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"IMPROVED METHODS FOR ENUMERATING STAPHYLOCOCCI AND DETECTING STAPHYLOCOCCAL ENTEROTOXIN IN MEAT FOODS"

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We have developed a new plating medium for the rapid identification and enumeration of coagulase positive staphylococci in meat foods which will enable us to recognize a possible health hazard as quickly as possible. The medium is prepared by adding rabbit plasma (5.0%), pork plasma (14.0%), bovine fibrinogen (0.1%), and potassium tellurite (0.01%) to Baird-Parker agar base. The coagulase produced by the growing Staphylococcus aureus organisms forms a visible fibrin halo around each colony, thus eliminating the need for the tube coagulase test and a saving of 24 hours. The accuracy of staphylococcal enterotoxin detection in meat products by the reversed-passive hemagglutination (RPH) technique has also been improved by the removal of interfering meat proteins from the extract. Trypsin digestion and ammonium sulfate precipitation have been particularly useful in minimizing false positive reactions and enhancing the reliability of the RPH test.

AMELIORATION DES METHODES DE DENOMBREMENT DES STAPHYLOCOCCI ET DE DETECTION DE L'ENTEROTOXINE STAPHYLOCCIQUE DANS LES ALIMENTS A BASE DE VIANDE.

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Résumé :

Pour détecter le plus rapidement possible les staphylococci coagulase positive dans les aliments à base de viande on a mis au point un nouveau milieu. On ajoute à la base gélosée du milieu de Baird-Parker du plasma de lapin, du plasma de porc, du fibrinogène de boeuf et du tellurite de potassium ; la coagulase produite lors de la croissance de staphylococcus aurens forme un halo visible de fibrine ; le test en tube de la coagulase n'est plus à faire, la détection de l'entérotoxine staphylococcique dans les produits à base de viande par la technique de l'hemaglutination passive inverse (RPH)a été rendu plus précise en éliminant de l'extrait les protéines interférantes, on a plus particulièrement utilisé la digestion trypsique et la précipitation au sulfate d'ammonium qui minimisent les réactions faussement positives et augmentent la fiabilité du test R.P.H.

IMPROVED METHODS FOR ENUMERATING STAPHYLOCOCCI AND DETECTING STAPHYLOCOCCAL ENTEROTOXIN IN MEAT FOODS

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With all of our modern technology, the prominence of the food poisoning syndrome is certainly not lessening. Increased surveillance, better reporting of food poisoning incidents and better methods of detecting the cause of food poisoning have contributed to this increasing problem.

The role staphylococci play in these outbreaks is significant. Staphylococcus food poisoning has risen from 14.4% of all outbreaks in 1966 to 28.8% of all outbreaks in 1971 involving 5,115 cases (Foodborne Outbreaks, CDC Annual Summary). Thus Staphylococcus aureus as an etiological agent was again able to hold first place in food poisoning in the United States.

MODE OF FOOD POISONING AND CELL COUNT SIGNIFICANCE

Staphylococcal food poisoning is an intoxication caused by a heat-stable enterotoxin which is a metabolic product of some strains of staphylococci. Researchers have agreed that growth must occur in the food to be consumed, but do not agree on any one definitive number. [1, 2, 3] Suffice it to say that numbers in excess of 1,000,000/g are most commonly quoted in the literature. In addition, the staphylococci must be able to produce at least one of the six enterotoxin types A, B, C, D, E, or F. Indeed, not only do these organisms have to grow unrestricted to extremely high numbers, but more important, they

must produce a detectable amount of enterotoxin. Thus, no product can be termed an actual health hazard by virtue of its viable staphylococcal cell count, but only by the finding of the enterotoxin itself.

This paper will discuss our more recent methods of enumerating viable staphylococcal cells and detecting staphylococcal enterotoxin in meat foods.

VIABLE CELL DETECTION AND ENUMERATION

In order to curb the possibility of a food poisoning, we must endeavor to detect a potential hazard in our own products before they reach the consuming public. This may be done by determining the viable S. aureus cell count. Current methods of enumeration involve spreading aliquots of decimal dilutions on suitable plating media to obtain the total staphylococcal count.[4] Since there is a strong, but by no means complete, correlation between enterotoxin production and the enzyme coagulase, a certain percentage of the colonies are picked, grown in a nutrient broth, and subsequently coagulase-tested with rabbit plasma. In this way the number of S. aureus organisms can be estimated and the potential hazard defined. This procedure is both time-consuming (78 hours) and costly in terms of labor.

What we have done to shorten the time involved is to incorporate animal plasma, both porcine and rabbit, into the plating medium. Coagulase produced by the growing S. aureus organisms will react with the plasma to form an opaque zone (fibrin halo) around each S. aureus colony. The number of

coagulase-positive staphylococci may then be counted directly on the plate, eliminating the need for the tube coagulase test.

Reports have appeared in the literature concerning the suitability of various animal plasmas for coagulase testing. Duthie and Lorenz [5] found that the plasmas of human, monkey, horse, cat, pig, fowl and rabbit were superior to the plasmas of cow, sheep, dog, guinea pig, and mouse, in that the former plasmas contained more coagulase-reacting factor (CRF). Although rabbit plasma is used in the official method for detecting S. aureus by the tube test [4], Orth et al.[6] found it undesirable to use it in the plate test. They found rabbit plasma to have a high plasmin activity which was activated by the enzyme staphylokinase, a product of S. aureus. The staphylokinase caused fibrinolysis or dissolution of the fibrin halo which, in turn, would lead to a false negative reaction. Porcine plasma was found to be more suitable since it combines a relatively fast clotting time with a low plasmin activity.[6] Indeed the plasmin -- plasminogen system of porcine plasma -- was never observed to be activated by staphylokinase. We have recently found that rabbit plasma does contribute some unknown factors which the porcine plasma does not; therefore, to obtain maximum sensitivity, we have included it in our medium to complement the CRF in the porcine plasma.

The medium we employ is prepared by adding 140 ml of sterile porcine plasma (for preparation see Appendix A) and 50 ml of sterile rabbit plasma (Difco Laboratories, Detroit, Michigan), both with 0.1% ethylenediamine tetraacetic acid (EDTA), to 1 liter of melted Baird-Parker agar base (Difco). Ten

milliliters of a 1.0% solution of sterile potassium tellurite (Difco) and 1.0 g of sterile bovine fibrinogen (The Sylvana Co., Millburn, New Jersey) are then added. Care must be exercised that these reagents are tempered to 40 C before addition, or the medium will prematurely solidify.

PORCINE-RABBIT PLASMA MEDIUM

Melted Baird-Parker Agar Base	63 g/800 ml H_2O
Porcine Plasma	140 ml
Rabbit Plasma	50 ml
Potassium Tellurite (0.1% solution)	10 ml
Bovine Fibrinogen	1 g

To gain optimum reactions, the agar is poured as thin as possible and allowed to harden. Spread plates may then be prepared from dilutions as normal.

We have found the porcine-rabbit plasma particularly useful because of the saving of time and labor. Since the number of coagulase-positive staphylococci can be read directly from the plate without going through the traditional tube coagulase tests, results can be obtained in 48 hours, whereas the traditional procedures take a minimum of 78 hours.

ENTEROTOXIN DETECTION

When the number of staphylococci is found to be inordinately high, or if staphylococcal food poisoning is suspected in a meat food-related illness, it becomes necessary to test for the staphylococcal enterotoxin itself.

Three methods of enterotoxin detection are currently available. They are the microslide method of Casman and Bennett[7] as modified by Zehran and Zehran[8], the radioimmunoassay of Johnson et al.[9], and the reversed-passive hemagglutination procedure (RPH) of Silverman et al.[10]

The RPH method is particularly attractive because it is easily performed and results may be obtained within one day, whereas seven days are required for the Casman and Bennett microslide technique. This is an important consideration in an industrial situation where many samples must be analyzed, or at least screened in a short amount of time. Our research has found the sensitivity of the RPH technique to be equal to that of the microslide in the order of 0.5 mg/100 g of suspect meat product.

The RPH method is begun by separating enterotoxin from the insoluble extractives by homogenizing the food sample with an equal volume of acetate-buffered saline, pH 4.6, after centrifugation; the resulting supernatant is given a chloroform extraction to remove the lipids present. A 2-ml aliquot of this extract is then absorbed with .2 ml packed sheep red blood cells (SRBC) to avoid nonspecific agglutination. The test, itself, consists of preparing duplicate serial dilutions of the sample in normal rabbit serum, one series of dilutions as a test and the other as a control. A control enterotoxin of known strength is also serially diluted to act as the standard. Antitoxin globulin-coated tanned SRBC are then added to one set of tubes, while unsensitized tanned SRBC are added to the control set. Because of the need for economy in using antitoxin globulin,

these tests are carried out in micro amounts. The microtiter technique employing "u" plates instead of tubes, and microdiluters instead of pipettes, use less of each reactant; also, large numbers of tests can be performed more rapidly with no loss in sensitivity. After two hours of incubation, the tests may be read. Toxin concentration in the sample is calculated by multiplying the reciprocal of the greatest dilution of sample agglutinating by the end point of the enterotoxin standard.

Our meat research program uncovered an undesirable lack of specificity with the RPH method, particularly when meat foods are analyzed. We first observed this phenomenon in pure cultures of S. aureus. We were finding isolates which, by the RPH procedure, were yielding substantial quantities of enterotoxin A, but were negative when tested on a microslide. Indeed, samples of raw meat and certain prepared meat products with no history of staphylococcal contamination were giving us positive results, not spontaneous agglutination of the SRBC, but nonspecific agglutination with the sensitized cells. These would indeed be classified as "false positive" reactions.

In an attempt to minimize the false positive reactions, Dr. Merlin Bergdoll has recommended a 0.1% trypsin digestion to correct the problem[11], since the interfering substance was determined to be a protein. However, this did not completely abate the problem of false positives in our laboratory. Thermally-processed comminuted meats, such as frankfurters and some types of dry sausage, did not respond to the trypsin digestion. However, we found that, in these cases where we were

dealing with heat-processed high protein products, the meat extract had to be precipitated at pH 6.0-6.2 with ammonium sulfate at a final concentration of one-third saturation. Since the isoelectric point of enterotoxin A is 6.8, and even higher for B and C [12, 13, 14], the enterotoxin itself will not be precipitated. There will be only a minimal loss of enterotoxin due to the physical trapping of the other precipitating proteins, if the ammonium sulfate is added slowly and with constant stirring. Once the ammonium sulfate has been added, the mixture may be centrifuged and the supernatant recovered. The supernatant may then be absorbed with packed SRBC and the test carried out with a greater degree of specificity. With these modifications, the test may still be carried out in one day.

However, we have not investigated all of the volatile products, such as milk, cheese, and bakery products, which lend themselves to rapid staphylococcal growth and enterotoxin development. I suspect that as is often the case, standard procedures may need some alteration and tailoring to the particular food product, such as we found to be true in enterotoxin detection in meat foods.

SUMMARY

Because there is an increase in reported food poisoning incidents attributed to staphylococci made possible by improved methodology and reporting, it is incumbent upon any food processor to initiate quality control procedures to protect the consumer as well as the integrity of his products. We have found that, in our work on meat foods vulnerable to staphylococcal growth, further improvements in methodology in assessing public health significance were needed.

We feel our improvements are of benefit. These improvements are:

1. A porcine and rabbit plasma plating medium which enables a laboratory to enumerate directly from the plate the number of coagulase-positive staphylococci within 48 hours; thus, eliminating the need for picking suspect colonies and coagulase testing by the commonly used tube method.

2. Increasing specificity in the reversed-passive hemagglutination technique for enterotoxin detection by precipitating competing proteins in meat food with a saturated solution of ammonium sulfate to one-third saturation at pH 6.0-6.2. This modification has decreased the number of false positives encountered in meat foods. It should be kept in mind that this is a screening method for rapid enterotoxin detection, and when positive results are found, or when a food poisoning incident is being investigated, it is prudent to confirm these results by one or more of the alternate procedures.

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APPENDIX A

PREPARATION OF STERILE PORCINE PLASMA

Porcine blood is collected at the time of slaughter into sterile 200-ml centrifuge bottles containing 5 ml of a sterile 4% solution of EDTA. This results in a final concentration of .1% EDTA in the blood which is adequate to prevent clotting. The blood is immediately chilled and returned to the laboratory where the plasma is separated from the erythrocytes by centrifugation in a Sorvall RC2-B refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Connecticut). Centrifugation at 750 rpm for 30 min at 0 C is generally adequate. During all of these manipulations great care must be taken in handling the blood because swine erythrocytes are extremely fragile and break upon the slightest trauma.

The plasma is then decanted and filter-sterilized through a .45 μ HAWP filter (Millipore Corporation, Bedford, Massachusetts). This preparation is stable for up to three months under refrigeration.