AGAR AND GRAM STAINING

1. WHERE DOES AGAR COME FROM?

As most students of microbiology will recall Agar, a critical tool in microbiology, is a polysaccharide extract from the cell wall of seaweeds belonging to the family of rhodophyceae. It is produced by boiling the seaweed and the resulting liquid extract is cooled, dried and sold as flakes or cakes. Originally called agar-agar, a Malay word for a local seaweed, these seaweeds are found all over the world in areas favorable to their growth. In spite of the importance of this group of seaweeds in the marine botanical life attaining more than 10,000 species, agar-agar is contained only in few defined ones such as: Gelidium and Gracilaria.

Large known natural layers of Gelidium are situated on the Atlantic coasts from south Morocco to south France, whereas those of Gracilaria are localized on the coast of Chile, South Korea, Japan and Argentina. Because of the reduced size of these agarophyte seaweeds from 10 to 35 cm, the harvesting and collecting can only be done manually during the summer by specialized divers going to 10 meters depth, or by pulling out the plants during the high tides of equinox.

A very manual labor-intensive infrastructure surrounds the diving, gathering, drying and the packaging of red seaweeds in specific coastal areas where Gelidium and Gracilara seaweeds grow. Modern day production of agar involves an extraction of the seaweed liquor under high pressure. The gel is then pressed to eliminate water and, once in cake form, is dried in large drying ovens. Agar is very hygroscopic and the drying cycle can be very long to drive off moisture to eventually achieve a moisture level of 15% prior to milling.

2. DOES AGAR CONTAIN BACTERIA?

The organic nature of agar means that bacteria are always found in this raw material and the process of manufacture, drying and milling provides an excellent environment for bacterial multiplication. With each batch of agar raw material, the original manufacturer provides a certificate of analysis with the viable bio-burden load. This raw material is then incorporated into many different media used in microbiology. Most of these media are in Petri dish plates or tubes and are never scrutinized under the microscope by Gram staining. The medium is sterilized by autoclave so the bio-burden in reality is not an issue. The critical factor in this case is the quality of the microbial growth on or in the medium rather than bio-burden. The use of agar in Amies Agar Gel transport medium is a rare example in which some agar is mixed with patient sample and is microscopically scrutinized. In this case, the microbiologist makes a report based on an assessment of patient sample plus foreign organic agar.

Culture media and transport media are always guaranteed sterile but the non-viable bio-burden load becomes a concern if the medium is examined microscopically. Ironically, agar that produces the most superior bacterial recovery when used in plated media may be totally unacceptable for use in Amies transport media due to the non-viable bio-burden count.

3. WHAT DO MANUFACTURERS DO TO AVOID NON-VIABLES?

Microbiology media manufacturers have to face the reality of a viable and non viable bio-burden in all agar raw materials they use. There is no such thing as “sterile agar raw material” Highly purified agars, like Noble Agar and Agarose, exist with lower bio-burden, but two issues preclude the use of these agars in transport medium: lack of adequate gel strength and cost.

In order to maintain the viability of fastidious bacteria and anaerobes, swab samples must be submerged deep into a column of agar gel transport medium. Agar has to be carefully selected with the correct gel strength to maintain a deep column of agar stable, intact and without any breaks or bubbles. Only an intact column of agar gel functions properly and impedes the penetration of atmospheric oxygen, which is toxic or harmful to anaerobes and fastidious bacteria like Neisseria gonorrhoeae. The barrier feature of the agar gel column is absolutely essential to obtain acceptable recovery of pathogenic bacteria at 24 or 48 hours. Agar gelling strength is one critical factor which cannot be simply overcome by adding more grams of agar because excessive agar is also inhibitory to bacteria. Purified agars are notoriously poor in their gelling strength property. Add to this the phenomenon that ionizing irradiation needed to sterilize the finished product causes a dramatic reduction in agar gelling strength and the agar column can breakdown. These factors, in practice, exclude the use of purified agars for swab transport medium production.

Cost is the other consideration when selecting agar raw material for transport medium. Bacteriology transport swabs have been regarded as one basic commodity product despite the key role they play in the Pre-analytical Phase. The product has had a long history of very low market price. The decision process for choosing a transport swab has largely been based on cost and is more often than not made by purchasing managers outside of the microbiology lab. This means that manufacturers have to be very careful and responsible about all costs of manufacture. If a highly purified agar could be found that met all the specifications of gel strength and resistance to ionizing irradiation then cost becomes the limiting factor.

Micro pore size filters (0.2 μ) for filtration of agar to remove dead cells is one process that can remove non-viable bacterial cells. The agar that results from this process tends to be saved for premium transport products. Transport medium manufacturers, like Copan, try to find the best middle ground without adding unreasonable extra cost. Copan extensively scrutinizes and selects each and every batch of agar to find the raw material with the lowest possible bio-burden. Copan first reviews the certificate of viable bacterial count for each lot of agar raw material submitted by a manufacturer and then performs many Gram stains to analyze and quantify the nonviable load before final acceptance of any agar lot for use in transport medium production. The target is to find an agar lot with the lowest possible bio-burden.

The water used for making transport medium can be one major source of non-viables. It is well known that water straight from the faucet or tap can often contain bacteria that can contaminate Gram stains or Acid Fast stains. Copan takes great care of the water used for the manufacture of its transport media. All water used is produced by Double Reverse Osmosis and is held in hot storage tanks with a completely closed supply loop. Using this system completely eliminates bacterial cells from water and the end-product is often referred to as “Ultra Pure Water.”

Copan takes all precautions during the manufacturing process to eliminate any possible opportunity for bacteria to enter the product or to multiply inside the product. All transport medium is sterilized by steam before dispensing aseptically under laminar flow into tubes and the final packaged product is terminally sterilized by lethal ionizing irradiation. Bio-burden collected during the manufacturing process is normally zero or close to zero, the only potential source of bio-burden including non viable bacteria is the raw material. The only significant source of non-viables in raw material is the agar component itself.

4. ARE THERE OTHER SOURCES OF NON-VIABLES THAT CAN BE DETECTED BY THE GRAM STAIN?

Other known sources of non-viable bacteria that can contaminate the Gram stain slide and have been seen during microscopic examination:

- Gram stain reagents (Crystal Violet, Iodine, Safranin and Neutral Red)
- Dirty glass slides
- Contaminated water used to rinse slides

When investigating non-viable organisms seen in Gram stains, it is always wise to eliminate every potential source of non-viables.

5. DO AGAR-BASED TRANSPORT MEDIA CONTAIN NON VIABLE BACTERIA?

All manufacturers examine and screen raw materials and finished product for nonviable bacteria. The goal is very low or zero nonviable bacteria, but the current state-of-the-art is that agar is not free of bacteria, this is one mission impossible unless manufacturers sterile filter all agar used in transport medium. Alternatively, the manufacturer's target is to achieve the lowest possible level of non-viables in the finished product. Assuming that low levels of bacteria may be present in agar, errors can be made when preparing a Gram stain smears that, by accident, exaggerate, magnify or skew this low level of bacteria. Refer to "What Can Go Wrong When Making a Gram Stain?" for clues to avoid non-viables.

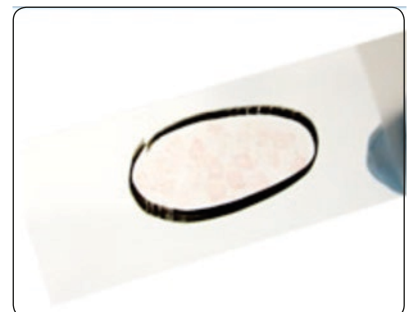
6. ARE NON-VIABLES AN ISSUE FOR LIQUID MEDIA?

The simple answer is no. As agar is the source of non-viable bacteria, Liquid transport medium, like Liquid Stuart and Liquid Amies, are generally free of non-viables. Liquid Stuart and Liquid Amies are essentially sterile balanced salt solutions with a pH buffer and reducing agent, sodium thioglycollate.

7. WHAT QC PROCEDURES DOES COPAN USE TO SCREEN TRANSPORT MEDIUM FOR NON-VIABLES?

Copan performs extensive QC analysis for non-viables on every lot of agar raw material and every lot of finished product. Gram staining protocols can be difficult to reproduce from one lab to another as this procedure is hard to quantify. Gram staining is more of an art form than a science, as each microbiologist has his/her particular style and technique for preparing a Gram stain. The volume of patient specimen and agar emulsion applied to a glass slide and the surface area over which the material is spread is not well defined and can vary dramatically. The smear can incorporate a very thick or very thin agar emulsion, and this is a major source of discrepancy. Copan has devised a volumetric and quantitative protocol for Gram staining agar to analyze the level of non-viables. By quantifying the Gram stain, removing technical variation and subjectivity, it will be possible to set a quality standard for non-viables that can be tested and verified in any lab in the world. Copan uses this as the basis for driving quality improvements in bio-burden with the target being an ultra clean agar gel specification. A simple volumetric protocol offers every laboratory a simple and standardized method for quality control of transport medium for non-viables that is volumetric and objective. The Copan QC procedure details precisely the volume of agar

GRAM STAIN RESULTS

EXCESSIVE AGAR	CORRECT METHOD
	

to analyze, the surface area to spread the emulsion and the number of microscopic fields to examine. Currently no published standard exists for analyzing agar for non-viables. The procedure that Copan uses is precise and more stringent than any anecdotal reference that may currently exist.

8. WHAT CAN GO WRONG WHEN MAKING A GRAM STAIN FROM AGAR TRANSPORT MEDIUM?

The first and most critical concept to grasp is that with swab samples the patient specimen is trapped inside the fiber matrix of the swab. The agar gel is just a distraction. The agar gel is simply a support medium that provides moisture so the organisms do not dry out and maintains optimum pH, Eh and osmotic pressure for microbial survival. The goal when making any Gram stain from a swab is to squeeze out some of the specimen that is trapped in the fiber. From the outset, the target is to avoid as much as possible the transfer of any agar onto the glass slide. Lift the swab out of the agar gel and try to remove the excess pieces of agar against the side walls of the swab transport tube before removing the applicator stick out of the tube.

The swab bud shape is formed around a plastic stick that resembles a narrow drinking straw. By design, the tip of the swab bud has the greatest amount of fiber, hence trapping the greatest volume of the patient sample. The best way to squeeze the patient sample onto the microscope slide is to place the glass slide flat on the lab bench. Hold the swab vertical (at approximately 90°) to the glass slide and push down on the tip for a few seconds. The liquid in the fiber tip should ooze out of the tip onto the glass slide. Use the swab then to spread the liquid over a marked area on the glass slide. By focusing on squeezing out sample from the swab tip, you will be sure that the material you Gram stain is the patient sample and not sterile agar.

Sometimes the patient sample discolors the agar gel transport medium. Blood can typically diffuse into the agar. This discoloration can also be a big distraction, and it can be a temptation for the technologist to deposit large amounts of agar on to the microscope slide. This is a big mistake. The agar gel is sterile and if you Gram stain volumes of agar instead of sample squeezed from the swab tip you risk missing completely the heart of the sample or giving a report on a specimen that was dramatically diluted with sterile agar.

The agar gel is simply a supporting phase for the bacteria; remember the majority of the sample is trapped in the fiber swab tip. If you focus on squeezing out the sample and avoiding the gel this will result in maximal specimen released onto the slide, onto the culture plate or into the assay. Remember how you are encouraged to squeeze out the swab tip for those rapid Strep A assays.

9. IS IT OK IF I CANNOT SEE MUCH VISIBLE STAINED MATERIAL ON MY MICROSCOPE SLIDE?

Sometimes when you squeeze the patient sample from the swab tip onto the slide it produces material that does not stain very heavily. This can create some doubt or uneasy feeling. Do not succumb to the temptation to layer large quantities of agar gel like marmalade onto the slide. This, for sure, will give you a nice strongly staining organic mass on your slide that you can see with the naked eye from 10 yards away. This slide preparation, however, contains less of the patient specimen, which will, in any case, be obscured by the excessive amount of darkly staining agar. At the same time, this dramatic increase in agar volume within the same surface area to be examined dramatically increases the chance of discovering and counting nonviable bacteria that originated from the agar and not from the patient.

10. HOW CAN I MINIMIZE THE CHANCE OF SEEING NON-VIABLES IN AGAR TRANSPORT MEDIUM?

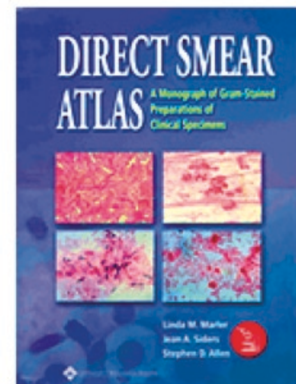
Focusing on squeezing sample from the swab tip and avoiding the agar support phase automatically and dramatically reduces the chance of seeing nonviable bacteria. This is good practice, in any case, as it improves the quality of the sample that you are actually examining microscopically.

11. ULTRA CLEAN AGAR FOR ALL FUTURE GRAM STAINING - A NEW QUALITY STANDARD

The starting agar raw material is not perfect; it comes already charged with bacteria. Manufacturers must carefully select the material with the lowest bio-burden, and then utilize this product in a process that must exclude any opportunity for bacterial growth. If the manufacturing process fails, it will result in an explosive quantity of non-viables that would be visible in large numbers in every microscopic field. Under normal circumstances, if the manufacturing process succeeds, then you can enjoy an end product that has low numbers of non-viables. The only way to truly remove all non-viables is to filter all agars just immediately prior to filling tubes to remove all debris and dead bacterial cells. Until now, most manufacturers have chosen to stay with careful microscopical analysis to select the best raw material and to QC the finished product. However there is a growing demand in clinical microbiology for ultra clean agar transport medium for Gram stain microscopy, so there is little or no chance of seeing non-viables. Technologists today do not want to have to hold back a report because a Gram stain revealed scanty cells that could be nonviable bacteria from the agar gel medium rather than from the patient. The alternative products, such as Liquid Stuart transport medium or specific anaerobic transport swabs, may not cover the spectrum of bacteria needed or are too expensive. If traditional fiber swabs are used, agar gel transport medium represents the most convenient and broad spectrum medium for maintaining aerobes and anaerobes. The future need therefore is for unequivocal ultra clean agar gel medium to allow stat Gram strains to be done with no risk of ambiguity. Copan has responded to this need by raising current product performance specifications so that selection criteria for agar raw material and criteria for agar product to be released for sale are significantly higher. Information on QC procedures Copan uses for detection and quantification of non-viables in agar transport is available upon request. With these procedures microbiologists will be able to perform QC in their own labs to determine the quality of their transport medium. An ultra clean agar standard for Amies Agar Gel Medium will be an important advance to improve Gram staining analysis on swabs.

12. CAN YOU RECOMMEND ANY REFERENCE BOOKS OR MATERIALS FOR TEACHING OR TRAINING HOW TO READ GRAM STAINS?

Marler, Linda, Siders, Jean and Allen,Stephen
Direct Smear Atlas - A Monograph of Gram-Stained Preparations of Clinical Specimens
Lippincott Williams & Wilkens ISBN 0-7817-2663-8



R. Quintiliani and R. Bartlett
The Gram-Stained Smear: An Important Tool In Presumptive Antibiotic Therapy
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