A Practitioner's Approach to Mastitis Microbiology— Acute and Subclinical

Douglas M. Vandamme, *D.V.M.*
Baldwin, Wisconsin 54002

Acute Mastitis

Selection of Cows and Quarters to Sample

At our clinic we sample all cases of acute mastitis we are called to the farm to treat. We also try to culture all chronic recurring cases as well. Our philosophy is such that if we know who we are fighting the battle is already half won. Knowing the ecology and pathogenesis of the organism often dictates which direction we will go in treatment such as dry treatment for *Staph aureus,* herd treatment for *Strep ag* etc. Each quarter is sampled individually in a whirlpak bag, except for subclinical mastitis with high cell counts which are composite sampled. We also have farmers bringing in samples for culture which are chosen from the monthly DHIA summary sheet because of high cell count but without clinical mastitis. When large numbers of cows with high cell counts are present in the herd or the dairy farmer is in danger of loosing his milk market we request a bulk tank culture followed by a whole herd culture if appropriate from information obtained by the bulk tank culture.

Rationale of Intensive Culturing

In cases of acute bovine mastitis, rapid treatment is essential to salvaging milk production in the affected quarter and often times in saving the life of the cow. Proper antibiotic therapy and other necessary procedures such as fluid therapy calcium administration must be instituted at the first sign of mastitis. Treatment success or failure is usually determined within the first twenty four hours of onset of signs. Acute cases of mastitis in the bovine generally are caused by coliform organisms although staphylococci and streptococci may be involved occasionally. The production of endotoxins from massive coliform growth and subsequent death account for a major part of the mastitis syndrome exhibited by the cow. Knowledge gained by identification of the causative organism and its sensitivity profile can help the practitioner solve many herd problems. For example, testing can determine if the infection is curable or likely to become chronic. Herd problems may be due to Klebsiella from dirty straw or contaminated wood shavings, Serratia from contaminated teat dip cups or other sources, *Escherichia coli* or Group D streptococci from environmental contamination, or *Strep. ag.* and *Staph. aureus.* from contaminated milking equipment. Susceptibility testing is necessary to choose an appropriate antibiotic and to eliminate ineffective therapy. At our clinic identification and susceptibility results are complete within 18 to 24 hours. Accumulated knowledge from large numbers of cultures gives us an estimation of which antibiotics are most likely to be effective against the organism in question and also establishes a pattern for the dairy involved. When culturing mastitis on the premises we can watch the cultures in the incubator and check on them every few hours and at the first sign of growth which is often at 6 to 8 hours we can begin the workup of the culture and consequently have the culture identification and susceptibility done in 18 to 24 hours. If no improvement is seen in the animal's temperature after 8 hours the farmer is encouraged to call and we Gram stain the culture or direct smear the milk to see if we can get an earlier diagnosis such as Gram negative rod, Gram positive cocci, or possibly a yeast. Then we may change our mode of therapy based on these findings. We also culture all owner treated cows that have had acute mastitis in the previous few days and have not responded to treatment or have worsened. We first Gram stain these samples at the time the culture is started since about one third will not grow any bacteria but will have a few bacteria on the Gram stain. A surprisingly high number, approximately one half to two thirds, will grow a pure culture, usually a coliform or yeast, after intramammary infusion or even broad spectrum antibiotics. We also culture chronic recurring cases but ask that samples be taken at the time of a flareup if meaningful results are to be obtained. Culturing also helps a dairy farmer decipher his problem be it *Strep ag, Staph aureau, Klebsiella* from wood shavings or sawdust or dirty straw, *Serratia* or *Pseudomonas* from teat dips, dipping cups or dirty contaminated equipment used in the milking process.

Manufacture of Culture Plates

Supplies needed:

1) Tryptose Agar Base
2) Mueller-Hinton Medium
3) Phenol Red Mannitol Agar (Difco only)
4) TKT-FC Agar Medium (Gibco only)
5) Petri dishes 15x100mm
6) Petri dishes 25x150mm
7) Hot plate
8) Autoclave
9) 125 Wheaton bottles with Teflon caps since these will withstand repeat autoclavings.

10) Prescription balance or postage scale
11) Pourite (ASP B1162-1)
12) Esculin Powder (sigma E8250)
13) Ferric Citrate 1% solution

Powdered media is weighted out and mixed with distilled water and heated in a metal coffee pot over a hot plate after two drops of Pourite are mixed with the water. Pourite is added to the water as a surface active agent to prevent bubbles on the surface of the media when pouring plates, especially a problem with blood plates since blood is added to the media just before pouring and mixing results in bubble formation. If blood agar is allowed to stand long enough for the bubbles to dissipate before pouring, the agar often solidifies partially in the bottle and reheating chocolatizes the agar or else lumps form on the plates; therefore we find Pourite to be a useful tool for us. The media is heated to boiling while stirring constantly and then poured into 125ml Wheaton bottles and autoclaved at 212 degrees F. for 30 minutes with the caps on loosely. After autoclaving the bottles are removed and caps tightened when cool and then marked with contents using a felt tipped marker and placed in the refrigerator until it is needed or some plates can be poured after autoclaving and cooling to the point where one can hold the bottle next to your cheek without burning yourself. If the plates are poured when the media is too hot much moisture condenses on the surface of the media and the colonies run together instead of being isolated. If the media is too cool before pouring it may partially solidify and form lumps in your plates. When more plates are needed a bottle of media is taken from the refrigerator, the caps loosened, and heated in the autoclave 20 to 30 minutes until the media is remelted and upon cooling fresh plates are poured. We usually pour between 1 to 2 liters of media in 125ml bottles at one time and keep it refrigerated after autoclaving. One 125ml bottle will pour about 7 to 8 plates 15x100mm containing 12-14ml of media.

We also make our own blood plates. The blood is drawn in a sterile manner in an ACD Solution container under vacuum from a healthy bovine animal that has not lactated if a heifer and has not recently been ill. We draw 3 to 4 containers of 125ml size each time we go to the farm to draw blood and it keeps suitably for 3 to 4 months under refrigeration. When the tryptose agar base is remelted, we cool it until we can hold it against the cheek without burning and then aseptically add 5ml of ACD blood which has been mixed well. Recap the media and rotate the blood agar and mix well and pour immediately. Do this in a room with no air vents and or air movement to prevent contamination. When the media is cooled and hardened we flame the plates for 2 to 3 seconds with a propane torch with a flared tip to sterilize the surface before closing the covers and refrigerating upside down. This is about as sterile environment as a veterinary clinic could expect to possess. We keep on hand some organisms of known hemolytic patterns and test each new lot of blood with these organisms. They are an incomplete beta hemolytic Staph, a Staph with a double zone of hemolysis, a beta hemolytic *Strep ag*, and an alpha hemolytic Strep and a nonhemolytic Strep. To date by choosing a nonlactating heifer that is healthy we have never had to discard any blood because of improper hemolytic patterns. The plates are bagged and stored upside down in the refrigerator as soon as they are hardened. We pour only 10 to 20 plates at a time always insuring fresh plates that are more likely to yield better and faster isolations, without any wastage due to contamination or drying out.

We also use phenol red manitol agar with 0.25% added dextrose along with our blood agar in all acute mastitis cultures to insure better isolation. We like the phenol red mannitol agar because we get faster isolations of organisms especially the Gram negative rods and yeasts grow much better on this media than on blood agar. Also the mannitol reaction helps to early identify *Pseudomonas, Proteus,* yeasts and a few other organisms that are mannitol negative. Most *Staphs, Streps,* and Enterobacteraciae are mannitol positive. With the small amount of dextrose added there is not enough carbohydrate to disturb the mannitol reaction but it does enhance the fastidious organisms growth. We are very happy with media since if we are to fail to get an isolation it will invariably be on the blood agar and seldom on the phenol red mannitol media. This media is not to be confused with phenol red mannitol salt agar which is selective for *Staph* only. The only drawback to phenol red mannitol agar is that no information regarding hemolytic patterns is afforded and this is very important when culturing mastitis causing organisms. The only time we do not use this media is when doing bulk tank cultures.

Sample Collection

We do not collect all samples and therefore we must educate our clients on how to do this for themselves. We evidently have succeeded since we rarely get samples resulting in mixed cultures or a high degree of *Bacillus* contamination. Client education for us means early detection and proper treatment, this means success in treating most acute mastitis cases.

If the teat is clean and dry simply brush off any loose dirt with your fingers, discard the first three to four squirts then collect about 15 to 30ml of milk in a whirlpak bag. If the teat is dirty clean off with an alcohol soaked cotton or disinfectant solution on a paper towel but make sure the teat is completely dried off or contamination will occur from liquid running down the side of the teat and into the collection vessel. Again discard the first 3 to 4 squirts of milk after cleaning the teat and collect 15 to 30ml of milk from the affected quarter or quarters. If a composite sample is submitted then it should contain about 3 to 4 squirts of milk from each quarter. The sample is brought to the clinic immediately or if not, it is refrigerated until it arrives. Samples can be held for two days under refrigeration or up to 1 week if frozen. If a dairyman treats a case of mastitis himself he is instructed to sample the quarter first and

refrigerate it if he does not want it cultured immediately and then go ahead and treat it himself. If it does not respond satisfactorily then he can submit the untreated sample for direct Gram stain and culture the following day. If we collect samples on the farm we get them on media within 3 hours or less. These collection methods along with fresh media, result in 90% recovery rate in chronic mastitis cases treated at least 2 weeks previous and 95% recovery rate for acute mastitis.

Plates are streaked by taking a swab of milk from the culture bag and swabbing ¼ to ⅓ of the plate and then taking a flamed loop and streaking into the swab line 3 times and then 4 to 6 extra streaks, continue to do this two more times. Streak one phenol red mannitol plate and one blood plate. Immediately place the plates upside down and incubate at 35 to 37 degrees C, and observe them beginning in six hours and every 2 hours thereafter for visible growth since in acute mastitis cases colonies are often visible on the primary isolation plates in 6 to 8 hours after incubation begins. Chronic mastitis cases are not checked until 12 to 18 hours post incubation. In cases of no growth cultures are kept for 3 days to check for slow growing organisms like *Corynebacterium, Nocardia,* some yeasts and *Mycobacterium* and molds and fungi.

Bulk Tank Sampling

The milk hauler is asked to fill an extra whirlpak bag of milk for 2 to 3 consecutive pickups and each time the farmer freezes the extra bag of milk and upon getting 3 he will submit them to the clinic in frozen form for bulk tank culturing. After thawing and mixing well we take 3 cc of milk from each bag with sterile pipettes and mix together in a sterile culture tube. Then 0.2ml of milk is placed in a sterile tube containing 1.8ml of sterile saline and this will then make 2.0ml of a 1:50 dilution. Then 0.2ml of this 1:50 solution is taken and added to another tube containing 1.8ml of sterile saline to make 2.0ml of a 1:500 solution. You are now ready to steak the plates. The three types of plates that are needed are MacConkey, TKT-FC, and blood agar. Three of each type are needed and are marked 1:5, 1:50, 1:500. The plates are then inoculated with sterile pipettes with the same amounts of the 1:50 and 1:500 dilutions previously made. The plates are then ready for spreading with a sterile glass rod bent in the shape of a hockey stick and sterilized between the spreading of the milk on each plate with alcohol dipping and flaming and cooling. Then the plates are placed upside down in the incubator til the next morning or 24 hours before reading. After incubation the plates are counted for total and differential numbers of bacteria. Three dilutions assures an accurately countable number of bacteria on one of the dilutions without having to redo the process the following day.

Blood Agar Plate—count all colonies for TOTAL bacteria.

MacConkey Plate—count all colonies which are coliforms.

TKT-FC Agar Plate—count all colonies with beta hemolysis, they are *Strep agalactia,* count all colonies with darkening around them, they are *Strep uberis,* since they are esculin positive.

Blood Agar Plate—count all colonies with large zones of beta hemolysis, they are *Staph aureus.*

The TKT-FC plates are made by using the TKT-FC Agar Base and adding 1cc of hemolysin and 5cc of ACD blood per 100ml of liquid medium. Hemolysin is made by growing a strongly beta hemolytic *Staph aureus,* in 10ml of tryptic soy broth for 3 to 4 days mixing daily then killing the culture by adding 5cc of chloroform and mixing well and several times during the next 12 hours. Let the mixture set in a tube rack for 24 hours for the chloroform to settle out and then the supernatant which contains the hemolysin but no live bacteria is ready to use in making TKT-FC plates. Mix well after each opening of the tube to prevent bacterial contamination. This hemolysin if kept refrigerated will usually remain potent for about 1 month.

The following chart depicts how we interpret out bulk tank results and our advise to the dairyman.

Type of Bacteria	Usual source of Infection	Major Means of Spread	Control Measures
Strep ag	infected udder of other cows	cow to cow by contaminated wash rag teat cups etc.	milk clean dry cows separate towels, dry, dry treat, teat dip, herd eradication.
Staph aureus	infected udder contaminated skin and bedding	cow to cow by contaminated wash rag milking equipment or inadequate equipment	milk clean dry cows, separate towels to wash and dry teat dip, dry cow teat and cull.
Nonag Strep	environment	environment to cow wet dirty lots, milking wet cows liner squawking	improve barn and lot sanitation, milk clean, dry cows, avoid liner slippage
Coliforms	environment	environment to cow wet lots, milking wet cows, liner slippage teat injuries and hot weather	improve barn and lot sanitation, milk dry cows, avoid liner slips
Staph epi	skin inhabitant	poor cow preparation milking wet cows,	milk clean dry cows, teat dip

Gram Staining

The most commonly encountered pathogens are *Staph, Strep, E. coli, Klebsiella,* yeasts, *Serratia,* and *Corynebacterium.* Occasionally *Nocardia, Pseudomonas,* and *Proteus* are seen. These organisms can be difficult to distinguish on a culture plate by gross morphology when very young at six to

eight hours of age, therefore Gram morphology is essential. Taking a glass slide and emulsifying a colony or two with water and drying it we then stain with a commercial Gram stain kit such as Difco's Bacto Gram Stain Kit. About 15 seconds on a dye and rinse cycle seems sufficient. We Gram stain direct milk smears if needed and when time permits since it can give us a tentative diagnosis if required. We also Gram stain the chronic cases that have been treated and do not respond, and all cases resulting in no significant growth in 24 hours, also acute cases treated in the last day or two without significant response and in those cases that have deteriorated significantly in the last eight hours. It can often times give us a quick clue before culture results can be finalized.

Catalase Testing

All Gram positive cocci are tested with a drop or two of 3% hydrogen peroxide to check for evolution of oxygen signifying the presence of the enzyme catalase which is present only in Staph and not in Strep. Be sure not to scrape into the blood agar if using colonies from a blood plate since erythrocyctes contain the enzyzme catalase also. We use colonies from the phenol red mannitol plate to assure we don't have any false positives.

Coagulase Testing

Coagulase positive staphylococci generally cause clinical mastitis with high cell counts. Coagulase negative staphs may or may not cause clinical mastitis but usually have lower cell counts than coagulase positive staph isolates. Therefore coagulase testing is a useful tool in separating the *Staph aureus* from the other *Staph sp.* Technically the current identification of *Staph aureus* is not by coagulase testing alone but also by biochemical fermentation patterns, however for all practical purposes we commit a few errors if we call all coagulase positive *Staphs, Staph aureus.* For coagulase testing we are using the Staphase test by API-Analytab Products, Inc. This test is a lyophylized rabbit plasma test. The lyophilized rabbit plasma comes in a plastic tube and cupule typical of API packaging and is reconstituted with 2 drops of distilled water and mixed well with 2 to 3 colonies of staph on a wooden applicator stick. It is then incubated at 35 to 37 degrees C. for 1 to 4 hours in a plastic humidity chamber supplied with the kit. Any gelling or coagulation in the four hour period is a positive reaction. The strip should be checked hourly and removed if positive before four hours since the enzyme staphylokinase may lyse the clot and it may read as a false negative. However, this is much less of a problem than with the tube coagulase which can take up to 24 hours. When using only the coagulase test for determining which cultures are *Staph aureus* one must bear in mind that 100% of *Staph intermedius* and about 50% of *Staph hyicus* are also coagulase positive but this can actually be beneficial since these are also known mastitis pathogens.

In our clinic we have been using the Staph-Ident strips by API for about 2 years and found them to be confusing and somewhat inadequate. The color reactions seem hard to read since differentiation of how yellow must yellow be for a positive reaction is difficult. We agree wholeheartedly with Harmon and coworkers of Kentucky that veterinary isolates of staphylococci are not adequately identified by the Staph-Ident system as it is now marketed.[1]

Occasionally a *Micrococcus sp.* will be encountered which looks like a staph but is coagulase negative. It could be therefore mistaken for a coagulase negative staph but not *Staph aureus* which would be coagulase positive. Also *Micrococcus sp.* is oxidative only and requires the presence of air to utilize glucose. *Staph* is fermentative and can utilize glucose beneath the surface without the aid of oxygen.

Streptococci Identification

Strep are the Gram positive cocci or coccobacilli that are catalase negative and may or may not show chaining on media. Chaining is more often noted when grown in liquid media or directly smeared from milk. The three most important *Strep sp.* causing bovine mastitis are *Strep ag, Strep uberis,* and *Strep dysgalactiae.* However, occasionally *Strep faecalis, Strep faecium, Strep bovis, Strep pneumonia,* and Lancefield Group "G" also cause mastitis. The following chart is useful in differentiating these organisms.

	Camp	Esculin	Lancefield Group	Hemolysis
Strep ag	+	−	B	Beta
Strep dysgal	−	−(+)**	C***	Alpha
Strep uberis	−(+)*	+	None	Alpha, Gamma
Strep faecalis	−	+	D	Alpha, Gamma
Strep faecium	−	+	D	Alpha, Gamma
Strep pneumonia	−	−	None	Alpha, Gamma
Group "G" Strep	—	—	G***	Beta
Strep bovis	−	+	D	Alpha, Gamma

* Most isolates of Strep uberis are CAMP negative but a few will be positive. We use this battery of tests at our clinic for differentiation of Streps we isolate. We also have an API 205 system for backup in case we have a Strep sp. we want identified.
** Up to 25% of Strep dysgalactiae can be esculin positive.
*** Group "G" and some beta hemolytic Group "C" streps will give a false club shaped CAMP reaction to the untrained eye.

The *Strep sp.* is first inoculated onto a CAMP-ESCULIN plate and both reactions take place simultaneously and are very inexpensive. A CAMP-ESCULIN plate is streaked down the center with a culture on an incomplete beta staph and then the streps are inoculated at a 90 degree angle to the staph and within 2 to 3mm of the staph streak and the strep growth will be a clear arrowhead zone which is a positive CAMP reaction. If the esculin reaction is positive there will be a darkening of the media along the growth of the strep line. Darkening of the media is caused by splitting of the esculin to esculetin which reacts with the iron to form a dark color. As many as 10 streps can be inoculated onto one plate at a time. Always use a control *Strep ag* and a Group "D"

positive strep to check your CAMP and ESCULIN reactions.

CAMP-ESCULIN plates are made as follows:

Add two drops of Pourite to 1 liter of distilled water and stir. Then add 40Gm. of Tryptose Agar Base and dissolve. Then add 1Gm. of Esculin powder and 10ml of 1% FerricCitrate Solution. This gives 0.1% Esculin and 0.01% Ferric Citrate in the agar. The mixture is brought to boiling and poured into Wheaton bottles to volume of 100ml and autoclaved. After autoclaving, cool to the same temperature as you would for making blood plates and add 3 to 4% ACD blood or washed cells and pour your plates. If stored, the blood is added to the media upon remelting and subsequent cooling of the media. Using only 3 to 4% blood in these plates gives a better hemolytic pattern for the CAMP reaction.

Esculin and Ferric Citrate added to the Tryptose Agar Base can be remelted without any stability problems. A 1% Ferric Citrate Solution can be stored in the dark in a refrigerator for at least a year. It is important to use Tryptose Agar Base for CAMP-ESCULIN testing because the CAMP and ESCULIN reactions are much more pronounced and easier to read than if other types of blood agar bases are used.

The CAMP and ESCULIN reactions are good for separating *Strep ag* from other *Strep sp.* However, several *Strep sp.* are CAMP(–) and Esculin (+). This presents a problem because some of these streps are penicillin resistant as well as cephalosporin resistant. Therefore it is important to be able to differentiate these species. Most fecal streps are penicillin G resistant as well as cephalosporin resistant, whereas *Strep uberis* can be penicillin resistant, according to current literature. I believe some of the confusion stems from the fact that some of the organisms called *Strep uberis* may in fact be Group "D" streps therefore simply stated Group "D" streps are often penicillin G and cephalosporin resistant but *Strep uberis* may in fact not be resistant to penicillin G or cephalosporins. We have used the API 20S system for streptococcus identification but have now discarded this system because it was too difficult to interpret and required technical assistance for proper identification along with more testing which was usually LanceField typing. The system just became too cumbersome and time consuming. This has been a typical response from other people who have used this system in their laboratories. We are now using the Phadebact Streptococcus Identification System by Pharmacia Diagnostics. All CAMP(–) Esculin(+) streps are tested for Group "D" antigens. If they are negative then they are *Strep uberis.* If they are positive then they are either enterococcal or nonenterococcal Group "D" strep. The enterococcal Group "D" streps are known commonly as fecal streps. When we are using the Streptococcus 20S ID system we found that greater than 50% of the Group "D" streps were identified as *Strep faecalis* or *Strep faecium.* Therefore I feel that most of the organisms called *Strep uberis* are in a catch all category and the majority are fecal streps and the minority are truly *Strep uberis.*

Identification of Gram Negative Rods

If a Gram stain reveals a Gram negative rod the culture is then inoculated onto a cytochrome oxidase disc or strip to differentiate the Enterobacteriaciae which are CO (–) from the nonEnterobacteriaciae which are CO (+). We use the Taxo N disc from BBL which is reported to be more sensitive for weak oxidase producers like *Pasteurella sp.* We then take enough colonies to make a density equal to a 3 to 5 McFarland in 5ml of sterile saline and inoculate an API 20E strip according to the directions. The strip is placed in a humidity chamber and incubated at 35 to 37 degrees C. for 8 to 24 hours. Originally the strips were to be read at 24 hours however we compared heavy inoculum density and incubating for 8 hours and found we got consistantly reliable results in the shorter time span. The API 20E will reliably identify the Gram negative organisms listed on both sides of the handout booklet. An API Profile Index is provided to identify the organisms by a seven digit system and a nine digit number system for nonfermenters.

Antibiotic Susceptibility Testing

The only approved method is the FDA approved Bauer-Kirby method, which we essentially use but deviate slightly to speed up the susceptibility results. We were originally inoculating a tube of Tryptic soy Broth to a 0.5 McFarland density standard with organisms instead of inoculating a few organisms and waiting for the organisms to multiply to create the correct density. We now use the new Prompt Sensitivity Standardizing System by 3M Company to directly pickup the correct number of organisms, namely 5×10^7 to 5×10^8 colony forming units per ml. The organisms are picked from typical isolated colonies on the original isolation plate with a wand which has a very precise grid on the end, which picks up only the correct density of organisms. The loaded wand is placed in the 0.5ml saline container supplied with the kit and the complete inoculated unit is vortexed for 10 seconds at high speed or hand shaken vigorously for 50 times.

Then a 25x150mm plate of Mueller-Hinton medium is swabbed with organisms from the Prompt tube. Swabbing is first in the form of a cross. Then the plates are swabbed completely by turning the plate 60 to 90 degrees each time. Then the inside of the plate is ringed with the swab to remove any excess moisture and get organisms to the edge of the plate. The swab should be rolled between the fingers as the swabbing is done to get even inoculation all over the plate. Then place the susceptibility testing discs on the plate at least 25mm apart to prevent interference by antibiotic diffusion. On a 150mm plate you can place approximately 12 to 13 discs. We use this size Mueller-Hinton plate for testing staph, Gram negative rods, and *Bacillus sp.* If we are doing susceptibility testing of streps or *Coryne bacterium sp.* we use 100mm plates because of the smaller number of antibiotics tested. However, for mastitis work susceptibility testing of strep and *Coryne sp.* need not be done because of

reliable antibiotic susceptibility patterns.

After inoculation of the organism and placement of discs the susceptibility test plate is placed upside down in the incubator and incubated for 12 to 18 hours at 35 to 37 degrees C. according to the Bauer-Kirby method. Using Prompt System by 3M Company susceptibilities can be read reliably at 8 hours except in the case of carbenicillin and nitrofurans which should be read at 18 hours. If needed at least some antibiotics can be chosen after 8 hours if emergencies require it. One of the most common errors I see made is the use of the wrong test disc for the penicillinase resistant penicillin group. Many laboratories are using cloxacillin or dicloxacillin discs and getting many resistant staph zone sizes because of the unavailability of correct zone sizes for this organism using these two previously listed test discs. The only disc to use when testing for the penicillin resistant staph is the Methicillin 5mcg disc. This is the class test disc for all the following antibiotics: nafcillin, oxacillin, cloxacillin, dicloxacillin as well as methicillin itself.

We use the following test discs for the listed organisms:

Staph	Strep and Corynebact	Gram Neg. rods	Bacillus
PenG	PenG	Vetisulid(SL 1.0)	PenG
Amp	Amp	Kana	Amp
Meth	Meth	Gent	Meth
Carb	Carb	Tet	Carb
Ceph	Ceph	SSS	Ceph
Novob	Novob	Amp	Novob
Clinda	Clinda	Ceph	Clinda
Erythro	Erythro	Cefox	Erythro
Fur		Carb	Fur
SSS		Chloro	SSS
Chloro		PolyB	Gent
Gent		Spect	Kana
Kana		SXT	Tet
Tet		Fur	SXT
SXT		Nolv	

Nolvasan discs are available from Fort Dodge Labs, Fort Dodge, Iowa. Spectinomycin discs are available from Diamond Labs, Des Moines, Iowa. Vetisulid discs are available as Sonilyn 1.0mgm discs from BBL.

Yeasts

Yeasts are present in our milk samples at the rate of about 2.5%. Since we culture all acute mastitis and also many chronic mastitis cases we pick these up usually with the first 24 hours on acute cases and definitely on the first culture in chronic or nonresponsive cases. The chance of obtaining yeasts is one of the best reasons for attempting culture in milk samples that have antibiotics present in them since yeasts are uninhibited by any antibiotics. Yeasts can easily be identified on direct milk smears that are Gram stained. Yeasts grow out well on phenol red mannitol agar within 12 to 24 hours. They will also grow on blood agar but the colonies are much smaller and slower to grow. Yeasts are also mannitol negative, at all times in our experience, and usually have a characteristic odor of fresh rising bread.

The usual method of susceptibility testing is not reliable for susceptibility testing yeasts therefore it should not be done. A new agar overlay technique has recently been reported to be of value and is currently used in research but is not practical for use in veterinary practice.[2] Also there is only one disc available and many new compounds effective against yeasts are not yet available as discs or infusion products. Some drugs used that are sometimes effective are iodine, chlorhexidine, miconazole, clotrimazole, and ketoconzole.

Several companies make diagnostic kits for the identification of yeasts genus and species, including the achlorophllic algae, Prototheca, which is not technically a yeast.

Other Mastitis Causing Bacteria

Corynebacterium pyogenes grows slowly on blood agar with characteristic tiny colonies and beta hemolysis and negative catalase reaction. The milk often has an offensive odor due to the concommitant growth of *Peptococcus indolicus* which is an anaerobe and will not grow on the culture plate. Often times the clinical use is so far advanced that even long term penicillin therapy will not cure the disease.

Corynebacterium bovis grows only on the original swab line of the culture plate because of its requirement for oleic acid. Often times it is difficult to determine if it is pathogenic or not. It is commonly found in the streak canal of many cows without any clinical mastitis or elevated cell counts and at other times it is the only organism culturable in an acute flareup of mastitis.

Bacillus sp. especially *Bacillus cereus* is an organism that will invariably cause severe mastitis and often result in the death of the cow if it gains entrance to the quarter. It is easily recognizable on a blood plate because of its large zone of hemolysis around a large, grayish-green, dry, ground-glass appearing colonies. Usually refractory to penicillin therapy but quite sensitive to other broad spectrum antibiotics. Early identification and treatment is important in this type of mastitis.

Nocardia sp. is associated with chronic granulomatous infection. Nocardia produces small whitish colonies on blood agar after 2 to 3 days that are powdery and dry and adhere to the media. Microscopically this organism branching filamentous and partially acid fast staining. Physically the udder will have several large palpable abscesses about the size of golf balls which is very characteristic of this type of mastitis. It is a very different feel than the typical meaty feeling of a staph or Coryne scarred or abscessed quarter.

Suggested Texts and Literature

Diagnostic Procedures in Veterinary Bacteriology and Mycology, Third ed. 1979. Carter, G.R. DVM Publisher-Charles C. Thomas Co. Springfield, Ill. $32.75.

Microbiological Procedures for Use in the Diagnosis of Bovine Mastitis 1981 National Mastitis Council 2nd ed. National Mastitis Council 1840 Wilson Blvd. Arlington, VA. 22201 703-243-6111 $2.00.

Manual of Clinical Microbiology, 3rd ed. 1980. Lennette, Balowes, Hausler, Truant-editors. American Society of Microbiology 1913 "I" St. NW Washington, D.C. 20006 $27.50.

References

1. Langlois, B.E., Harmon, R.J., Akers, K. J. Clin. Microbiology, Nov. 1983. pp. 1212-1219. *Identification of Staph Sp. of Bovine Origin with Staph-Ident.* 2. McDonald, J.S., Richard, J.L., Anderson, A.J., Fichtner, R.E. *In Vitro Sensitivity of Yeasts Isolated from Infected Bovine Mammary Glands* Am. J. Vet Res Vol 41, No. 12, pp. 1987-1990, Dec. 1980. 3. Barnes-Pallesen, Fran, Director New York State Mastitis Lab. Itheca, N.Y. Several uncited references in this paper are personal communications.

Questions & Answers:

Question: Would we have had different results if we had been able to take a stage of lactation into account?

Answer: We tried to do just that but it ended up with such very, very small samples in any reasonable stage for a reasonable duration of numbers of days that we just weren't able to do anything with it.

Answer: For the culturing of our acute samples of mastitis, we are charging $15 for a complete identification and sensitivity of the organism in that acute sample. For our bulk tank culturing we are charging $2 and that includes for the herd a composite susceptibility for the bulk of *Staph. aureus* and the *Staph.* species, but for that *Strep.* you were talking about, that needed to be worked up through group D, we would charge $15 for identification.

Question: What is the cost of manufacturing a blood plate?

Answer: The last time I figured out the cost of manufacturing a blood plate and a phenolred manitol plate, for the two plate we would probably have about 50 cents, considering we manufactured them ourselves. I think we would probably have closer to $1 in the two plates if we were to buy them from a commercial source. For the susceptibility testing discs, you have to figure that you've got about 4-5 cents per disk that you decide to use in your testing, and for a plate, a susceptibility testing plate, you've probably got about 75-80 cents, that is our cost for manufacturing one plus probably anywhere from 40-50 cents for the susceptibility testing disks that are placed on there, and then for the theta back test we have approximately $1 in that test that is a very expensive test solution. But you can get about 40-50 tests out of one bottle. So essentially it is not that expensive. If you were to use much of it you were to waste it or it expired and outdated on you, all these solutions that the theta back company makes are all dated for about a year to a year and a half dating on them. Campexculin plate will cost us to make probably in the neighborhood of 25 cents. So adding this all together we probably would have in the neighborhood of about $3-$4. And then our time. Time was not included on manufacturing or working up any of this. This was just materials cost.

Question: What happens to the cell counts in those cows?

Answer: Cell counts don't respond immediately to cleared infections or to treatments, so those cell counts don't automatically drop. This was the reason that we explicitly made our program one where we would only treat a single time during any one lactation or on the basis of elevated somatic cell count, because they don't come back to normal very rapidly.

Answer: Obviously if you develop a sensitivity to iodines or to chlorhexadine or any of those products, you have to switch products. Well, that's not necessarily so. If you can go to rubber gloves, which you are not always able to do in a larger dairy, but certainly on selected milkers I've seen it where they've gone to rubber gloves and reduced the irritation. That's one thing you might suggest. The other thing is switching products. When you switch products, if you've got a little problem in which products you're going to use in terms of backflushing, there are some acid sanitizers out, non halogen. Backdrop is one we can work with that looks very good. It doesn't have the germicidal activity, but certainly has an adequate organic material removal action that does reduce organisms. Other than that in the teat dip area, certainly I think you give something away when you go to the non-iodines. I've never had a lot of good luck with chlorine or clorox as a teat dip. We've used it in selected instances, but we get into some teat irritation with it, so you might want to try the chlorhexadine dips which are good, except that the trouble with chlorhexadine dips is that you have a weakness in the spectrum pseudomonas, and we've had some real pseudomonas problems. In 75% of our herds we cannot get pseudomonas out of an occasional cow, out of water, and this sort of thing, so it is always in the environment. Extended use of something like that, if it has a weak spectrum, always worries me. But for short term use and good monitoring you could probably go to something like chlorhexadine teat dip, in an acid sanitizer as a backflush.

Answer: Where we have made comparative studies in the lab and observations in the field both on teat swabs and clinical incidence, I would say that the particular product probably has about the same efficacy as 2500 parts of iodine, a little more efficacy as a 1000 parts of iodine, but certainly no where near the efficacy that 10,000 parts does. If you look at clinical outbreaks, I think we've had clinical outbreaks at a higher level than we would desire with the 1000 parts iodine, even the 2500 parts iodine, and the other product you mentioned. So I would say in a high contagion environment you want to go with your better dips and I still think in that environment your 1% teat dips are superior. In the premilking dips, it has a severe cleaning action. It has glycerin in it, however it is a little harder to wipe off. So I would say in a premilking dip the low iodines in that product again are probably equivalent in their bacterial reduction. Less desirable than the 1%, but we've had more residues with the 1% than Dave indicated in his particular studies, the higher risk of residues, in commercial dairies where they don't do as good a

job of wiping them off.

Question: If a cow comes in with dirty teats in a dairy where you are predipping, what would your sanitation recommendation be? To wash, wipe, dip, and wipe, or do you just dip and wipe? Should you only predip a clean teat?

Answer: We did a series of studies where we grossly contaminated free stalls and had manure kicked up on the cows udder when they entered the parlor to do the treatments. Our work would show that you should wash, dip, dry. Some people would say wash, dry, dip, dry, dip, and then dry. But our work said you've got to do something to remove the gross contamination and that is water, then come along with premilking dip, and then follow up with dry towel. But the main objective, whatever you do in the end, is to have her clean and dry, so that's the procedure that we would use.

Second Answer: You like to have cows come in in a relatively clean condition and go ahead and finish the cow off with this predip or the preflusher I mentioned, because those in themselves will not really remove any adhered material.

Question: What about the anticoagulant effect on hemolysis?

Answer: As far as the anticoagulant having any effect on hemolysis we haven't found any. I have talked with Fran Barnes from New York and she said they at one time did use this. They now have the capability of making washed cells which I think is preferable, but I don't know about the young calves that haven't nursed. We have always used just young calves or young heifers, probably a year of age, or whatever. We don't randomly select our animals. We go to the same farm that has good sanitary conditions, raises very healthy calves, with very few problems, and I don't know, maybe we have been more lucky than anything else, but with the acid citrate dextrose or ACD blood I see no problems today. I suppose we could have some next week. As far as using heparain as an anticoagulant, I don't have any experience in using anything other than ACD as an anticoagulant. I think probably we use our blood for up to three months that we collect. If it is the dextrose that keeps their blood cells, the RVCs, healthy enough to make good blood agar plates and get good hemolysis, I don't know. This is what we have done and had good luck with it.

Question: Have you used chlorhexadine as a backflush?

Answer: I've never had any experience using chlorhexadine in a backflush situation, only quaternary ammoniums we have. And after a years period of time we did get a very high buildup of pseudomonas in the system and increase in pseudomonas mastitis in the quads. I would suspect our results might be similar to quads but I don't really know because I have never used it. The chlorhexadine tends to be very alkaline in its primary state and I don't really know what factors pH would play in chlorhexadine in the system. Do you have any experience with that, Walt? maybe Dave? None of us on the panel has any experience with chlorhexadine in a backflush situation. It would be interesting to try it. I don't know. The other question was, how do you adjust pH of water? If you have a very alkaline water and probably the easiest way to determine is to add whatever sanitizer you have on the farm and determine, how much you can adjust the pH level of that water. The thing that determines the pH really is the bicarbonate ion in the water and it is not related to water hardness, necessarily. The one thing you could do if you have fairly alkaline water, and let's say you want to operate at 25 ppm and you were not able to get the pH down, you might select a product that has a higher acid ratio, because the acid content of the products on the market do, very considerably, all the way from about 40% acid down to as low as 5%. There is a lot of variation there. You might pick a higher acid product. The other factor you could do is actually increase the amount of product being used which also concurrently increases the level of iodine that is going into the system, so you might have to go up to 50 parts of iodine in order just to get your pH down and you'd have a better relationship in terms of bacteriocidal activity, too. The third option would be to get a double injection system where you could actually inject possibly boric or citric acid in the system so you are getting a separate source of additional acid in order to get your pH down. We did backflush trials. We found that getting a very acid pH in itself gives you bacteriocidal activity, and so that is why I made the statement that if some point you get a product that is very acid and good detergent activity, probably the addition of the halogen is not that important.