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Editorial

Contents

With the progress of science, **Journal of Hygiene Science** continues to focus on the latest trends and significant matters in the field of microbiology and disinfection. Along with this it also makes you aware about the history and existing techniques of microbiology. Hope you have enjoyed our first effort.

The first of the modern antibiotics and still one of the most of the useful antibiotic is penicillin. We bring you an overview of commercial production of penicillin in Mini Review section. The antibiotic industry did not exist in 1941 and penicillin was the first antibiotic to be produced industrially. Many general laboratories still lack computers for the main microbiology laboratory functions. The Current Trends section offers an insight into the computerization of microbiology laboratory and explains its essentiality in the betterment of microbiology laboratory. Compared to manual system it is more efficient, rapid and flexible. This time our In Profile section is dedicated to Joseph Lister who has introduced the postoperative antisepsis surgery. He is known as the 'Father of Antiseptic Surgery'. *Clostridium difficile* is a ubiquitous bacterium found in soil, hospital environments, childcare facilities and nursing homes. It is the most frequent etiologic agent for health-care associated diarrhea. In our Bug of the Month section we have discussed about the pathogenesis and clinical symptoms of the *C. difficile* associated diseases and have also suggested the diagnosis method, treatment and control measures to prevent the spread of this health-care-associated diarrhea.

Water pollution has become a serious environmental hazard in recent years. Water-borne diseases are any illness caused by drinking water contaminated by human or animal faeces, which contain pathogenic microorganisms. So to maintain the water quality for drinking and other purpose routine monitoring for enteropathogens is necessary. Did You Know section gives you a close view of water analysis by using *Escherichia coli* as an indicator organism. Bacteria are too small and transparent so to make them more visible by imparting contrast, they are stained. The Gram stain has been used for many years in the qualitative differentiation of bacteria. Our Best Practices section allows you to take a look in detail of Gram staining methods. The article has also analyzed the errors in technique that can alter Gram stain results. Have a look at our Encyclopedia and In Focus pages. Relax Mood section will refresh you from today's busy schedule.

We are eagerly waiting for your enthusiastic response.

Penicillin Production

- An overview

Antibiotics are used to prevent bacterial infections. The large-scale production of an antibiotic depends on a fermentation process. During fermentation, large amounts of the antibiotic-producing organism are grown. During fermentation, the organisms produce the antibiotic material, which can then be isolated for use as a drug. For a new antibiotic to be economically feasible, manufacturers must be able to get a high yield of drug from the fermentation process, and be able to easily isolate it. Extensive research is usually required before a new antibiotic can be commercially scaled up.

Historical Aspects

Penicillin is a fungal secondary metabolite, which is used as an antibiotic. The most popular mechanism for Penicillin is suggested by blocking the synthesis of the membrane peptidoglycan in gram-positive type bacteria, resulting in cell lysis. In 1928, Alexander Fleming made one of the most important contributions to the field of antibiotics. In an experiment, he found that a strain of green *Penicillium notatum* mold inhibited the growth of bacteria on an agar plate. This led to the development of the first modern era antibiotic, penicillin. Later Howard Florey and associates discovered a new strain of *Penicillium,* which produced high yields of penicillin. This allowed large-scale production of penicillin, which helped launch the modern antibiotics industry.

The first generation penicillin products from these fungi were benzyl penicillin (penicillin G) and penicillins V, X, F and K. The penicillin base unit is 6-aminopenicillanic acid (6-APA; a 5-membered thiazolidine ring system fused to the square rigid - Lactam ring). The functional group was identified as the - Lactam ring. Semi-synthetic penicillin was developed in the late 50's with the commercial production of benzyl penicillin, in which the fermentation process is induced in the above *Penicillium* mould by adding phenylacetic acid (C6H5.CH2.COOH). Since then large scale production of penicillin has developed. The yield of penicillin was monitored and many changes to the fermentation and extraction stages have occurred in the last 50 years to optimize penicillin production.

Industrial production of Penicillin

Penicillin is produced from fungi. It is only produced when the fungi are under stress. The mycelial density tends to be high when fungi tend to cluster together, a common feature of fungi. This high mycelial density produces great stress on the fungal population. To ensure the fungi are in a stressed environment they are previously inoculated to occupy about 10% of the total volume container and cultured for 30-40 hours.

This culture preparation is known in industry as seed preparation (inoculum preparation) and takes place in a smaller fermenter, which is churned with an impeller, aerated, cooled and supplied with water and nutrients.

Strain selection and preservation

The most important factor for the success of any fermentation industry is a selection of strain. The selected strain should posses the following characters to achieve the desired product in industry: 1) The strain should be high yielding 2) It should have stable biochemical characteristics. 3) It should not produce undesirable substances. 4) It should be easily cultivated on large scale by employing cheapest raw materials.

The fungus *Penicillium notatum* originally used by Fleming for penicillin production, gave poor result. Moreover, many strains of this fungus were developed which produced more penicillin than the original one. *Penicillium chrysogenum* was selected for the commercial production of penicillin. From this one ancestral fungus each penicillin manufacturer has evolved high yielding production strain by a series of mutagenic treatments, each followed by the selection of improved variants. These selected variants have proved capable of producing amounts of penicillin far greater than those produced by the 'wild' strain, especially when fermented on media under particular control regimes developed in parallel with the strains.

Production strains are stored in a dormant form by any of the standard culture preservation techniques. Thus, a spore suspension may be mixed with a sterile, finely divided, inert support (soil or sand is traditional) and desiccated. Alternatively, spore suspensions can be lyophilised in appropriate media or stored under liquid nitrogen.

Raw materials

The compounds that make the fermentation broth are the primary raw materials required for antibiotic production. This broth is an aqueous solution made up of all of the ingredients necessary for the proliferation of the microorganisms. Typically, it contains a carbon source like molasses, or soy meal, both of which are made up of lactose and glucose sugars. These materials are needed as a food source for the organisms. Nitrogen is another necessary compound in the metabolic cycles of the organisms. For this reason, an ammonia salt is typically used. Additionally, trace elements needed for the proper growth of the antibiotic-producing organisms are included. These are components such as phosphorus, sulfur, magnesium, zinc, iron, and copper introduced through water-soluble salts.

The medium initially placed in the fermenter is a complete one

but designed only to support the desired amount of early growth. The preferred nitrogen source is corn steep liquor (CSL), a by-product of the maize starch-producing industry. This material was originally found to be useful for the penicillin fermentation specifically but is now recognized as valuable in many fungal antibiotic fermentation media. Apart from its primary purpose in supplying cheap and readily available nitrogen, CSL also contains a useful range of carbon compounds, for example lactic acid, inorganic ions and growth factors in short it is virtually a complete growth medium in itself. However, a further carbon source, usually a sugar derivative, is also included in the medium. Phenyl acetic acid supplies the side chain of penicillin G: without PAA, the organism synthesizes only small quantities of this penicillin. So addition of PAA stimulates the production of penicillin.

When glucose was absent from the medium, the amino acids and peptides in the nutrient were utilized in preference to lactose as the carbon source during the growth phase of the fermentation. This resulted in the liberation of ammonia, which served as the nitrogen source during the next phase of the fermentation and also caused a sharp rise in pH. It was necessary to control this rise in pH by adding glucose as a replacement for part of the lactose in media containing yeast extract, or fish soluble. The medium containing glucose gave higher yields of penicillin than the medium without glucose. When glucose was present in the medium the pH was maintained in a region more suitable for penicillin synthesis. However in the absence of glucose, the pH rose above 8 during the penicillin-producing phase. Because the medium containing glucose had a greater supply of rapidly utilizable carbon available during the growth phase and the pH was maintained in a region more suitable for growth, more mycelial nitrogen was present in this fermentation and a good penicillin yield resulted. The length of time that the fermentation continues in the penicillin-producing phase is determined by the concentration of lactose in the medium. When all the lactose is exhausted, autolysis sets in, thereby terminating the penicillin-producing phase of the fermentation. Therefore the amount of lactose in a medium is one of the factors determining the penicillin yields on that medium.

Inoculum preparation

Before fermentation can begin, the desired antibiotic-producing organism must be isolated and its numbers must be increased by many times. To do this, a starter culture from a sample of previously isolated, cold-stored organisms is created in the lab. In order to grow the initial culture, a sample of the organism is transferred to an agar-containing plate. The initial culture is then put into shake flasks along with food and other nutrients necessary for growth. This creates a suspension, which can be transferred to seed tanks for further growth. The aim is to develop for the production stage fermenter, a vessel of 25-250 m^3 capacity, a pure inoculum in sufficient volume and in the fast-growing (logarithmic) phase so that a high population density is soon obtained. The seed tanks are steel tanks designed to provide an ideal environment for growing microorganisms. They are filled with the all the things the specific microorganism would need to survive and thrive, including warm water and carbohydrate foods like lactose or glucose sugars. Additionally, they contain other necessary carbon sources, such as acetic acid, alcohols, or hydrocarbons, and nitrogen sources like ammonia salts. Growth factors like vitamins, amino acids, and minor nutrients round out the composition of the seed tank contents. The seed tanks are equipped with mixers, which keep the growth medium moving, and a pump to deliver sterilized, filtered air. After about 24-28 hours, the material in the seed tanks is transferred to the primary fermentation tanks.

Fermentation

The fermentation tank is essentially a larger version of the steel, seed tank, which is able to hold about 30,000 gallons. It is filled with the same growth media found in the seed tank and also provides an environment to growth. Here the microorganisms are allowed to grow and multiply. During this process, they excrete large quantities of the desired antibiotic. Requirements for the good penicillin production are the following: growth of a good crop of mycelium; maintenance of the mycelium with an adequate air supply; a pH value below 8 but not much below 7; conditions of partial carbohydrate starvation; and presence of phenyl acetic acid or other suitable precursor. For the growth phase, a pH below 7 is desirable, and readily available carbon sources must be present. The partial carbohydrate starvation required during the penicillin-forming phase is ordinarily brought about either by the use of a poorly available energy source such as lactose, or by slowly feeding a readily available source such as glucose.

Oxygen supply-The penicillin fermentation needs oxygen, which is supplied as filtersterilised air from a compressor. As oxygen is poorly soluble in water, a number of stratagems are adopted to assist its passage into the liquid phase and through that into the mould mycelium. In a conventional fermenter, air is introduced at the bottom of the vessel via a ring 'sparger' that breaks the flow into a myriad of bubbles to increase the transfer area. These bubbles lose oxygen as they rise up the tank and, at the same time, carbon dioxide and other gaseous metabolites diffuse into them. Impellers mounted on a rotating vertical shaft driven by a powerful electric motor further assist the transfer of oxygen. Baffles are also included to achieve the correct blend of shear and of bulk circulation from the power supplied, and generally to promote intimate contact of cells and nutrients. Aeration is a major expense as very large amounts of energy are consumed. This has led to considerable research into novel; energetically more efficient methods of aeration and the next generation of fermenters may include some that are radically different in design. Temperature control- The production of penicillin G is very sensitive to temperature, the tolerance being less than 1°C. Heat is

generated both by the metabolism of nutrients and by the power dissipated in stirring, and has to be removed by controlled cooling. Coils, through which chilled water can be circulated within the vessel, are generally used to effect the necessary heat reduction. The optimum temperature of penicillin production is 23-27ºC.

Defoaming and instrumentation- Microbiological systems stirred vigorously and aerated usually foam, so provision has to be made for adding defoaming agents. To prevent foaming during fermentation, anti-foaming agents such as lard oil, octadecanol, and silicones are used.

The vessel is fitted with several probes to detect foaming, to monitor temperature and to record parameters such as pH. The rate of airflow through the fermenter is measured, and the exhaust gases that emerge from the top of the vessel may also be analyzed.

Media additions-Not all the nutrients required during fermentation are provided initially in the culture medium and provision is therefore also made to add these while the fermentation is in progress, usually via automatic systems that allow a preset programme of continuous or discrete additions.

Transfer and sampling systems- Finally, appropriate pipe work is provided to transfer the inoculum to the vessel, to allow the taking of routine samples during fermentation, and to transfer the final contents to the extraction plant when fermentation is complete. Sampling is essential to monitor the amount of growth, the running levels of key nutrients and the penicillin concentration. It is necessary also to check that there has been no contamination by unwanted microorganisms.

Steps of penicillin production

Penicillin is produced on large scale in a commercially devised fermenter, which provides optimum growth conditions to *Penicillium chrysogenum* for maximum yield. Following are the steps involved for the production of penicillin.

i) Inoculate 100 ml medium in 500 ml Erlenmeyer flask with spores of *Penicillium chrysogenum* strain and incubate at 25°C by keeping them on a rotary shaker.
ii) After 4 days, transfer the content of flask to another flask (4-liter capacity) containing 2 liters of medium and incubate 2 days.
iii) Transfer the content to a stainless steel tank (800-liter capacity) containing 500 liters of medium. This tank is equipped in such way that it could provide the optimum conditions for fungal growth.
iv) After 3 days, use the contents for inoculation of about 1,80,000 liter medium kept in fermenter (2,50,000 liter capacity). The later is equipped with automatic devices to optimum growth conditions.
v) After three to five days, the maximum amount of antibiotic will have been produced and the isolation process can begin. Depending on the specific antibiotic produced, the fermentation broth is processed by various purification methods.

Recovery of penicillin

There are ten steps in the recovery of Penicillin:

(a) Broth Filtration, (b) Filtrate Cooled, (c) Further Filtration, (d) Extraction of Penicillin with Solvent, (e) Carbon Treatment, (f) Transfer back to Aqueous Phase, (g) Solvent Recovery, (h) Crystallization, (i) Crystal Washing, (j) Drying of Crystals.

(a) Broth Filtration - By analyzing a fermentation broth at the time of harvesting it will be discovered that the specific product may be present at a low concentration in an aqueous solution that contains intact micro-organisms, cell fragments, soluble and insoluble medium components and other metabolic products. In the first stage, the main objective is to remove large solid particles and microbial cells by either centrifugation or filtration. Filtration is the most versatile and most established method for removing insoluble from our broth. In filtration, the microorganisms are captured in a concentrated cake, which looks like sand, sludge or paste. Many factors influence which type of filtration will take place; viscosity and density of filtrate, solid: liquid ratio, size and shape of particles, scale of operation, need for aseptic conditions, need for batch or continuous operation and the need for pressure or vacuum suction to ensure an sufficient for rate for liquid. The Rotary Vacuum Filter is the most common piece of equipment used for the extraction of penicillin, and is used in continuous processing. Rotary Vacuum Filter designs vary, but usually outline as follows:

The Filter Drum: Cylindrical, hollow drum, which carries the filter cloth. On the inside it is segmented into rows to which a vacuum can be applied or shut off in sequence as the drum slowly revolves.

Trough: Filter is partially immersed in through which contains the penicillin broth. The trough is sometimes fitted with an agitator to maintain solids in suspension.

Discharge Nodes: Filter cakes are produced from the filtration of penicillin broth. Because of this a node is devised to scrap off the cake after filtration. When this happens the vacuum is broken.

The filter drum, partially submerged in the trough of broth, rotates slowly. Filtrate and washings are kept separate by the segments in the drum. The liquid is drawn through the filter and a cake of solids builds up on the outer surface. Inside the drum, the filtrate is moves from the end of the cylindrical drum onto a storage tank. As our penicillin cells move from the broth, the vacuum is used to remove as much moisture as possible from the cake, and to hold the cake on the drum. The section at the node/knife, which scrapes off the filtrate, can get air pressure to burst out, helping contact with the node. Rotary vacuum filters are expensive, but they are convenient and do provide a considerable degree of mechanization.

(b) Filtrate Cooled - From filtration the penicillin rich solution is cooled to 5°C. As penicillin G only has a half-life 15 minutes at pH 2 at 20°C, this helps reduce enzyme and chemical degradation

during the solvent extraction step.

(c) Further Filtration- Further filtration again takes place using the Rotary Vacuum Filter.

(d) Extraction of Penicillin with Solvent- For penicillin recovery, it is standard practice to use liquid-liquid countercurrent extraction processes. The basis to which liquid-liquid extraction, also called solvent extraction, works is that the extraction agent and the liquid in which the extract is dissolved are not perfectly miscible. Liquid-liquid extraction is suitable for the recovery of penicillin because of its operation at low temperatures, greater selectivity and is less expensive compared to distillation, evaporation and membrane technology.

(e) Carbon Treatment- Penicillin rich solution is then treated with 0.25 - 5% activated carbon to remove pigments and impurities. Activated carbon is an amorphous solid, and absorbs molecules from the liquid phase through is highly developed internal pore structure. It is obtained in powered, pelleted or granular form and is produced from coal, wood and coconut shells.

(f) Transfer back to Aqueous Phase- Using a second Podbielniak Centrifugal Contractor; the penicillin rich solvent is passed into a fresh aqueous phase. This is done in the presence of Potassium or Sodium hydroxide to bring the pH back to 5.0-7.5, creating the penicillin salt.

(g) Solvent Recovery-The penicillin solvent is usually recovered by distillation. Distillation is carried out in three phases: Evaporation, Vapour-liquid separation in a column and condensation of the vapour. Firstly the solvent is vapourized from the solution, then the low boiling volatile components are separated from the less volatile components in a column, and finally condensation is used to recover the volatile solvent fraction. Solvent recovery is an important process, as solvent is a major expense in the penicillin extraction process.

(h) Crystallisation - Crystals are highly organized inert matters. If grown without external interference, they grow in polyhedral shapes and exhibit many degrees of symmetry. Penicillin G is an odourless, colourless or white crystal, or crystalline powder. Crystallization is essentially a polishing step that yields a highly pure product. It is done through phase separation from a liquid to a solid. Super saturation refers to a state in which there are more dissolved solids in the solvent than can ordinarily be accommodated at that temperature at equilibrium. Super saturation can be achieved usually by cooling, drowning, solvent evaporation, or by chemical reaction. Since the solubility of penicillin in its aqueous solution decreases with decreasing temperature, as the solution cools, its saturation increases until it reaches super saturation and crystallization begins. Drowning is also common of recovery of penicillin G. It is the addition of a nonsolvent to the solution to decreases the solubility of the solid. A chemical reaction can be used to alter the dissolved solid to decrease its solubility in the solvent, thus working toward super saturation. Then crystallization is done in two phase- primary nucleation and secondary nucleation. Batch crystallization is the most the most used method for polishing antibiotics, including penicillin G. Batch crystallizers simply consist of tanks with stirrers and are sometimes baffled. They are slowly cooled to produce super saturation. Further cooling until the desired crystals are obtained encourages seeding causes nucleation and growth. While the crystallization procedures product of very high purity, improves appearance and has a low energy input, the process can be time consuming due to the high concentration of the solutions during crystallization. It can also be profoundly affected by trace impurities and batch crystallization can often give poor quality, nonuniform product.

(i) Crystal Washing- Penicillin G crystals formed after crystallization are essentially pure in nature but adsorption and capillary attraction cause impurities from its mother liquor on their surfaces and within the voids of the particulate mass. Because of this the crystals must be washed and pre-dried in a liquid in which they are relatively insoluble. This solvent should be miscible with the mother solvent. For this purpose we use anhydrous propanol, n-butanol or another volatile solvent.

(j) Drying of Crystals- Drying can stabilize many heat sensitive products like penicillin. The drying of penicillin must be carried out with extreme care to maintain its chemical and biochemical activity, and ensure that it retains a high level of activity after drying. There are many methods for drying penicillin:

Lyophilization: another name for freeze-drying. The wet penicillin is frozen to solidify it. Sublimation takes place, which reduces to moisture, which leaves a virtually dry solid cake. Finally, secondary drying takes place where the bound moisture is reduced to the final volume. These three stages do overlap somewhat.

Spray Dryers: the precise atomization of solutions in seeded in a controlled drying environment for spray drying to take place. Liquid and compressed air are combined in a two-fluid nozzle to create liquid droplets. Warm air streams dry the droplets and a dry powder is created. This is a continuous process and the transition from liquid to powder is almost instantaneous.

Vacuum Band Dryers: thin wet layers of penicillin crystals are fed onto a slow rotating heated drum. Radiant heat dries the layer and scalpels remove the product from the end.

Quality control

Quality control is of utmost importance in the production of antibiotics. Since it involves a fermentation process, steps must be taken to ensure that absolutely no contamination is introduced at any point during production. To this end, the medium and all of

the processing equipment are thoroughly steam sterilized. During manufacturing, the quality of all the compounds is checked on a regular basis. Of particular importance are frequent checks of the condition of the microorganism culture during fermentation. These are accomplished using various chromatography techniques. Also, various physical and chemical properties of the finished product are checked such as pH, melting point, and moisture content. If oxygen availability falls below a critical level, penicillin G biosynthesis is greatly reduced although culture growth continues. Thus, if growth in the fermenter proceeds unchecked at the rate prevailing in the seed stages, the culture would become very dense and its aeration potential would fall below that required for penicillin production. Accordingly, conditions are so adjusted that fast growth is achieved only until the cell population has reached the maximum density that the vessel can support. Further net growth is constrained by deliberately limiting the supply of a key nutrient (in practice, a sugar). The cells can then be stimulated to an overproduction of penicillin G while restricting the amount of mycelial growth: thus a stable cell population of maximal productivity can be sustained. In the United States, antibiotic production is highly regulated by the Food and Drug Administration (FDA). Depending on the application and type of antibiotic, more or less testing must be completed. For example, the FDA requires that for certain antibiotics each batch must be checked by them for effectiveness and purity. Only after they have certified the batch can it be sold for general consumption.

References

Fred V. Soltero and Marvin J. Johnson. 1952. The Effect of the Carbohydrate Nutrition on Penicillin Production by *Penicillium chrysogenum* Q-1 761. Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison, Wisconsin. 52-56.

Hugo, WB. Russell, A D., *Pharmaceutical Microbiology* 2nd Ed. 1981

Kenneth B. Raper and Dorothy I. Fennell. 1946. The Production of Penicillin X in Submerged Culture. *J Bacteriol*. **51(6):** 761-777.

Encyclopedia

Anaerobe

An anaerobe or anaerobic organism is an organism that does not require oxygen for growth. Anaerobes can be differentiated in,

- Obligate anaerobes- It dies when exposed to atmospheric levels of oxygen. Obligate anaerobes may use fermentation or anaerobic respiration.
- Facultative anaerobes- It uses oxygen when it is present. In the presence of oxygen, facultative anaerobes use aerobic respiration; without oxygen some of them ferment, some use anaerobic respiration.
- Aerotolerant organisms- It can survive in the presence of oxygen, but they are anaerobic because they do not use oxygen as a terminal electron acceptor. Aerotolerant organisms are strictly fermentative.

Antibiotic

An antibiotic is a chemotherapeutic agent that inhibits or abolishes the growth of micro-organisms, such as bacteria, fungi, or protozoans. It is usually obtained from microorganisms, that inhibit the growth of or destroy certain other microorganisms.

The great number of diverse antibiotics currently available can be classified in different ways, e.g., by their chemical structure, their microbial origin, or their mode of action. Their effective range also frequently designates them. Eg. Penicillin, Tetracycline, Aminoglycoside etc.

The mass production of antibiotics began during World War II with streptomycin and penicillin. Now most antibiotics are produced by staged fermentations in which strains of microorganisms producing high yields are grown under optimum conditions in nutrient media in fermentation tanks holding several thousand gallons. The mold is strained out of the fermentation broth, and then the antibiotic is removed from the broth by filtration, precipitation, and other separation methods. In some cases new antibiotics are laboratory synthesized, while many antibiotics are produced by chemically modifying natural substances; many such derivatives are more effective than the natural substances against infecting organisms or are better absorbed by the body, e.g., some semi synthetic penicillins are effective against bacteria resistant to the parent substance.

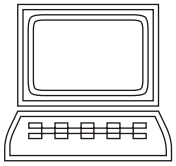
Asepsis

Asepsis is the practice to reduce or eliminate contaminants (such as bacteria, viruses, fungi, and parasites) from entering the operative field in surgery or medicine to prevent infection.

The modern concept of asepsis evolved in the 19th century. Semmelweis showed that washing the hands prior to delivery reduced puerperal fever. After the suggestion by Louis Pasteur, Lister introduced the use of carbolic acid as an antiseptic and reduced surgical infections rates.

Aseptic technique

Aseptic technique refers to a procedure that is performed under sterile conditions. This includes medical techniques and laboratory techniques, such as with microbiological cultures. Aseptic technique is the name given to the procedures used by microbiologists to prevent microbial contamination of themselves, which may result in infection, contamination of the environment they are working in (e.g. fomites), and contamination of the specimen they are working on, which is especially important when a pure culture is desired.



Computer is not an optional but an essential in microbiology laboratory

The automation of laboratory systems is of increasing importance and is critical in research settings because the laboratory not only needs to produce accurate results but also must do so in a timely fashion since there are often clinical implications for the research subjects. The first microbiology computer systems originated in large hospitals and, in the United Kingdom (UK). Many general laboratories still lack computers for the main microbiology laboratory functions. Data processing in microbiology the data are largely textual, and the results are obtained in a multi step fashion. Various data processing systems have been described. Some use punched cards with off site processing and others use either a self-contained laboratory computer or one connected on line to a hospital mainframe computer. Microcomputers have recently been used for data processing in smaller laboratories and for more specific tasks.

Objectives and functional requirements

In computerized systems there are some advantages and disadvantages compared with manual systems. The positive attributes of a computer include more efficient and rapid data storage, rapidity of data recall, flexibility, speed and ease of analysis of stored data and the potential for rapid communication of results via ward or clinic based computer terminals. The following objectives were required of the system.

- Each specimen processing area should be supported by a largely autonomous computer subsystem, so that the whole system is not totally reliant on any one unit of computer equipment.
- The system should be developed and introduced in stages: the hardware purchased in small cost units as required, and the software assimilated into the laboratory with little change to existing work patterns. It should be developed by at least two members of the laboratory staff to allow full cover for system maintenance and changes in routine-for example, alterations in antibiotic reporting.
- It should allow all staff easily to adapt to, understand, and be in control of the system without specialized training. Data entry should be uncomplicated, and the use of codes and other restrictions should be kept to a minimum. All relevant instructions must be displayed on the computer screen, with full error trapping and explanatory prompts in the data entry sections. Existing clerical staff or technical staff as required should enter the patient details easily.
- The system must be as flexible as possible, with results keyed into the computer(s) directly as the work is processed and allowing result amendments and report printout at any time of the day. The system must be capable of easily generating provisional reports, extra copies of reports, and non-standard reports.
- Eliminating filing, photocopying, manually prepared day sheets, and the repeated manual transcription of results should speed up work processing.
- Reports, day sheets, and visual display unit (VDU) formats should be clear and concise with day sheets in surname alphabetical order including the progress of unfinished work.
- It should be possible to revert easily to a manual system in the event of major breakdown.
- It should provide simple and accurate means to identify patients and indicate redundant or duplicate specimens.

The functions of a laboratory computer system can be split into three groups, (1) data acquisition, (2) data analysis and (3) data reporting.

(1) Data acquisition

The system must allow patient data to be admitted directly or, more advantageously, via other pathology or hospital systems.

The system should be able to recognize when a specimen relates to a previously received request, or a request for repeat investigation, so that all specimens from the same patient are linked in a single file. Labels may be printed, either on specimen receipt or before for attachment to the request form, specimen and culture plates.

(2) Data analysis

Test results should be entered. Limit checks may be used to highlight abnormal results. Incomplete test/specimen lists is displayed to enable overdue reports to be investigated by senior technical staff.

(3)Data reporting

Provision of interim reports for clinicians by VDU, paper or direct download of reports should be present. Workload/billing data of varying complexity, depending on the needs of the whole organization should be provided.

Hardware and software

Laboratory workers should be aware of the functional capacities of their system and its limitations. Hardware and software are two frequently used terms related to computer. Hardware is the collective term for those parts of the computer, which physically exist and can be seen, while software refers to the programs, which run on the hardware. With most laboratory computer systems the main computer or processing unit is remote from the users and may be able to handle enquiries from a large number of terminals at the same time. For a computer to operate as a laboratory computer one of the pivotal functions is storage of data.

Error trapping

Comprehensive checks are employed in all data entry routines to avoid errors and nonsense data. Each individual key press is checked and invalid non-alphanumeric keys are ignored. The final composite entry is also checked for general legality (length etc) and by more specific criteria, which vary with each input request-for example, a routine accessed by laboratory reference number will accept only a five-digit number within predetermined limits. The entry of erroneous data results in an audible bleep and a VDU reminder of the requirements.

Formatting

Much attention was given to producing VDU, report form, and day sheet formats, which were clear and concise. Any abbreviated or coded keyboard entries immediately appear as decoded text on the VDU. Report forms and day sheets are printed in full text without codes. All data entry sections display patient details and all previously entered results

Application of computers in microbiology laboratory

A microbiology computer or a laboratory computer with microbiology functions can perform multiple tasks. Word processing programs can be used in microbiology for a number of administrative functions. Besides the obvious functions of facilitating correspondence and writing manuscripts, a word processor is invaluable for preparing and updating procedure manuals. Quality control recording sheets and records can be generated with such a program, changing parameters as necessary. Ongoing records of quality control discrepancies can be placed into the program and then printed out as a single report when needed. The computer could be programmed to flag certain dates for checking on back orders or initiating inquiries. Computers can be used to calculate workload units. Raw data can be entered daily as numbers of specimens processed or numbers of tests performed and calculations can be analyzed and stored for later retrieval. Computers can also directly decrease the manual labour involved in logging in new specimens by printing labels for culture media, printing work cards and many more. Barcode labeling of specimens, read by computer assisted electronic devices, can be used to identify specimens and to order tests correctly. Computers also have advantages in producing listings, charts and graphs to help convey information to clinicians about hospital infection, and provide statistical packages for analyzing trends in infection by hospital ward or consultant. Computers can also be used for more complex nosocomial infection surveillance, not only in analyzing trends in infection but also in high-risk patients. Computers can provide advance searches to define seasonal trends in infection, e.g. *Salmonella* isolation in an infectious diseases ward. In addition to handling management functions, the computer can aid in reporting results. Results immediately accessible to the patient units will have much greater impact on patient care as well as cut down on communication lags and errors. Laboratory computers that interface with hospital computers can deliver patient demographic data directly to the laboratory and provide direct billing capability. Nurse epidemiologists in another location for more efficient operation could retrieve epidemiological data entered in the laboratory.

Implementation of computer and managing changes

A laboratory with a computer is faced with problems associated with the need to develop and maintain additional computing skills. In general, increased clerical staff time has to be found for inputting data to the system. Staff time has to be found for updating and developing the system in line with advances in laboratory techniques or new demands for information.

Implementing a new computer system can be a stressful and unpleasant experience, which, if performed badly, may have long-term impact on the efficiency of a laboratory. Ideally each step should be carefully evaluated and explained, but in practice considerable determination may be required to push through change. Key personnel should be able to work together and communicate well with the other members of the department. Most of the problems within the department or between the departmental implementation group and the commercial suppliers or programmers result from poor communication. Inadequate training and professional arrogance on the part of laboratory and computer staff also impede implementation. All systems need to be tested to detect faults or 'bugs'.

As microbiology databases contain information on the origin of specimens, the type of specimen and the workload associated with each specimen, it may be possible to extrapolate likely changes in work patterns over several years. These can be compared with likely financial constraints and staff availability to highlight when staff changes may need to be made. Specific management computer systems can be used to estimate staff time and consumable costs in processing specific specimen types.

Computers have become increasingly used in all microbiology laboratories. While it is common for laboratory staff to be confused by the jargon surrounding their use, it should be remembered that computers are tools to be used like any other piece like laboratory equipment. Computers will not make poorly organized departments efficient or solve the problems of under or inappropriate staffing, but they may provide the information to help solve these problems and to provide a better service.

References

A.P. MacGowan, Susan Y. McCulloch and A.M. Lovering. Computers in medical microbiology. *Mackie & McCartney Practical Medical Microbiology*. Fourteenth edition.

R. Ashley, B. Downing. 1985. Microcomputer system for multistep specimen processing and reporting in a microbiology laboratory. *J Clin Pathol.* **38:** 215-221.

Joseph Lister

Birth: April 5, 1827
Death: February 10, 1912
Nationality: English
Known for: Inventor of antiseptic surgery

By the middle of the nineteenth century, post-operative sepsis infection accounted for the death of almost half of the patients undergoing major surgery. A common report by surgeons was: operation successfully but the patient died. Being a surgeon Joseph Lister introduced his postoperative antisepsis. Lister did not discover a new drug but he did make the like between lack of cleanliness in hospitals and deaths after operations. For this reason, he is known as the 'Father of Antiseptic Surgery'.

Joseph Lister was born in Upton, Essex, England, on April 5, 1827. He was the second of three children born to Joseph Jackson Lister, a very successful wine merchant and amateur scientist and Isabella Harris. He came from a prosperous Quaker home in Upton, Essex. Young Joseph attended Quaker schools in Hertfordshire and London. Following matriculation, he obtained a Bachelor of Arts degree at the University of London in 1847. After his graduation in arts he enrolled in the faculty of medical science, 1848. Lister was a brilliant student and graduated a bachelor of medicine with honours in 1852.

Lister became first assistant surgeon to James Syme, at the University of Edinburgh in Scotland. In January 1860, Joseph and Agnes moved to Glasgow where he was appointed Regius Professor at the university there. Lister was met by extreme filth and unfavorable conditions in his wards in Glasgow, and lack of cooperation from his colleagues in keeping the area clean. Earlier concept of sepsis (infection) was believed due to the bad air contact or miasma in the air. Lister became aware of a paper published by Louis Pasteur which showed that rotting and fermentation could occur without any oxygen if micro-organisms were present. Lister confirmed this with his own experiments. Lister recognized the relationship between Pasteur's research and his own. He considered that microbes in the air were likely causing the putrefaction and had to be destroyed before they entered the wound. Pasteur suggested three methods: to filter them out, to heat them up, or expose them to chemical solutions. The first two were inappropriate in a human wound so Lister experimented with the third. Lister needed to find a suitable chemical to kill the germs. He learned that carbolic acid was being used as an effective disinfectant to treat sewage. Beginning in 1865, Lister used carbolic acid to wash his instruments, and the bandages used in the operation. Lister also sprayed the air with carbolic acid to kill airborne germs. He also made surgeons wear clean gloves and wash their hands before and after operations with 5% carbolic acid solutions but later it was found that carbolic acid cause contact dermatitis. After more than a year of using and refining these techniques, Lister had sufficient data to show that his methods were a success. He published his findings in a series of articles on the *Antiseptic Principle of the Practice of Surgery* in the medical journal, *The Lancet*, in 1867. Then Lister left Glasglow and returned to the University of Edinburgh and continued to develop improved methods of antisepsis and asepsis. As the germ theory of disease became more widely accepted, it was realised that infection could be better avoided by preventing bacteria from getting into wounds in the first place. This led to the rise of sterile surgery. Some consider Lister "the father of modern antisepsis." Over the next 12 years, Lister's methods gradually gained acceptance. Doctors from Denmark and Germany were the first to implement Lister's antiseptic principle, and they met with stunning success. By 1875, Lister was receiving international acclaim in Europe. However, the majority of English doctors still misunderstood Lister's work and therefore failed to accept its usefulness. It was not until Lister was appointed Professor of Surgery at King's College Hospital in London in 1877 that he began to win over the English doctors. By 1879, Lister's principle of antiseptic surgery had gained almost universal acceptance. Lister moved from Scotland to King's College Hospital, in London, and became the second man in England to operate on a brain tumor. He also developed a method of repairing kneecaps with metal wire and improved the technique of mastectomy.

In 1883 Lister was created a baronet and made Baron Lister of Lyme Regis in 1897. He was also appointed one of the 12 original members of the Order of Merit in 1902. In his later years, the scientific community in recognition of his great contribution to medicine gave Lister many prestigious positions. These included Vice-President of the Royal College of Surgeons, President of the Royal Society, and President of the British Association for the Advancement of Science. In 1891, Lister had helped to establish the British Institute of Preventative Medicine. In 1903, this was renamed the Lister Institute in his honour.

He had retired in 1893 after a long and outstanding career. Lister died on 10 February 1912 at his country home in Walmer, Kent at the age of 84. Although the materials and procedures used have changed over the years, the antiseptic principle itself remains today as the cornerstone of modern surgery. The importance of Lister's antiseptic principle is emphasized by eminent creation scientist, Dr. Henry Morris, who says, 'This development is probably second only to Pasteur's contribution to the saving of human lives'.

References

S. W. B. Newsom. 2003. Pioneers in infection controlJoseph Lister. *Journal of Hospital Infection.* **55(4):** 246-253.

Enjoy the humour

Susie's husband had been slipping in and out of a coma for several months. Things looked grim, but she was by his bedside every single day. One day as he slipped back into consciousness, he motioned for her to come close to him. She pulled the chair close to the bed and leaned her ear close to be able to hear him.

"You know" he whispered, his eyes filling with tears, "you have been with me through all the bad times. When I got fired, you stuck right beside me. When my business went under, there you were. When we lost the house, you were there. When I got shot, you stuck with me. When my health started failing, you were still by my side. "And you know what?"

"What, dear?" she asked gently, smiling to herself.

"I think you're bad luck."

Thoughts to live by

- The difference between a successful person and others is not a lack of strength, not a lack of knowledge, but rather a lack in will. (Vince Lombardi)
- Patience, persistence and perspiration make an unbeatable combination for success. (Napolean Hill)
- To get rich never risk your health. For it is the truth that health is the wealth of wealth. (Richard Baker)
- In the arithmetic of love, one plus one equals everything, and two minus one equals nothing. (Mignon McLaughlin).
- What we are today comes from our thoughts of yesterday, and our present thoughts build our life of tomorrow: our life is the creation of our mind. (Buddha, Founder of Buddhism)

Track your brain

Rearrange the letters to make familiar words. Place one letter in each box or circle. Use the letters in the circles to make words that answer the question below.

SERIES I

(a) T B R A C E I A

(b) P S I S E S

(c) G S U D E L

(d) O L E C N

What do you called the envelope or slime layer surrounding the cell wall of certain microorganisms?

SERIES II

(a) A I A R M L A

(b) O I V D I R

(c) X N O T I

(d) A G L E A

Name of the substance which is used to set dyes in Gram Staining.

Check your Answers on Page 16

Clostridium difficile

Clostridium difficile is a bacterial enteric pathogen that causes a broad range of clinical disease from asymptomatic colonization or mild diarrhea to life-threatening pseudomembranous colitis. It was first described in 1935 as a component of the fecal flora of healthy newborns and was initially not thought to be a pathogen. The species was named "difficile" because initially it was hard to culture. Early studies showed that *C. difficile* could be isolated from the gastrointestinal tracts of most neonates; thus, it was believed to be a commensal organism. Early investigators noted that the bacterium produced a potent toxin; the role of *C. difficile* in antibiotic-associated diarrhea and pseudomembranous colitis was not elucidated until the 1970s.

Bacteriology

Clostridium difficile or CDF is a species of bacteria of the genus *Clostridium* which are Gram-positive, anaerobic, spore-forming rods (bacillus). *Clostridium* shows optimum growth when plated on blood agar at human body temperatures. When the environment becomes stressed, however, the bacteria produce spores that tolerate the extreme conditions that the active bacteria cannot. It is a commensal bacterium of the human intestine in a minority of the population. Patients who have been staying long-term in a hospital or a nursing home have a higher likelihood of being colonized by this bacterium. The organism forms heat-resistant spores; it can remain in the hospital or nursing home environment for long periods of time.

Epidemiology

C. difficile is a ubiquitous bacterium found in soil, hospital environments, child care facilities and nursing homes. This Gram-positive bacillus can spread via fecal oral route; patient-to-patient transmission is well known in hospitals. Whereas the incubation period for *C. difficile*-associated disease is difficult to determine accurately, diarrhea associated with it may occur within days or up to eight weeks after therapy with medications that alter the gastrointestinal (GI) flora. Acquisition of *C. difficile* occurs primarily in the hospital setting, where the organism has been cultured from bed rails, floors, windowsills, and toilets, as well as the hands of hospital workers who provide care for patients with *C. difficile* infection. A wide range of oral antibiotics (eg, penicillins, clindamycin, cephalosporins) and chemotherapeutic agents (eg, fluorouracil, methotrexate) can alter the natural GI flora and favour the emergence of *C. difficile*. Infants and children are more likely to carry *C. difficile* asymptomatically in the GI tract than adults.

Pathogenesis

C. difficile is transmitted from person to person by the fecal-oral route. The organism is ingested either as the vegetative form or as hardy spores. Once spores are ingested, they pass through the stomach unscathed because of their acid-resistance. They change to their active form in the colon and multiply. In the small intestine, spores germinate into the vegetative form. *C. difficile* associated disease can arise if the normal flora of large intestine has been disrupted by antibiotic therapy. Pathogenic strains of *C. difficile* produce two distinct toxins. Toxin A is an enterotoxin, and toxin B is a cytotoxin. Receptor-bound toxins gain intracellular entry where they catalyze a specific alteration of Rho proteins, small glutamyl transpeptidase (GTP)binding proteins that assist in actin polymerization, cytoskeletal architecture, and cell movement. Finally, toxin A exerts its effect on leukocytes by altering the chemotaxis of neutrophils, the activation of macrophages and mast cells, and the induction of inflammatory mediator release. The end result of toxin activity in the intestine is fluid secretion, mucosal damage and interstitial inflammation. Both toxin A and toxin B appear to play a role in the pathogenesis of *C. difficile* colitis in humans. The major host factors predisposing patients to the development of symptomatic *C. difficile*-associated diarrhea include antibiotic therapy, advanced age, number and severity of underlying diseases, and faulty immune response to *C. difficile* toxins. Patients at highest risk for fulminant disease include those who recently received immunosuppressive therapy or recently underwent surgical procedures, and those with a history of *C. difficile* associated diarrhea.The ability to mount an immune response is not protective against *C. difficile* colonization, but it is associated with decreased morbidity, mortality, and recurrence of *C. difficile*-associated diarrhea.

Clinical manifestation

The incubation period from ingestion of *C. difficile* to manifestation of disease has not been established. Symptoms can appear immediately after beginning antimicrobial therapy, or they may not develop until several weeks after it is completed. The clinical presentation of *C. difficile* is a continuum that includes asymptomatic carriage, diarrhea, colitis, pseudomembranous colitis, and fulminant colitis. Most often, CDAD (*Clostridium difficile* associated disease) presents as mild to moderate nonbloody diarrhea, sometimes accompanied by low abdominal cramping. Systemic symptoms are typically absent, and physical examination is remarkable only for mild abdominal tenderness. Colitis tends to present with more severe symptoms, including profuse watery diarrhea and abdominal pain and distention. Fever, nausea, and dehydration are often present. Once severe or systemic symptoms develop, appropriate treatment is crucial to prevent progression to more severe disease. Patients with severe colitis are at increased risk of developing paralytic ileum and toxic mega colon. These may lead to a paradoxical decrease in diarrhea. Watery diarrhea is the disease most frequently associated with *C. difficile* in children. Because the toxins produced by *C. difficile* can cause intestinal cell water secretion, it seems logical that watery diarrhea may result. However, because the organism is found so frequently in asymptomatic children, it is difficult to prove that *C. difficile* is

the cause of this syndrome, which is often mild. Pseudomembranous colitis is a very serious disease; a role for *C. difficile* in this disease has been clearly established. Characteristic features include progressively severe diarrhea, abdominal pain, fever, leukocytosis, systemic toxicity, and stool containing blood, mucus and leukocytes. The most severe manifestation of pseudomembranous colitis is toxic mega colon, which may lead to intestinal perforation.

Recurrence is one of the most frustrating and challenging complications of CDAD. There is no significant way to distinguish between a clinically second episode of CDAD reinfection and a relapse.12% to 24% of patients develop a second episode of CDAD within 2 months of the initial diagnosis. If a patient has two or more episodes of CDAD, the risk of additional recurrences increases to 50% to 65%.

Diagnosis

C. difficile should be suspected in any adult with antimicrobial-associated diarrhea, and CDAD can occur up to several months after antimicrobial treatment is ended. Only watery or loose stools should be tested for *C. difficile* because the rate of colonization is high. A positive result in a normal stool sample proves that the patient is colonized with *C. difficile* but not necessarily infected. There are a variety of tests for *C. difficile,* each with advantages and disadvantages. Factors to be considered when selecting a diagnostic test include turnaround time, sensitivity, specificity, cost, whether there is an ongoing outbreak, and availability. Since the original observations that *C. difficile* are responsible for antibiotic associated colitis, several diagnostic tests have been developed that detect the cytotoxin(toxin B) and enterotoxin (toxin A) produced by *C. difficile. C. difficile* may be recovered frequently from the stool of symptomatic children, but disease due to this organism can only be established if its toxin(s) is identified. The 'gold standard' toxin bioassay evaluates the cytotoxicity of cells in tissue culture. This requires 24 to 48 h, but it has a high sensitivity and specificity. Along with cytotoxin detection, culture has been a mainstay in the laboratory diagnosis of CDAD. A bacterial culture requires up to 72 hours under anaerobic conditions, and it has an overall sensitivity of approximately 95%. The primary advantage of anaerobic culture is that it lends itself to molecular typing of strains, which may be useful in an outbreak. Enzyme immunoassays, available in most clinical laboratories, are fast and require less technical expertise than tissue culture. Other rapid tests that provide answers in minutes to hours include enzyme immunoassays, polymerase chain reaction, latex agglutination and immunocard. Each of these tests is rapid and highly specific, but they all suffer from a lack of sensitivity. Endoscopy is done to diagnose pseudomembranous colitis. Sensitivity of this process is low.

Treatment

The treatment of *C. difficile*-associated diarrhea depends on the clinical presentation. Otherwise healthy adults, the first step is to discontinue the precipitating antibiotic, if possible, and administer fluids and electrolytes to maintain hydration. With this conservative therapy, diarrhea can be expected to resolve in 15 to 23 percent of patients. Specific pharmacotherapy for *C. difficile*-associated diarrhea should be initiated in older patients, patients with multiple medical problems, and patients in whom antibiotics need to be continued. Specific treatment also should be initiated if diarrhea persists despite discontinuation of the precipitating antibiotic or if there is evidence of colitis (i.e., fever, leukocytosis, characteristic findings of colitis on CT scanning or endoscopy). Treatment of symptomatic patients with metronidazole or vancomycin for 10 days is effective; metronidazole is preferred to reduce risk of vancomycin resistance among other organisms in hospitals. If medical therapy fails or perforation or toxic mega colon develops, surgical intervention with colectomy and ileostomy is indicated but carries a high mortality rate.

Control

In considering the possible avenues of infection control, it is useful to examine two approaches that are distinct but related, namely (1) efforts directed at interrupting horizontal spread of *C. difficile*, and (2) efforts to minimize the possibility that organism exposure will result in clinical infection. The rationale for the use of these methods is that *C. difficile* is spread from patient to patient by direct contact with hospital personnel, presumably via their hands. The following guidelines are recommended when dealing with the patients with *C. difficile* associated disease:

- Good hand washing technique is effective against the prevention of *C. difficile* transmission. Washing hands with high-level disinfectant reduce the chance of positive culture rates.
- Medical professionals should use laboratory coats and disposable gloves.
- Medical and nursing staffs should be educated regarding the disease and its epidemiology.
- Environmental contamination of *C. difficile* is, due to persistence of spores that can be highly resistant to routine disinfectants and can survive on dry surfaces for many weeks or months. The rate of surface contamination increases in proportion to the *C. difficile* status, severity of diarrhea, and incontinence of patients in the area. Disinfection is effective in reducing the number of C. *difficile* positive cultures from the environment.
- More aggressive control measures, such as treatment of asymptomatic carriers, might be considered in a severe epidemic.

References

Dale N. Gerding *et al*. 1995. *Clostridium difficile* associated diarrhea and colitis. *Infection control and hospital epidemiology*. **16(8):** 459-477.

Rebecca H. Sunenshine & L.C. McDonalad. 2006. *Clostridium difficile* associated disease : New challenges from an established pathogen. *Cleveland clinic journal of medicine*. **73:** 187-197.

Escherichia coli as an indicator organism for water analysis

Fresh water streams, lakes, ground water or coastal ocean water is considered as polluted when some condition makes the water unsafe for human recreation or consumption. Water can be polluted either by the toxic chemicals or by the pathogenic microorganisms. The largest single source of potentially pathogenic microbes in water is fecal contamination. To maintain the water quality for drinking and other purpose routine monitoring for enteropathogens is necessary. Testing for each organism separately would be extremely costly and time consuming. Therefore a simple rule is followed: if a water sample contains any microorganism common to animal intestines, it should not be consumed, because it may contain enteric pathogens. If these common organisms are absent, bacterial pathogens are unlikely to be present. This concept of indicator organisms was introduced in late 19th century.

Theodor Escherich identified *Escherichia coli*, originally known as *Bacterium coli*, in 1885. *E. coli* is widely distributed in the intestine of humans & warm blooded animals and is the predominant facultative anaerobe in the bowel and part of the essential intestinal flora that maintains the physiology of the healthy host. In 1892, Shardinger proposed the use of *E. coli* as an indicator of fecal contamination. So the presence of *E. coli* in water became accepted as indicative of recent fecal contamination and the possible presence of pathogens. Certain criteria should exist before an indicator organism can be considered reliable in predicting a health risk:

- The organism must be exclusively of fecal origin and consistently present in fresh fecal waste.
- It must occur in greater numbers than the associated pathogen.
- It must be more resistant to environmental stresses and persist for a greater length of time than the pathogen.
- It must not proliferate to any great extent in the environment.
- Simple, reliable and inexpensive methods should exist for the detection, enumeration and identification of the indicator organism.

Escherichia coli was originally proposed as an indicator organism. Other enteric bacteria show similar phenotypic characteristics of *E. coli* and difficult to distinguish. As a result, the term "coliform" was coined to describe this group of enteric bacteria. This group of organisms is aerobic or facultatively anaerobic, Gram negative, non-sporulating, rod shaped bacteria that can ferment lactose at 35-37ºC with the production of acid and gas within 24-48 hours. Fecal coliforms are distinguished from other coliforms by their ability to ferment lactose with acid and gas formation at elevated temperature (44.5-46ºC).

Another group of bacteria has been recognized as an indicator of dangerous pollution and characteristic of sewage and animal fecal wastes, known as fecal streptocooci. In comparison to *E. coli,* they are usually present in lower numbers in feces, but survive better in the aqueous environment and are about twice as resistant to disinfectant.

Another group that has been investigated in this respect is *E. coli* specific viruses, the so-called coliphages. A second group of indicators that is even more resistant are the anaerobic spore formers belonging to the genus *Clostridium*.

Three primary techniques are used for the routine detection and enumeration of indicator bacteria. They are the most probable number (MPN), membrane filter and plate count technique. Additionally, presence-absence test for coliform bacteria have been approved for NPDWR (National Primary Drinking Water Regulations, US) reporting purposes.

MPN test- the most probable number method is a statistical multi step assay consisting of presumptive, confirmed and completed phases. The MPN test uses a specified number of tubes containing a specific medium(lactose broth) and sample water. After incubation each tube is examined for growth of the target organism with acid and gas production. The first or presumptive test is a screening test to sample water for the presence of coliform organisms. If the presumptive test is negative, no further testing is performed, and the water source is considered microbiologically safe. If any tube in the series shows acid and gas, the water is considered unsafe and the confirmed test is a second screening procedure in which a Gram-negative selective medium is used (like EMB agar) to confirm the presence of coliforms. The completed test is performed on a typical, well-isolated colony to confirm gas production in lactose and to determine the morphology and Gram reaction of the isolate from a nutrient agar slant.

Membrane filter test- the membrane filtration (MF) method requires filtering a sample of appropriate volume through a membrane filter of sufficiently small pore size to retain the organism sought. Then the filter is placed on an appropriate agar medium and incubated.Then typical colonies are counted and the number is reported as the number of colonies per 100ml of sample.

Heterotrophic plate count methods provide a standardized means of determining the density of aerobic and facultatively anaerobic heterotrphic bacteria in water. Two methods are followed for plate count techniques: (a) pour plate and (b) spread plate. A count is taken of the growing colonies and reported as colony forming units (CFU) per ml of water sample. Plate count agar and tryptone glucose extract agar are recommended for either method. Violet red bile agar is also used for the isolation and identification of coliforms.

The presence-absence (P-A) test is a presumptive detection test for coliforms in water. The test is a simple modification of the multiple tube procedure. USEPA approved methods require the use of 100ml of sample and the bromocresol purple indicator. The sample is incubated for 24 to 48 hours at 35±0.5ºC. A yellow colouration indicates the formation of acid from lactose fermentation and the test is positive.

References

Atlas, Ronald M. and Richard Bartha. 1993. Microbial Ecology: Fundamentals and Applications. Benjamin/Cummings. Redwood City, CA.

E W Rice, E E Geldreich, and E J Read. 1989. The presence-absence coliform test for monitoring drinking water quality. *Public Health Rep*. **104(1):** 54-58.

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

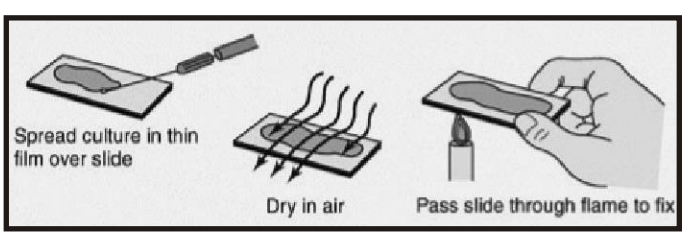
Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50-90% of cell wall), which stain purple and Gram-negative bacteria have a thinner layer (10% of cell wall), which stain pink. Gram-negative bacteria also have an additional outer membrane which contains lipids, and is separated 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 color. Counterstain, which is usually positively-charged safranin or basic fuchsin, is applied last to give decolorized Gram-negative bacteria a pink or red color.

Gram Staining 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.



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 upto 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 shaked off from

the surface.

- The slide is decolourized with 95% of ethanol. The exact time to stop is when the solvent is no longer colored 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 where as *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.

References

G.J. Hucker. 1920. A new modification and application of Gram stain. *New York Agricultural Experiment Station, Geneva, New York*. 395-397.

Forbes BA, Sahm DF, Weissfeld AS. *Bailey & Scott's Diagnostic Microbiology*. 11th ed. St. Louis: Mosby; 2002:97-99,122-125.

As you have gone through the article on Did You Know related to *E. coli* as an indicator organism **Microxpress** recommends following Kits for water quality testing.

Water Quality Testing Kits

- PA Coliform Test Kit
  For the detection of presence or absence of coliform bacteria from water samples.
- Rapid Coliform Test Kit
  For rapid detection of *E. coli* and coliforms from water samples on the basis of enzyme substrate reaction.
- Rapid Coliform Test Kit
  For rapid identification and differentiation of enterococci from water samples.
- Rapid H_2S Test Kit
  For simultaneous detection of *Salmonella*, *Vibrio* species, *Citrobacter* from water samples.
- Presumptive Identification Test Kit for *E. coli*
  Test for fluorescence detection of *E. coli* on the basis of enzyme substrate reaction.
- Test kit for *E. coli*
  Test for presumptive identification of *E. coli* on the basis of enzyme substrate reaction and Indole test.
- *E. coli* Identification Kit
  A panel of 12 tests for identification of *E. coli* (Kit contains sterile broth for MR test, Voges Proskauer test, Citrate utilization, Indole test, Glucurornidase utilization, Nitrate reduction, ONPG utilization and six different carbohydrates Glucose, Lactose, Sucrose, Sorbitol).

In this issue Best Practices article focused on Gram staining and Microxpress recommends following Kit for Gram staining.

- Modified Gram's Stain Kit (containing A, B and C)
  To differentiate between Gram positive and Gram negative.

The healthcare environment contains a diverse population of microorganisms, but only few are significant pathogens for susceptible humans. Microorganisms are present in great numbers in moist organic environments, but some can also persist in dry conditions. The surface and environment are potential reservoirs for the pathogen and plays a major role in spreading contamination & health care associated disease. **BioShields** offers a complete solution for environment and surface disinfection.

Environment and Surface Disinfectant Range

Silvicide™

It is an aerial fumigant and surface & water disinfectant solution from BioShields Environment and Surface disinfectant range. Silvicide™ can be used for the daily disinfection, maintenance and cleaning of critical areas of all hospitals. They can also be used with fogging machines in laboratories, pharmaceuticals, food and dairy industry. Silvicide™ contains 0.01% w/v silver nitrate IP and 10% w/v Hydrogen peroxide IP. Hydrogen peroxide along with silver ions has good synergistic effect. Silvicide™ has following special features:

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SERIES I

B	A	C	T	E	R	I	A
S	E	P	S	I	S		
S	L	U	D	G	E		
C	L	O	N	E			
C	A	P	S	U	L	E	

SERIES II

M	A	L	A	R	I	A
V	I	R	O	I	D	
T	O	X	I	N		
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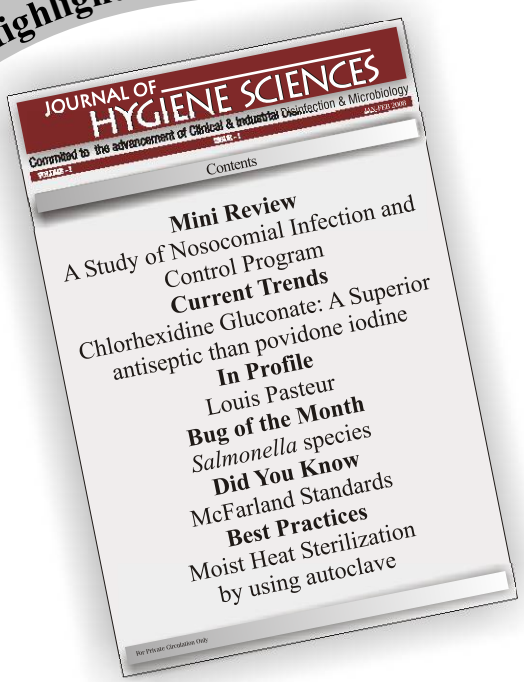
Highlights of the coming issue



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