypoxia promotes anaerobic subcutaneous infections and decreases the bactericidal activity of neutrophils, 1 arterial disease decreasing the blood supply to the wound and consequently the influx of endogenous and exogenous factors (antibiotics) involved in the fight against infection, 1 the particular anatomy of the foot, divided into several compartments, explaining the rapid spread of infection.

1c) What clinical classifications are available to guide the management of diabetic foot infections? Many wound classifications have been proposed. The University of Texas classification (UT classification) is easy to use and should now be used as the reference wound classification. It comprises four grades according to depth and four stages according to the presence or absence of infection and/or arterial disease (Table 1B). A complementary classification of wound infections has been defined by the International Consensus on the Diabetic Foot (Table 1A). This classification comprises four grades, from grade 1 (no infection) to grade 4 (severe sepsis).

Table 1B

	Grade 0 Completely epithelialized lesion	Grade 1 Superficial wound	Grade 2 Wound penetrating to tendon or capsule	Grade 3 Wound penetrating to bone or joint
Stage A Not infected Not ischemic	0A (0%)	1A (0%)	2A (0%)	3A (0%)
Stage B Infected	0B (12.5%)	1B (8.5%)	2B (28.6%)	3B (92%)
Stage C Ischemic	0C (25%)	1C (20%)	2C (25%)	3C (100%)
Stage D Infected Ischemic	0D (50%)	1D (50%)	2D (100%)	3D (100%)

Question 2: How to document acute diabetic foot infection?**2a) How to obtain reliable microbiological data?****2a1) What are the indications for bacteriological specimens?**

Bacteriological specimens are only indicated in the case of clinically confirmed infection, starting at grade 2 of the International Consensus classification. Bacteriological specimens should not be obtained systematically from wounds with no clinical signs of infection.

2a2) What are the methods of microbiological isolation?

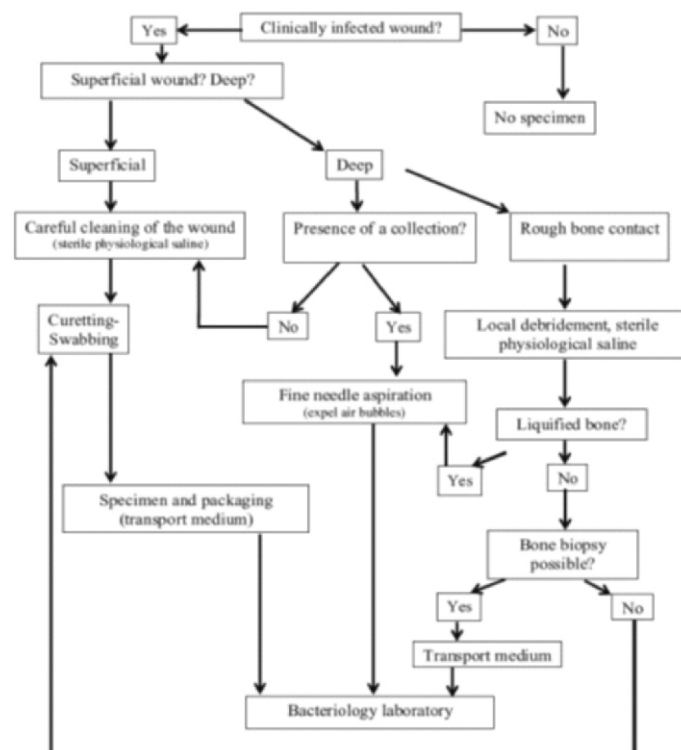
Protocols designed jointly by clinicians and microbiologists are essential to obtain clinically useful results. The objective is to obtain isolation and identification of the micro-organism(s) responsible for the infection from a specimen, while avoiding contamination by the commensal flora that colonizes the skin. No consensus has been reached concerning the best technique for microbiological isolation. Before taking any specimen, the wound must be prepared. Debridement with a sterile curette or scalpel is essential. The wound must then be cleaned with gauze soaked in sterile physiological saline. Antiseptics can be used, but they must be eliminated by sterile physiological saline before taking the specimen. Fig. 1 summarizes the choice of specimens to be performed as a function of the type of wound. Repeated specimens should be taken in the event of an unfavorable course or when the patient presents severe sepsis. Specimens must be sent to the microbiology laboratory as rapidly as possible (collaboration between clinicians, nurses and couriers), in transport medium. Specimens must be inoculated on conventional media and incubated at 37 °C. For specimens derived from deep tissues and aspirations, cultures must also be performed under anaerobic conditions. There is a poor correlation between the results of Gram stain and the results of culture of tissue biopsies.

2a3) Interpretation of the results and epidemiology

Interpretation of the results must take into account the conditions of collection of the specimen, the specimen transport time and transport conditions and the type of bacteria isolated. Firstline treatment should disregard the least virulent or commensal bacteria (coagulase-negative staphylococci, Corynebacteria, Pseudomonas aeruginosa, enterococci). There is no 100% reliable microbiological method to distinguish between pathogenic and nonpathogenic micro-organisms at the present time. When there is a doubt, specimens must be repeated and these bacteria will be taken into consideration when they are isolated on several occasions or when the patient's septic state is worrisome.

Gram-positive aerobic bacteria are the most frequent microorganisms; in this group, *S. aureus* is the micro-organism most frequently isolated. Gram-negative aerobic bacteria, essentially Enterobacteriaceae, are generally detected in chronic or previously treated infections. *P. aeruginosa* is frequently isolated after a long stay in hospital or when the wound is treated by moist dressings. Strict anaerobic bacteria are often associated with aerobic bacteria. The ecology of multiresistant bacteria must be taken into account, especially Methicillin-resistant *Staphylococcus aureus* (MRSA) which constitutes a very serious problem, although isolation of MRSA is not synonymous with increased virulence. Other bacteria must also be taken into account: Enterobacteriaceae resistant to third generation cephalosporins, multiresistant *P. aeruginosa* and other environmental bacteria.

Fig.1-Flow chart of the specimens to be taken as a function of the type of wounds identified in a diabetic subject

**2b) What is the value of laboratory examinations?**

No laboratory marker is sufficiently sensitive and specific to confirm the diagnosis of infection or colonization of a diabetic foot wound. Laboratory markers are often misleading, even in the case of severe lesions.

Question 3: Apart from microbiological factors, what other risk factors should be investigated?**3a) Mechanical factors**

Diabetic neuropathy predisposes to the development of foot deformities and gait abnormalities with the appearance of pressure zones and reactive hyperkeratosis. Continued walking induces the appearance of a zone of inflammatory detachment underneath the zone of hyperkeratosis, leading to mal performance that penetrates into deeper planes and predisposes to the development of infection. The main mechanical factor worsening foot wounds is therefore continued weightbearing. Other mechanical factors have also been identified: poorly fitting shoes, nail diseases, acquired deformities (hallux valgus or quintus varus), oedema (predisposing to poor distal arterial perfusion) and prolonged bed-rest. These mechanical risk factors must be identified and eradicated.

3b) Peripheral vascular disease (PVD)

Evaluation of the underlying vascular state is essential in any diabetic patient with an infected wound. This evaluation is based on clinical examination and complementary investigations:

3b1) Clinical examination must look for signs of intermittent claudication, rest pain, that may often not be experienced by the patient due to diabetic neuropathy, with inspection of the foot, auscultation of arteries and palpation of pedal pulses.

3b2) Complementary investigations

a. Ankle-Brachial Index (ABI) must be systematically determined in all diabetic patients. It corresponds to the ratio between systolic blood pressure measured at the ankle and in the arm (brachial). The ABI may reveal PVD in a number of asymptomatic patients. Normal values are between 0.90 and 1.30. An ABI < 0.90 confirms the diagnosis of PVD. Interpretation of the ABI may be limited by medial sclerosis of ankle arteries which makes the arteries poorly compressible or incompressible, thereby falsely raising the systolic pressure. An ABI > 1.30 is indicative of incompressibility.

b. Arterial Doppler examination of the lower limbs is highly recommended in diabetic patients over the age of 40 years and/or who have suffered from diabetes for more than 20 years and/or in the presence of known coronary artery disease or atheroma of the supra-aortic vessels. This examination must be systematically performed in the case of diabetic foot infection. B-mode ultrasound identifies stenoses and occlusions, analyses the arterial wall and measures the external diameter of the artery at the site of the stenosis and at a presumably healthy site. Doppler studies provide hemodynamic analysis of flow at the stenosis and in the distal runoff. Arteriography remains the reference examination for the anatomical evaluation of PVD and to guide revascularization. MR angiography and CT angiography (performed without direct arterial injection and without injection of iodinated contrast agent for MR angiography) can be alternatives to arteriography of the lower limbs to evaluate the lesions, especially distal and calcified lesions.

c. Other examinations

Toe blood pressure (TBP), which can almost always be measured in diabetic patients, is indicated in the case of neuropathy associated with medial arterial sclerosis, when ABI is = 1.30. PVD is defined by a 50 mmHg difference between ankle systolic pressure and TBP or by a toe/brachial systolic pressure index < 0.55. A TBP less than 30 mmHg corresponds to critical ischemia and a revascularization procedure must be envisaged. Transcutaneous partial pressure of oxygen (TcPO₂) is an index of the severity of skin ischemia and the probability of spontaneous healing even in the presence of medial sclerosis. The normal value of TcPO₂ measured on the dorsum of the foot is about 50 mmHg in diabetic patients. For a TcPO₂ greater than 30 mmHg, healing is possible in more than 90% of cases. A value less than 20–30 mmHg indicates critical ischemia with a healing rate < 30%. It is falsely lowered in the case of edema of the dorsum of the foot or infection. As a value > 30 mmHg confirms the absence of severe ischemia in a case of diabetic foot infection, this examination must be performed in the presence of arterial disease.

Question 4: What treatment modalities are available?**4a) What is the value of a multidisciplinary approach?**

Diabetic foot is a complex disease requiring global management of the patient and not only the foot. A multidisciplinary approach is essential and requires good coordination between all health care professionals involved.

4b) Which strategies should be implemented?**4b1) The role of blood glucose control**

There are many arguments in support of maintaining blood glucose as close to normal as possible. Insulin therapy is usually required to ensure blood glucose control.

4b2) The importance of mechanical off-loading

Complete and permanent off-loading of the wound must be ensured as strictly as possible. Various modalities are available: bed rest, wheelchair (keeping the affected leg horizontal), removable or non-removable casts. The patient's strict compliance with off-loading, tolerance and the condition of the offloading device must be closely monitored.

4b3) Medical debridement

Mechanical debridement consists of excising necrotic soft tissues, devitalized and contaminated tissues and slough, leaving only healthy tissue in order to promote wound healing. The presence of arterial disease must be eliminated before performing any debridement procedure. In predominantly neuropathic ulcers, debridement must be continued until healthy tissue is reached, but in ischemic ulcers, debridement must be performed very cautiously and must be limited to a simple drainage. Ideally, debridement should be performed after or during revascularization. Debridement allows complete visualization of the wound, exposure of any extensions, better drainage of exudates and collection of deep bacteriological specimens and it also promotes healing. It must always be performed before application of any topical agents and must be repeated as often as necessary.

4b4) Antiseptics and topical antibiotics

Topical antibiotics have no place in the topical treatment of infected foot wounds in diabetic patients. However a topical antiseptic with novel molecule like PHMB (poly-hexamethylene- biguanide) shows challenging results.

4b5) Dressings

No consensus has been reached concerning the type of dressing to be used on infected diabetic foot wounds. The principle of daily dressings to allow close surveillance of the infected wound is generally accepted. In the case of cellulitis, the edges of the inflammatory zone must be marked with a felt-tip pen to follow the course. Adhesive or occlusive dressings must not be used on infected wounds. The dressing must be adapted to the volume of exudate. Regardless of the type of dressing applied, detailed wound care protocols must be established and the course of healing must be objectively documented by regular measurement of the wound as well as photographs.

4b6) Control of edema

It has been shown that reduction of edema increases the healing rate of debrided diabetic foot infections.

4b7) Tetanus vaccine status must be systematically verified**4b8) Other treatments**

Hyperbaric oxygen therapy and growth factors are not currently recommended as treatment for diabetic foot infections. Hyperbaric oxygen therapy can be considered in the case of severe arterial disease (critical ischemia) not suitable for revascularization.

4c) What surgical strategies are available?

4c1) Revascularization procedures Revascularization procedures have two main objectives: to ensure salvage of a limb when viability is compromised by severe ischemia and to allow healing of ulcers.

In the case of severe (critical) ischemia associated with signs of infection, coldness of the foot, pallor, absent pulses, presence of necrosis and suggestive signs on vascular investigations (ankle blood pressure < 50 mmHg or TcPO₂ < 30 mmHg or TBP < 30 mmHg), revascularization must be systematically considered. The treatment of infection (off-loading, debridement, antibiotic therapy) must be started immediately and revascularization must be considered once the infection has been controlled. In the case of surgical exposure, the revascularization procedure should be performed as soon as possible to avoid extension of infection, absence of healing, or even life-threatening deterioration. Ideally, the revascularization should be performed at the same time as the debridement procedure. In the case of more moderate ischemia, a less severe clinical situation and less unfavorable vascular investigations (ankle blood pressure > 70 mmHg, TcPO₂ > 30 mmHg and TBP > 50 mmHg), revascularization can be deferred and proposed secondarily, especially in the case of delayed healing despite control of infection and well conducted medical treatment. In every case, it is essential to evaluate the patient's arterial status to assess the need for a revascularization procedure, which could reduce healing time and reoperations. The criteria for revascularization take into account: the patient's general state (operability), the potential for ulcer healing, the quality of the arterial distal runoff and the site of the lesions (arteriography or possibly MR angiography in patients with renal failure). The indications for revascularization depend on the level of the lesions. Aorto-iliac lesions are treated by angioplasty or bypass graft. Femoropopliteal or tibial lesions should preferably be treated by angioplasty, which do not prevent the possibility of secondary bypass grafts. Multisegmental lesions, the most frequent situation, require a combination of angioplasty and bypass grafts. Distal bypass grafts have transformed the prognosis of serious trophic disorders of the diabetic foot and are not performed sufficiently frequently. They must be performed only after infection has been controlled, preferably with venous material or allografts. Lumbar sympathectomy is not indicated in the treatment of diabetic PVD with or without infected lesions.

4c2) Orthopaedic surgery

Conservative surgery can be considered in two circumstances: Emergency, in the case of limb-threatening or life-threatening infection, abscess complicated by a compartment syndrome or necrosis, or necrotizing cellulitis. Emergency surgery must be as conservative as possible. Amputations, even minor, must be exceptional. Deferred, in the absence of improvement in response to well conducted medical treatment. This procedure must be performed after vascular assessment and revascularization, if necessary, and must be as conservative as possible. Amputation surgery sometimes still remains the only option in the case of severe, deep infection, especially in a context of ischemia. The choice of the level of amputation depends on the vascular status, while taking every effort to preserve heel weightbearing with the prosthesis. No amputation must be performed without first performing complementary vascular investigations.

Question 5: What are the specificities of osteomyelitis of the diabetic foot?

5a) What is the definition of osteomyelitis of the diabetic foot?

Bone infection is frequent in diabetic patients, present in 30–80% of cases depending on the severity of the infection. It may consist

of isolated osteomyelitis, especially of the toes and calcaneus, or more often bone and joint infection, while isolated septic arthritis is rare. Bone or joint infection generally occurs by contiguous spread from a skin wound, while a hematogenous origin of osteomyelitis or osteoarthritis of the foot is exceptional in diabetic patients.

5b) What are the signs suggestive of osteomyelitis of the foot in a diabetic?

Osteomyelitis must be suspected in the following cases: resistance to treatment, recurrent infection of an ulcer, especially when it is localized over a bony prominence, unfavorable or persistent course despite optimal management and a satisfactory arterial blood supply. Other clinical signs are also in favor of osteomyelitis:

Rough bone contact when probing with a sterile, blunt metal probe introduced through the ulcer, although the value of this procedure has recently been questioned; Bone exposure; the edematous, erythematous “sausage” appearance of a toe or abnormal mobility of a toe are also suggestive of bone and joint infection.

6a) Detection of diabetic patients with a high risk of foot problems

This consists of identifying risk factors (history of ulceration or amputation, sensory loss of the foot demonstrated by the monofilament test, PVD and foot deformities). These risk factors identify patients according to their level of risk, as defined in the International Consensus classification whose predictive value has been demonstrated by a prospective study.

6b) Prevention measures

6b1) Education

Patient education is essential right from grade 1 and must include the patient's family, comprising practical and adapted advice (awareness of sensory loss and its consequences, awareness of poor blood supply and its consequences, high risk situations, self-examination of the feet, atraumatic footwear and hygiene and maintenance of the feet (nails, hyperkeratosis, fungal infections). Nurse education must emphasize the importance of regular examination of the feet of diabetic patients, scoring of the risk of foot problems, setting up of preventive strategies based on patient education and foot care.

6b2) Other measures

Podiatric care (removal of hyperkeratoses and nail care), good quality shoes, fashioning of orthopaedic shoes and adapted orthoses are essential, as these disorders are responsible for ulceration of the diabetic foot.

References

Management of diabetic foot infections, Organized by the Société de Pathologie Infectieuse de Langue française (SPILF) with the participation of the following scientific societies: Association de Langue Française pour l'Étude du Diabète et des Maladies Métaboliques (ALFEDIAM), Société Française de Chirurgie Vasculaire, Société Française de Microbiologie, Collège Français de Pathologie Vasculaire, Médecine et maladies infectieuses 37 (2007).

Mycobacterium Tuberculosis

Introduction

Humans are very susceptible to the Tuberculosis infection but are remarkably resistant to the Tuberculosis disease; which is dependent largely on the state of the hosts immune system. Of all the Mycobacterial species *Mycobacterium tuberculosis* remains the most common cause of pulmonary tuberculosis and remains the most virulent of all the Mycobacterial species.

The disease, as now well known, is highly contagious. Although the disease involves all susceptible individuals, the incidence is higher amongst disadvantaged minorities. Industrialization, increased crowded housing and nutritional deprivation have influenced the spread. With the emergence of HIV and resultant immunocompromise, TB has emerged as a major killer not only in the third world countries but is also resurging in the western world. According to World Health Organisation (W.H.O.) reports, each year an estimated eight million new cases of Tuberculosis occur, leading to three million deaths; and almost a third of the world's population is infected by the causative organism, *Mycobacterium tuberculosis*.

According to a study, in India, the number of Tuberculosis patients is increasing at the rate of 1.5 million per year, and a quarter of these are sputum positive. Thus, about 40 per cent of all Indians are infected with *Mycobacterium tuberculosis*.

With the emergence of the multiple drug resistant strains due to poorly administered therapeutic measures and patient non-compliance, *Mycobacterium tuberculosis* is challenging its containment, on the basis of empirical treatment alone.

Brief Microbiology

The genus *Mycobacterium* is composed of slow growing organisms, which are "acid fast". Currently about 55 species of Mycobacteria are recognized. They are non-motile, slightly curved or straight rods (0.2 – 0.6 X 1-10 mm) and may occasionally demonstrate branching. The organisms are aerobic and have a gram positive cell wall, although they do not gram stain well.

The Mycobacteria contain a lipid rich cell surface which includes true waxes and glycolipids. 60-90 carbon, long chain Mycolic acids, unique to the Mycobacterial cell wall are responsible for their:

- Acid fastness;
- Failure to react with gram stains ;
- Resistance to the action of antibodies and complement.

The four species in the *Mycobacterium tuberculosis* complex are *M. tuberculosis*, *M. microti*, *M. africanum* and *M. bovis*. Laboratories can use biochemical tests for differentiation between isolated strains.

Diagnosis of Mycobacterium tuberculosis infection

The diagnosis of Tuberculosis is often made on the basis of clinical symptoms, chest X-ray and sputum AFB, since available tests based on immunological principles for Mycobacterium tuberculosis diagnosis have yet to overcome the problem of poor sensitivity and specificity associated with them. For the time being, speedy and appropriate laboratory diagnosis of Tuberculosis infection through AFB staining, culture and sensitivity have more and more important role to play in sensitive detection and appropriate treatment of patients with Tuberculosis. However, sample collection, preparation, processing techniques and detection methods employed have a profound effect on the sensitivity and specificity of the results for the detection of *Mycobacterium tuberculosis* infection by AFB and culture methods.

Specimen Selection

A critical factor in the ability of laboratories to isolate *Mycobacterium tuberculosis* is obtaining appropriate specimen for AFB smear and culture. Approximately 85% of the TB cases are pulmonary. However many patients cannot produce sputum spontaneously and alternative respiratory tract specimens such as induced sputum, gastric lavage or fiberoptic bronchoscopy may be needed. As the proportion of patients with extra-pulmonary form of Tuberculosis is increasing, adequate specimen from extra pulmonary sites need to be provided.

Recommendations for Sample Collection for Mycobacterial isolation and Acid Fast Staining

Specimen type	Specimen requirements	Special instructions	Unacceptable specimen
Abscess contents, aspirated fluid	As much as possible in syringe with Luer tip cap	Cleanse skin with alcohol before aspirating sample. Laboratory may provide 7H9 broth/Kirchner medium for transport of small volumes of aspirates	Dry swab
Blood	10ml SPS (yellow top) blood collection tube or 10ml isolator tube	Disinfect site as for routine blood culture. Mix tube contents immediately after collection. SPS is preferred anticoagulant. Heparinized blood is also acceptable	Blood collected in EDTA, which greatly inhibits Mycobacterial growth even in trace amounts. Coagulated blood.

Specimen type	Specimen requirements	Special instructions	Unacceptable specimen
Body fluids (pleural, pericardial, Peritoneal).	As much as possible (10-15ml min.) in sterile container or syringe with Luer tip cap. Collect bloody specimens into SPS blood collection tubes	Disinfect site with alcohol if collecting by needle & syringe.	-----
Bone	Bone in sterile container without fixative or preservative	-----	Specimen submitted in formalin.
Bone marrow	As much as possible in SPS blood collection tube or 1.5ml in pediatric isolator tube.	Collect aseptically. Mix SPS tube contents following collection	-----
Bronchoalveolar lavage or bronchial washings	≥5ml in sterile container.	Avoid contaminating bronchoscope with tap water. Saprophytic mycobacteria may produce false positive culture or smear results.	-----
Bronchial brushings	Sterile container or Middlebrook 7H9 broth. Or Krichner medium.	-----	-----
CSF	≥2ml in sterile container.	Use maximum volume attainable.	-----
Gastric Lavage fluid	≥5-10ml in sterile container. Collect in the morning soon after the patient awakens in order to obtain sputum swallowed during sleep.	Collect fasting early morning specimen on three consecutive days. Use sterile saline Adjust to neutral pH with 100mg of sodium carbonate immediately following collection. Laboratory should provide collection tube containing sodium carbonate	Specimen that has not been neutralized
Lymph node	Node or portion on sterile container without fixative or preservative	Collect aseptically and avoid indigenous microbiota. Select caseous portion if available. Do not immerse in saline or other fluid or wrap in gauze.	Specimen submitted in formalin
Skin lesion	Submit biopsy specimen in sterile container without fixative or preservative. Submit aspirate in syringe with Luer tip cap.	Swabs in transport medium (Amies or Stuarts) are acceptable only if biopsy sample or aspirate is not obtainable. For cutaneous ulcer, collect biopsy sample from periphery of lesion, or aspirate material from under margin or lesion.	Dry swab
Smear on slides	Smear specimen over 1.5 by 1.5cm area of clear slide.	Heat fix smears. Transport in slide container taped closed and labeled BIOHAZARD	-----
Sputum	5-10ml in sterile, wax-free disposable container. Collect an early morning specimen from deep productive cough on at least three consecutive days. Do not pool specimens. For follow up of patients on therapy, collect at weekly intervals beginning three weeks after initiation of therapy.	For expectorated sputum, instruct patient on how to produce sputum specimen as distinct from saliva or nasopharyngeal discharge. Have patient rinse mouth with water before collecting sputum to avoid contaminating specimen with food particles, mouthwash, or oral drugs, which may inhibit the growth of mycobacteria. For induced sputum, use sterile hypertonic saline. Indicate on request if specimen is induced sputum.	24 hour pooled specimens, saliva.
Stool	≥ 1g in sterile wax-free disposable container.	Collect specimen directly into container directly into container or transfer from bedpan or plastic wrap stretched over toilet bowl. Wax from container may produce false positive smear.	Frozen specimen. Utility of culturing stool for acid-fast bacilli remains controversial.

Sample Concentration and Decontamination

Specimens obtained from sterile sites such as CSF, peritoneal or pleural fluids do not require decontamination. However most specimens for AFB smear and culture are from the respiratory tract and do contain mixed microbial flora.

Successful recovery of Mycobacteria depends upon properly collected specimen and suppression of contaminating bacteria.

Since mucous traps AFB and protects other organisms from effective decontamination a combination of 2% NaOH (decontaminant) and 0.5% N-acetyl- L-cysteine (mucolytic agent) is preferably employed. Neutralization of strong decontaminating solutions before using the sample for AFB stain and culture is usually accompanied with sequential buffered wash of the concentrated sample because if the pH of the concentrate remains alkaline or acidic it can destroy the culture medium and prevent the growth of Mycobacteria and staining efficiency of the AFB smears. The buffered wash also helps in reducing the specific gravity of specimen and sediments the Mycobacterium more effectively.

Another important aspect post decontamination is the specimen concentration and relative centrifugal force applied to the specimen.

Improvement in correlation between specimen showing a positive smear for AFB and a positive culture has been demonstrated by increasing the centrifugal force applied to pellet the specimen.

Effect of Centrifugal Force on positive smears / cultures for Mycobacteria

Specimen	Relative Centrifugal Force (g)		
	1260	3000	3800
Positive smears	1.8%	4.5%	9.6%
Positive cultures	7.1%	11.2%	11.6%
Correlation of positive smears/ cultures	25%	40%	82%

Thus proper decontamination and preparation of specimen is crucial to AFB detection by culture and AFB staining.

The AFB Smear

The sensitivity of AFB smear for specimen from extra pulmonary sites is lower than from sputa. The lipid-containing cell walls of Mycobacteria have a unique characteristic in binding carbol fuchsin stain so tightly that it resists de-staining with strong decolorizing agents such as strong alcohols and strong acids. This "acidfast" staining reaction of Mycobacteria, along with their unique beaded and slightly curved shape, is a valuable aid in the early detection of infection and monitoring of therapy.

It has been estimated that there must be 10,000 acid-fast bacilli per milliliter of sputum to be detected by microscopy. Patients with extensive disease will shed large numbers of Mycobacteria and show a good correlation between a positive smear and a positive culture. In patients with minimal or less advanced disease, the correlation of positive smears to positive cultures may range from 30 to 80 per cent.

Acid-fast stains performed on a weekly basis are also useful in following the response of patients to drug therapy. After drugs are started, cultures will become negative before smears, indicating that the bacilli are injured sufficiently to prevent replication but not to the point of preventing binding of the stain. With continued drug treatment, more organisms are killed and fewer shed, hence monitoring the number of stainable organisms in the sputum during treatment can provide an early and objective measure of response.

It should be noted that in patients receiving antimycobacterial therapy not all stainable organisms are viable. Should the number of organism fail to decrease after therapy is started, the possibility of drug resistance must be considered.

Additional cultures should be taken and drug susceptibility studies obtained.

Two types of acid-fast stains are frequently used:

1. Carbol fuchsin based stains;
2. Fluorochrome based stains.

1. The carbol fuchsin stains, so called because of the reagent formed by mixing of the stain Basic Fuchsin with the disinfectant Phenol (carbolic acid). Carbol fuchsin stained Mycobacteria appear bright red / pinkish against a bluish background.

Two procedures using carbol fuchsin based stains are in common use:

- (a) Three component Ziehl-Neelsen, or "hot stain", and
- (b) Three component Kinyoun or "cold stain".

The Kinyoun stain is a modification of the classical Ziehl-Neelsen "hot stain". The classical Ziehl-Neelsen "hot stain" requires application of heat to the fixed smears flushed with the stains during staining process, where as the Kinyoun stain does not require the application of heat and is less tedious to perform and standardize.

Recent advances in staining techniques have been reported where the cold Kinyoun stain has been further modified to accommodate the decolorizer within the counter stain. The novel two component two step stain is time, labor and cost saving, more user friendly and easy to standardize. It also has good correlation with the classical Ziehl-Neelsen "hot stain" and AFB cultures.

2. The fluorochrome based stains for AFB comprise of Auramine O, sometimes used in combination with a second fluorochrome stain, Rhodamine.

Smears stained with Auramine O can be scanned using a 25 x objective. Fluorochrome-stained Mycobacteria appear bright yellow against a dark background obtained by counterstaining with potassium permanganate, thereby permitting the slide to be scanned under the lower magnification without losing sensitivity. The sharp visual contrast between the bright colored Mycobacteria and the dark background offers a distinct advantage in scanning a much larger area of the slide during the same time necessary for looking at the carbol fuchsin stain.

When using the Auramine stain, a significantly larger area of the smear can be scanned in the same period of time used to scan a carbol fuchsin-stained smear.

Enthusiasm for the carbol fuchsin and fluorochrome staining methods varies between laboratories, with different professionals strongly partial to one method or the other.

Specificity for *Mycobacteria* seems to be the same for both.

The crucial factors in maximizing smear sensitivity and specificity are:

- Centrifugation of digested fluid specimen at a minimum of 3000 g;
- The smear should be prepared on a new clean undamaged glass slide;
- Scanning of at least 300 fields per slide;
- The reporting of the AFB smear should be preferably done according to the C.D.C., USA method, or as per the National Reference Institution norms.

Quantitation Scale for Acid-Fast Bacillus Smears according to stain used

Carbolfuchsin (X 1,000)	Fluorochrome (X 250)	Quantity Reported
No AFB/300 fields	No AFB/30 fields	No AFB seen
1-2 AFB/300 fields	1-2 AFB/30 fields	Doubtful; repeat test
1-9 AFB/100 fields	1-9 AFB/10 fields	Rare (1+)
1-9 AFB/10 fields	1-9 AFB/ field	Few (2+)
1-9 AFB/ field	10-90 AFB/ field	Moderate (3+)
>9 AFB/ field	>90 AFB/ field	Numerous (4+)

However, Indian Reference Institutions recommend reporting after 5 minutes of examination covering about 100 fields. Grading is done as follows:

Number of Acid Fast Bacilli observed	Report
No Acid Fast Bacilli	Negative
1-10 Acid Fast Bacilli	Actual Number
>10 Acid Fast Bacilli	+
Masses of Acid Fast Bacilli in several fields	++

Smears with fewer than 3 AFB per slide account for about 85% of false positive smear reporting and are considered doubtful. A repeat specimen should be registered. However *Mycobacterium tuberculosis* infection must be considered for any patient with repeat smear AFB positive regardless of the number of AFB observed.

Factors Influencing Sensitivity & Specificity of AFB Smears False Positive Results

● Acid fast particles other than tubercle bacilli

Occasionally, a sputum specimen or smear may contain particles that are acid-fast i.e., when treated with the Ziehl-Neelsen method, they retain the red stain (carbolfuchsin) and resist

decolorization with acid-alcohol. These red particles may sometimes resemble tubercle bacilli. They include certain food particles (e.g., waxes, oils), precipitates, other micro-organisms, inorganic materials and artifacts.

Food particles: To eliminate these, the patient should rinse their mouth with pure water and clean their teeth (without using tooth-paste or disinfectant) before producing the sputum specimen. It is even better if the patient produced the specimen before breakfast or on an empty stomach.

Precipitated stains: Though these are quite easy to differentiate from acid-fast bacilli, they may hamper reading or occasionally mislead an inexperienced microscopist. Precipitates can be removed by filtration of staining solutions. However, it is safer to use freshly prepared solutions, filled into carefully cleaned bottles, rather than stale staining solutions.

Saprophytic acid-fast bacilli: These occur in soil and water, and may occasionally get into the specimen or smear during processing. This can be avoided by using distilled or boiled water from scrupulously clean containers.

Mycobacterium kansasii or Nocardia species: These occasionally occur in specimens. When they cause pulmonary disease, they are usually present in large numbers.

Spores of Bacillus subtilis: These are very rare, mostly of ovoid shape, and larger than tubercle bacilli.

Fibers & pollens: Fibers, including those of wood, cotton, filter paper and bamboo, usually occur singly, most often in only one microscopic field. The pollen of certain pine trees is seen as short, coccoid rods occurring very rarely in specimens.

Scratches on the slide: Scratches may sometimes retain the red stain and confuse beginners. They are usually seen in parallel rows, are generally longer than acidfast bacilli, and are undulated. They can be identified easily, because they are found in a deeper layer on the slide, below the smear disappearing when the cells (e.g., leucocytes) in the smear get focused on.

● Contamination through the transfer of bacilli from one smear to another

It may happen that acid-fast bacilli are transferred accidentally from a positive slide to a negative one, when several slides are treated simultaneously in staining or decolorisation tanks. This can be avoided by processing each slide separately, e.g., on a rack. Such racks are usually made of wire and can be decontaminated easily by flaming.

Acid-fast bacilli may also be transferred accidentally when the glass rod or dropper used for placing immersion oil on the slide touches the surface of a positive slide and rubs off some material. The same can happen when blotting paper is used for drying several stained smear consecutively. Therefore the blotting paper should not be used at all, or for no more than one slide. The oil dropper should not touch the smear, and the oil should be allowed to drip freely on to the slide. For the same reason, the surface of the slide should not be rubbed with the oil immersion objective. Before a new slide is examined, the oil should be wiped off the lens with a piece of cotton tissue or, even better, with special lens-cleaning paper.

When microscopy is used for the detection of acid-fast bacilli, slides should never be used more than once.

False Negative Results

False negative results are commonly due to deficiencies in the preparation of the smear, in staining, and in scanning. Adequate collection of the specimen and subsequent selection of sputum particles are essential to the preparation of a smear and should receive special attention.

Deficiencies leading to false negative results include the following:

● Inadequate sputum collection

The patient is sometimes not told clearly enough what constitutes a proper sputum specimen and how he should produce one. It must be made clear to him that saliva and nasopharyngeal discharge are unsuitable for examination. Patients should be encouraged and given time to produce bronchial sputum from the “depths of the chest”. If repeated attempts have failed, tickling of the inner surface of the epiglottis or trachea with a swab, or intratracheal instillation of 5-10 ml of cool saline or sterile water may provoke a vigorous cough with sputum. Other techniques to stimulate the production of sputum, such as aerosol induction, gastric aspiration, and bronchoscopy, require more complex equipment or special skills.

If a patient discharges acid-fast bacilli in his sputum, these are more likely to be found in a specimen produced in the early morning than in one produced later in the day. If early-morning sputum is required, the patient should be given a container and instructed to place in it the very first sputum he produces in the morning, before breakfast and before taking any medicaments.

● Improper storage of sputum specimens and stained smears

Acid-fast bacilli may lose their acid-fastness as a result of exposure of the specimen to direct sunlight, radiation (e.g., ultraviolet light), excessive heat, or storage for more than a week in hot and dry conditions.

If Ziehl-Neelsen stained smears have to be stored for re-examination, the immersion oil must be washed from the smears with xylol because the immersion oil removes the stain from the acid-fast bacilli.

Fluorochrome stained smears will lose their fluorescence with storage.

● Failure to select suitable sputum particles for smear preparation

Tubercle bacilli are most likely to be found in little blobs (“lentils”) of greenish-grey or yellowish matter of a thick, creamy consistency. (Such blobs usually consist of dead caseous tissue eliminated from a cavity in the lung). If the sputum is not treated by a special concentration procedure involving centrifugation, these blobs have to be carefully separated from the rest of the sputum and transferred to a slide. They can be seen more easily in the sputum against a dark background.

● Inadequate preparation of smear or staining of slides

False negative results may be obtained also when:

- (a) Too little material has been spread on the slide, so that the smear is too thin;
- (b) The smear is too thick, so that sufficient light cannot pass through it;
- (c) The slide has been over heated when fixing the smear;
- (d) The smear has not been sufficiently fixed and parts of the material have been washed off;
- (e) The staining with carbol fuchsin was too short or was overdone by boiling;
- (f) The counterstaining was too intensive, so that the acid-fast bacilli have been obscured;
- (g) Staining and counterstaining times have not been followed precisely.

● Inadequate examination of the smear

If the scanning is done erratically or too briefly, too few fields may be examined.

(Occasionally the examiner is unable to distinguish the red-stained acid-fast bacilli because of color blindness or other visual disturbances).

● Other reasons for false results

Administrative errors: Such errors may include:

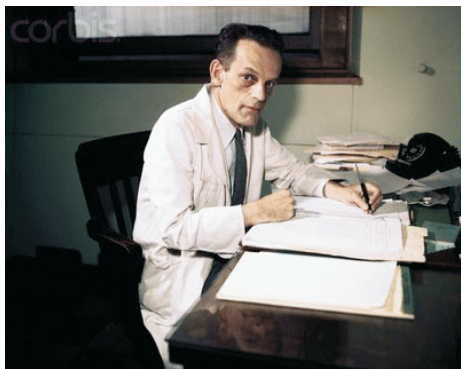
- (a) Misidentification of patients, misspelling of names, or confusion of names or of codes numbers of specimens and slides;
- (b) Mistakes in labeling containers;
- (c) False recording of reporting.

Reading errors: Reader or observer error, which is mainly due to visual or psychological reasons, occurs in practically all diagnostic, clinical and laboratory work. The nature of this phenomenon, sometimes called the “human factor”, is to a large extent unknown. Nevertheless, under certain conditions it is measurable. The degree and frequency of error-overreading as well as under-reading varies from one person to another and also within the same individual at different times.

Inter-individual reader variations in smear microscopy has been repeatedly studied and its frequency has been found relatively low compared, for instance, with inter individual error in say, chest radiography.

It seems likely that many reader errors would be avoided if each microscopist were properly trained and strongly advised to report what he actually saw, and never what he thought he was expected to see. However, discrepancies in the results of smear microscopy are far more often due to deficient sputum collection and smear preparation than due to reader error.

To be continued in the next issue....

Max Theiler

Born: January 30, 1899, Pretoria, South Africa

Died: August 11, 1972, New Haven, Connecticut, United States

Books: The Arthropod-borne Viruses of Vertebrates: An Account of the Rockefeller Foundation Virus Program, 1951-1970

Education: Pretoria Boys High School, King's College London, University of Cape Town

Awards: Nobel Prize in Physiology or Medicine, Lasker-DeBakey Clinical Medical Research Award

Max Theiler was a South African-American virologist and doctor. He was awarded the Nobel Prize in Physiology or Medicine in 1951 for developing a vaccine against yellow fever in 1937.

Max Theiler (pronounced Tyler) was a leading scientist in the development of the **yellow-fever vaccine**. His early research proved that yellow-fever virus could be transmitted to mice. He later extended this research to show that mice that were given serum from humans or animals that had been previously infected with yellow fever developed immunity to this disease. From this research, he developed two different vaccines in the 1930s, which were used to control this incurable tropical disease. For his work on the yellow-fever vaccine, Theiler was awarded the Nobel Prize in medicine or physiology in 1951.

Theiler was born on a farm near Pretoria, South Africa, on January 30, 1899, the youngest of four children of Emma (Jegge) and Sir Arnold Theiler, both of whom had emigrated from Switzerland. His father, director of South Africa's veterinary services, pushed him toward a career in medicine. In part to satisfy his father, he enrolled in a two-year premedical program at the University of Cape Town in 1916. In 1919, soon after the conclusion of World War I, he sailed for England, where he pursued further medical training at St. Thomas's Hospital Medical School and the London School of Hygiene and Tropical Medicine, two branches of the University of London. Despite this rigorous training, Theiler never received the M.D. degree because the University of London refused to recognize his two years of training at the University of Cape Town.

Theiler was not enthralled with medicine and had not intended to become a general practitioner. He was frustrated by the ineffectiveness of most medical procedures and the lack of cures for serious illnesses. After finishing his medical training in 1922, the 23-year-old Theiler obtained a position as an assistant in the Department of Tropical Medicine at Harvard Medical School.

His early research, highly influenced by the example and writings of American bacteriologist Hans Zinsser, focused on amoebic dysentery and rat-bite fever. From there, he developed an interest in the yellow-fever virus.

Yellow fever is a tropical viral disease that causes severe fever, slow pulse, bleeding in the stomach, jaundice, and the notorious symptom, "black vomit." The disease is fatal in 10–15% of cases, the cause of death being complete shutdown of the liver or kidneys. Most people recover completely, after a painful, extended illness, with complete immunity to reinfection. The first known outbreak of yellow fever devastated Mexico in 1648. The last major breakout in the continental United States claimed 435 lives in New Orleans in 1905. Despite the medical advances of the twentieth century, this tropical disease remains incurable. As early as the eighteenth century, mosquitoes were thought to have some relation to yellow fever. Cuban physician Carlos Finlay speculated that mosquitoes were the carriers of this disease in 1881, but his writings were largely ignored by the medical community. Roughly 20 years later, members of America's Yellow Fever Commission, led by Walter Reed, the famous U.S. Army surgeon, concluded that mosquitoes were the medium that spread the disease. In 1901, Reed's group, using humans as research subjects, discovered that yellow fever was caused by a blood-borne virus. Encouraged by these findings, the Rockefeller Foundation launched a world-wide program in 1916 designed to control and eventually eradicate yellow fever.

By the 1920s, yellow fever research shifted away from an all-out war on mosquitoes to attempts to find a vaccine to prevent the spread of the disease. In 1928, researchers discovered that the Rhesus monkey, unlike most other monkeys, could contract yellow fever and could be used for experimentation. Theiler's first big breakthrough was his discovery that mice could be used experimentally in place of the monkey and that they had several practical research advantages.

One unintended research discovery kept Theiler out of his lab and in bed for nearly a week. In the course of his experiments, he accidentally contracted yellow fever from one of his mice, which caused a slight fever and weakness. Theiler was much luckier than some other yellow-fever researchers. Many had succumbed to the disease in the course of their investigations. However, this small bout of yellow fever simply gave Theiler immunity to the disease. In effect, he was the first recipient of a yellow-fever vaccine.

In 1930, Theiler reported his findings on the effectiveness of using mice for yellow fever research in the respected journal *Science*. The initial response was overwhelmingly negative; the Harvard faculty, including Theiler's immediate supervisor, seemed particularly unimpressed. Undaunted, Theiler continued his work, moving from Harvard University, to the Rockefeller Foundation in New York City. Eventually, yellow-fever researchers began to see the logic behind Theiler's use of the mouse and followed his lead. His continued experiments made the mouse the research animal of choice. By passing the yellow-fever virus from mouse to mouse, he was able to shorten the incubation time and increase the virulence of the disease, which enabled research data to be generated more quickly and cheaply. He was now certain that an attenuated live vaccine, one weak

enough to cause no harm yet strong enough to generate immunity, could be developed.

In 1931, Theiler developed the mouse-protection test, which involved mixing yellow-fever virus with human blood and injecting the mixture into a mouse. If the mouse survived, then the blood had obviously neutralized the virus, proving that the blood donor was immune to yellow fever (and had most likely developed an immunity by previously contracting the disease). This test was used to conduct the first worldwide survey of the distribution of yellow fever.

A colleague at the Rockefeller Foundation, Dr. Wilbur A. Sawyer, used Theiler's mouse strain, a combination of yellow fever virus and immune serum, to develop a human vaccine. Sawyer is often wrongly credited with inventing the first human yellow-fever vaccine. He simply transferred Theiler's work from the mouse to humans. Ten workers in the Rockefeller labs were inoculated with the mouse strain, with no apparent side effects. The mouse-virus strain was subsequently used by the French government to immunize French colonials in West Africa, a hot spot for yellow fever. This so-called "scratch" vaccine was a combination of infected mouse brain tissue and cowpox virus and could be quickly administered by scratching the vaccine into the skin. It was used throughout Africa for nearly 25 years and led to the near total eradication of yellow fever in the major African cities.

While encouraged with the new vaccine, Theiler considered the mouse strain inappropriate for human use. In some cases, the vaccine led to encephalitis in a few recipients and caused less severe side effects, such as headache or nausea, in many others. Theiler believed that a "killed" vaccine, which used a dead virus, wouldn't produce an immune effect, so he and his colleagues set out to find a milder live strain. He began working with the Asibi yellow-fever strain, a form of the virus so powerful that it killed monkeys instantly when injected under the skin. The Asibi strain thrived in a number of media, including chicken embryos. Theiler kept this virus alive for years in tissue cultures, passing it from embryo to embryo, and only occasionally testing the potency of the virus in a living animal. He continued making subcultures of the virus until he reached strain number 176. Then, he tested the strain on two monkeys. Both animals survived and seemed to have acquired a sufficient immunity to yellow fever. In March 1937, after testing this new vaccine on himself and others, Theiler

announced that he had developed a new, safer, attenuated vaccine, which he called 17D strain. This new strain was much easier to produce, cheaper, and caused very mild side effects.

From 1940 to 1947, with the financial assistance of the Rockefeller Foundation, more than 28 million 17D-strain vaccines were produced, at a cost of approximately two cents per unit, and given away to people in tropical countries and the United States. The vaccine was so effective that the Rockefeller Foundation ended its yellow-fever program in 1949, safe in the knowledge that the disease had been effectively eradicated worldwide and that any subsequent outbreaks could be controlled with the new vaccine. Unfortunately, almost all yellow-fever research ended around this time and few people studied how to cure the disease. For people in tropical climates who live outside of the major urban centers, yellow fever is still a problem. A major outbreak in Ethiopia in 1960–1962 caused 30,000 deaths. The World Health Organization still uses Theiler's 17D vaccine and had mounted efforts to inoculate people in remote areas.

The success of the vaccine brought Theiler recognition both in the U.S. Over the next ten years, he received the Chalmer's Medal of the Royal Society of Tropical Medicine and Hygiene (1939), the Lasker Award of the American Public Health Association, and the Flattery Medal of Harvard University (1945).

In 1951, Theiler received the Nobel Prize in medicine or physiology "for his discoveries concerning yellow fever and how to combat it."

After developing the yellow-fever vaccine, Theiler turned his attention to other viruses, including some unusual and rare diseases, such as Bwamba fever and Rift Valley fever. His other, less exotic research focused on polio and led to his discovery of a polio-like infection in mice known as encephalomyelitis or Theiler's disease. In 1964, he retired from the Rockefeller Foundation, having achieved the rank of associate director for medical and natural sciences and director of the Virus Laboratories. In that same year, he accepted a position as professor of epidemiology and microbiology at Yale University in New Haven, Connecticut. He retired from Yale in 1967.

Theiler married in 1938 and had one daughter. Theiler died on August 11, 1972, at the age of 73.

JOKES



Wife: "How would you describe me?"

Husband: "ABCDEFGHIIJK."

Wife: "What does that mean?"

Husband: "Adorable, beautiful, cute, delightful, elegant, fashionable, gorgeous, and hot."

Wife: "Aw, thank you, but what about IJK?"

Husband: "I'm just kidding!"

Teacher: "Kids, what does the chicken give you?"

Student: "Meat!"

Teacher: "Very good! Now what does the pig give you?"

Student: "Bacon!"

Teacher: "Great! And what does the fat cow give you?"

Student: "Homework!"

A child asked his father, "How were people born?" So his father said, "Adam and Eve made babies, then their babies became adults and made babies, and so on." The child then went to his mother, asked her the same question and she told him, "We were monkeys then we evolved to become like we are now." The child ran back to his father and said, "You lied to me!" His father replied, "No, your mom was talking about her side of the family."

Wife: "I look fat. Can you give me a compliment?"

Husband: "You have perfect eyesight."

Three men were sitting together bragging about how they had set their new wives straight on their domestic duties.

The first man had married a woman from Italy and boasted that he had told his wife she was to do all the dishes and house cleaning that needed to be done. He said that it took a couple days but on the third day he came home to a clean house and the dishes were all washed and put away.

The second man had married a woman from France. He bragged that he had given his wife orders that she was to do all the cleaning, all the dishes, and the cooking. He told them that the first day he didn't see any results, but the next day it was better. By the third day, his house was clean, the dishes were done, and he had a delicious dinner on the table.

The third man had married an Irish girl. He boasted that he told her his house was to be cleaned, the dishes washed, the cooking done and the laundry washed. And this was all entirely her responsibility. He said the first day he didn't see anything and the second day he didn't see anything, but by the third day some of the swelling had gone down so he could see a little out of his left eye!

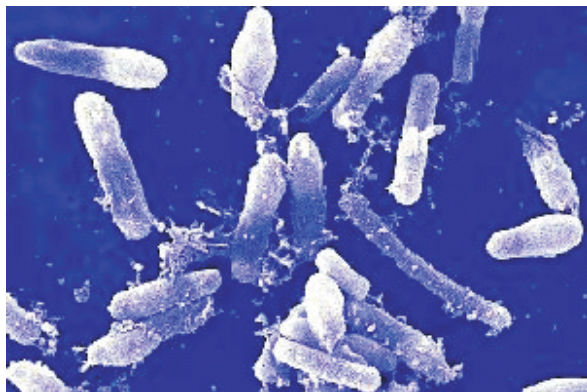
Husband: Darling!!! Tumhara Naam Haath Pe Likhu Ya Dil Pe??

Wife: Idhar Udhar Kyun Likhte Ho ? Agar Sachcha Pyar Karte Ho Toh Seedha Apne Property Ke Papers Pe Likho !!!

Great Quotes on Sales

1. "Every choice you make has an end result." – Zig Ziglar
2. "The difference between try and triumph is just a little umph!" – Marvin Phillips
3. "The secret of man's success resides in his insight into the moods of people, and his tact in dealing with them." – J. G. Holland
4. "Leadership is doing what is right when no one is watching." – George Van Valkenburg
5. "Do not let what you cannot do interfere with what you can do." – John Wooden
6. "What helps luck is a habit of watching for opportunities, of having a patient but restless mind, of sacrificing one's ease or vanity, or uniting a love of detail to foresight, and of passing through hard times bravely and cheerfully." – Victor Cherbuliez
7. "Timid salesmen have skinny kids." – Zig Ziglar
8. "I have never worked a day in my life without selling. If I believe in something, I sell it, and I sell it hard." – Estée Lauder
9. "You don't close a sale; you open a relationship if you want to build a long-term, successful enterprise." – Patricia Fripp
10. "If we had no winter, the spring would not be so pleasant: if we did not sometimes taste of adversity, prosperity would not be so welcome." – Josh Billings
11. "People don't buy for logical reasons. They buy for emotional reasons." – Zig Ziglar
12. "In order to succeed, we must first believe that we can." – Nikos Kazantzakis
13. "Enthusiasm is the inspiration of everything great. Without it no man is to be feared, and with it none despised." – Christian Nestell Bovee
14. "If you are not taking care of your customer, your competitor will." – Bob Hooley
15. "There are no shortcuts to any place worth going." – Beverly Sills
16. "Catch a man a fish, and you can sell it to him. Teach a man to fish, and you ruin a wonderful business opportunity." – Karl Marx
17. It's easier to explain price once than to apologize for quality forever. – Zig Ziglar
18. "Art is making something out of nothing and selling it." – Frank Zappa
19. "On any given Monday I am one sale closer and one idea away from being a Millionaire." – Larry D. Turner
20. "The man who will use his skill and constructive imagination to see how much he can give for a dollar, instead of how little he can give for a dollar, is bound to succeed." – Henry Ford
21. "If you learn from defeat, you haven't really lost." – Zig Ziglar
22. "As you travel down life's highway... whatever be your goal, you cannot sell a doughnut without acknowledging the hole." – Harold J. Shayler
23. "The most unprofitable item ever manufactured is an excuse." – John Mason
24. "Success is the ability to go from failure to failure without losing your enthusiasm." – Winston Churchill

Bacillus Cereus



Domain	: Bacteria
Phylum	: Firmicutes
Class	: Bacilli
Order	: Bacillales
Family	: Bacillaceae
Genus	: Bacillus
Species Group	: <i>Bacillus cereus</i> group

Description and significance

Bacillus cereus is a large, 1 x 3-4 μm , Gram-positive, rod-shaped, endospore forming, facultative aerobic bacterium. It was first successfully isolated in 1969 from a case of fatal pneumonia in a male patient and was cultured from the blood and pleural fluid. 16s rRNA comparison reveals *Bacillus cereus* to be most related to *Bacillus anthracis*, the cause of anthrax, and *Bacillus thuringiensis*, an insect pathogen used as pesticide. Although they have similar characteristics, they are distinguishable as *B. cereus* is most motile, *B. thuringiensis* produces crystal toxins, and *B. anthracis* is nonhemolytic.

B. cereus is mesophilic, growing optimally at temperatures between 20°C and 40°C, and is capable of adapting to a wide range of environmental conditions. It is distributed widely in nature and is commonly found in the soil as a saprophytic organism. *B. cereus* is also a contributor to the microflora of insects, deriving nutrients from its host, and is found in the rhizosphere of some plants.

As a soil bacterium, *B. cereus* can spread easily to many types of foods such as plants, eggs, meat, and dairy products, and is known for causing 2-5 % of food-borne intoxications due to its secretion of emetic toxins and enterotoxins. Food poisoning occurs when food is left without refrigeration for several hours before it is served. Remaining spores of contaminated food from heat treatment grow well after cooling and are the source of food poisoning.

In addition, *Bacillus cereus* is an opportunistic human pathogen and is occasionally associated with infections, causing periodontal diseases and other more serious infections. Immunocompromised patients are susceptible to bacteremia, endocarditis, meningitis, pneumonia, and endophthalmitis. Its potential to cause systemic infections are of current public health and biomedical concerns. Thus, the genome sequence of *Bacillus cereus* is significant in order to establish genetic background information for future investigations. Sequencing its genome is vital to expand understanding of its pathogenicity for treatment and for the development of antimicrobial drugs. Additionally,

since *Bacillus cereus* strains are so genetically closely related to *B. anthracis*, genomic comparisons between the two species are important to the study of *B. anthracis* virulence.

Cell structure and metabolism

4.1 Cell Structure

Bacillus cereus is a 1 x 3-4 μm , rod shaped, Gram- positive bacterium. Its cell structure consists of an inner membrane and a thick peptidoglycan which functions to maintain cell shape [10]. The polysaccharide portion makes up 50% percent of the cell wall and consists of a neutral polysaccharide composed of N-acetylglucosamine, N-acetylmannosamine (ManNac), N-acetylgalactosamine and glucose in a molar ratio of 4: 1: 1: 1. The acidic portion of the cell wall is characteristic in having a repeating tetrasaccharide unit. 5% of the cell wall is made up of teichoic acids consisting of N-acetylglucosamine, galactose, glycerol, and phosphorus in a molar ratio of 1: 1.4: 1: 1. The linkage between the polysaccharide and peptidoglycan is a muramic acid 6-phosphate. The peptidoglycan of some *B. cereus* strains are unique with only a few oligomers present, the cross-linked mucopeptides are dimmers, and many of the mucopeptide lack the N-acetyl group. These distinguishing features affect cell surface charge which contributes to the attachment of an outer capsule or an S-layer in pathogenic strains.

Clinical isolates of *B. cereus* have a glycoprotein S-layer over its peptidoglycan which consists of proteinaceous paracrystalline arrays and covers the cell surface. The S-layer is involved in the virulence of *B. cereus* and functions to promote interactions with human polymorphonuclear leucocytes. It also enables *B. cereus* to adhere to laminin, type I collagen, fibronectin, and fibrinogen of the epithelium, and thus has a role in increasing interaction between *B. cereus* and its host. In addition, this proteinaceous layer enhances its resistance to radiation.

B. cereus is motile by means of flagella and exhibits two types of motility including swimming and swarming, depending on the environment. Single cells exhibit swimming motility by means of short flagellated rods. On the other hand, swarming is a collective movement of swarm cells with flagellum that is observed to be three to four times longer, and also forty times more flagellated than single swimming cells.

4.2 Spore Structure

B. cereus spore formation occurs when nutrients are scarce within the environment and germinate into vegetative cells once they are available. Therefore, spore structure is important to the survival of this bacterium. *B. cereus* spores consist of an inner core surrounded by the inner membrane, and outer cortex surrounded by the outer membrane with an additional exterior coat. The spore coat is made of proteins and small amounts of lipids and carbohydrates which contribute to its resistance to oxidizing agents and chemicals by blocking toxic molecules. In addition, the outer spore structure allows them to be heat and γ -radiation resistant. Spore germination is commonly in response to L-alanine which stimulates germination events including hydrations of spores, loss of Ca^{2+} and dipicolinic acid, and metabolism.

4.3 Metabolism

B. cereus is a facultative aerobe so it can utilize oxygen as a

terminal electron acceptor, but also has methods of anaerobic respiration as a mechanism of energy release. Whole genome sequencing revealed genes encoding for metabolic enzymes such as NADH dehydrogenases, succinate dehydrogenase, complex III, non-proton-pumping cytochrome bd quinol oxidases, and proton-pumping oxidases such as cytochrome c oxidase and cytochrome aa3 quinol oxidase.

In aerobic respiration, reducing equivalents produced from glycolysis and the Krebs cycle are reoxidized by the electron transport chain, creating a proton motive force and ATP by ATP synthase. In anaerobic respiration, *B. cereus* utilizes fermentation to generate energy. Fermentation recycles NAD⁺ by reducing pyruvate and produces lactate and ethanol. ATP is generated by substrate level phosphorylation.

B. cereus can metabolize a variety of compounds including carbohydrates, proteins, peptides and amino acids for growth and energy. Some of the major products produced from carbon sources such as sucrose or glucose during anaerobic respiration include L-lactate, acetate, formate, succinate, ethanol, and carbon dioxide. During nitrate respiration, nitrate reductase converts nitrate into nitrite which is converted to ammonium by nitrite reductase.

Pathology

Bacillus cereus causes two types of food poisoning in humans including diarrhoeal syndrome and emetic syndrome. Food poisoning results from its production of enterotoxins in the gastrointestinal tract. The dosage of ingested *B. cereus* spores leading to diarrhoeal syndrome is 105–107 g l of ingested food, and 105–108 g l of ingested food for emetic syndrome. Enterotoxins associated with diarrhoeal syndrome are unresistant to the acidic conditions of the stomach. However, the cereulide peptide toxin associated with emetic syndrome is more resistant to acidic conditions and remains active at 121 °C.

Virulence factors associated with diarrhoeal syndrome involve three enterotoxins including hemolysin BL (HBL), non-hemolytic enterotoxin (NHE), and cytotoxin K. The main virulence factor of *B. cereus* is HBL which is made of the three proteins B, L1, and L2. Symptoms of diarrhoeal syndrome include watery diarrhea, abdominal cramps, and pain occurring 6–15 hours after ingestion which may last for twenty-four hours. The emetic syndrome is caused by cereulide peptide toxin which is secreted during stationary phase. This toxin has a ring structure, dodecadepsipeptide, which consists of four amino acids, repeating three times, and oxy acids. Symptoms associated with emetic syndrome include nausea and vomiting within half an hour to six hours after ingestion of food and also lasts for about twenty-four hours.

Although *B. cereus* is commonly known to cause food-borne intoxications, it has been reported to cause local and systemic infections, as an opportunistic pathogen, especially among immunocompromised patients, newborns, and patients with surgical wounds. *B. cereus* can cause ocular infections such as keratitis, endophthalmitis, and panophthalmitis. The main virulence factor in *B. cereus* endophthalmitis is HBL which can result in the detachment of the retina and blindness. In addition, *B. cereus* can cause gangrene, bovine mastitis, pyogenic infections, cellulitis, infant death, septic meningitis, periodontal disease, lung abscesses, and endocarditis. However, these infections are less common. Virulence factors associated with non gastrointestinal infections include hemolysins and phospholipase C. Hemolysin III causes erythrocyte lysis. Phospholipase C causes tissue damage by stimulating degranulation of human

neutrophil, and breaks down the subepithelial matrix affecting the healing of tissue in infections.

Application to Biotechnology

7.1 Biological Control Agent

Biological control agents are alternatives to chemical pesticides that are capable of suppressing plant pests and can also enhance plant growth. Most strains of *Bacillus cereus* produce toxins known to cause food-borne illnesses. However, there are strains that do not produce the HBL enterotoxin. Thus, antifungal compounds of *Bacillus cereus* strains have been developed as a useful biological control agent in the suppression of fungi and crop disease.

7.2 *Bacillus cereus* Strains

Bacillus cereus B4 is used as a pesticide to deter fungi from rotting seedling plants. This strain produces three types of metabolites that enable it to suppress certain plant diseases and promote plant growth. The metabolites include Kanosamine, 3, 4-dihydroxy benzoate, and 2 keto-4 methylthiobutyrate. The metabolite 3,4-DOHB improves crop strength and results in a healthier and larger root system.

Bacillus cereus DGA34 was isolated from the environment and is useful in fighting off fungal damping disease in crops. It naturally produces the antibiotic zwittermicin A. by fermentation which is found in the supernatant fluid of its culture medium. This antibiotic is effective in fighting a wide range of fungi and bacteria, and reduces symptoms of damping-off disease and root rot.

Bacillus cereus UW85 produces two antibiotic toxins including zwittermicin A. and antibiotic B. *Bacillus cereus* UW85 is used to protect alfalfa seeds from dampening off caused by *Phytophthora medicaginis*, cucumber fruits from *Pythium aphanidermatum*, peanuts from *Sclerotinia minor*, and tobacco seedlings from *Phytophthora nicotianae*. These are just a few *B. cereus* strains with current United States patents as biocontrol agents for crop diseases. There are currently many *Bacillus cereus* strains that are still under review.

Current Research

8.1 Biofilms of *B. cereus*

The ability of *B. cereus* to form biofilms on surfaces can cause potential contamination problems within the food industry. Therefore, biofilm formation of several *B. cereus* strains are currently being studied to prevent potential food contamination and to ensure safety during production. In a recent study, microtiter assay and assays on stainless steel were completely or partially submerged in liquid in order to observe *B. cereus* biofilm formation. Since stainless steel is commonly used for pipes and tanks in the food industry, additional tests were conducted to investigate *B. cereus* biofilm formation from spores on stainless steel coupons. The results from both tests were similar. It appears that *B. cereus* biofilms preferentially form within the air-liquid interface. This tendency is due to the availability of oxygen in this region, causing bacterial movement toward oxygen. In addition, spore formation was more rapid in the suspension phase of biofilm formation suggesting that biofilms are a cavity for sporulation. The results show that *B. cereus* biofilms may develop within storage and piping systems when either partially filled or when liquid residues remain during production. In addition, increase in spore formation by *B. cereus* within biofilms can potentially cause recontamination and equipment failure during food production.

8.2 Effects of Porcine Bile on *B. cereus*

Resistance to bile is important to the survival of *B. cereus* within the small intestine where it can proliferate and release enterotoxins. On going research projects are being conducted to test *B. cereus* and factors that may affect its growth and release of enterotoxins once ingested. A recent study tested the effects of porcine bile (PB) on *B. cereus* and on its HBL enterotoxin in the small intestine by using intestine media with different food types. Different concentrations of porcine bile were added to the gastric media to simulate acid stress. Results show that the growth of *B. cereus* was affected by the type of food in the small intestine media which may be explained by the protective effect of different types of food against porcine bile. For instance, components of food such as fiber can bind to bile salts reducing their toxic effects on *B. cereus*. In addition, bile salts may be sequestered by food components that lower cholesterol levels. It was concluded that the tolerance of *B. cereus* to porcine bile and its ability to grow and produce toxins is dependent on the type of food and on bile concentrations in the small intestine. Additional studies are being conducted to test the effects of bile on *B. cereus* enterotoxin as well as the effects of different carbon sources on enterotoxin release.

8.3 Virulence Factors of *B. cereus*

B. cereus has been a growing and established opportunistic human pathogen. Therefore, current research is being conducted to understand its pathogenicity and virulence factors in order to find potential targets for antimicrobial drugs. In a recent study conducted, *B. cereus fur* gene, a transcriptional regulator that is responsible for bacterial iron uptake and metabolism, was shown to reduce virulence in the pathogenic *fur* mutant. Results in this investigation revealed a decrease in the regulation of iron uptake with three times more intercellular iron in the *fur* mutant than in

the wild-type, resulting in a greater amount of oxidizing free radicals. The virulence of *B. cereus fur* mutant was measured in an insect infection which revealed the significance of iron metabolism regulation in bacterial pathogens by its reduced virulence. This recent study gives insight to the importance of the *fur* gene in the regulation of iron concentrations for cell growth, survival, and pathogenesis. The reduced virulence of *B. cereus fur* mutants in this experiment demonstrates the potential of the *fur* gene to be a good target for antimicrobial drugs as a conserved protein among pathogenic bacteria.

8.4 *B. cereus* Endophthalmitis

B. cereus causes endophthalmitis which can lead to blindness. There is currently no universal treatment for this disease. Various research projects are being conducted by the Callegan Lab to understand its pathogenicity and virulence in order to develop better treatment and antibiotics.

One current research project is studying the role of *B. cereus* proteases in endophthalmitis. It appears that strains without metalloproteases InhA and InhA2, immune inhibitors, were more virulent than the wild-type strain. Further studies are being conducted to determine why.

Another ongoing research project involves the study of the *plcR* gene which regulates toxin production and has an important role in *B. cereus* endophthalmitis pathogenesis. The *plcR* mutant strain exhibited a reduction in virulence which resulted in 20% retained retinal function. The effects of blocking the peptide *PapR* as a potential form of treatment is being investigated.

Cell wall structures of *B. cereus* including peptidoglycan, S-layer, capsules, and teichoic acids are being studied for their ability to cause intraocular inflammation. Recognition of these cell wall structures by retinal cells are being researched.

Disinfectants in Drinking Water: Chloramine, Chlorine and Chlorine Dioxide

To protect drinking water from disease-causing organisms, or pathogens, water suppliers often add a disinfectant, such as chlorine, to drinking water. However, disinfection practices can be complicated because certain microbial pathogens, such as *Cryptosporidium*, are highly resistant to traditional disinfection practices. Also, disinfectants themselves can react with naturally-occurring materials in the water to form byproducts, such as

trihalomethanes and haloacetic acids, which may pose health risks.

A major challenge for water suppliers is how to control and limit the risks from pathogens and disinfection byproducts. It is important to provide protection from pathogens while simultaneously minimizing health risks to the population from disinfection byproducts.

What are disinfectants, how are they used, and what are their health effects in drinking water at levels above the maximum residual disinfectant level?

Disinfectant (Chemical Abstract Service Registry Number)	Definition and uses	Service Registry Number)
Chloramine (as Cl ₂) (10599-90-3)	Chloramine (as Cl ₂) is a water additive used to control microbes, particularly as a residual disinfectant in distribution system pipes. It is formed when ammonia is added to water containing free chlorine. Monochloramine is one form of chloramines commonly used for disinfection by municipal water systems. Other chloramines (di- and tri-) are not intentionally used to disinfect drinking water and are generally not formed during the drinking water disinfection process.	Some people who use water containing chloramine in excess of the maximum residual disinfectant level could experience irritating effects to their eyes and nose, stomach discomfort or anemia.
Chlorine (as Cl ₂) (10049-04-4)	The gaseous or liquid form of chlorine (Cl ₂) is a water additive used by municipal water systems to control microbes. It is relatively inexpensive and has the lowest production and operating costs and longest history for large continuous disinfection operations. Chlorine is a powerful oxidant.	Some people who use water containing chlorine well in excess of the maximum residual disinfectant level could experience irritating effects to their eyes and nose. Some people who drink water containing chlorine well in excess of the maximum residual disinfectant level could experience stomach discomfort.
Chlorine dioxide (as ClO ₂) (10049-04-4)	Chlorine dioxide is a water additive used to control microbes and can be used to control tastes and odors. It rapidly disappears from stored water.	Some infants, young children, and fetuses of pregnant women who drink water containing chlorine dioxide in excess of the maximum residual disinfectant level could experience nervous system effects. Some people who drink water containing chlorine dioxide well in excess of the MRDL for many years may experience anemia.

This health effects language is not intended to catalog all possible health effects for disinfectants. Rather, it is intended to inform consumers of some of the possible health effects associated with disinfectants in drinking water when the rule was finalized.

What are EPA's drinking water regulations for disinfectants?

In 1974, Congress passed the Safe Drinking Water Act. This law requires EPA to determine the level of residual disinfectants in drinking water at which no adverse health effects are likely to occur. These non-enforceable health goals, based solely on possible health risks and exposure over a lifetime, with an adequate margin of safety, are called maximum residual disinfectant level goals (MRDLG). Contaminants are any physical, chemical, biological or radiological substances or matter in water. EPA sets MRDLGs based on the best available science to prevent potential health problems.

Based on the MRDLG, EPA has set enforceable regulations for disinfectants, called a maximum residual disinfectant level (MRDL), at the following levels:

Disinfectant	MRDLG	MRDL
Chloramine	4 milligrams per liter (mg/L) or 4 parts per million (ppm)	4.0 mg/L or 4 ppm as an annual average
Chlorine	4 mg/L or 4 ppm	4.0 mg/L or 4 ppm as an annual average
Chlorine Dioxide	0.8 mg/L or 800 parts per billion (ppb)	0.8 mg/L or 800 ppb

MRDLs are set as close to the health goals as possible, considering cost, benefits and the ability of public water systems to detect and remove contaminants using suitable treatment technologies. In this case, the MRDL equals the MRDLG, because analytical methods or treatment technology do not pose any limitation. States may set more stringent drinking water MRDLGs and MRDLs for disinfectants than EPA.

The following drinking water regulations apply to disinfectants and disinfection byproducts:

■ **Stage 1 Disinfectants and Disinfection Byproducts Rule (Stage 1 DBP)** (December 16, 1998)

The Stage 1 Disinfectants and Disinfection Byproducts Rule

reduces exposure to disinfection byproducts for customers of community water systems and non-transient non-community systems, including those serving fewer than 10,000 people, that add a disinfectant to the drinking water during any part of the treatment process.

■ **Stage 2 Disinfectants and Disinfection Byproducts Rule (Stage 2 DBP)** (December 15, 2005)

Stage 2 DBP rule builds upon earlier rules that addressed disinfection byproducts to improve your drinking water quality and provide additional public health protection from disinfection byproducts.

The Safe Drinking Water Act requires EPA to periodically review the national primary drinking water regulation for each contaminant and revise the regulation, if appropriate, based on new scientific data. EPA will include the Disinfectants and Disinfection Byproducts rules in future review cycles.

How will I know if disinfectants are in my drinking water?

Public water systems using surface water or ground water under the direct influence of surface water are required to maintain a detectable disinfectant residual in the distribution system. When routine monitoring indicates that disinfectant levels are above the MRDL, your water supplier must take steps to reduce the amount of disinfectant so that it is below that level. For chlorine dioxide, water suppliers must notify their customers as soon as practical, but no later than 24 hours after the system learns of the violation. For chloramine and chlorine, water suppliers must notify their customers as soon as practical, but no later than 30 days after the system learns of the violation. Additional actions, such as providing alternative drinking water supplies, may be required to prevent serious risks to public health.

How are disinfectants controlled in my drinking water?

The following treatment method(s) have proven to be effective for removing chloramines, chlorine, and chlorine dioxide to below their MRDLs: control of treatment processes to reduce disinfectant demand and control of disinfection treatment processes to reduce disinfectant levels.

Antiseptics and Areas of concern (Part-1)

Alcohols

Although several alcohols have been shown to be effective antimicrobials, ethyl alcohol (ethanol), isopropyl alcohol (isopropanol, propan-2-ol) and n-propanol are the most widely used. Alcohols exhibit rapid broad-spectrum antimicrobial activity against vegetative bacteria (including mycobacteria), viruses, and fungi but are not sporicidal. They are, however, known to inhibit sporulation and spore germination, but this effect is reversible. Because of the lack of sporicidal activity, alcohols are not recommended for sterilization but are widely used for both hard surface disinfection and skin antisepsis. Lower concentrations may also be used as preservatives and to potentiate the activity of other biocides. Many alcohol products include low levels of other biocides (in particular chlorhexidine), which remain on the skin following evaporation of the alcohol, or excipients (including emollients), which decrease the evaporation time of the alcohol and can significantly increase product efficacy. In general, isopropyl alcohol is considered slightly more efficacious against bacteria and ethyl alcohol is more potent against viruses; however, this is dependent on the concentrations of both the active agent and the test microorganism. For example, isopropyl alcohol has greater lipophilic properties than ethyl alcohol and is less active against hydrophilic viruses (e.g., poliovirus). Generally, the antimicrobial activity of alcohols is significantly lower at concentrations below 50% and is optimal in the 60 to 90% range. Little is known about the specific mode of action of alcohols, but based on the increased efficacy in the presence of water; it is generally believed that they cause membrane damage and rapid denaturation of proteins, with subsequent interference with metabolism and cell lysis. This is supported by specific reports of denaturation of *Escherichia coli* dehydrogenases and an increased lag phase in *Enterobacter aerogenes*, speculated to be due to inhibition of metabolism required for rapid cell division.

Anilides

The anilides have been investigated primarily for use as antiseptics, but they are rarely used in the clinic. Triclocarban (TCC; 3,4,4-trichlorocarbanilide) is the most extensively studied in this series and is used mostly in consumer soaps and deodorants. TCC is particularly active against gram-positive bacteria but significantly less active against gram-negative bacteria and fungi and lacks appreciable substantivity (persistency) for the skin. The anilides are thought to act by adsorbing to and destroying the semipermeable character of the cytoplasmic membrane, leading to cell death.

Chlorhexidine

Chlorhexidine is probably the most widely used biocide in antiseptic products, in particular in handwashing and oral products but also as a disinfectant and preservative. This is due in particular to its broad-spectrum efficacy, substantivity for the skin, and low irritation. Of note, irritability has been described and in many cases may be product specific. Despite the

advantages of chlorhexidine, its activity is pH dependent and is greatly reduced in the presence of organic matter. A considerable amount of research has been undertaken on the mechanism of the antimicrobial action of this important bisbiguanide, although most of the attention has been devoted to the way in which it inactivates nonsporulating bacteria. Nevertheless, sufficient data are now available to examine its sporostatic and mycobacteriostatic action, its effects on yeasts and protozoa, and its antiviral activity. Chlorhexidine is a bactericidal agent. Its interaction and uptake by bacteria were studied initially by Hugo et al., who found that the uptake of chlorhexidine by *E. coli* and *S. aureus* was very rapid and depended on the chlorhexidine concentration and pH. More recently, by using [¹⁴C] chlorhexidine gluconate, the uptake by bacteria and yeasts was shown to be extremely rapid, with a maximum effect occurring within 20s. Damage to the outer cell layers takes place but is insufficient to induce lysis or cell death. The agent then crosses the cell wall or outer membrane, presumably by passive diffusion, and subsequently attacks the bacterial cytoplasmic or inner membrane or the yeast plasma membrane. In yeasts, chlorhexidine “partitions” into the cell wall, plasma membrane, and cytoplasm of cells. Damage to the delicate semipermeable membrane is followed by leakage of intracellular constituents, which can be measured by appropriate techniques. Leakage is not per se responsible for cellular inactivation but is a consequence of cell death. High concentrations of chlorhexidine cause coagulation of intracellular constituents. As a result, the cytoplasm becomes congealed, with a consequent reduction in leakage, so that there is a biphasic effect on membrane permeability. An initial high rate of leakage rises as the concentration of chlorhexidine increases, but leakage is reduced at higher biocide concentrations because of the coagulation of the cytosol. Chlorhexidine was claimed by Harold et al. to be an inhibitor of both membrane-bound and soluble ATPase as well as of net K⁺ uptake in *Enterococcus faecalis*. However, only high biguanide concentrations inhibit membrane-bound ATPase, which suggests that the enzyme is not a primary target for chlorhexidine action. Although chlorhexidine collapses the membrane potential, it is membrane disruption rather than ATPase inactivation that is associated with its lethal effects. The effects of chlorhexidine on yeast cells are probably similar to those previously described for bacteria. Chlorhexidine has a biphasic effect on protoplast lysis, with reduced lysis at higher biguanide concentrations. Furthermore, in whole cells, the yeast cell wall may have some effect in limiting the uptake of the biguanide. The findings presented here and elsewhere demonstrate an effect on the fungal plasma membrane but with significant actions elsewhere in the cell. Increasing concentrations of chlorhexidine (up to 25 mg/ml) induce progressive lysis of *Saccharomyces cerevisiae* protoplasts, but higher biguanide concentrations result in reduced lysis. Work to date suggests that chlorhexidine has a similar effect on the trophozoites of *Acanthamoeba castellanii*, with the cysts being less sensitive. Furr reviewed the effects of chlorhexidine and

other biocides on *Acanthamoeba* and showed that membrane damage in these protozoa is a significant factor in their inactivation. Mycobacteria are generally highly resistant to chlorhexidine. Little is known about the uptake of chlorhexidine (and other antiseptics and disinfectants) by mycobacteria and on the biochemical changes that occur in the treated cells. Since the MICs for some mycobacteria are on the order of those for chlorhexidine-sensitive, gram-positive cocci, the inhibitory effects of chlorhexidine on mycobacteria may not be dissimilar to those on susceptible bacteria. *Mycobacterium avium* intracellularly is considerably more resistant than other mycobacteria. Chlorhexidine is not sporicidal. Even high concentrations of the bisbiguanide do not affect the viability of *Bacillus* spores at ambient temperatures, although a marked sporicidal effect is achieved at elevated temperatures. Presumably, sufficient changes occur in the spore structure to permit an increased uptake of the biguanide, although this has yet to be shown experimentally. Little is known about the uptake of chlorhexidine by bacterial spores, although coatless forms take up more of the compound than do "normal" spores. Chlorhexidine has little effect on the germination of bacterial spores but inhibits outgrowth. The reason for its lack of effect on the former process but its significant activity against the latter is unclear. It could, however, be reflected in the relative uptake of chlorhexidine, since germinating cells take up much less of the bisbiguanide than do outgrowing forms. Binding sites could thus be reduced in number or masked in germinating cells. The antiviral activity of chlorhexidine is variable. Studies with different types of bacteriophages have shown that chlorhexidine has no effect on MS2 or K coliphages. High concentrations also failed to inactivate *Pseudomonas aeruginosa* phage F116 and had no effect on phage DNA within the capsid or on phage proteins; the transduction process was more sensitive to chlorhexidine and other biocides than was the phage itself. This substantiated an earlier finding that chlorhexidine bound poorly to F116 particles. Chlorhexidine is not always considered a particularly effective antiviral agent, and its activity is restricted to the lipid-enveloped viruses. Chlorhexidine does not inactivate nonenveloped viruses such as rotavirus, HAV, or poliovirus. Its activity was found by Ranganathan to be restricted to the nucleic acid core or the outer coat, although it is likely that the latter would be a more important target site.

Alexidine

Alexidine differs chemically from chlorhexidine in possessing ethylhexyl end groups. Alexidine is more rapidly bactericidal and produces a significantly faster alteration in bactericidal permeability. Studies with mixed-lipid and pure phospholipid vesicles demonstrate that, unlike chlorhexidine, alexidine produces lipid phase separation and domain formation. It has been proposed that the nature of the ethylhexyl end group in alexidine, as opposed to the chlorophenol one in chlorhexidine, might influence the ability of a biguanide to produce lipid domains in the cytoplasmic membrane.

Triclosan

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether; exhibits particular activity against gram-positive bacteria. Its efficacy against gram-negative bacteria and yeasts can be significantly enhanced by formulation effects. For example, triclosan in combination with EDTA caused increased permeability of the outer membrane. Reports have also suggested that in addition to its antibacterial properties, triclosan may have anti-inflammatory activity. The specific mode of action of triclosan is unknown, but it has been suggested that the primary effects are on the cytoplasmic membrane. In studies with *E. coli*, triclosan at subinhibitory concentrations inhibited the uptake of essential nutrients, while higher, bactericidal concentrations resulted in the rapid release of cellular components and cell death. Studies with a divalent-ion-dependent *E. coli* triclosan mutant for which the triclosan MIC was 10-fold greater than that for a wildtype strain showed no significant differences in total envelope protein profiles but did show significant differences in envelope fatty acids. Specifically, a prominent 14:1 fatty acid was absent in the resistant strain, and there were minor differences in other fatty acid species. It was proposed that divalent ions and fatty acids may adsorb and limit the permeability of triclosan to its site of action. Minor changes in fatty acid profiles were recently found in both *E. coli* and *S. aureus* strains for which the triclosan MICs were elevated; however, the MBCs were not affected, suggesting, as for other phenols, that the cumulative effects on multiple targets contribute to the bactericidal activity.

To be continued in the next issue....

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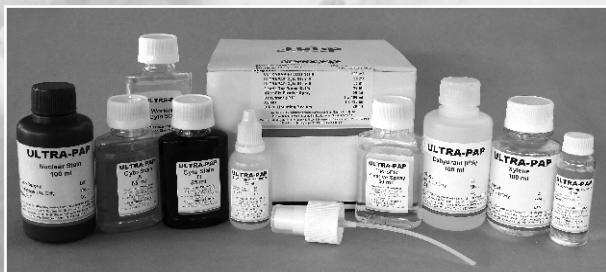
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Highlights of the coming issue

