Editorial

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In Profile Scientist – Prof. Udupi Ramachandra Rao, was an internationally renowned space scientist who made original contributions to the development of space technology in India and its extensive application to communications and remote sensing of natural resources.

Bug of the month – *Bartonella quintana* infection (historically called 'trench fever') is a vector-borne disease primarily transmitted by the human body louse *Pediculus humanus humanus*. The infection is associated with a wide variety of clinical conditions, including chronic bacteraemia, endocarditis, lymphadenopathy and bacillary angiomatosis. Since the 1990s, it has been recognised as a reemerging pathogen among impoverished and homeless populations — so-called 'urban trench fever' — living in unsanitary conditions and crowded areas predisposing them to infestation with ectoparasites that may transmit the infection.

Did You Know? - Since 2000, progress in malaria control has resulted primarily from expanded access to vector control interventions, particularly in sub-Saharan Africa. However, these fragile gains are threatened by emerging resistance to insecticides among *Anopheles* mosquitoes. Left unchecked, insecticide resistance could lead to a substantial increase in malaria incidence and mortality. Urgent action is required to prevent the further development of resistance and to maintain the effectiveness of existing vector control interventions.

Best Practices - Gram staining is an empirical method differentiating bacterial species into two large groups (Gram positive and Gram negative) based on the chemical and physical properties of their cell walls. The method is named after its inventor, the Danish scientist Hans Christian Gram (1853-1938), who developed the technique in 1884 to discriminate between *pneumococci* and *Klebsiella pneumoniae* bacteria.

Blood Culture

Blood culture is a microbiological culture of blood. It is employed to detect infections that are spreading through the bloodstream. This is possible because the bloodstream is usually a sterile environment. The laboratory detection of bacteremia and fungemia remains one of the most important functions of clinical microbiology laboratories. A positive blood culture establishes or confirms that there is an infectious etiology of the patient's illness. Moreover, it provides the etiologic agent and allows antibiotic susceptibility testing for optimization of therapy.

Introduction

The blood does not normally have any bacteria or fungi in it. A blood culture is a test that checks for foreign invaders like bacteria, yeast, and other microorganisms in your blood. Having these pathogens in your bloodstream can be a sign of a blood infection, a condition known as Bacteremia. This type of infection involves the blood that circulates within your entire body. Bacteria that start on your skin or in your lungs, urine, or gastrointestinal tract are common sources of blood infections.

An infection can spread to your blood and become systemic if it's severe or if your immune system is not able to keep it contained. A systemic infection is known as Sepsis. A blood infection may also develop when the immune system is weak. This can occur in infants and older adults, and from disease or from medicines.

The laboratory detection of bacteremia and fungemia using **blood culture vials (Colorcult™)** is one of the most simple and commonly used investigations to establish the etiology of bloodstream infections. Rapid, accurate identification of the bacteria or fungi causing bloodstream infections provides vital clinical information required to diagnose and treat sepsis. Sepsis is a complex inflammatory process that is largely under recognized as a major cause of morbidity and mortality worldwide. There are an estimated 19 million cases worldwide each year, meaning that sepsis causes 1 death every 3-4 seconds. Colorcult™ culture vials comprise of highly nutritious media intended for the growth of significant pathogenic microorganisms including fastidious microorganisms present in blood and other body fluids. The samples to be tested are inoculated in Colorcult™ culture vials. These vials are kept in incubator and monitored periodically for change in color of the CO_2 sensor which is at the base of the bottle for positive reporting.

Positive   Negative
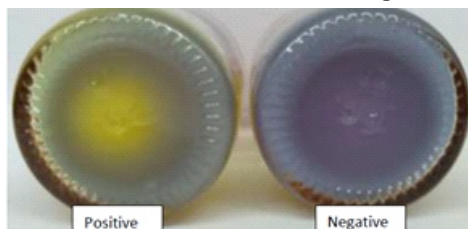
Definitions of Important terms in Blood Culture

Bacteremia: the presence of bacteria in the blood. It may be transient, intermittent or continuous.

Blood culture: blood specimen submitted for culture of microorganisms. It enables the recovery of potential pathogens from patients suspected of having bacteremia or fungemia.

Blood culture series: a group of temporally related blood cultures that are collected to determine whether a patient has bacteremia or fungemia.

Blood culture set: the combination of blood culture bottles (one aerobic and one anaerobic) into which a single blood collection is inoculated.

Bloodstream Infection (BSI): an infection associated with bacteremia or fungemia.

Contaminant: a microorganism isolated from a blood culture that was introduced during specimen collection or processing and is not considered responsible for BSI (i.e., the isolates were not present in the patient's blood when the blood was sampled for culture).

Contamination: presence of microorganisms in the bottle that entered during sampling but were not actually circulating in the patient's bloodstream.

Fungemia: the presence of fungi in the blood.

Sepsis: life-threatening organ dysfunction caused by a dysregulated host response to infection.

Septicemia: clinical syndrome characterized by fever, chills, malaise, tachycardia, etc. when circulating bacteria multiply at a rate that exceeds removal by phagocytosis.

Septic episode: an episode of sepsis or septic shock for which a blood culture or blood culture series is drawn.

Septic shock: a subset of sepsis in which underlying circulatory and cellular metabolism abnormalities are profound enough to substantially increase mortality.

Colorcult™ Blood culture media

Colorcult™ culture media contains various types of proteins and other nutrients necessary to support the growth of fastidious and non-fastidious microorganisms present in blood and other body fluids. Sodium Polyanethol Sulfonate (SPS) is a polyanionic anticoagulant, which inhibits complements and lysozyme activity, interferes with phagocytosis and inactivates many antibiotics. It is generally considered to enhance the rate and speed of bacterial isolations by counter-acting the bacterial inhibitors of human blood. After thorough and gentle mixing, the specimen is added to the Colorcult™ culture vial. The vial is incubated and observed for color change of CO_2 sensor as evidence of microbial growth.

Each vial contains a chemical sensor at the bottom which can detect increase in CO_2 produced by the growth of microorganisms. The sensor is monitored visually for color change which is proportional to the amount of CO_2 present. A positive color change indicates the presumptive presence of viable microorganisms in the vial. Resins have been incorporated in the Colorcult™ culture vials to enhance recovery of organisms without a need for special processing. Growth in the Colorcult™ culture vials is indicated by the color change of the chemical sensor at the bottom from blue to bright yellow. For vials with no growth shows no color change of the chemical sensor. They remain blue in color. The vials should be observed for 5-7 days for positive color change before reporting it negative.

Colorcult™ Blood Culture Vials Available in 3 Variants:

Colorcult™ Aerobic Culture Vial (203031050030)

Colorcult™ Pediatric Culture Vial (203031070030)

Colorcult™ Anaerobic Culture Vial (203031060030)

When should a blood culture be performed?

Blood cultures should always be requested when a bloodstream infection or sepsis is suspected. Clinical symptoms in a patient which may lead to a suspicion of a bloodstream infection are:

- undetermined fever (≥38°C) or hypothermia (≤36°C)
- shock, chills, rigors
- Severe local infections (meningitis, endocarditis, pneumonia, pyelonephritis, intra-abdominal suppuration).
- Abnormally raised heart rate
- low or raised blood pressure
- raised respiratory rate

Blood should be collected:

As soon as possible after the onset of clinical symptoms; ideally, prior to the administration of antimicrobial therapy. If the patient is already on antimicrobial therapy, recovery of microorganisms may be increased by collecting the blood sample immediately before administering the next dose and by inoculating the blood into bottles containing specialized antimicrobial neutralization media.

What volume of blood should be collected?

The optimal recovery of bacteria and fungi from blood depends on culturing an adequate volume of blood. The collection of a sufficient quantity of blood improves the detection of pathogenic bacteria or fungi present in low quantities. This is essential when an endovascular infection (such as endocarditis) is suspected.

Blood culture bottles are designed to accommodate the recommended blood to-broth ratio with optimal blood volume. Commercial continuously monitoring blood culture systems may use a smaller blood-to-broth ratio due to the addition of sodium polyanetholsulfonate (SPS) which inactivates inhibitory substances which are present in blood.

Adults:- For an adult, the recommended volume of blood to be obtained per culture is 20 to 30 ml. Since each set includes an aerobic and an anaerobic bottle, each bottle should be inoculated with approximately 10 ml of blood. This volume is recommended to optimize pathogen recovery when the bacterial/fungal burden is less than 1 Colony Forming Unit (CFU) per ml of blood, which is a common finding.

Pediatric:- The optimal volume of blood to be obtained from infants and children is less well prescribed, however, available data indicate that the yield of pathogens also increases in direct proportion to the volume of blood cultured. The recommended volume of blood to collect should be based on the weight of the patient. Specially formulated blood culture bottles are commercially available for use in children Colorcult™ Pediatric Vial.

Which media to use?

Microorganisms causing bloodstream infections are highly varied (aerobes, anaerobes, fungi, fastidious microorganisms) and, in addition to nutrient elements, may require specific growth factors and/or a special atmosphere. In cases where the patient is receiving antimicrobial therapy, specialized media with antibiotic neutralization capabilities should be used. Antibiotic neutralization media have been shown to increase recovery and provide faster time to detection versus standard media.

Which bottle should be inoculated first?

Inoculate the anaerobic bottle first to avoid entry of air. If the amount of blood drawn is less than the recommended volume, then approximately 10 ml of blood should be inoculated into the aerobic bottle first, since most cases of bacteremia are caused by aerobic and facultative bacteria. In addition, pathogenic yeasts and strict aerobes (e.g., *Pseudomonas*) are recovered almost exclusively from aerobic bottles. Any remaining blood should then be inoculated into the anaerobic bottle.

How many days of incubation are recommended?

The current recommendation, and standard incubation period, for routine blood cultures performed by continuous-monitoring blood systems is five days. However, published data suggest that three days may be adequate to recover over 97% of clinically significant microorganisms.

Is it a contaminant or a true pathogen?

Contamination of blood cultures during the collection process can produce a significant level of false-positive results, which can have a negative impact on patient outcome. A false positive is defined as growth of bacteria in the blood culture bottle that were not present in the patient's bloodstream, and were most likely introduced during sample collection. Contamination can come from a number of sources: the patient's skin, the equipment used to take the sample, the hands of the person taking the blood sample, or the environment.

Collecting a contaminant-free blood sample is critical to providing a blood culture result that has clinical value.

Certain microorganisms such as coagulase-negative staphylococci, viridans group streptococci, *Bacillus* spp, *Propionibacterium* spp., *diphtheroids*, *Micrococcus* spp. rarely cause severe bacterial infections or bloodstream infections. These are common skin contaminants, and a though they are capable of causing serious infection in the appropriate setting, their detection in a single blood culture set can reasonably be identified as a possible contaminant without clinical significance. However, it is important to consider that coagulase-negative staphylococci are the primary cause of both catheter and prosthetic device-associated infections and may be clinically significant in up to 20% of cases. However, even when the best blood collection protocols are used, it may not be possible to reduce the contamination rate below 2%.

Impact of contamination rates

A contaminated blood culture can result in unnecessary antibiotic therapy, increased length of hospitalization and higher costs. It has been found that each false positive result can lead to: Increased length of stay - on average 1 day. 39% increase in intravenous antibiotic charges. 20% increase in laboratory charges. 3 days longer on antibiotics.

Environmental Monitoring & Its Importance

Environmental conditions may directly or indirectly affect the quality of the finished product. An environmental monitoring program hasto be properly constituted to be of any practical use. Environmental control describes the systems functionally ensuring that clean rooms operate within predetermined limits whereas environmental monitoring describes the techniques used to measure the effectiveness of the environmental control systems and defines the procedure necessary in the event of limits being exceeded. Environmental control, particularly in sterile manufacture, is achieved by means of many factors: well designed and efficiently operated facilities and air handling systems, by the use of integral HEPA filters, well designed and well-made garments for staffs, by reliable disinfection regimes, and by rigid adherence to aseptic disciplines. The purpose of routine monitoring of aseptic manufacturing facilities is to obtain some measure of the level of control being achieved. The ideal is that monitoring should be done in a way that will promptly reveal any failure of the control systems to meet their intended purpose.

All pharmaceutical manufacturing environments merit a level of environmental monitoring. The greatest emphasis and the tightest limits are applied to sterile manufacturing facilities. When different areas within sterile manufacturing facilities serve different purposes, so the environmental monitoring programs differ. In Europe, microbiological limits applying to various grades of manufacturing clean room are specified in the Guide to Good Manufacturing Practice for Medicinal Products. Monitoring should be done when the facilities are manned and operational. In chapter of <1116> of United States Pharmacopeia is followed for the microbiological environmental monitoring. The manufacturer should decide the type of suitable environmental monitoring program for his/her own facility, since each plant is unique. Most manufacturers therefore develop their own in-house standards. Such standards must be based on knowledge of the normal background levels of contamination.

One of the major routes of contamination in any pharmaceutical production facility is the air. Biological Air Sampling is routinely used to monitor the populations of air borne particles of the surrounding area. In the context of microbiological assessment air sampling is the collection of air borne microbial contaminants that may impact on product spoilage, product safety and human health. Air Sampling has the following significance:

- Measures the number of viable airborne particles (i.e. the concentration of microorganisms in the air).
- Evaluation of the effectiveness of control methods.
- Compliance status with respect to various occupational health standards.
- Routine surveillance.

Clean Room Classification

The design and construction of clean rooms and controlled environments are covered in Federal Standard 209E. This standard of air cleanliness is defined by the absolute concentration of airborne particles. The application of Federal Standard 209E to clean rooms and other controlled environments in the pharmaceutical industry has been used by manufacturers of clean rooms to provide a specification for building, commissioning and maintaining these facilities. Data available in the pharmaceutical industry provide no scientific agreement on a relationship between the number of nonviable particulates and

the concentration of viable microorganisms. The criticality of the number of nonviable particulates in the electronic industry makes the application of Federal Standard 209E a necessity, while the pharmaceutical industry has a greater concern for viable particulates than total particulates as specified in Federal Standard 209E. The rationale that the fewer particulates present in a clean room, the less likely it is that airborne microorganisms will be presently accepted and can provide pharmaceutical manufacturers and builders of clean rooms and other controlled environments with engineering standards in establishing a properly functioning facility.

Federal Standard 209E, as applied in the pharmaceutical industry is based on limits of all particles with sizes equal to or larger than 0.5µm. Following table describes airborne particulate cleanliness classes in Federal Standard 209E as adapted to the pharmaceutical industry. The pharmaceutical industry deals with Class M 3.5 and above. According to Federal Standard 209E (United States) USP classification of clean room is given as follows:

Class Name		Particles equal to and larger than 0.5 µm	
S1	U.S. Customary	(m^3)	(ft^3)
M1	-	10.0	0.283
M1.5	1	35.3	1.00
M2	-	100	2.8
M2.5	10	353	10.0
M3	-	1,000	28.3
M3.5	100	3,530	100
M4	-	10,000	283
M4.5	1,000	35.300	1,000
M5	-	100,000	2,830
M5.5	10,000	353,000	10,000
M6	-	1,000,000	28,300
M6.5	100,000	3,530,000	100,000
M7	-	10,000,000	283,000

Table: Airborne Particulate Cleanliness Classes* (* Adapted from U.S. Federal Standard 209E)

The number in the classification description represents the limit of particles >= 0.5 µm that may be present in a one cubic meter sample of air. Therefore, lower the number in the class description, cleaner the air. Class 100 rooms are considered to be critical areas or zones where sterile product or containers come in direct contact with the environment. Class 10,000 describes the air quality in rooms immediately outside of Class 100. These rooms are usually storage rooms, corridors and other rooms with service and support functions within the aseptic core. Class 100,000 describes the air quality for preparation areas, compounding areas and any other area immediately adjacent to the aseptic suite.

Clean areas for the manufacture of sterile products are classified according to the required characteristics of the environment. Each manufacturing operation requires an appropriate environmental cleanliness level in the operational state in order to

minimize the risks of particulate or microbiological contamination of the product or materials being handled. In order to meet "in operation" conditions these areas should be designed to reach certain specified air cleanliness levels in the "at rest" occupancy state. This latter state is the condition where the installation is complete and the production equipment has been installed and is operating, but no operating personnel are present. The "in operation" state is the condition where the installation is functioning in the defined operating mode and the specified numbers of personnel are present.

According to EU GMP- guidelines classification of clean room is given as follows:

GMP CLASS	Max. Number of particles. If not in operation (1/M^3)		Max. Number of particles in operation (1/M^3)	
Particle Size	0.5 µm	5 µm	0.5 µm	5 µm
A	3,500	1	3,500	1
B	3,500	1	350,000	2,000
C	350,000	2,000	3,500,000	20,000
D	3,500,000	20,000	n.d.*	n.d.*

*n.d. = not defined

Four grades are described as follows:
- Grade A: The local zone for high-risk operations, e.g., filling and making aseptic connections. Normally such conditions are provided by laminar airflow workstations.
- Grade B: In aseptic preparation and filling, the background environment for the Grade A zone.
- Grade C & D: Clean areas for carrying out less critical stages in the manufacture of sterile products.

In order to reach the B, C & D air grades, the number of air changes should be appropriate for the size of the room and the equipment & personnel present in it. At least 20 air changes per hour are usually required for a room with a good airflow pattern and appropriate HEPA filters.

The basic limitation of particulate counters is that they measure particles of 0.5µm or larger. While airborne microorganisms are not free floating or single cells, they frequently associate with particles of 10-20µm. Microbial counts within controlled environments vary with the sampling location and the activities being conducted during sampling.

Test frequencies (air sampling) for environmental monitoring:
The frequency of air monitoring depends on the criticality of the specified sites. Following table (acc to USP) shows the frequencies of sampling in decreasing order of frequency of sampling and in relation to the criticality of the area of the controlled environment being sampled.

Aseptic production (clean room area)	Evaluation frequency
Class 100	Every shift
Class 1,000	Daily
Class 10,000	2 times per week
Class 100,000 (Non-product/container product)	1 time per week

Microbiological Air Sampling: One of the Methods of Environmental Monitoring
Environmental monitoring can be done by various methods: microbiological air sampling, microbiological surface sampling, physical particle monitoring, monitoring of pressure differentials, etc. Following construction of a clean room, it must be tested to ensure that it is providing the required quality of environment. These verification tests are rigorously performed and are similar to the tests used to monitor clean room subsequently. The monitoring tests ensure that the clean room continues to provide satisfactory operation. To ensure that the pharmaceutical clean room is providing the required environmental standards, the following are determined.

Air Quality
The air supplied to the clean room must not contribute to particulate or microbial contamination within the room. The HEPA filters for the inlet air must be tested to ensure that neither the filter fabric nor the filter seals are leaking. Air handling equipment for any controlled or critical area should be sized to provide adequate volumes of incoming and exhaust air in order to achieve and maintain appropriate air quality. Air quality should be commensurate with the type of process (aseptic or non-sterile), risk of product exposure to the environment, and criticality of microbial limit expectations.

Air Movement
Adequate ventilation throughout the clean room can be determined by air movement tests. These are carried out at the time of clean room validation. The outflow of air from a clean room with a higher standard of cleanliness to an area with a lower standard is indicated by the pressure differential between the rooms.

Air Velocity
The velocity of the air at several points in a clean room area of critical importance should be determined. This is done both at validation of the clean room and at timed intervals. The procedure involves the use of an anemometer.

Airborne Particulate and Microbial Monitoring
The particle count and the microbial bioburden of the clean room provide the basis for the air classification system for grading a clean room as discussed above. The points for sampling and the number of samples taken at each position are determined by the size and the grade of the clean room. There should be very few viable organisms present in the clean room air.

Operators within the clean room disperse large numbers of skin particles. Many of these particles are contaminated with bacteria. Sampling for microbial contamination is necessary when people are present in the clean room during production. Monitoring of the microbial contamination during production will ensure that that both the use of clean room clothing by the operators and the air ventilation system are producing the required environmental standards.

Air sampling can be done in two ways: active air sampling and passive air sampling. Active air sampling is intended to provide an index of the number of microorganisms per unit volume of air space in clean rooms. If a good air handling system, with integral HEPA filters in place, serves the clean room air borne, microbial contamination arises from personnel operating within the clean room.

Most active air sampling should be done when the clean room is operational. If a clean room has been non-operational for a few days (e.g. a long weekend) or a few weeks (e.g., a scheduled shut

down for vacation or maintenance), it is beneficial to start sampling a few days prior to production start-up. All active air samplers will disrupt airflow to some extent. They should be located carefully, and when they are operated, they must not counteract protective airflow patterns in significant parts of the clean room.

It is generally accepted by scientists that air borne microorganisms in controlled environments can influence the microbiological quality of the intermediate or final products manufactured in these areas. Different types of air samplers are used for sampling procedure.

Slit-to-Agar Air Sampler (STA) - The principle of the slit sampler is that of inertial impaction; particles moving in an air streamhave an individual inertia and may be deflected onto a surface where they may be trapped by impaction. Slit samplers are provided with pumps that draw air from the area being sampled through a narrow slit.

The effect of the slit is to increase the velocity of the airflow and hence the inertial velocity of the any particle being carried in the air stream. The accelerated particles are directed onto the surface of a plate of basal medium (e.g. nutrient agar) that is rotated continuously and progressively. Pumps provided with slit samplers usually operated at fix rates, and an on/off button controls the volume of air sampled.

Sieve Impactor - The apparatus consists of a container designed to accommodate a Petri dish containing a nutrient agar. The cover of the unit is perforated, with the perforations of a predetermined size. A vacuum pump draws a known volume of air through the cover and the particles in the air containing microorganisms impact on the agar medium in the Petri dish. Some samplers are available with a cascaded series of containers containing perforations of decreasing size.

Sieve impactors are available in single or multistage designs, which facilitate both enumeration and sizing of aero biologic contaminants. All single stage systems impact microorganisms aspirated by the sampler through a matrix of multiple inlet orifice, directly on to an agar medium for development from a single plate with no further subculture steps required for enumeration. In the case of multistage impactors, each vertically stacked stage contains an individual agar plate. As the sample air transits the device at a constant volume, sample velocities increased at each stage, resulting in gradient deposition and accurate sizing of microorganisms of smaller diameters and lower mass.

Advantages of sieve samplers are generally high particle deposition rates, and ability to size particles and vary sampling time and volume. Sieve impactors demonstrate superior collection efficiencies when compared to other methods of aero biologic testing.

Centrifugal Sampler - This type of sampler contains a propeller or turbine that pulls a known volume of air into the instrument and then pushes it outward onto a nutrient agar strip. After sampling the strip is placed back into its original sterile plastic cover and incubated for a specified time at a specified temperature range. Centrifugal samplers operate by accelerating air with entrained particles until a critical velocity is reached for a given particle size. The blades of an impeller force the air onto agar surface. The volume of air sampled may be difficult to determine because the air mixes as it enters and exits the sampler. Centrifugal sampler may disrupt the linearity of airflow in the controlled zone where it is placed for sampling.

Sterilizable Microbiological Atrium - The unit is a variant of the single stage sieve impactor. The unit's cover contains uniformlyspaced orifices approximately 0.25 inch in size. The base of the unit accommodated one Petri dish containing a nutrient agar. Vacuum pump controls the movement of air through the unit, and a multiple unit control centre as well as a remote sampling probes are available.

Surface Air System Sampler - This integrated unit consists of an entry section that accommodates an agar contact plate. Immediately behind the contact plate is a motor and turbine that pulls air through the unit's perforated cover over the agar contact plate and beyond the motor, it is exhausted. Multiple mounted assemblies are also available.

Gelatin Filter Sampler - The unit consists of a vacuum pump with an extension hose terminating in a filter holder that can be located remotely in the critical space. The filter consists of random fibers of gelatin capable of retaining airborne microorganisms. After a specified exposure time, the filter is aseptically removed and dissolved in an appropriate diluent and then plated on an appropriate agar medium to estimate its microbial content.

Passive air sampling is done by means of settle plates - Petri dishes containing agar medium are exposed to the environment for a prolonged period of time and microorganisms are deposited on the agar surface by gravity. Large and heavy particles tend to settle out due to gravitational forces; with increasing air movement only the very heaviest particle settle out. This limits the value of the method in laminar flow protected areas or other clean rooms where still air is not intended. This method is used only for qualitative purpose. This method is inefficient since organisms are deposited on plates by chance. This method is also affected by airflow and particle sizes in the environment that may not be controllable.

Andersen's Air Sampler: A Popular Sampler for the Collection and Enumeration of Viable Air Borne Particles

Andersen's Sampler was designed to count viable airborne particles are counted. Andersen's Sampler consists of a series of six stages through which the sample of air or aerosol is consecutively drawn. The device is pressure sealed with gaskets and three adjustable spring fasteners. Each stage contains a plate perforated with 400 holes and immediately below a Petri dish of agar culture medium. Air is drawn through the device at the rate of 1 cubic foot per minute (cfm) and a jet of air from each of these holes plays on the surface of the medium. The size of the holes is constant for each stage; consequently, the jet velocity is uniform in each stage but increases in each succeeding stage.

When the velocity imparted to a particle is sufficiently great, its inertia will overcome the aerodynamic drag and the particle will leave the turning stream of air and be impinged on the surface of the medium; otherwise the particle remains in the stream of air and proceeds to the next stage. The sampler is so designed that when operated at 1 cfm any airborne particle, a fraction of 1 micron or larger, will be collected on one stage or another,depending on its aerodynamic dimensions. If the particle size spectrum of the aerosol sample is sufficiently broad, particles will be collected on all stages of the sampler. Each succeeding stage will remove a top fraction (largest particles) of the remaining particles; the last stage completes the collection of bacterial particles.

The flow rate of 1 cfm is achieved with a small vacuum pump or with a vacuum system. The collection and assessment of aerosol samples is very simple. Six Petri dishes, each containing enough agar medium appropriate for the microorganisms which may be encountered, are placed in the instrument and the sample of air is drawn; the plates are then removed, inverted in their covers, incubated and counted. When samples of aerosols are being

drawn from a chamber, airflow through the instrument should not be interrupted until air washing with clean air from outside the chamber has replaced all of the sample air within the sampler. Air should not be drawn through the sampler unless Petri plates are in place because this may lodge dirt in the small holes of the lower stages.

Colonies on plates are scattered over the plates and should be counted in the usual manner, except when the plates are heavily loaded, in which case counting may be done through a dissecting type microscope before the colonies merge. A number of fields or segments of the plate are counted, and the total number of colonies for the plate is calculated. In stages 3-6, the colonies conform to the pattern of jets and are counted by either the 'positive hole' method or by the microscope method.

The positive hole method is essentially a count of the jets which delivered viable particles to the Petri plates and conversion of this count by use of the positive hole conversion table (Feller's correction table). This table is based upon the principle that as the number of viable particles being impinged on a given plate increases, the probability of the next particle going into an empty hole decreases. The values in the table were calculated from the basic formula (Feller, 1950): $P_r = N\,[1/N + 1/N-1 + 1/N-2 \ldots \ldots 1/N-r\ r+1]$

Where P_r is the expected number of viable particles to produce, r is positive holes and N is the total number of holes per stage (400). The above formula assumes that the flow of particles stops at the instant a particle enters the rth hole. Since, in the actual case of sampling, the flow of particles stops at random, the expected number of particles present if r positive holes are observed, would be equal to or greater than P_r but less than P_{r+1} and the average would be $(P_r + P_{r+1}^{-1})\,/2$. This correction has been applied in the construction of the table.

Media Recommended for Environmental Monitoring

As per USP chapter <1116>Soyabean Casein Digest Agar (SCDA) is the standard medium for sampling or quantitation of microorganisms in controlled environments. Incubationtemperature is 30-35°C. Yeasts and moulds may also be specifically sought out. Sabouraud Dextrose Agar is used especially for yeasts and moulds. Incubation temperature is maintained at 20-25°C.

Sometimes media are supplemented with additives to overcome or to minimize the effects of sanitizing agents or of antibiotics if used or processed in these environments. The duration of incubation generally recommended is 48-72 hours. Environmental control media should be validated for their ability to support growth throughout their shelf lives. Media are often prepared, sterilized and stored for days and weeks before melting and pouring as environmental monitoring plates.

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Prof. Udupi Ramachandra Rao (1984-1994)

Prof U R Rao, was an internationally renowned space scientist who made original contributions to the development of space technology in India and its extensive application to communications and remote sensing of natural resources.

He was the Chairman of the Governing Council of the Physical Research Laboratory at Ahmedabad and the Chancellor of the Indian Institute of Space science and Technology at Thiruvananthapuram. After working as a Faculty Member at MIT and Assistant Professor at University of Texas at Dallas where he carried out investigations as a prime experimenter on a number of Pioneer and Explorer spacecrafts, Prof. Rao returned to India in 1966 as Professor at the Physical Research Laboratory, Ahmedabad.

Convinced of the imperative need to use space technology for rapid development, Prof. Rao undertook the responsibility for the establishment of satellite technology in India in 1972. Under his guidance, beginning with the first Indian satellite 'Aryabhata' in 1975, over 18 satellites were designed and launched for providing communication, remote sensing and meteorological services.

After taking charge as Chairman, Space Commission and Secretary, Department of Space in 1984, Prof. Rao accelerated the development of rocket technology, resulting in the successful launch of ASLV rocket and the operational PSLV launch vehicle, which can launch 2.0 ton class of satellites into polar orbit. Prof. Rao initiated the development of the geostationary launch vehicle GSLV and the development of cryogenic technology in 1991.

Prof Rao had published over 350 scientific and technical papers covering cosmic rays, interplanetary physics, high energy astronomy, space applications and satellite and rocket technology and authored many books. He was also the recipient of D.Sc. (Hon. Causa) Degree from over 25 Universities including University of Bologna, the oldest University in Europe.

Prof Rao was awarded 'Padma Bhushan' by the Government of India in 1976, which is the third highest Civilian Award and 'Padma Vibhushan' in 2017 which is the second highest Civilian Award. Prof. U.R. Rao became the first Indian Space Scientist to be inducted into the highly Prestigious "Satellite Hall of Fame" at Washington DC, USA on March 19, 2013. Prof. U.R. Rao became the first Indian Space Scientist to be inducted into the highly Prestigious "IAF Hall of Fame" at Guadalajara, Mexico.

Awards

He was the recipient of many national and international awards, such as :

National Awards

- 1975 Karnataka Rajyotsava Award
- 1975 Hari Om Vikram Sarabhai Award
- 1975 Shanti Swarup Bhatnagar Award in the Space science & technology field
- 1976 Padma Bhushan
- 1980 National Design Award
- 1980 Vasvik Research Award in the Electronic Sciences & Technology field
- 1983 Karnataka Rajyotsava Award
- 1987 PC Mahalnobis Medal
- 1993 Om Prakash Bhasin Award in the Energy & Aerospace field
- 1993 Meghnad Saha Medal
- 1994 P.C. Chandra Puraskar Award
- 1994 Electronics Man of the Year Award by ELCINA
- 1995 Zaheer Hussain Memorial Award
- 1995 Aryabhata Award
- 1995 Jawaharhal Nehru Award
- 1996 SK Mitra Birth Centenary Gold Medal
- 1997 Yudhvir Foundation Award
- 1997 Rabindranath Tagore Award of Viswa Bharati University
- 1999 Gujar Mal Modi Award for Science & Technology
- 2001 Nadoja Award from Kannada University, Hampi
- 2001 Life Time Contribution Award in Engineering of INAE
- 2002 Sir M. Visvesvaraya Memorial Award
- 2003 Press Bureau of India Award
- 2004 Star of India Award from Vishwabharathy Foundation, Hyderabad
- 2004 Special Award 2004, Karnataka Media Academy
- 2005 Bharat Ratna Rajiv Gandhi Outstanding Leadership Award
- 2007 Life Time Achievement Award of Indian Space Research Organisation
- 2007 Distinguished Scientist Gold Medal of the Karnataka Science & Technology Academy.
- 2007 Vishwamanava Award by Vishwamanava Samsthe
- 2007 A.V. Rama Rao Technology Award
- 2008 Jawaharlal Nehru Birth Centenary Award for 2007-2008 from ISCA
- 2017, 25 Jan, Padma Vibhushan

International Awards

- 1973 Group Achievement Award by NASA, USA
- 1975 Medal of Honour by Academy of Sciences, USSR
- 1991 Yuri Gagarin Medal of USSR
- 1992 Allan D Emil Award on International Cooperation
- 1994 Frank J Malina Award (International Astronautical Federation)
- 1996 Vikram Sarabhai Medal of COSPAR
- 1997 Outstanding Book Award of the International Academy of Astronautics for the Book Space Technology for Sustainable Development
- 2000 Eduard Dolezal Award of ISPRS
- 2004 Space News magazine named him as one of the Top 10 International personalities who have made a substantial difference in civil, commerce and military space in the world since 1989
- 2005 Theodore Von Karman Award which is the highest Award of the International Academy of Astronautics.
- 2013 Inducted into Satellite Hall of Fame by Society of Satellite Professionals International'
- 2016 Inducted into Hall of Fame by International Astronautical Federation.

Jokes

A guy goes in for a job interview and sits down with the boss.
The boss asks him, "What do you think is your worst quality?"
The man says "I'm probably too honest."
The boss says, "That's not a bad thing, I think being honest is a good quality."
The man replies, "I don't care about what you think!"

HR: "What's your biggest weakness?"
Me: "Interviews"
HR: "And besides that?"
Me: "Follow up questions"

Boss: Can you come to office on Sunday there's some work to finish.
Me: Sure, however, I'll be late at work as public transport on Sunday's is really bad.
Boss: Sure. That should be fine. By when would you reach.
Me: "Monday!"

My boss arrived at work in a brand-new Lamborghini. "Wow," I said. "That's an amazing car."
He replied, "If you work hard, put all your hours in, and strive for excellence, I'll get another one next year."

Bartonella quintana

Introduction

Bartonella quintana infection (historically called 'trench fever') is a vector-borne disease primarily transmitted by the human body louse *Pediculus humanus humanus*. The infection is associated with a wide variety of clinical conditions, including chronic bacteraemia, endocarditis, lymphadenopathy and bacillary angiomatosis. Since the 1990s, it has been recognised as a reemerging pathogen among impoverished and homeless populations — so-called 'urban trench fever' — living in unsanitary conditions and crowded areas predisposing them to infestation with ectoparasites that may transmit the infection. Primary prevention of trench fever relies on measures for avoiding infestation with body lice.

The pathogen

- *B. quintana* is slow-growing Gram-negative bacterium with short rods morphology (0.3 to 0.5 mm wide and 1.0 to 1.7 mm long).
- *B. quintana* has a circular chromosome (≈1.6 Mb) recognised as a genomic derivative of *Bartonella henselae*, the causative agent of cat scratch disease.

Clinical features and sequelae

- The incubation period is usually between 15 and 25 days but has been reported as shorter, up to six days, under experimental conditions.
- Clinical manifestations of *B. quintana* infection include the classical recurrent fever 'trench fever', chronic bacteraemia, endocarditis and, among immunocompromised individuals, bacillary angiomatosis. Lymphadenopathy and ocular complications are occasionally reported.
  - Trench fever or *quintana* fever (5-day fever) is a recurrent fever among non-immunocompromised individuals. Fever episodes lasting for one to five days are associated with nonspecific and varying symptoms such as severe headache, tenderness or pain in the shin, weakness, anorexia or abdominal pain. Splenomegaly is common. The number of periodic fever episodes varies (from one to five in general) and are separated with asymptomatic periods of four to six days. Episodes usually decrease in severity over time. Although it causes prolonged disability, the mortality of trench fever is low.
  - Chronic bacteraemia is possible due the ability of *B. quintana* to cause intraerythrocytic paratism that can be asymptomatic. This has been demonstrated in both immunocompromised and immunocompetent patients.
  - *B. quintana* infection is a significant cause of blood culture-negative endocarditis. Fever is frequently reported. Valvular vegetations are often visible on echocardiography. Laboratory confirmation assays can be performed on cardiac valve if surgery is required.

Bacillary angiomatosis is a proliferative vascular disease due to both *B. quintana* and *B. henselae*. The disease is characterised by angioproliferative lesions of skin (sarcoma-like skin lesions) or various organs (spleen, liver, bone marrow and lymph nodes). *Bacillary angiomatosis* due to *B. quintana* was mainly reported among immunodeficiency virus-infected persons in the 1990s, but can affect other immunocompromised individuals.

- Differential diagnoses vary according to the clinical presentation, and include:
  - Trench fever: louse-borne and tick-borne relapsing fever, infection due to *B. henselae* (cat scratch disease), typhus, malaria, typhoid and non-typhoid salmonellosis.
  - Endocarditis: HACEK group infections, fastidious growing streptococci endocarditis, legionellosis and Q fever.
  - Bacillary angiomatosis: Kaposi sarcoma, pyogenic granuloma, or Peruvian wart (*verruga peruana*) due to *Bartonella bacilliformis*.
  - Lymphadenopathy: infectious (fungal infection, tularaemia, tuberculosis, plague, lymphogranuloma venereum (LGV), AIDS, and syphilis) and non-infectious causes (lymphoma, leukemia and other neoplasms).

Epidemiology

- Historically, trench fever was described in relation to outbreaks among soldiers during the first and second world wars. Since then, few cases have been documented, mainly in Europe and Russia. The disease is considered to have a worldwide distribution based on serological evidence and molecular identification (Africa and South-east Asia notably).
- During the 1990s, *B. quintana* infections and outbreaks were reported among the homeless in USA and Europe (France) leading to the infection being recognised as a re-emerging disease among deprived populations. The main risk factors for infection are impoverished, overcrowded and unhygienic conditions, chronic alcoholism, cat-contact, and body louse infestation. The disease is therefore primarily observed among homeless people.
- Small case series of *B. quintana* infections were reported among immunocompetent patients without body louse infestation: a family cluster with pigeon mites (*Dermanyssus* sp.) as the presumed vector in Czech Republic (2007) and three pediatric cases of lymphadenopathy in Italy (2014).
- Humans are considered the main host for this organism but several publications have reported isolation or molecular identification of the bacteria in mammals (macaques, cats and dogs).

Transmission

- *B. quintana* is transmitted from human to human by the body louse *Pediculus humanus humanus*. However head lice, *Pediculus humanus capitis*, have been found to be infected, but their role as a vector has not been established.
- *B. quintana* DNA has been identified from several arthropods (ticks, bed bugs, cat- and rodent- fleas) but the epidemiological role of these arthropods in transmission of the pathogen is not established yet.
- When feeding on an infected human, the body louse ingests *B. quintana* which multiplies in the intestinal tract of the body louse. Body louse infection is lifelong. *B. quintana* is excreted in body louse faeces.
- Infected dried body louse faeces can remain infectious for 12 months and new cases can arise for some time even after elimination of the louse population.

- Human infection probably results from inoculation of *B. quintana* from contaminated louse faeces when scratching and itching. The transmission does not invoke the death of the louse, therefore an individual louse can spread the disease to several persons.
- On average a mature body louse lives for 20–30 days.

Diagnostics

- The laboratory diagnostic of choice is isolation in culture from blood or tissues on specific media under specific conditions.
- Due the low-growing characteristic of *B. quintana*, diagnosis is often based on supportive diagnostic tests such as serology or polymerase chain reaction (PCR) based genomic assays.
- Regarding serological test, indirect immunofluorescence assay is the reference method. However cross reactions are possible, notably with other *Bartonella* species. High levels of antibodies are usually detected among immunocompetent patients with endocarditis related to *B. quintana*. Serologic testing cannot stand alone as a means to confirm *Bartonella* infection and should be interpreted in the context of the clinical presentation, immunological status of the patient and results of others supporting laboratory test.
- Immunohistochemical tests are supportive of the diagnosis of bacillary angiomatosis or identification with biopsies (cardiac valve, lymph node, skin or other tissue).
- PCR–based genomic assays on blood and tissues can distinguish *Bartonella* species in targeting specific genes.

Infection control, personal protection and prevention

- Primary prevention of *B. quintana* relies on measures to avoid infestation with body lice.
- Body louse infestations are linked to low socioeconomic status, over-crowding and poor personal hygiene.
- Body lice are transmitted primarily by direct contact with an infested person; transmission of the body lice also occurs through fomites, clothes or bedding. Lice multiply rapidly and a population can increase by 11% per day.
- Body lice are highly susceptible to cold and desiccation. They are found on clothing close to the human skin. Discarding infested clothes is an effective way to control the infestation. If this is not possible, clothes should be washed at a temperature above 60°C. Oral ivermectin for body delousing has been proposed for populations with low compliancy but does not protect from lice reinfestation.
- In outbreak situations, dusting powder with an appropriate insecticide has been applied to obtain a rapid decrease of infested persons with some lasting benefits.
- Although there are no reports of transmission, a risk of *B.quintana* infection by substances of human origin (SoHO) cannot be excluded because of possible donations by asymptomatic donors with bacteraemia. In addition, a case of possible transfusion transmission of the closely related *B. henselae* has been reported. Due to possible transmissibility through SoHO, infected donors should be deferred for at least two weeks after signs and symptoms have resolved and a course of effective treatment has been completed. Donation of organs, cells and tissues from deceased donors with a trench fever is not recommended.

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Malaria Mosquitoes Develop Resistance to Insecticide

Since 2000, progress in malaria control has resulted primarily from expanded access to vector control interventions, particularly in sub-Saharan Africa. However, these fragile gains are threatened by emerging resistance to insecticides among *Anopheles* mosquitoes. Left unchecked, insecticide resistance could lead to a substantial increase in malaria incidence and mortality. Urgent action is required to prevent the further development of resistance and to maintain the effectiveness of existing vector control interventions.

Insecticide resistance already widespread

The WHO *Global report on insecticide resistance in malaria vectors: 2010–2016* showed that resistance to the 4 commonly used insecticide classes – pyrethroids, organochlorines, carbamates and organophosphates – is widespread in all major malaria vectors across the WHO regions of Africa, the Americas, South-East Asia, the Eastern Mediterranean and the Western Pacific.

According to the latest *World malaria report*, resistance to at least 1 of the 4 insecticide classes was detected in 73 of the 81 malaria endemic countries that provided data for 2010–2018, an increase of 5 countries compared with the previous reporting period 2010–2017. In 26 countries, resistance was reported to all main insecticide classes.

However, our understanding of the extent of the problem is incomplete, because:

- many countries do not carry out adequate routine monitoring for insecticide resistance in local vectors; and
- monitoring data are often not reported in a timely manner.

WHO investigation: LLINs still provide significant protection against malaria

In 2011, WHO spearheaded a large, multi-country evaluation to assess the impact of insecticide resistance on malaria vector control interventions – primarily long-lasting insecticidal nets (LLINs), the mainstay of malaria prevention. The 5-year evaluation was conducted at more than 250 locations across five countries: Benin, Cameroon, India, Kenya and Sudan.

Researchers found that people who slept under LLINs in the evaluation areas had significantly lower rates of malaria infection than those who did not use a net, even though mosquitoes showed resistance to pyrethroids (the only insecticide class used in LLINs) in all of these areas. The study reaffirms the WHO recommendation of universal LLIN coverage for all populations at risk of malaria.

While these findings are encouraging, WHO continues to highlight the urgent need for new and improved tools in the global response to malaria. To prevent an erosion of the impact of core vector control tools, WHO also underscores the critical need for all countries with ongoing malaria transmission to develop and apply effective insecticide resistance management strategies.

Global action plan developed

Issued in May 2012, the WHO *Global plan for insecticide resistance management in malaria vectors* (GPIRM) is a plan of action for all stakeholders engaged in the fight against malaria. The document provides comprehensive technical recommendations to manage insecticide resistance in different situations.

The GPIRM calls for the following key actions to be undertaken:

- plan and implement insecticide-resistance management strategies in malaria-endemic countries;
- ensure proper, timely monitoring of entomological and resistance monitoring and effective management of data;
- develop new, innovative vector-control tools;
- fill gaps in knowledge on mechanisms of insecticide resistance, and the impact of current approaches to management of insecticide resistance; and
- ensure that enabling mechanisms (advocacy, and human and financial resources) are in place.

More action urgently needed

To date, 40 countries have completed insecticide resistance monitoring and management plans, in line with the GPIRM. WHO continues to work with governments of endemic countries, donor organizations, United Nations agencies, and research and industry partners to implement the 5-pillar strategy contained in the GPIRM; this includes ongoing support for the development of new and innovative vector-control tools and strategies.

Endemic countries are urged to develop and implement comprehensive insecticide-resistance management strategies, and ensure timely entomological and resistance monitoring.

WHO global database on insecticide resistance in malaria vectors

Established in 2014, this WHO database consolidates information from countries, scientific publications and partners on the status of insecticide susceptibility of *Anopheles* mosquitoes in malaria-endemic countries. It is the largest and most up-to-date collection of insecticide bioassay data reviewed and standardized for analysis; the data is collected through a DHIS2 module specifically designed for this purpose. Such data is needed to inform national and global malaria vector-control policy and implementation.

References:

- Guidelines for malaria vector control
- Global plan for insecticide resistance management in malaria vectors
- Global report on insecticide resistance in malaria vectors: 2010–2016
- Test procedures for insecticide resistance monitoring in malaria vector mosquitoes (Second edition)

Gram Staining

Bacteria are too small and too transparent to be well-described using light microscopy and a wet mount. To make them more visible by imparting contrast, they are stained. Most cellular staining that takes place falls into three categories:

(a) Simple staining – A single stain is used to make them visible under the light microscope.

(b) Differential staining – A differential staining uses more than one dye and stains different kinds of organisms different colors. It is employed to differentiate different group of bacteria. Eg- Gram staining.

(c) Special staining – These are procedures can be used to identify capsules, endospores, flagella, and essentially any molecule made by a microbe using dyes linked to antibodies.

Historical aspects

Gram staining is an empirical method differentiating bacterial species into two large groups (Gram positive and Gram negative) based on the chemical and physical properties of their cell walls. The method is named after its inventor, the Danish scientist Hans Christian Gram (1853-1938), who developed the technique in 1884 to discriminate between *pneumococci* and *Klebsiella pneumoniae* bacteria. In the original method of Gram, the smear was stained with aniline-gentian violet, treated with Lugol's iodine, decolourized with absolute alcohol and counterstained with Bismarck brown. Later it was modified by Hucker in 1921 and modifications give better result.

Principle

Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50-90% of cell wall), which stain purple and Gram-negative bacteria have a thinner layer (10% of cell wall), which stain pink. Gram-negative bacteria also have an additional outer membrane, which contains lipidsand is separated from the cell wall by the periplasmic space. There are four basic steps of the Gram stain, which include applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture, followed by the addition of a mordant (Gram's iodine), rapid decolorization with alcohol or acetone, and counterstaining with safranin or basic fuchsin.Crystal violet (CV) dissociates in aqueous solutions into CV^+ and chloride (Cl) ions. These ions penetrate through the cell wall and cell membrane of both Gram-positive and Gram-negative cells. The CV^+ ion interacts with negatively charged components of bacterial cells and stains the cells purple. Iodine (I or I_3) interacts with CV^+ and forms large complexes of crystal violet and iodine (CVI) within the inner and outer layers of the cell. When a decolorizer such as alcohol or acetone is added, it interacts with the lipids of the cell membrane. A Gram-negative cell will lose its outer membrane and the peptidoglycan layer is left exposed. The CVI complexes are washed from the Gram-negative cell along with the outer membrane. In contrast, a Gram-positive cell becomes dehydrated from an ethanol treatment. The large CVI complexes become trapped within the Gram-positive cell due to the multilayered nature of its peptidoglycan. The decolorization step is critical and must be timed correctly; the crystal violet stain will be removed from both Gram-positive and negative cells if the decolorizing agent is left on too long (a matter of seconds). After decolorization, the Gram-positive cell remains purple and the Gram-negative cell loses its purple colour. Counterstain, which is usually positively-chargedsafranin or

basic fuchsin, is applied last to give decolorized Gram-negative bacteria a pink or red colour.

GramStaining Protocol
Preparation of smear

Before performing gram staining preparation of smear is important. Smear preparation involves the following steps:

- A drop of the suspended culture is transferred to examine on a slide with an inoculation loop. If the culture is to be taken from a Petri dish or a slant culture tube, a drop of water is added on the slide and aseptically transferred a minute amount of a colony from the petri dish. To be visible on a slide, organisms that stain by the Gram method must be present in concentrations of a minimum of 10^4 to 10^5 organisms/ml of unconcentrated staining fluid. At lower concentrations, the Gram stain of a clinical specimen seldom reveals organisms even if the culture is positive.

- The culture is spread with an inoculation loop to an even thin film over a circle of 1.5 cm in diameter, approximately the size of a dime.

- Then it is dried in air and fixed it over a gentle flame, while moving the slide in a circular fashion to avoid localized overheating. The applied heat helps the cell adhesion on the glass slide to make possible the subsequent rinsing of the smear with water without a significant loss of the culture. Heat can also be applied to facilitate drying the smear. However, ring patterns can form if heating is not uniform.

Spread culture in thin film over slide   Dry in air   Pass slide through flame to fix
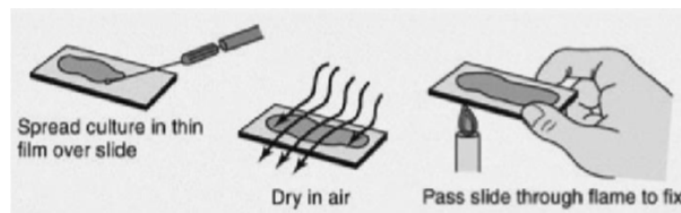
Gram staining

After preparing the smear following steps should be performed for gram staining:

- Crystal violet stain is added over the fixed culture and waited up to 60 seconds. The stain is poured off and gently rinsed the excess stain with a stream of water.

- Gram's iodine solution was added on the smear waited 30-60 seconds. The iodine solution is poured off and the slide is rinsed with running water. Excess water is shakedoff from the surface.

- The slide is decolourized with 95% of ethanol. The exact time to stop is when the solvent is no longer coloured as it flows over the slide. Further delay will cause excess decolourization in the gram-positive cells, and the purpose of staining will be defeated.

- Then it is counterstained with safranin (or basic fuchsin) for 40-60 seconds. The solution is washed off with water. Then the slide is blotted dry to remove the excess water.

Interpretation of Gram staining results and precautions

The slides should be observed under microscope to examine the Gram staining results. Organisms that retain the violet-iodine

complexes after washing in ethanol stain purple and are termed Gram-positive, those that lose this complex stain red from the safranin counter stain are termed Gram negative. The Gram stain will not detect organisms, which exist within host cells (e.g., *Chlamydia* spp), organisms with no cell wall (e.g.,*Mycoplasma* spp and *Ureaplasma* spp), and organisms too small to be seen with light microscopy (e.g., spirochetes). Mycobacteria usually will not stain, and *Legionella* spp stain only when taken directly from culture. Gram-negative bacteria that stain poorly with safranin include *Campylobacter* spp, *Legionella* spp, *Bacteroides* spp, *Fusobacterium* spp, and *Brucella* spp. Certain conditions are known to damage the cell wall, causing gram-positive bacteria to falsely appear gram-negative or gram-variable. These include antibiotic treatment, cultures more than 48 hours old, inflammatory responses in the host, and autolytic enzymes (e.g., *S. pneumoniae*). To minimize ambiguous results, specimens should be collected before the patient begins antibiotic therapy. Also, Gram stains should be performed on colonies taken from culture media that do not contain antibiotics, preferably on colonies that are 18-24 hours old. Finally, correct interpretation of Gram stains requires a theoretical background of bacteria and their morphology, because improper technique or sub optimal reagents can cause unreliable results. Errors in technique that can alter Gram stain results include the following:

- Fixation with excessive heat alters cell morphology and makes organisms more susceptible to over-decolourization.
- Low concentrations of crystal violet make gram-positive organisms more susceptible to over-decolorization.
- Insufficient exposure to iodine and lack of available iodine can prevent crystal violet from bonding firmly with the cell wall, thus making gram-positive organisms more susceptible to over-decolorization. To ensure reliable Gram stain results, only fresh iodine should be used.
- Prolonged decolourization, especially with acetone, can cause gram-positive bacteria to appear gram-negative. Insufficient decolourization can make gram-negative organisms falsely appear gram positive.
- Insufficient counterstaining can fail to stain gram-negative bacteria and background material, whereas excessive counterstaining will leach the crystal violet-iodine complex from gram-positive bacteria and stain them with safranin, thus making them falsely appear gram-negative.
- Prolonged washing between any of the steps can cause over-decolourization.
- Precleaned or degreased glass slides should be used for Gram staining. Storing slides in a jar with 95% ethanol will ensure clean slides.

- The amount of the mordant available is important to the formation of the crystal violet - iodine complex. The lower the concentration, the easier to decolorize (0.33% - 1% commonly used). QC of the reagent is important as exposure to air and elevated temperatures hasten the loss of Gram's iodine from solution.
- As the counter stain is also a basic dye, it is possible to replace the crystal violet - iodine complex in gram-positive cells with an over-exposure to the counter stain. The counter stain should not be left on the slide for more than 30 seconds.

Analytical Considerations

- Appearance of the reagents should be checked daily. If crystal violet has precipitated or formed crystal sediment, it should be refiltered before use even when purchased commercially. Some stains, especially basic fuchsin and safranin, can become contaminated. When suspected, either culture or start with fresh material in a clean bottle.
- Evaporation may alter reagent effectiveness; working solutions should be changed regularly if not depleted with normal use.
- Daily when a new lot is used, it should be checked by staining with the *Escherichia coli* (ATCC 25922) or *Staphylococcus aureus* (ATCC 25923). *E. coli* should be appeared as pink gram-negative bacilli whereas *S. aureus* as purple cocci.

Applications of Gram Staining

Gram staining is a common procedure in the traditional bacteriological laboratory. The technique is used as a tool for the differentiation of Gram-positive and Gram-negative bacteria, as a first step to determine the identity of a particular bacterial sample. Gram stains are performed on body fluid or biopsy when infection is suspected. It yields results much more quickly than culture and is especially important when infection would make an important difference in the patient's treatment and prognosis; examples are cerebrospinal fluid for meningitis and synovial fluid for septic arthritis.

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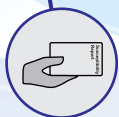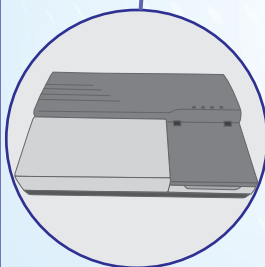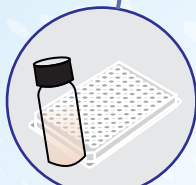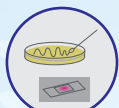
Highlights of the coming issue