IDENTIFICATION OF *STAPHYLOCOCCUS AUREUS* FROM THE BOVINE UDDER BY THE FLUORESCENT ANTIBODY TECHNIQUE

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INTRODUCTION

Mastitis is the most costly dairy cattle disease not under satisfactory control. Recent reports (1,19) indicate that it is costing the Nation's dairy farmers between $225 million and $500 million annually, and there is no firm evidence that the disease is on the decline. In fact, mastitis caused by Staphylococcus aureus appears to be on the increase. The importance of S. aureus from a mastitis standpoint is enhanced by the fact that a large reservoir of antibiotic-resistant strains has emerged in recent years.

Laboratory methods such as the direct microscopic method afford some information about the bacteria responsible for a given case of mastitis; however, these methods are of limited value as diagnostic tools because most micrococci and even some streptococci may be confused with S. aureus due to similarities in morphology.

Cultural techniques currently being used in the isolation of S. aureus from mastitic milk require from 12-48 hours. If a complete serological and biochemical characterization is necessary after primary isolation, results may not be available for several days. Failure to identify rapidly the infective agent and institute proper therapy may result in additional dissemination of the disease; thus increasing the chances of permanent udder damage in the alveolar system, or duct system, or both.

New diagnostic techniques, that will permit the specific identification of S. aureus in mastitic milk in the shortest possible time with a minimum outlay of labor and facilities, are urgently needed. A technique potentially capable of meeting these requirements is the fluorescent antibody technique originally described by Coons et al. (12). This technique possesses all the specificity of the conventional antigen-antibody system, yet it permits visualization of fluorescent antibody-coated organisms when they are illuminated with ultraviolet light and viewed through a specially equipped microscope.

Taking cognizance of the above, this study was undertaken to determine the feasibility of staining and identifying S. aureus in mastitic milk with a specific, staphylococcal antiserum and a commercial, fluorescein-labeled, sheep antiserum to rabbit globulin.

REVIEW OF LITERATURE

General Applications of the Fluorescent Antibody Technique

Coons et al. (12) used fluorescein-labeled antibodies in 1942 to demonstrate the presence of soluble pneumococcal polysaccharide in sections of tissue from infected mice. This work represented a significant advance in the field of diagnostic microbiology. Since 1950, research papers dealing with the fluorescent antibody technique have appeared in increasing numbers and the bibliography on the subject has become voluminous.

Briefly stated, the fluorescent antibody technique involves labeling an antibody with a fluorescent dye, adsorbing the labeled antibody to a homologous antigen, and observing the antigen-antibody complex with the fluorescence microscope. If the antibody has been adsorbed to the antigen, the antibody-coated antigen will fluoresce.

There are two principal staining techniques that are used in fluorescent antibody studies. The first technique was reported by Coons and Kaplan (13) and involves a one-step procedure in which a conjugated antibody is placed on a smear or tissue section and allowed to react for a designated period of time. The slide is then washed in buffered saline, mounted in buffered glycerol, and examined. This procedure is known as the direct staining technique. A second technique known as the indirect staining technique was developed by Weller and Coons (40) and involves a

two-step procedure. Using the indirect technique, the smear or tissue section is first reacted with a specific nonfluorescent antiserum prepared in the rabbit. After washing in buffered saline, the preparation is reacted with fluorescent antiserum against rabbit globulin and washed again in buffered saline, mounted with buffered glycerol, and examined. Usually, the latter antiglobulin is prepared in the sheep or goat. The indirect staining technique has the advantage that only one fluorescent antiglobulin is required for each type of animal in which specific antisera are produced.

Most of the early fluorescent antibody studies were conducted using fluorescein isocyanate as the labeling agent; however, in 1958 Riggs et al. (33) and Marshall, Eveland, and Smith (24) published papers describing the synthesis and use of the isothiocyanate derivative of fluorescein as a labeling agent. Fluorescein isothiocyanate is superior to the original compound because it is not water labile and it can be purchased commercially and stored with little difficulty.

Any fluorochrome, which is conjugated with antibody, must conform to certain specifications. Smith (35) listed three of the more important specifications: (1) it must not alter the immunologic specificity of the antibody; (2) it must form an irreversible combination with the antibody; and (3) it must be readily available and easy to use. Fluorescein isothiocyanate meets these specifications and has, therefore, found widespread acceptance as a labeling agent.

The fluorescent antibody technique has been used experimentally in a surprisingly large number of areas. Some of the areas, in which the technique has found application, are mentioned briefly below in an

effort to demonstrate its versatility. The technique has been used to identify bacteria in smears made from growing cultures (5,6,26,27,42); to study the antigenic constitution of Salmonella typhosa (37) and Paramecium aurelia (2); to group serologically actinomyces (34), Shigella flexneri (21), and streptococci (25); to diagnose pertussis in nasopharyngeal smears (14); to detect bacterial and viral antigens in animal and plant tissues (3,13,29,38); to stain pathogens in suspensions made from human stools (11,15); to stain malaria parasites in blood (39); to detect parasitic protozoa in peritoneal exudate (16); to stain yeast (17); to detect air-borne Pasteurella tularensis (20); to demonstrate the presence of specific bacteria in rumen contents (18); to detect Leptospira pomona in guinea pig and bovine urine (41); to demonstrate the presence of Hemophilus influenzae in cerebrospinal fluid sediments (30); to demonstrate the antigenicity of inclusion bodies of canine distemper (28); to identify Histoplasma capsulatum in sputum (23); to demonstrate that human growth hormone is localized in specific cells of the human anterior pituitary gland (22); and in many other areas too numerous to mention.

Fluorescent Antibody Studies with Staphylococci

In addition to the applications mentioned above, fluorescent antibodies have been used to study and identify staphylococci. The first fluorescent antibody study pertaining to staphylococci was reported in 1959 by Carter (4). Specific antisera were prepared against two strains of S. aureus (Cowan types I and II) and conjugated with fluorescein isothiocyanate. The titers of both antisera were 1:1280 with homologous and heterologous antigen. The two fluorescent antisera were first used

to test 253 cultures of gram-positive cocci isolated from food products. Of these cultures, 44 were coagulase-positive while 43 stained with type I antiserum and 44 stained with type II antiserum. It was interesting to note that all of the coagulase-negative cultures failed to stain with either of the fluorescent antisera. Carter also inoculated ten Loeffler blood serum slants with nose and throat swabs and stained the growth with the two antisera. Seven of the ten cultures showed the presence of fluorescent staphylococci. Coagulase-positive staphylococci were isolated from six of the ten cultures.

Smears were also made from a number of cheese and dried milk samples and stained directly with the two antisera. Portions of the same samples were cultured on selective media for the isolation of coagulase-positive staphylococci. The demonstration of fluorescent cocci in smears was confirmed, with two exceptions, by the isolation of coagulase-positive staphylococci. In another trial, large numbers of fluorescent cocci were demonstrated by staining smears made from two samples of cheese that had been implicated epidemiologically in a food poisoning outbreak typical of that caused by S. aureus. Viable coagulase-positive staphylococci could not be isolated from either of the two cheese samples.

Wolfe and Cameron (43) were apparently the first workers to report that some staphylococci will stain with fluorescein-labeled globulin from nonimmunized rabbits. These workers further reported that fluorescein-labeled, Group A antistreptococcus globulin will stain some coagulase-positive staphylococci. Pittman and Moody (31) have also reported that Group A streptococcus fluorescent antibody and labeled,

normal rabbit serum will stain some coagulase-positive staphylococci. Heat-fixed smears of viable cell suspensions were allowed to react with labeled, Group A streptococcus antiserum or labeled, normal rabbit serum globulin. A total of 14 S. aureus cultures and 4 coagulase-negative Staphylococcus epidermidis cultures was stained and examined with the fluorescence microscope. Staphylococcus aureus consistently stained brilliantly with both the labeled, Group A streptococcus antiserum and the labeled, normal rabbit serum; however, S. epidermidis did not stain with either serum. Agglutination titers ranging from 1:40 to 1:2560 were reported for S. aureus with each of the sera; however, only a few very low reactions occurred with S. epidermidis.

Cohen, Cowart, and Cherry (8) conducted a rather comprehensive study of antibodies against S. aureus in nonimmunized rabbits. In one phase of the study, normal serum from specific-pathogen-free rabbits was fractionated and the globulin portion was labeled with fluorescein isothiocyanate. The labeled globulin failed to stain 14 coagulase-negative strains of S. aureus; however, some coagulase-positive strains stained brilliantly, while other strains either failed to fluoresce or showed questionable reactions. It should be noted, also, that in several instances there was a lack of agreement between fluorescent staining and agglutination reactions.

More recently, Cohen et al. (9) have reported the presence of staphylococcal antibodies in germfree mice as demonstrated by serum-gel diffusion, agglutination, and fluorescent antibody tests. Pools of sera from germfree and conventional mice were made and each pooled serum was conjugated with fluorescein isothiocyanate. Heat-fixed smears of

S. aureus were stained for 45 minutes in a moisture chamber using the direct staining procedure. Some strains were stained by dilutions of conjugated serum as high as 1:2100, whereas, other strains did not stain even when undiluted serum was used. As was the case with preimmune rabbit serum, there was a lack of agreement between fluorescent staining and agglutination reactions.

The study most nearly related to the present investigation was one conducted by Smith (35,36). Six strains of coagulase-positive S. aureus were used to prepare antisera in rabbits. Four of the strains were isolated from either mastitic milk or normal milk, while the remaining two strains were departmental stock cultures. Titers of the antisera ranged from 1:1280 to 1:5120 by the tube agglutination test using homologous antigen.

Milk films used for staining were prepared using an 11 per cent suspension of nonfat dry milk. Staphylococci from agar slants were included in some of the films. All films were dried at 45 C for 5 minutes, fixed in 95 per cent ethanol, stained with antibody that had been labeled with fluorescein isothiocyanate, and washed in phosphate-buffered saline. Examination of the milk films under the fluorescence microscope revealed that many of the milk films had disintegrated during the staining and washing treatments. Also, the milk adsorbed a large amount of the fluorochrome and the resulting background fluorescence made it impossible to detect fluorescing staphylococci. Thus, it was obvious that fluorescein-labeled antibody could not be used to detect bacteria in milk films. Therefore, an aliquot of antibody was conjugated with rhodamine isothiocyanate and milk films were prepared and stained in the manner described

for fluorescein-labeled antibody. In general, results obtained with the rhodamine-labeled antibody were similar to those obtained with the fluorescein-labeled antibody.

Since the adsorption of the fluorochrome by the milk prevented detection of fluorescing staphylococci, Smith decided to attempt to remove the nonspecific staining agents from the antibody. Efforts to remove these agents by adsorption of the fluorescein-labeled antibody with Dowex 2 chloride and acetone-dried mouse liver powders were unsuccessful. A number of attempts were made to alter the milk and remove or destroy the component(s) responsible for fluorescence. Such attempts met with little or no success. Consequently, the investigator turned his attention to the problem of disintegration of milk films. Numerous variations of the fixing solution were used and the technique finally selected was as follows: The slides were immersed in xylene for 2 minutes, and air-dried; immersed in 95 per cent ethanol for 2 minutes, and air-dried; and finally immersed in 2 N NaOH for 5 minutes. The slides were then gently dipped three or four times in each of two beakers of water, drained, and air-dried.

The indirect staining technique was employed in an effort to overcome the background fluorescence problem experienced with the direct staining technique. Smears were prepared in the manner described above and each slide was flooded with a small amount of unlabeled, staphylococcal antiserum and placed in a moisture chamber for 20-30 minutes. Slides were immersed in either phosphate-buffered saline (pH 7.2, 0.05 M phosphate) or distilled water (pH 7.0), and air-dried. Next, a drop of commercial, fluorescein-labeled, sheep antiserum to rabbit globulin was

applied to the smear and the slides were placed in the moisture chamber for another 20 minutes. Excess serum was removed and the slides were washed in phosphate-buffered saline (pH 8.0) for 15 minutes, drained dry, mounted with a drop of buffered glycerol and a cover glass, and examined with the fluorescence microscope. When examined under ultraviolet illumination, cells of S. aureus fluoresced brightly with the yellow-green fluorescence typical of fluorescein. Various control slides were used and some showed a small amount of fluorescence, but not to a degree that would interfere with interpretation of the results. The surprising result of the indirect staining technique was the marked lack of interferring background fluorescence.

One of the problems encountered by Smith was that organisms, other than S. aureus in the reconstituted milk, often stained with the fluorescent globulin. Consequently, he conducted a series of experiments designed to study the nonspecific staining. An assortment of bacteria that might be found normally in nonfat dry milk was selected for the specificity experiments. Included in the assortment were Bacillus cereus, Bacillus subtilis, Bacillus coagulans, Aerobacter aerogenes, Streptococcus lactis, Streptococcus faecalis, Lactobacillus casei, and S. aureus. Smears were prepared from water suspensions of washed cells and stained by the indirect staining technique. All of the bacteria adsorbed a small amount of the fluorescent antibody; however, none of the heterologous organisms stained as brightly as did S. aureus. Bacillus cereus adsorbed enough of the fluorescent globulin to fluoresce brightly; however, differences in morphology were sufficient to distinguish it from S. aureus.

Since only the homologous strain for the antiserum had been stained previously, Smith decided to determine whether or not a single immune serum could be used to detect a large selection of staphylococcal strains. Therefore, a collection of 72 cultures from bovine sources, human sources, and cheese sources was made. Only 12 of the 72 cultures failed to adsorb enough antibody to permit specific identification. No common property, such as being coagulase negative or all of bovine origin, could be established for the 12 strains that failed to react. Of these 12, there were 3 strains from bovine sources, 2 from human sources, and 7 from cheese sources. Smith concluded that the cultures which did not ferment mannitol or produce coagulase might possibly be members of the genus Micrococcus rather than the genus Staphylococcus. It was interesting to note that the percentage of negative reactions (16.6) to the fluorescent antibody test corresponded fairly closely to the 23 per cent that failed to react to the agglutination test with the same unlabeled antiserum.

Quantitative studies were conducted to determine the minimal number of S. aureus cells that could be detected, and to determine whether the fluorescent antibody technique could be used to determine quantitatively the number of S. aureus cells in nonfat dry milk.

In the first quantitative experiment, nonfat dry milk suspensions were prepared which contained 3.6×10^7, 3.6×10^6, 3.6×10^5, and 3.6×10^4 staphylococcal cells per milliliter. Smears were prepared from each of the above suspensions and stained, and direct microscopic counts were made of fluorescent staphylococci on each slide. The results were then compared to the number of staphylococci originally added to the milk. Examination of the films showed 2.3×10^7, 2.8×10^6, and 4.5×10^5

fluorescent cells, respectively, for the first three suspensions. There were not enough fluorescent staphylococcal cells visible in films prepared from the fourth suspension to give reliable counts.

In the second quantitative experiment, suspensions which contained 5×10^7 and 10×10^7 staphylococci per milliliter were prepared. Examination of the stained films indicated counts of 6.05×10^7 and 13×10^7 fluorescent staphylococci per milliliter, respectively.

Cohen and Oeding (10) reported recently on the use of the fluorescent antibody technique to identify seven antigenic factors of coagulase-positive staphylococci. The fluorescent antibody results were compared to agglutination reactions with the same antigenic factors and the same cultures. Thirteen serologically different strains of coagulase-positive staphylococci were used in the study. The serological type of each strain was based on agglutination in antisera for the antigenic factors a, b, c, e, h, i, k, m, and n.

Detailed procedures were given for the preparation of staphylococcal suspensions for inoculating rabbits and for the preparation of the seven factor sera. The sera used in the fluorescent antibody phase of the study were fractionated with $(NH_4)_2\ SO_4$ and labeled with fluorescein isothiocyanate. These workers also found that labeled sera could be used for agglutination tests as well as for fluorescent antibody tests.

Both the agglutination and fluorescent antibody tests were performed on cultures from 5-hour Nutrient Agar slants. Smears of the cultures were prepared for fluorescent antibody staining by suspending the growth from the slant in saline and transferring a loopful to a glass slide. All slides were heat-fixed and stained for 45 minutes in a moisture chamber, after which the slides were rinsed, dried, and mounted

in buffered glycerol.

Correlation between agglutination and fluorescent antibody tests were almost complete for factors a, b, i, and k. However, the fluorescent antibody reagent for factor c yielded a broader spectrum of activity than the corresponding agglutination serum, while the fluorescent antibody reagent for factor m stained fewer strains than were agglutinated in m serum.

Labeled reagents for factors a, c, h, i, and k were tested to determine whether or not they would stain four different strains of Group A streptococci. None of the streptococci stained significantly. Moreover, none of the streptococci stained significantly when reacted with labeled, unadsorbed, S. aureus immune globulins. These workers concluded that the fluorescent antibody system was as reliable as the agglutination system for differentiating strains of staphylococci.

de Repentigny and Sonea (32) have reported also on staphylococcal fluorescence; however, the study did not involve the use of fluorescent antibodies. A large number of bacterial species were examined in unstained smears with a fluorescence microscope and 31 species showed primary fluorescence (autofluorescence). All eight sources of staphylococci fluoresced brightly and at the same high intensity, a phenomenon not observed with the other bacterial species.

EXPERIMENTAL PROCEDURE AND RESULTS

Cultures and Materials

A total of 18 cultures of S. aureus was isolated initially for use in the early exploratory studies. Each culture was isolated from a different cow with chronic mastitis. One culture was isolated from the Oklahoma State University dairy herd while the remaining 17 cultures were isolated from the North Louisiana Hill Farm Experiment Station dairy herd, Homer, Louisiana. An additional 82 cultures were isolated approximately 5 months after the isolation of the first 18 cultures. The 82 additional cultures were isolated for use in the final phases of the study, thus, a total of 100 staphylococcal cultures were used during the conduct of the study. The latter group of cultures was isolated from four additional dairy herds in North Louisiana making a total of six dairy herds that were represented in the 100 cultures.

Media used throughout the conduct of the study were purchased from Difco[1]. All cultures were isolated on Staphylococcus Medium no. 110. They were characterized as to chromogenesis, hemolysin production, coagulase production, and ability to ferment mannitol. The results are given in Table I.

Chromogenesis was determined after incubation on Staphylococcus

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[1]Difco Laboratories, Detroit 1, Michigan.

TABLE I

CHARACTERIZATION OF STAPHYLOCOCCAL CULTURES USED IN THE STUDY

Culture no.	Chromogenesis	Hemolysin production	Coagulase production	Fermentation of mannitol
S- 1	Orange	+	+	+
S- 2	Orange	+	-	+
S- 3	Orange	+	+	+
S- 4	Orange	+	+	+
S- 5	Orange	+	+	+
S- 6	Orange	+	-	+
S- 7	Orange	+	-	+
S- 8	Orange	-	+	+
S- 9	Orange	+	+	+
S-10	White	+	-	-
S-11	Orange	+	+	+
S-12	Orange	+	+	+
S-13	Orange	+	+	+
S-14	Orange	+	+	+
S-15	Orange	+	+	+
S-16	White	-	-	-
S-17	White	+	-	-
S-18	Orange	+	-	+
S-19	Orange	+	+	+
S-20	White	-	-	+
S-21	Orange	+	+	+
S-22	Orange	+	+	+
S-23	Orange	+	+	+
S-24	White	-	+	+
S-25	Orange	+	+	+
S-26	Orange	+	+	+
S-27	White	-	+	-
S-28	Orange	+	+	+
S-29	Orange	+	+	+
S-30	Orange	+	+	+
S-31	Orange	+	+	+
S-32	White	+	+	+
S-33	White	-	-	-
S-34	Orange	+	+	+
S-35	Orange	+	+	+
S-36	Orange	+	+	+
S-37	Orange	+	+	+

TABLE I (continued)

Culture no.	Chromogenesis	Hemolysin production	Coagulase production	Fermentation of mannitol
S-38	White	-	-	-
S-39	White	+	+	+
S-40	Orange	+	+	+
S-41	Orange	+	+	+
S-42	Orange	+	+	+
S-43	White	-	-	-
S-44	White	-	-	-
S-45	Orange	+	+	+
S-46	Orange	+	+	+
S-47	Orange	+	+	+
S-48	Orange	+	+	+
S-49	White	-	-	-
S-50	Orange	+	+	+
S-51	Orange	+	+	+
S-52	Orange	+	+	+
S-53	Orange	+	+	+
S-54	Orange	+	+	-
S-55	Orange	+	+	+
S-56	Orange	+	+	+
S-57	Orange	+	+	+
S-58	Orange	+	+	-
S-59	White	-	+	-
S-60	Orange	-	-	+
S-61	Orange	+	+	+
S-62	White	+	-	+
S-63	Orange	+	+	+
S-64	Orange	+	+	+
S-65	Orange	+	+	+
S-66	Orange	+	+	+
S-67	Orange	+	+	+
S-68	Orange	+	+	+
S-69	Orange	+	+	+
S-70	Orange	+	+	+
S-71	Orange	+	+	+
S-72	White	-	-	-
S-73	Orange	+	+	+
S-74	White	-	-	-
S-75	White	-	+	-
S-76	Orange	+	+	+
S-77	Orange	+	+	+
S-78	Orange	+	+	+

TABLE I (continued)

Culture no.	Chromogenesis	Hemolysin production	Coagulase production	Fermentation of mannitol
S-79	White	-	+	-
S-80	White	-	-	-
S-81	Orange	+	+	+
S-82	Orange	+	+	+
S-83	Orange	+	+	+
S-84	Orange	+	+	+
S-85	Orange	+	+	+
S-86	White	-	+	-
S-87	White	-	-	-
S-88	Orange	+	+	+
S-89	Orange	+	+	+
S-90	Orange	-	-	+
S-91	Orange	+	+	+
S-92	Orange	+	+	+
S-93	White	-	-	+
S-94	White	-	-	-
S-95	Orange	+	+	+
S-96	Orange	+	+	+
S-97	Orange	+	+	+
S-98	Orange	+	+	+
S-99	Orange	+	+	+
S-100	Orange	+	+	+

Medium no. 110 for 48 hours at 37 C. Table I indicates that 76 of the 100 cultures were chromogenic while 24 were nonchromogenic. All cultures that showed any evidence of being chromogenic have been reported by the author as orange in color. Chromogenesis in the 76 cultures varied from a light orange to a deep golden color.

Hemolysin production was determined on Tryptose Blood Agar Base containing 5 per cent fresh, defibrinated blood. No differentiation was made between the hemolysins, and each culture is reported either as hemolytic or nonhemolytic. There was, however, a preponderance of alpha and beta hemolysins in the 78 hemolytic cultures.

Coagulase production, which is generally considered to be the best single indicator of potential pathogenicity, was determined using fresh rabbit plasma. The coagulase test was conducted by emulsifying a small amount of an 18-hour culture in 0.2 ml of Brain Heart Infusion in a Wasserman tube and adding 0.5 ml of plasma. The tubes were placed in a water bath at 37 C for 3 hours. Inspection of Table I reveals that 78 of the cultures were coagulase positive while 22 were coagulase negative.

Mannitol fermentation was determined on Mannitol Salt Agar following 36 hours incubation at 37 C. Cultures which fermented the mannitol, thus producing acid, were surrounded by yellow zones; whereas cultures that did not ferment mannitol were surrounded by red or purple zones. Eighty of the cultures fermented mannitol while 20 did not.

A close inspection of Table I indicates that 11 of the 100 cultures were nonpigmented, nonhemolytic, coagulase negative, and nonmannitol fermenting. It is possible that at least some of the 11 cultures were

members of the Genus Micrococcus rather than the Genus Staphylococcus. However, it should be noted that each of the cultures grew luxuriously on the Staphylococcus Medium no. 110, which is a selective medium for staphylococci.

All cultures of S. aureus were maintained on Nutrient Agar slants at 5 C and transfers were made at 30-day intervals.

Preparation of Immune Antisera

Initially, culture S-1 was used as an immunizing antigen to prepare immune antisera. Cultures S-5 and S-19 were used later in the study. The methods used in each instance were identical. The three strains were grown overnight at 37 C in Brain-Heart Infusion Broth and 3 ml of the broth were transferred to each of four prescription bottles containing Brain-Heart Infusion Agar. The prescription bottles were rotated to spread the broth evenly over the entire surface of the agar. Following incubation for 20 hours at 37 C, the growth was washed from the surface of each bottle. The suspensions were transferred to sterile, 25 ml, screw cap, test tubes and centrifuged at 2200 rpm for 30 minutes. The sediments from tubes containing the same culture were pooled and the cells were washed twice with 0.85 per cent saline. Washing was accomplished by suspending the cells in sterile saline and centrifuging. Following the second washing, the cells were suspended in saline to an optical density of 1.8 on a Bausch and Lomb Spectronic 20 Colorimeter at 5900 Å. Formaldehyde was added to a concentration of 0.4 per cent and the bacterins were incubated at 37 C for 24 hours after which 0.5 ml of each bacterin was spread over a blood agar plate and incubated for 24 hours at 37 C. No growth was evident on any of the plates indicating

that all bacteria had been killed.

Immune sera were prepared by injecting rabbits in the marginal ear vein with the formalin-killed antigens. An initial injection of 0.5 ml was given, followed 4 days later by 1.0 ml on each of 3 successive days. The rabbits were granted a 4-day rest period and the injection cycle was continued through the fourth week. A test bleeding was conducted 10 days after the final injection and the antibody titer was determined by the standard tube agglutination test.

Antigens for the agglutination test were prepared in the manner described above except that they were diluted to an optical density equivalent to a McFarland no. 4 standard. Antibody dilutions from 1:20 to 1:5120 were prepared using a two-fold dilution series. All tubes were incubated at 52 C for 3 hours, placed in the refrigerator overnight, and read the following morning. Titers of antisera ranged from 1:320 for culture S-5 to 1:2560 for cultures S-1 and S-19. Thus, only the antisera to the latter two cultures were used. The antisera were obtained by bleeding the rabbits by cardiac puncture, allowing the blood to clot, and decanting the serum which contained the antibody.

Instrumentation

The microscope used in the present investigation was the AO-Spencer[2] Fluorestar, Model 14TG-FW equipped with an N.A. 1.40 wide angle, aplanatic condenser (with a dark field stop) in a centerable mount. The microscope was equipped with 10X, wide-field eyepieces and 10X, 20X, 43X and 97X (with funnel stop) achromatic objectives.

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[2]American Optical Company, Instrument Division, Buffalo 15, New York.

The light source was an Osram HBO, 200 watt, high pressure, mercury arc lamp in the AO-Spencer Fluorolume Illuminator, Model 645. A number of exciter and barrier filter combinations was tried in the initial phases of the study. The combination finally chosen was a Shott BG-12, 3 mm thick, exciter filter and a Shott OG-1, barrier filter. The exciter filter was inserted in a filter holder that was an integral part of the illuminator. By placing a special ground glass plate in the chamber opposite the exciter filter, it was possible to view a slide under incandescent illumination and switch immediately to ultraviolet illumination simply by sliding the filter holder to the opposite position. The barrier filter, which was an ultraviolet-absorbing filter necessary for the isolation of the yellow-green fluorescence characteristic of fluorescein, was inserted into the body tube of the microscope.

To prevent breakage of the exciter filter, a heat-absorbing glass filter was inserted in a special chamber between the light source and the exciter filter.

The microscope was equipped with an AO-Spencer, 35 mm, photomicrographic camera, Model 635. Photographs were taken on Kodak, Panatomic X, fine grain, black and white film and Kodak, Kodachrome II, Professional Type A color film at exposures of approximately 3 minutes.

All microscopic observations were made with a funnel stop in the 97X objective and with Cargille Type A, very low fluorescence, immersion oil[3], both above and below the glass slide.

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[3]American Optical Company, Buffalo 15, New York.

Initial Exploratory Studies

Glass slides, 0.9-1.1 mm thick, were used throughout the conduct of the study. The microscope cover glasses selected were no. 1, 22 mm X 22 mm. New slides were prepared for use by wiping them thoroughly with lens paper and flaming them on both sides. Slides, that had been used previously for fluorescent antibody studies, were washed thoroughly with a suitable cleaning product, rinsed with distilled water, dried, and stored in a dust-free container. Prior to use, the washed slides were wiped with lens paper and flamed in the same manner as new slides. Cover glasses were wiped carefully with lens paper just prior to use.

Initially, all attempts to stain S. aureus were made by emulsifying a small amount of growth, from a 24-hour culture, in a small drop of 0.85 per cent saline and staining by the indirect staining technique. The technique was as follows: Each smear was air-dried, heat-fixed, and covered with a drop of S-1 antiserum for 20 minutes. The slides were then placed in moisture chambers for 30-40 minutes. The moisture chambers consisted of culture dishes that were lined with moistened filter paper. Upon removal from the moisture chambers, each slide was washed in 0.1 M phosphate-buffered, 0.85 per cent saline (pH 8.0) for approximately 15 seconds. The slides were drained dry and a drop of fluorescein-labeled, sheep antiserum to rabbit globulin[4] was placed over the smear. The slides were placed in the moisture chambers for approximately 20 minutes, removed and placed in buffered saline for approximately 15 minutes with occasional vertical agitation. Both the unlabeled antiserum and the labeled, sheep antiserum to rabbit globulin were added to the smears using small capillary pipettes. The drop of

[4]The Sylvana Company, Milburn, New Jersey.

antiserum was spread evenly over the entire smear by holding the pipette horizontal to the slide and using the thin end to spread the antiserum.

Following the second rinsing the slides were air-dried and the smears were mounted with a drop of glycerol saline (9 parts glycerol, 1 part 0.85 per cent saline buffered at pH 8.0 with phosphate buffer) and a cover glass. Controls consisted of (1) smears that were not reacted with either unlabeled or labeled antiserum, and (2) smears that received only the labeled, sheep antiserum to rabbit globulin. Examination of the treatment smears under ultraviolet light revealed fluorescing staphylococci, though the fluorescence varied with the culture used. The staphylococci in the first control smears showed little or no autofluorescence, while the staphylococci in the second control smears fluoresced weakly.

On the basis of results obtained with cultures S-1 through S-18 in 0.85 per cent saline, cultures S-5 and S-15 were selected for use in studies involving milk. Milk used for preparing smears was taken from the bulk milk tank at the North Louisiana Hill Farm Experiment Station dairy. The milk was considered satisfactory for purposes of the experiment because the official plate count was consistently between 3,000 and 10,000 bacteria per milliliter, and because the microscopic factor of the microscope being used was 318,000 with the oil immersion objective. Smears were prepared and fixed using essentially the same methods reported by Smith, McCoy, and Wilson (36) for nonfat dry milk. A small amount of growth from a 24-hour culture was emulsified in a drop of milk on a clean slide and the smear was dried for 5 minutes at

45 C. Smears were placed in xylene for 2 minutes, and air-dried; placed in 95 per cent ethanol for 5 minutes, and air-dried; placed in 2 N NaOH for 5 minutes, rinsed by gently dipping the slides four times in each of two beakers of water, and air-dried. Finally, the smears were reacted with unlabeled antiserum for 20-30 minutes; washed in phosphate-buffered saline for 1 minute; reacted with labeled, sheep anti-rabbit globulin for 20 minutes; washed in phosphate-buffered saline for 15 minutes; air-dried, and mounted in the manner described above.

A total of five control slides was prepared and examined. They consisted of (1) milk alone, (2) milk plus S-5, (3) milk plus S-5 plus unlabeled antiserum, (4) milk plus S-5 plus labeled antiserum, and (5) milk plus unlabeled antiserum plus labeled antiserum. Examination of the slides did not reveal any fluorescence on the first three control slides. However, both of the latter two control slides showed some fluorescence, though it was not enough to cause any real concern. The fluorescence on control slide no. 4 was due to the fact that both the staphylococci and milk adsorbed some of the conjugate, while fluorescence on control slide no. 5 was due entirely to milk adsorbing some of the conjugate. Staphylococci in smears which consisted of milk plus S-5 plus unlabeled antiserum plus labeled antiserum fluoresced far brighter than the milk constituents and were easily distinguishable.

The most serious problem encountered in the early trials involving milk was that of the milk smears tending to dislodge during the fixing, staining, and washing processes. This problem was overcome by (1) making thinner smears, (2) drying the smears more thoroughly, (3) placing the drop of antiserum at the edge of the smear and spreading it across the

smear rather than placing it directly on the smear, and (4) being more cautious during the washing steps. A second problem encountered was the fact that the cover glass, which was placed over the smear, was easily dislodged during manipulation of the slide under the microscope. The edge of the cover glass was sealed with melted paraffin to overcome this problem. The paraffin served to stabilize the cover glass and prevented the glycerol saline from flowing out from under the cover glass.

The next series of trials was undertaken to determine whether or not some other combinations of length of time designated for fixing, staining, and washing might be more desirable than those used in previous trials.

Fixing times varied from 1-5 minutes for xylene and 95 per cent ethanol, and from 2-8 minutes for 2 N NaOH. Washing times varied from 1-10 minutes for unlabeled antiserum, and from 5-30 minutes for labeled antiserum. Staining times varied from 10-30 minutes for unlabeled antiserum and from 10-60 minutes for labeled antiserum. Various combinations of all staining and washing times were tried. The results of the trials indicated that none of the fixing techniques studied were superior to the one reported by Smith et al. (36). However, some changes were made in the washing and staining times. Generally, washing the smears after treatment with unlabeled antiserum for 1-2 minutes was sufficient (Table IV). Washing the smears for 5-15 minutes in two changes of phosphate-buffered saline, after exposure to labeled antiserum, was satisfactory. On the basis of these trials, it was concluded that the length of the washing periods was not an extremely critical factor with the fluorescent antibody technique. It was decided that smears would be washed for 1-2 minutes after exposure to unlabeled antiserum and for 10 minutes after

exposure to labeled antiserum.

The optimum reaction time for unlabeled antiserum was from 15-20 minutes (Table V) and the author chose to use 15 minutes in future trials. The optimum staining time for labeled antiserum was 15-30 minutes and a 20 minute staining time was selected for use in future trials. There might have been a slight increase in fluorescence with staining times longer than those selected; however, it was felt that the slight increase in fluorescence was not sufficient to compensate for the increased time required.

The effect of age of culture on staining ability was studied briefly. Two trials were conducted, one using 24-hour cultures and a second using the same cultures after 7 days in the refrigerator at 5 C. None of the 19 cultures examined showed any appreciable change in staining ability between 1 and 8 days of age (Table VI). Cohen and Oeding (10) demonstrated that there was a correlation, in certain instances, between incubation time and staining ability. This was particularly true between 5-hour and 24-hour incubations. It is possible, therefore, that differences in staining ability could have been found if cultures incubated for less than 24 hours had been compared.

The system used for scoring intensity of fluorescence in this study is as follows: 4+ = cells very sharply outlined, very bright yellow-green fluorescence; 4- = cells very sharply outlined, bright yellow-green fluorescence; 3+ = cells sharply outlined, medium yellow-green fluorescence; 3- = cells sharply outlined, light yellow-green fluorescence; 2+ = cells not sharply outlined, light green fluorescence; 2- = cells not outlined, dull green fluorescence; 1+ = cells not outlined, faint green fluorescence; 1- = cells barely visible, very faint green

fluorescence; - = no visible fluorescence.

Comparison of Direct and Indirect Staining Techniques

Most of the trials conducted during the initial phases of the study employed the indirect staining technique. Consequently, a series of trials was conducted to determine the feasibility of using the direct staining technique to identify staphylococci in milk. Fluorescein-labeled, staphylococcal antisera used in the direct staining trials were purchased commercially[5]. The staining procedure consisted of spreading a drop of the labeled, staphylococcal antiserum over the smears and placing the slides in moisture chambers for 15 minutes after which they were washed for 10 minutes in two changes of phosphate-buffered saline (pH 8.0). Control slides were prepared as follows: (1) milk alone; (2) milk plus labeled antiserum; (3) milk plus S-5 or S-15; and (4) milk plus S-5 or S-15 plus unlabeled antiserum plus labeled antiserum. Autofluorescence of controls nos. 1 and 3 was negligible although control no. 2 adsorbed some of the dye and fluoresced at an intensity from 1+ to 2+. Control slide no. 4, which was an inhibition control, fluoresced at an intensity of 2+ in most trials. The inhibition procedure is based on the immunologic phenomenon of blocking specific antigen-antibody reactions by exposing the antigen to unlabeled, homologous antiserum prior to applying the labeled antiserum. The theory being that if the bacteria and antibody are homologous, the bacteria will become saturated with the antiserum and the labeled antiserum will be unable to react with the antigen when it is applied. The fact that the bacterial cells fluoresced at an intensity of 2+ indicated that inhibition was incomplete. Cherry

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[5]The Sylvana Company, Milburn, New Jersey.

et al. (7) indicate that this situation is to be expected. They reported that bright staining is encountered when blocking has not occurred and less bright staining is encountered when blocking has occurred. Most of the treatment slides, which consisted of milk plus S-5 or S-15 and labeled, staphylococcus antiserum, fluoresced at an intensity of 3+ or 4-.

In another series of trials, four lots of commercial, fluorescein-labeled, staphylococcal antiserum[6], that had been purchased between 1961 and 1964, were used to stain cultures S-1 through S-18 (Table VII). These trials were conducted in the hope that one lot of the antiserum might prove to be superior at staining staphylococci. However, this was not the case because all four lots stained the cultures to the same average intensity.

Background fluorescence was judged to be more of a problem with the direct staining technique than with the indirect staining technique. The differences were not great, although the slight reduction in background fluorescence using the indirect staining technique was sufficient to permit better visualization of fluorescing staphylococci. One may obtain an idea of the intensity of background fluorescence encountered with the indirect staining technique by examining Figures 1b, and 2b. Smith (35), in his studies on staining staphylococci in nonfat dry milk, noted that milk in smears stained by the direct staining technique adsorbed sufficient fluorescein to make detection of fluorescent staphylococci difficult.

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[6]The Sylvana Company, Milburn, New Jersey.

A direct comparison was made of several cultures stained by both the direct and indirect staining techniques. In most instances, cultures stained by the latter technique fluoresced more intensely. On the basis of results obtained with the two staining techniques, the author concluded that the indirect staining technique would be used exclusively throughout the remainder of the study.

The results of staining staphylococci by the indirect staining technique are demonstrated in Figures 1 and 2. Note that milk particles (denoted by arrows) caused some problems in visualizing staphylococci under incandescent illumination. The milk particles were not a serious problem under ultraviolet illumination because they either failed to fluoresce, or they fluoresced at a relatively weak intensity as compared to that of the staphylococci which exhibited bright peripherial fluorescence and resembled clusters of donuts.

Comparison of Experimental Antiserum with Commercial Antiserum

Several trials were conducted, using the indirect staining technique, to compare the staining ability of unlabeled S-1 antiserum and commercial, unlabeled, staphylococcal antiserum[7]. Four different, commercially available, fluorescein-labeled, anti-rabbit globulins were also included in the trials in an effort to determine whether or not a particular combination of unlabeled antiserum and labeled, anti-rabbit globulin would cause the staphylococci to fluoresce more intensely.

The results of the trials indicated that there was a slight increase

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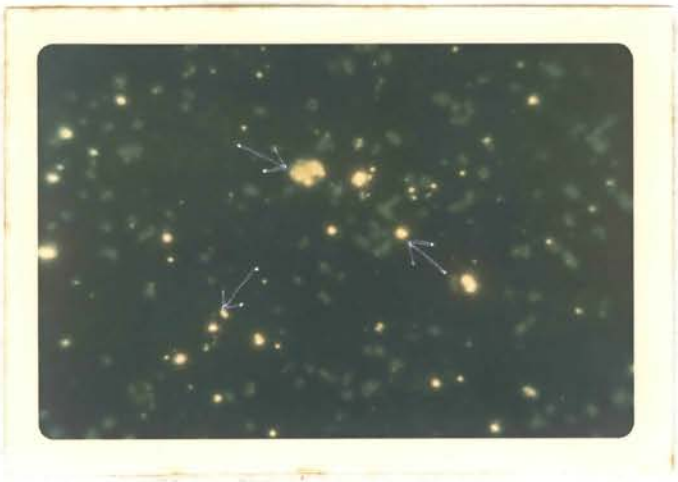
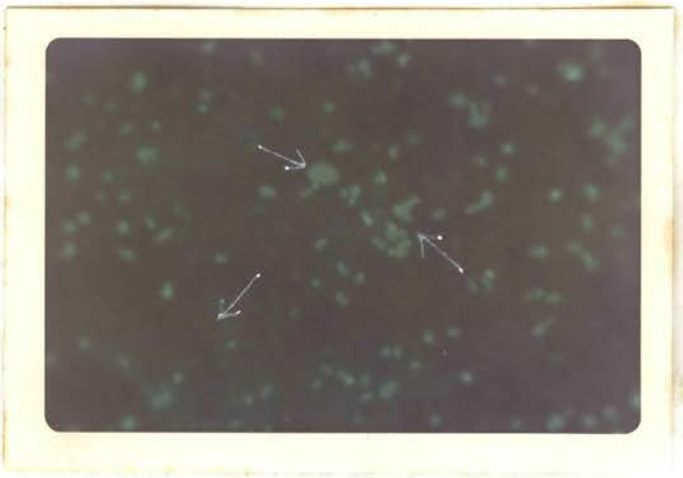
[7]The Sylvana Company, Milburn, New Jersey.

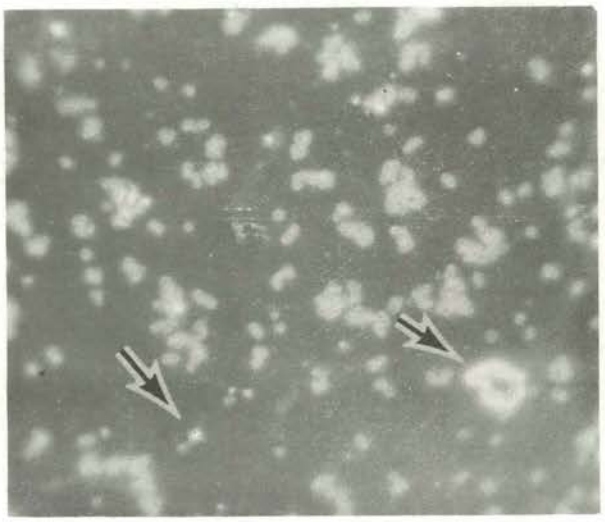


a. Incandescent illumination

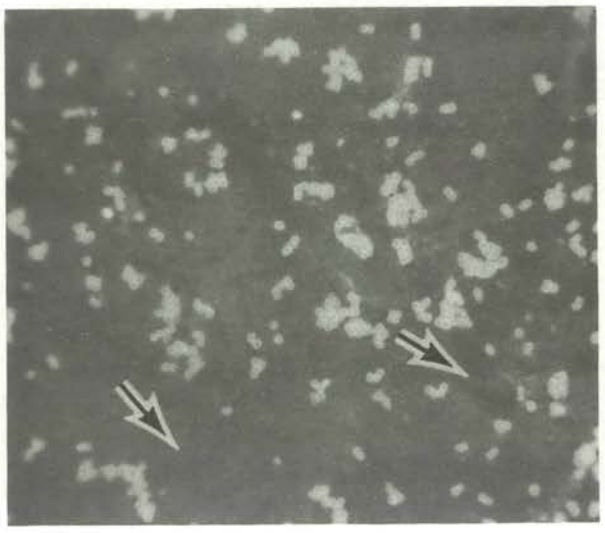


b. Ultraviolet illumination

Figure 1. A color comparison of incandescent and ultraviolet illumination of S. aureus in milk smears stained by the indirect staining technique.



a. Incandescent illumination



b. Ultraviolet illumination

Figure 2. A black and white comparison of incandescent and ultraviolet illumination of S. aureus in milk smears stained by the indirect staining technique.

in fluorescence (Table VIII) in favor of the experimental antiserum. There were no indications of an increase in fluorescence from any of the eight combinations of unlabeled antisera and labeled, anti-rabbit globulins. The author elected to use the experimental antiserum in the balance of the study because of the slight increase in fluorescence. The relatively high cost of the commercial antiserum was considered prohibitive, also.

Comparison of Commercial, Fluorescein-Labeled, Anti-Rabbit Globulins

A comprehensive search was made to determine which of the five commercially available, fluorescein-labeled, anti-rabbit globulins[8,9,10,11,12] would provide the highest intensity of fluorescence when used to stain cultures S-1 through S-18. Smears were prepared and stained by the indirect staining technique. The globulin fraction obtained from Sylvana (Table IX) consistently produced the highest intensity of fluorescence. Background fluorescence was a problem, at times, with all of the globulins; and there was no indication that the use of any one globulin resulted in less background fluorescence. Since the Sylvana globulin had produced more intense fluorescence than the other globulins, it was selected for use in the final phases of the study.

The next logical step, after deciding on the most desirable fluorescein-labeled globulin, was to determine whether or not the globulin could be diluted without materially reducing its staining ability.

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[8]Baltimore Biological Laboratory, Baltimore 18, Maryland.
[9]Difco Laboratories, Detroit 1, Michigan.
[10]Microbiological Associates, Bethesda, Maryland.
[11](Globulin fraction). The Sylvana Company, Milburn, New Jersey.
[12](Serum fraction). The Sylvana Company, Milburn, New Jersey.

Consequently, trials were conducted comparing undiluted globulin with globulin diluted 1:4. The dilution trials indicated that there was no appreciable decrease in fluorescence of S. aureus in smears when the globulin was diluted 1:4 (Table X). Therefore, a dilution rate of 1:4 was adopted for the labeled, sheep anti-rabbit globulin used in the remainder of the study. The dilutions were made using sterile, phosphate-buffered saline (pH 7.0). It was encouraging to note that the use of globulin diluted 1:4 resulted in less background fluorescence than undiluted globulin.

Sensitivity of Staphylococcal Cultures to Agglutination in Experimental Antisera

The objective of this study was to determine if staphylococci from the bovine udder could be stained and identified by the fluorescent antibody technique. If the objective was to be realized, it was necessary that a large percentage of staphylococcal cultures, isolated from the bovine udder, react serologically with one, or a very few, antisera. Therefore, 100 staphylococcal cultures recently isolated from the bovine udder were tested for their ability to agglutinate in the experimental antisera.

The antigens used in the agglutination tests were prepared according to the following procedure: All cultures were transferred to fresh agar and incubated for 18-24 hours after which each culture was used to inoculate sterile broth in 25 ml, screw cap, test tubes. The inoculated broth was incubated 18-24 hours and centrifuged at 2400 rpm for 20 minutes. The packed cells were harvested and washed twice with sterile, 0.85 per cent saline. Harvested cells were heat-killed by suspending them

in sterile saline and placing the tubes in a water bath at 65 C for 30 minutes. The killed cells were washed a final time, suspended in saline to an optical density equivalent to a McFarland no. 3 standard, and used immediately in agglutination tests.

Only 90 cultures were tested for their ability to agglutinate in S-1 antiserum because of a shortage of the antiserum. However, all 100 cultures were tested for their ability to agglutinate in S-19 antiserum and in normal rabbit serum. The data obtained from the agglutination trials are summarized in Table II.

Inspection of the data reveals that 79 of 90 cultures, or 88 per cent, agglutinated in S-1 antiserum. This compared to 96 of 100, or 96 per cent, that agglutinated in S-19 antiserum. Thus, for purposes of identifying staphylococci of bovine origin by the fluorescent antibody technique, it would appear that S-19 antiserum would be more satisfactory since it showed the broadest range of activity. The percentages reported here are somewhat higher than those reported by Smith (35), although, it should be noted that a majority of the cultures used by Smith were from cheese sources and human sources.

Further inspection of the data reveals that 23 of 100 cultures autoagglutinated in normal rabbit serum. Most of the autoagglutinations were at serum dilutions of 1:20 or 1:40; however, 3 of the cultures autoagglutinated at serum dilutions of 1:160. It was interesting to note that 16 of the 23 cultures autoagglutinating had been isolated from the same herd. Close examination of the 16 cultures revealed that all 16 were chromogenic, mannitol fermenting, and coagulase positive while 15 of the 16 cultures produced at least 1 hemolysin.

TABLE II

AGGLUTINATION TITERS OF STAPHYLOCOCCI IN VARIOUS ANTISERA

Culture no.	Reciprocal of highest serum[a] dilution agglutinating the antigen		
	AS-1	AS-19	NAS
S- 1	160	160	-
S- 2	-	80	-
S- 3	20	40	-
S- 4	80	160	-
S- 5	160	160	-
S- 6	160	160	-
S- 7	80	160	-
S- 8	160	160	40
S- 9	160	80	-
S-10	160	160	-
S-11	80	160	-
S-12	40	160	-
S-13	160	160	-
S-14	80	80	-
S-15	-	40	-
S-16	20	40	-
S-17	-	40	-
S-18	80	80	-
S-19	160	160	-
S-20	160	160	-
S-21	80	80	-
S-22	160	160	-
S-23	40	40	-
S-24	160	160	-
S-25	160	160	-
S-26	160	160	-
S-27	80	160	-
S-28	80	160	20
S-29	160	160	-
S-30	40	160	-
S-31	160	160	-
S-32	160	160	-
S-33	160	80	-
S-34	160	160	40
S-35	160	160	-
S-36	160	160	160
S-37	160	160	-
S-38	160	160	-
S-39	160	80	-
S-40	160	160	-

TABLE II (continued)

Culture no.	Reciprocal of highest serum[a] dilution agglutinating the antigen		
	AS-1	AS-19	NAS
S-41	160	160	-
S-42	160	160	160
S-43	160	160	-
S-44	-	-	-
S-45	160	160	80
S-46	-	20	-
S-47	80	80	-
S-48	160	160	-
S-49	160	160	-
S-50	-	40	-
S-51	160	160	-
S-52	80	80	-
S-53	-	80	-
S-54	160	160	-
S-55	20	40	-
S-56	-	80	-
S-57	160	160	-
S-58	40	80	-
S-59	40	160	-
S-60	80	160	-
S-61	80	40	20
S-62	-	80	-
S-63	160	80	20
S-64	80	20	40
S-65	40	-	-
S-66	80	80	20
S-67	80	80	20
S-68	80	160	40
S-69	160	160	80
S-70	160	160	-
S-71	160	160	-
S-72	-	-	-
S-73	160	160	40
S-74	80	160	-
S-75	160	160	-
S-76	80	80	-
S-77	160	160	-
S-78	160	160	40
S-79	160	160	-
S-80	160	160	-
S-81	160	160	160
S-82	80	80	20

TABLE II (continued)

Culture no.	Reciprocal of highest serum[a] dilution agglutinating the antigen		
	AS-1	AS-19	NAS
S-83	160	160	20
S-84	160	160	20
S-85	80	160	40
S-86	80	160	-
S-87	80	160	-
S-88	80	160	20
S-89	160	160	-
S-90	-	40	-
S-91	X	160	-
S-92	X	160	40
S-93	X	80	-
S-94	X	-	-
S-95	X	160	-
S-96	X	160	-
S-97	X	160	20
S-98	X	160	-
S-99	X	160	-
S-100	X	160	-

[a]Legend: AS-1 = antiserum prepared against culture S-1; AS-19 = antiserum prepared against culture S-19; NAS = normal rabbit antiserum.

X: Agglutinations not conducted due to a shortage of the antiserum.

Specificity of Indirect Staining

Species Specificity. Previous trials with the fluorescent antibody technique had involved only one experimental antiserum and only 18 cultures. Although agglutination tests had indicated that a large percentage of staphylococci isolated from the bovine udder would react serologically with the two experimental antisera, it remained to be determined whether or not the same cultures would fluoresce when stained by the indirect staining technique. Consequently, a series of fluorescent antibody experiments was conducted using the 100 staphylococcal cultures used in the agglutination tests. The data from the experiments are presented in Table III. Only 90 of the 100 cultures were tested with S-1 antiserum due to a shortage of the antiserum.

The second, third, and sixth columns in Table III represent control smears that were prepared and examined. These controls were necessary in order to interpret properly the microscopic observations. The second column indicates that 48 of the 100 cultures exhibited some autofluorescence. In most instances, the intensity of the autofluorescence was very weak and none of the cultures exhibited the bright autofluorescence reported by deRepentigny and Sonea (32). The third column indicates that the staphylococci adsorbed some of the fluorescein-labeled, sheep anti-rabbit globulin; although, the intensity of fluorescence was quite weak.

When examining smears, the author counted as negative all cultures that did not fluoresce at an intensity of 3- or above. Examination of the sixth column shows that 33 of the 100 cultures, which were reacted with normal rabbit serum and stained with labeled, sheep anti-rabbit globulin, fluoresced at an intensity of 3- or above. In fact, 16 of the

TABLE III

INDIRECT STAINING REACTIONS OF STAPHYLOCOCCAL CULTURES

Culture no.	Treatments[a]				
	Milk+ culture	Milk+culture +FAS	Milk+culture +AS-1 +FAS	Milk+culture +AS-19 +FAS	Milk+culture +NAS +FAS
S- 1	1-	1+	4+	4+	3+
S- 2	1-	1+	4-	4+	2-
S- 3	-	1+	4+	4+	2+
S- 4	1-	1+	4-	4-	3-
S- 5	-	1+	4+	4-	2+
S- 6	-	2-	3+	4-	2+
S- 7	-	2+	3+	4-	2-
S- 8	-	1+	4-	3+	1+
S- 9	-	1-	3+	3-	2-
S-10	1-	1+	2+	3+	2+
S-11	-	1-	3+	3+	3+
S-12	-	1-	4-	3+	3+
S-13	-	1+	3+	4-	3-
S-14	-	1+	3+	3+	2+
S-15	-	1+	4-	4+	2-
S-16	-	1-	4-	4+	1-
S-17	1-	1-	4+	4+	2-
S-18	1-	1+	3+	4-	2-
S-19	-	1-	3+	4+	2-
S-20	-	1+	3+	3-	2+
S-21	1+	1+	4-	3+	1+
S-22	-	2-	3+	3-	1+
S-23	1+	2-	2+	2+	2+
S-24	1+	2-	3-	3-	2+
S-25	2-	1+	2+	3-	2-
S-26	-	1+	2+	3-	1+
S-27	1+	1+	2+	3-	2+
S-28	1+	1+	3+	3+	2-
S-29	-	1+	4-	4-	2+
S-30	1-	1+	3+	4-	2-
S-31	-	1+	4+	4+	3+
S-32	-	2-	4+	4+	4-
S-33	1+	1+	4+	4+	4-
S-34	1-	1+	3+	3+	2-
S-35	-	1-	4-	4+	1+
S-36	-	1-	3+	2+	1+
S-37	-	1+	4-	4+	2-
S-38	-	1+	4-	4-	1+
S-39	-	1-	4-	4-	3-

TABLE III (continued)

Culture no.	Treatments[a]				
	Milk+ culture	Milk+culture +FAS	Milk+culture +AS-1 +FAS	Milk+culture +AS-19 +FAS	Milk+culture +NAS +FAS
S-40	1-	1-	3+	4-	2-
S-41	-	1+	4-	4+	2+
S-42	1-	1+	4+	4+	2+
S-43	1+	1+	3+	4-	2+
S-44	1-	1+	3+	4-	2+
S-45	-	1-	4-	4-	2-
S-46	-	1+	3+	2+	2-
S-47	1-	-	4-	3+	1+
S-48	-	1+	3+	3-	1+
S-49	1-	1+	3+	3+	2-
S-50	-	2-	3+	3+	3-
S-51	-	2-	4-	4-	3-
S-52	1-	1+	3+	3+	2-
S-53	1-	1+	4-	4-	2+
S-54	1-	1+	2+	3+	2+
S-55	1-	1+	4-	4+	2+
S-56	1+	1+	3+	4-	2+
S-57	1+	2-	4+	4-	2+
S-58	-	2-	4-	3+	2-
S-59	-	2+	4-	4+	3-
S-60	-	2+	4-	4+	3-
S-61	-	2-	3+	3+	3-
S-62	1-	2+	4-	3-	2+
S-63	1-	1+	3-	3+	3-
S-64	-	2-	2+	4-	2+
S-65	-	1+	3+	2+	2+
S-66	-	2-	3-	2-	2-
S-67	-	2-	2+	3+	2+
S-68	1-	2-	3+	2+	2+
S-69	1+	1+	4-	4+	3-
S-70	1-	1-	3+	3-	1+
S-71	-	1+	3+	3+	3-
S-72	-	1+	3+	3+	2+
S-73	-	2-	4-	4+	3-
S-74	1-	1+	3-	4-	2+
S-75	1-	2-	3-	4-	2+
S-76	1+	2-	4+	4+	4-
S-77	-	2-	4-	4+	2+
S-78	-	2+	4+	4-	3-
S-79	-	1+	4-	4+	2+
S-80	-	2-	4-	4-	2+
S-81	-	2+	4+	4+	3+

TABLE III (continued)

Culture no.	Treatments[a]				
	Milk+ culture	Milk+culture +FAS	Milk+culture +AS-1 +FAS	Milk+culture +AS-19 +FAS	Milk+culture +NAS +FAS
S-82	-	2-	4-	4-	3-
S-83	1+	2-	4+	4+	3+
S-84	1-	1+	4+	4-	3+
S-85	1+	2-	4+	4-	3-
S-86	1+	2+	3+	3+	2+
S-87	2-	2-	4-	4-	2+
S-88	-	2-	3+	4-	2+
S-89	1+	2-	4+	4-	3+
S-90	1+	2-	4-	4+	4-
S-91	1+	1+	X	4-	2+
S-92	1-	2-	X	4+	3-
S-93	1-	2-	X	4+	3+
S-94	-	2-	X	4+	3+
S-95	1-	2-	X	4+	3+
S-96	-	1+	X	4+	3+
S-97	-	1+	X	3-	2+
S-98	1+	2-	X	3+	2-
S-99	-	1+	X	2+	3-
S-100	1-	1+	X	3+	2-

[a]Legend: FAS = Fluorescein-labeled sheep anti-rabbit serum; AS-1 = unlabeled antiserum prepared against culture S-1; AS-19 = unlabeled antiserum prepared against culture S-19; NAS = unlabeled normal rabbit serum.

X: Test not conducted due to a shortage of the antiserum.

cultures fluoresced at an intensity of 3+ or above. Thus, it would appear that the normal rabbit serum, which was obtained from three-month-old rabbits, contained some antibodies against staphylococci.

The data in columns four and five indicate that both S-1 and S-19 antisera were effective at staining staphylococci. The S-1 antiserum stained 82 of 90 cultures, representing 91 per cent, while S-19 antiserum stained 93 of 100 cultures, representing 93 per cent. However, these figures are somewhat misleading because a close examination of the data reported in the last three columns of Table III reveals that several of the treatment smears fluoresced at an intensity equal to, or only slightly above, the intensity of the final control listed in column six. Thus, some of the treatment smears should possibly be classed as doubtful or suspicious.

Intergeneric Specificity. Specificity of staphylococcal staining was studied in the presence of a variety of microorganisms that might be encountered in mastitic milk. The bacteria selected included Aerobacter aerogenes, Bacillus subtilis, Corynebacterium bovis, Corynebacterium pyogenes, Escherichia coli, Micrococcus luteus, Pseudomonas aeruginosa, Serratia marcescens, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus faecalis, and Streptococcus uberis, in addition to S. aureus.

Smears were prepared by placing a small amount of a 24-hour culture in a drop of milk and spreading it on a glass slide. Both pure cultures and mixtures of the organisms were used. The smears were fixed and stained by the indirect staining procedure. All of the strains adsorbed some of the antibody as evidenced by fluorescence observed when the smears were examined in the fluorescence microscope (Table XI).

With one exception, the fluorescence was relatively weak as compared to that of S. aureus and the low intensity of the fluorescence and differences in morphology made it practically impossible to confuse the other strains with S. aureus. The exception was Str. agalactiae. However, since it appeared in chains, as shown in Figure 3, it was easy to differentiate from S. aureus. In fact, Str. agalactiae found in mastitic milk is often in even longer chains than those shown in the figure; thus confusion of the two organisms is almost impossible.

In an attempt to remove the nonspecific or common antibodies responsible for the cross-staining reactions, the antiserum was adsorbed with Dowex[13]. Adsorption was accomplished by placing a small amount of Dowex in a centrifuge tube containing 5 ml of unlabeled antiserum and placing the tube in the refrigerator for 2 hours at 5 C. The contents of the tube were mixed gently at 15-20 minute intervals. Following adsorption, the antiserum was poured into a dialysis bag which was placed in a flask of sterile, phosphate-buffered, 0.85 per cent saline (pH 7.2). The flask was placed in the refrigerator for 24 hours after which the antiserum was again used to stain the various bacterial strains. There was no distinguishable difference between the intensity of fluorescence of bacteria stained with the adsorbed antiserum and those stained with the unadsorbed antiserum.

Methods of Concentrating Staphylococci in Milk

The study has demonstrated thus far that the fluorescent antibody technique can be used to identify specifically S. aureus in milk smears;

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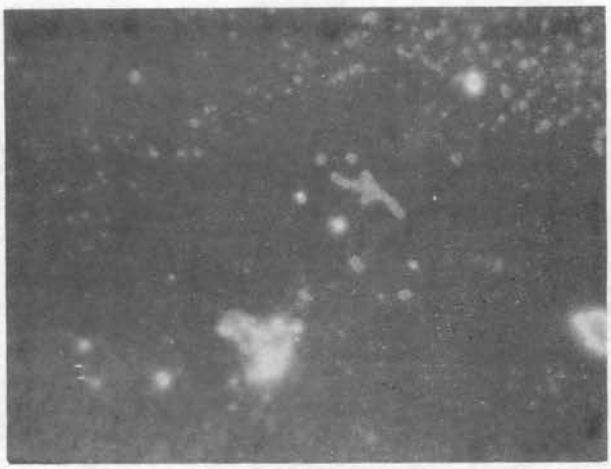
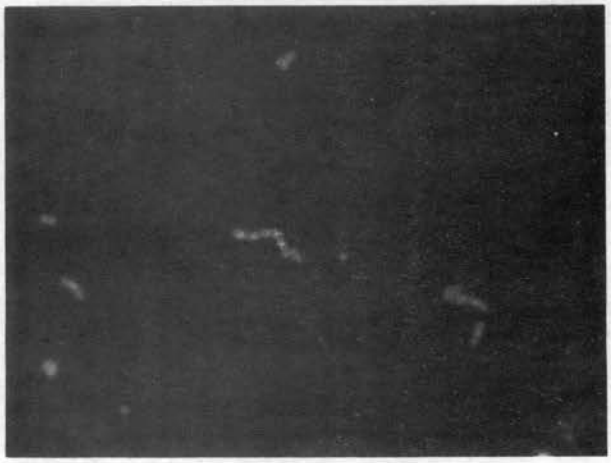
[13]Dowex 2-X4 chloride, 20-50 mesh. I. T. Baker Chemical Company, Phillipsburg, New Jersey.



a. Incandescent illumination

b. Ultraviolet illumination



c. Ultraviolet illumination

Figure 3. A comparison of incandescent and ultraviolet illumination of Str. agalactiae in milk smears stained by the indirect staining technique.

however, all previous trials were conducted using smears that were heavily inoculated with staphylococci. Therefore, a number of trials were conducted using milk from cows with clinical or subclinical staphylococcal mastitis. Examination of the smears revealed that the staphylococci could not be identified with any certainty because of the relatively low numbers of staphylococci present. Therefore, it was obvious that staphylococci in mastitic milk must be concentrated into a very small volume if the fluorescent antibody technique was to find acceptance as a diagnostic tool in mastitis work.

Two techniques, viz., centrifugation and filtration, were employed in attempts to concentrate staphylococci in mastitic milk and in milk purposely inoculated with staphylococci. The first attempts to concentrate staphylococci involved milk that had been heavily inoculated with culture S-5. The inoculated milk was placed in 15 ml, screw cap, centrifuge tubes and centrifuged for 30 minutes at 2400 rpm. A major problem encountered at this point involved the butterfat on top of the sample. The butterfat proved difficult to remove and the method finally selected involved "ringing" it with a sterile needle to free it from the walls of the tube. The top portion of the sample, including the butterfat, could then be decanted, leaving approximately 0.3 ml of milk in the bottom of the tube.

Smears were prepared by placing a drop of the centrifugate on each of two slides. One slide of each pair was stained with Newman-Lampert stain no. 2, while the other slide was stained with fluorescent antiserum. Smears were also prepared from the portion of the sample that was decanted. Fluorescing staphylococci were observed in smears

stained by the fluorescent antibody technique; however, background fluorescence was a serious problem. Comparisons of counts made of staphylococci in smears prepared from the centrifugate and the decanted portion, and stained with Newman-Lampert stain, indicated that the centrifugate contained approximately 30X more staphylococci, per volume, than the decanted portion.

Since centrifuging had been unsatisfactory as a means of concentrating staphylococci, the author decided to combine it with filtration. Milk was again inoculated with staphylococci, centrifuged, and decanted in the manner described above. The centrifugate was diluted in 30 ml of 0.1 per cent Triton X-100[14] and held at 48 C. A 20 ml, Leur-lock, syringe was filled with the mixture. The syringe was attached to a filtering apparatus[15] containing a membrane filter[16]. Pressure was applied to the barrel of the syringe to force the mixture through the filter. Several filterings were made and an average of only 7 ml passed through the filter before it clogged. Following removal from the holder, filters were placed in xylene for 2 minutes, attached to a glass slide with small stainless steel clips, and stained and washed in the manner described previously for the indirect staining technique. After the stained filters had dried, they were prepared for fluorescence microscopy by covering each one with a drop of immersion oil[17] and a cover glass. Upon adding the immersion oil to the filter, it became completely transparent.

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[14]Hartman-Leddon Company, Philadelphia, Pennsylvania.
[15]No. XX30 01200. Millipore Filter Corporation, Bedford, Massachusetts.
[16]Type DA, pore size 0.65 μ, disc diameter 13 mm. Millipore Filter Corporation, Bedford, Massachusetts.
[17]American Optical Company, Buffalo 15, New York.

Examination of the mounted filters under ultraviolet illumination revealed that it was impossible to visualize fluorescing staphylococci on the filter because the filter itself adsorbed some of the fluorescein dye and fluoresced at an intensity that served to mask the fluorescence of the staphylococci.

Considerable time and effort was exhausted trying to perfect a technique that would permit the visualization of fluorescing staphylococci on membrane filters. In some trials, leucocytes were removed from mastitic milk by passing the warm Triton mixture through a larger membrane filter[18] prior to filtering the bacteria out with a 0.65 μ filter. In other trials, a black, nonfluorescing filter[19] was used in the hope that the black would serve to mask some of the fluorescence resulting from the filter's adsorbing the fluorescein dye. All trials were equally unsuccessful and attempts to stain staphylococci on membrane filters were reluctantly abandoned.

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[18]Type SM, pore size 5.0 μ. Millipore Filter Corporation, Bedford, Massachusetts.

[19]Type AA, pore size 0.8 μ. Millipore Filter Corporation, Bedford, Massachusetts.

DISCUSSION

The objective of this study was to determine the feasibility of staining and identifying S. aureus in mastitic milk. This objective was not fully realized because techniques developed during the study could not be applied to clinical mastitis samples. However, techniques were developed that permitted the specific identification of S. aureus (of bovine origin) in milk smears and in the presence of a wide variety of microorganisms that might be encountered in mastitic milk. Thus, the study has served a most useful purpose in that it has demonstrated that the fluorescent antibody technique has great potential as a diagnostic tool in mastitis work, though further studies will be necessary to perfect the technique to a point that will permit its application to clinical mastitis samples.

During the early phases of the study, a large number of different fixing, staining, washing, and mounting procedures was tried in an effort to find the combination of procedures that would provide the most uniformly satisfactory results. The procedures finally adopted and used routinely in the course of the study are given below:

(1) Glass slides were wiped with lens paper, flamed, and cooled prior to use. Following use, the slides were washed with a suitable cleaning product, rinsed in distilled water, dried, and stored in a dust-free container.

(2) Smears were prepared by transferring a loopful of milk to a glass slide and adding a small amount of growth from a 24-hour culture of S. aureus. The milk and culture were mixed and spread over an area approximately 2 cm^2 in size.

(3) The smears were dried at 40-45 C for 5-7 minutes; placed in xylene for 2 minutes, and air-dried; placed in 95 per cent ethanol for 5 minutes, and air-dried; placed in 2 N NaOH for 5 minutes, rinsed gently by dipping the slides four times in each of two beakers of water, and air-dried. Caution was exercised during manipulation of the slides in the aqueous solutions to prevent disintegration of the smears.

(4) A drop of unlabeled, staphylococcal antiserum was placed adjacent to each smear using a small capillary pipette. The pipette was then held horizontal to the slide and the thin end was used to spread the antiserum over the entire smear. Placing the drop of antiserum directly on the smear usually resulted in disintegration of the smear at that spot during the spreading operation that followed. The slides were placed in moisture chambers for 15 minutes. The moisture chambers consisted of inverted culture dishes that were lined with moistened filter paper. Following removal from the moisture chambers, excess antiserum was drained off and the slides were washed for 1-2 minutes in 0.1 M phosphate-buffered, 0.85 per cent saline (pH 8.0), and air-dried.

(5) A drop of fluorescein-labeled, sheep antiserum to rabbit globulin was spread over each smear using the procedure

given above for unlabeled, staphylococcus antiserum. The slides were placed in the moisture chambers for 20 minutes; after which excess serum was drained off and the slides were washed for 10 minutes in two changes of 0.1 M phosphate-buffered, 0.85 per cent saline (pH 8.0), dipped in a beaker of distilled water, and air-dried. The slides were mounted with a drop of glycerol saline (9 parts of glycerol, 1 part 0.85 per cent saline buffered at pH 8.0 with phosphate buffer) and a cover glass. The cover glass was sealed with melted paraffin to prevent disloding during microscopic examination, and to prevent the glycerol saline from flowing out from under the cover glass.

(6) The smears were examined with the fluorescence microscope with Cargille Type A, very low fluorescence, immersion oil, both below and above the glass slide. The use of sufficient immersion oil was found to be very important to good fluorescence microscopy. When an inadequate amount of immersion oil was used, or when it contained air bubbles, visualization of the staphylococci was seriously impaired.

(7) Necessary controls were employed in each trial in order that the microscopic observations could be interpreted properly. The author feels that the following controls are necessary when examining staphylococci in milk smears: (1) milk alone; (2) milk plus staphylococci; (3) milk plus fluorescein-labeled, sheep antiserum to rabbit globulin; (4) milk plus staphylococci plus fluorescein-labeled, sheep antiserum to rabbit globulin;

and (5) milk plus staphylococci plus unlabeled, normal rabbit serum plus fluorescein-labeled, sheep antiserum to rabbit globulin.

Background fluorescence was a serious problem at times - particularly with the direct staining technique. This problem was reduced (1) by making thinner smears, (2) by making observations (whenever possible) at the periphery of the smear where less milk solids were present, and (3) by using the indirect staining technique rather than the direct staining technique. Background fluorescence was not completely eliminated by the above; however, it was reduced to a level at which it could be tolerated without materially interferring with the visualization of fluorescing staphylococci. Smith (35) experienced a serious background fluorescence problem, also, and was able to overcome it only by adopting the indirect staining technique.

A system for scoring the intensity of fluorescence was established. The system may not find application in other laboratories, though it did serve a most useful purpose in the present investigation.

Both the direct and the indirect staining techniques were compared, and the latter technique proved the more desirable for the present investigation. The indirect staining technique is a two-step procedure as opposed to the one-step direct staining technique. The unlabeled, specific antiserum used in the indirect staining technique plays a dual role. It acts as an antibody in the first step and as an antigen in the second step where the labeled, sheep antiserum to rabbit globulin is used. Thus, when using the indirect staining technique, only one labeled antiserum is required for each species of animal in which specific antisera are produced.

A total of five different commercially available, fluorescein-labeled, anti-rabbit globulins were compared to determine which one would provide the highest intensity of fluorescence. The labeled globulin purchased from Sylvana Company consistently produced the highest intensity of fluorescence and was selected for use. Dilution trials indicated that the globulin could be diluted 1:4 with sterile, phosphate-buffered saline (pH 7.0) without materially reducing its staining ability. Therefore, the practice of routinely diluting the globulin 1:4 prior to use was adopted.

A total of 100 staphylococcal cultures, recently isolated from mastitic milk, was used in fluorescent antibody and agglutination trials involving S-1 and S-19 staphylococcal antisera, and normal rabbit serum. The results of the trials indicated that the sensitivity of the fluorescent antibody technique for identifying S. aureus of bovine origin was high since approximately 90 per cent of the cultures exhibited bright fluorescence. The agglutination trials indicated that a similar percentage of the cultures agglutinated in the experimental antisera. Although the percentage of positive reactions in the fluorescent antibody and agglutination trials were of the same magnitude, it should be noted that the same cultures were not involved in each instance. The most disturbing part of these trials was the fact that a sizeable percentage of the cultures autoagglutinated in, and stained with, normal rabbit serum which was employed as a control on the two experimental antisera. One could only conclude that the normal rabbit serum contained antibodies against staphylococci even though it was obtained from healthy three-month-old rabbits. Other workers (8, 31, 43) using the direct

staining technique, have noted labeled serum from nonimmunized rabbits can be used to stain staphylococci. Cohen et al. (8) have theorized that the presence of antibodies in the sera from apparently healthy non-immunized animals may be related to the complex mechanism of resistance to infection.

The staining of control smears by normal rabbit serum caused some problems in interpreting the data because 30 of the 190 smears stained with experimental antisera fluoresced at an intensity equal to, or only slightly above, that of the normal serum control. Thus, some cultures stained with the experimental antisera should be classed possibly as suspicious rather than positive. These data point up the need for a thorough study of existing antibodies in nonimmunized rabbits prior to using them for the production of staphylococcal antisera that is to be used in fluorescent antibody studies.

Studies with milk from cows with clinical or subclinical staphylococcal mastitis indicated that S. aureus could not be specifically identified due to the relatively low number of organisms present. Leucocytes in smears prepared from mastitic milk adsorbed some of the antiserum and fluoresced at a medium intensity. They were not considered a serious problem.

Attempts to concentrate staphylococci by centrifuging 15 ml of mastitic milk for 30 minutes at 2400 rpm were unsuccessful. Possibly, these attempts would have been successful if a centrifuge had been available that would have permitted centrifuging at higher speeds. Efforts at filtering staphylococci from the centrifugate that had been diluted in warm Triton[18] were successful, though attempts to stain the staphylococci directly on the filters failed because the filters

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[18]See footnote 14, page 46.

adsorbed some of the fluorescein dye and fluoresced at an intensity that prevented visualization of fluorescing staphylococci. In an effort to overcome this problem, filters that had been used to filter staphylococci were inverted on a clean slide and carefully smeared in an attempt to dislodge some of the organisms. Smears prepared in this manner were heat-fixed and stained by the indirect staining technique. Fluorescing staphylococci were visible; however, this procedure was not considered satisfactory because only a fraction of the staphylococci present on the filter could be transferred to the slide.

Filtration studies also indicated that most leucocytes could be selectively filtered by employing a filter with a pore size of 5.0 μ prior to filtering the staphylococci on a filter with a pore size of 0.65 μ.

SUMMARY

This study was conducted to determine the feasibility of staining and identifying Staphylococcus aureus in mastitic milk using specific staphylococcal antisera and commercial, fluorescein-labeled, sheep antiserum to rabbit globulin.

A total of 100 cultures of S. aureus were isolated from cows with clinical or subclinical staphylococcal mastitis and characterized as to chromogenesis, hemolysin production, coagulase production, and ability to ferment mannitol. Three of the cultures were used to prepare staphylococcal antisera in rabbits, although only two of the antisera were used in the study.

The direct staining technique and the indirect staining technique were compared and the latter technique was selected for use in the study since it stained the cultures more intensely and resulted in less background fluorescence.

Five commercially available, fluorescein-labeled, anti-rabbit globulins were compared to determine which one would stain the cultures to the highest intensity. The globulin fraction obtained from Sylvana Company proved superior to the other globulins and was selected for use in the study. Further studies revealed that the globulin could be diluted 1:4 with sterile, phosphate-buffered saline (pH 7.0) without reducing the intensity of fluorescence.

A system for scoring the intensity of fluorescence was developed and it served a most useful purpose throughout the conduct of the study.

Both the agglutination and the fluorescent antibody trials produced firm evidence that broad-spectrum staphylococcal antisera had been produced. Approximately 90 per cent of all cultures agglutinated in, or stained with, the experimental antisera. In fact, 96 per cent of the cultures agglutinated in S-19 antiserum, while 93 per cent of the cultures fluoresced intensely when stained with the same antiserum.

Specificity of staphylococcal staining was demonstrated by staining S. aureus in the presence of a variety of microorganisms that might be encountered in mastitic milk.

Attempts to concentrate staphylococci by centrifugation and filtration were only partially successful. Most of the staphylococci in a sample could be concentrated by combining centrifugation and filtration; however, attempts to stain staphylococci on membrane filters failed because the filters adsorbed some of the fluorescein dye and fluoresced at an intensity that served to mask the fluorescence of the staphylococci. Staining of staphylococci on filters will apparently have to await the development of a filter that will not adsorb the fluorescein dye.

Using the indirect staining technique, it would be possible, theoretically, to identify a single S. aureus cell on a milk smear; providing the entire smear was examined with the fluorescence microscope.

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A P P E N D I X

TABLE IV

EFFECT OF DIFFERENT WASHING TIMES ON STAINING INTENSITY OF S. AUREUS STAINED BY THE INDIRECT STAINING TECHNIQUE

Milk + culture no.	Time (in minutes) allotted to different steps				
	Unlabeled antiserum	First washing	Labeled antiserum	Second washing	Staining intensity
S-5	15	1	15	5	3+
S-5	15	2	15	5	3+
S-5	15	5	15	5	4-
S-5	15	10	15	5	4-
S-5	15	1	15	10	4+
S-5	15	2	15	10	4+
S-5	15	5	15	10	4+
S-5	15	10	15	10	4+
S-5	15	1	15	15	4+
S-5	15	2	15	15	4+
S-5	15	5	15	15	4-
S-5	15	10	15	15	4-
S-5	15	1	15	30	4+
S-5	15	2	15	30	4+
S-5	15	5	15	30	4-
S-5	15	10	15	30	3+

TABLE V

EFFECT OF DIFFERENT STAINING TIMES ON STAINING INTENSITY OF *S. AUREUS* STAINED BY THE INDIRECT STAINING TECHNIQUE

Milk + culture no.	Time (in minutes) allotted to different steps				Staining intensity
	Unlabeled antiserum	First washing	Labeled antiserum	Second washing	
S-5	10	1-2	10	10	3+
S-5	15	1-2	10	10	3+
S-5	20	1-2	10	10	4-
S-5	30	1-2	10	10	4-
S-5	10	1-2	15	10	4-
S-5	15	1-2	15	10	4-
S-5	20	1-2	15	10	4-
S-5	30	1-2	15	10	4-
S-5	10	1-2	20	10	4-
S-5	15	1-2	20	10	4+
S-5	20	1-2	20	10	4+
S-5	30	1-2	20	10	4-
S-5	10	1-2	30	10	4+
S-5	15	1-2	30	10	4+
S-5	20	1-2	30	10	4-
S-5	30	1-2	30	10	4-
S-5	10	1-2	60	10	4-
S-5	15	1-2	60	10	4+
S-5	20	1-2	60	10	4+
S-5	30	1-2	60	10	4+

TABLE VI

EFFECT OF AGE OF CULTURES ON STAINING INTENSITY OF S. AUREUS

Culture no.	Age of cultures	
	1 day	8 days
S- 1	4+	4+
S- 2	4-	4-
S- 3	3+	3+
S- 4	4-	4+
S- 5	3+	4-
S- 6	4+	4-
S- 7	4-	4-
S- 8	4+	4-
S- 9	4+	4-
S-10	4-	3+
S-11	3+	4-
S-12	4+	4+
S-13	4+	4+
S-14	3+	3+
S-15	4+	3+
S-16	4-	4+
S-17	4+	4-
S-18	4-	4+
S-19	4+	3+

TABLE VII

DIRECT STAINING OF S. AUREUS WITH FOUR LOTS OF FLUORESCEIN-LABELED, STAPHYLOCOCCAL ANTISERUM[a]

Culture no.	Staining intensity with antisera purchased in 1961	1962	1963	1964
S- 1	4-	2+	3+	3+
S- 2	3+	2-	2+	2+
S- 3	2+	2+	3-	2+
S- 4	3+	3-	2+	2+
S- 5	3+	2+	2+	3-
S- 6	2+	2-	3-	2+
S- 7	3-	2+	2+	2+
S- 8	2+	2+	2+	2+
S- 9	3-	2+	2+	2+
S-10	2+	2+	2+	2+
S-11	3+	3+	2-	2+
S-12	2+	2+	2-	2+
S-13	3-	2+	2+	2+
S-14	2+	2+	2+	2+
S-15	2-	2-	2+	3+
S-16	2+	3+	3+	2+
S-17	2+	2+	2+	2+
S-18	2+	2+	2+	2+
S-19	3-	2+	2+	2+

[a]All antisera purchased from Sylvana Company, Milburn, New Jersey.

TABLE VIII

COMPARISON OF STAINING INTENSITY WITH EXPERIMENTAL ANTISERUM AND COMMERCIAL ANTISERUM

Culture no.	Experimental antiserum[a]	Commercial antiserum[b]
S- 1	4+	3-
S- 2	4-	2+
S- 3	4+	4-
S- 4	4+	4+
S- 5	4+	3+
S- 6	4+	4+
S- 7	4+	4-
S- 8	4+	4-
S- 9	4+	4+
S-10	4+	4+
S-11	4-	4+
S-12	4-	4-
S-13	4+	4+
S-14	4+	4+
S-15	4+	4+
S-16	4+	4+
S-17	4+	4+
S-18	4+	4-
S-19	4+	3+

[a]Antiserum prepared against culture S-1.
[b]Staphylococcal antiserum purchased from Sylvana Company, Milburn, New Jersey.

TABLE IX

COMPARISON OF COMMERCIAL, FLUORESCEIN-LABELED, ANTI-RABBIT GLOBULINS

Culture no.	Intensity of fluorescence with commercial globulins				
	1[a]	2[b]	3[c]	4[d]	5[e]
S- 1	4-	4+	3-	2+	3+
S- 2	3-	4-	3+	2+	2+
S- 3	4+	4+	3+	3-	3+
S- 4	4+	4+	4+	3+	2+
S- 5	4+	4+	4+	2+	3+
S- 6	4-	4+	3+	3+	3+
S- 7	4-	4+	4-	3+	3+
S- 8	4+	4+	4+	3+	3+
S- 9	4-	4+	3+	4-	3-
S-10	3+	4+	3+	3+	3+
S-11	4-	4-	3+	3+	3+
S-12	3+	4-	3+	3+	3-
S-13	4-	4+	2+	3-	3-
S-14	3+	4+	4-	3+	4-
S-15	4+	4+	3+	3+	3+
S-16	4-	4+	3+	3+	3+
S-17	4+	4+	3+	3-	4-
S-18	4-	4+	3+	3+	3-
S-19	4-	4+	3+	3-	4+

[a](Serum fraction) Sylvana Company, Milburn, New Jersey.
[b](Globulin fraction) Sylvana Company, Milburn, New Jersey.
[c]Baltimore Biological Laboratory, Baltimore 18, Maryland.
[d]Difco Laboratories, Detroit 1, Michigan.
[e]Microbiological Associates, Bethesda, Maryland.

TABLE X

STAINING INTENSITY OF LABELED GLOBULIN DILUTED 1:4 WITH STERILE, PHOSPHATE-BUFFERED SALINE

Culture no.	Undiluted antiserum	Diluted antiserum
S- 1	4+	4+
S- 2	4-	4-
S- 3	4-	3+
S- 4	3+	4-
S- 5	4-	3+
S- 6	4+	4+
S- 7	4+	4-
S- 8	4+	4+
S- 9	4-	4+
S-10	4-	4-
S-11	4+	3+
S-12	4+	4+
S-13	4+	4+
S-14	4+	3+
S-15	4+	4+
S-16	4+	4-
S-17	4+	4+
S-18	4+	4-
S-19	4+	4+

TABLE XI

INDIRECT STAINING OF A VARIETY OF BACTERIA COMMONLY FOUND IN MASTITIC MILK

Culture	Milk + culture	Milk + culture + FAS[a]	Milk + culture +AS-1[b] + FAS	Milk + culture +S-5+AS-1+FAS	
				Culture	S-5
A. aerogenes	1-	1+	2+	2+	4-
B. subtilis	1-	1+	2+	2+	4-
C. bovis	-	2-	3+	3+	4+
C. pyogenes	-	2-	3-	3-	4-
E. coli	-	2-	2+	2+	4+
M. luteus	-	1-	3+	2-	4-
P. aeruginosa	1-	1+	2-	2-	4-
S. marcescens	-	1+	2-	2-	4-
Str. agalactiae	-	1+	4-	4-	4+
Str. dysgalactiae	1+	2-	2+	2+	4-
Str. faecalis	-	1-	3-	2+	4-
Str. uberis	1-	1+	2-	2-	4-

[a]FAS = Fluorescein-labeled, sheep anti-rabbit serum.
[b]AS-1 = Unlabeled antiserum prepared against culture S-1.

VITA

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