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Reduction of Nonspecific Background Staining in the Fluorescent Treponemal Antibody-Absorption Test

MERRITT E. ROBERTS, JAMES N. MILLER,[1] AND GERALD F. BINNINGS
Space Division, Aerojet-General Corporation, El Monte, California 91734

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The nonspecific background fluorescence which occurs with the fluorescent treponemal antibody-absorption test for syphilis was found to result from a reaction between serum-treated *Treponema pallidum* organisms and the conjugated anti-human γ-globulin. It was also shown that β-lipoprotein and albumin were the important contributing factors in human serum. Various dilutions of 2.5% trypsin in phosphate-buffered saline specifically reduced background fluorescence under proper test conditions. By employing a trypsin digestion method, a semiautomated procedure utilizing a visual readout has been postulated as feasible.

The use of a label that fluoresces in ultraviolet light and that can be conjugated to antibody to demonstrate its specific combination with bacterial cell antigens was introduced by Coons and his associates (2). After the publication of this report, fluorescent conjugates were used in the diagnosis of many diseases; in 1960, Deacon and his associates (3) applied the principle to the diagnosis of syphilis. They described a fluorescent treponemal antibody (FTA-200) test for syphilis which involved the use of an indirect fluorescent antibody technique with *Treponema pallidum* (Nichols strain) as antigen. In preliminary studies, these investigators observed that the specificity and sensitivity of the procedure compared favorably with the *Treponema pallidum* immobilization (TPI) test, and that the technique was relatively simple to perform. Fife and his associates (5) found that a significant amount of nonspecific fluorescence occurred in the FTA-200 procedure described by Deacon and that considerable skill and experience were required to distinguish moderately reactive from nonreactive sera. By use of agitated rinsings, Fife et al. were able to reduce the nonspecific fluorescence sufficiently to allow them to recommend the FTA-100 test in preference to the FTA-200; in addition, they found that the FTA-100 test correlated much better with the TPI test.

Since humans are exposed to nonpathogens which have common antigens with *T. pallidum*, the relative nonspecificity of the FTA procedure precluded its use in the routine diagnosis of syphilis. This problem was solved when Hunter and her associates (10) showed that a 1:5 dilution of serum with an extract of Reiter treponemes resulted in an increase in specificity. The mechanism involved was presumed to be the consequence of nonspecific antibody binding by Reiter antigen. The high specificity and sensitivity of the fluorescent treponemal antibody-absorption (FTA-ABS) technique prompted our interest in the possible automation of the test.

When we began an investigation of the FTA-ABS test by a method (21) suggested by the National Communicable Disease Center (NCDC), it became obvious that the nonspecific background was so great as to render the procedure impractical for automation in which a final readout would be made by instrumentation. As a result, an investigation was begun to determine the role each of the reagents played in the total nonspecific background. The results of these investigations, the methods used to control the nonspecificity of the FTA-ABS procedure, and the procedural modifications necessary for such control are the subjects of this paper.

MATERIALS AND METHODS

Preparation of the T. pallidum antigen. The Nichols strain of *T. pallidum* was obtained from J. N. Miller of the UCLA Medical School. The antigen was prepared according to the method described by Miller et al. (17). The harvested treponemal suspension was standardized according to the NCDC method (21).

Preparation of the sorbent. The Reiter treponemes were obtained from J. N. Miller of the UCLA Medical School. The sorbent was prepared and standardized as described by Stout et al. (20), with the exception that the buildup volume from test tube to production bottles was 10% of the volume in each case.

[1] Present address: Department of Medical Microbiology and Immunology, University of California at Los Angeles School of Medicine, Los Angeles, Calif.

Preparation of the human γ-globulin antiserum. The antisera were prepared with New Zealand rabbits as described by Proom (19), with the exception that γ-globulin was used instead of serum. After each bleeding, the rabbits were allowed to rest for 2 to 3 weeks; then they were reinjected and again bled. This cycle was repeated four times before the rabbits were exsanguinated.

Conjugation of the anti-human γ-globulin. The anti-human sera from the rabbits were maintained at −20°C until conjugated with fluorescein isothiocyanate (FITC). At this time, the sera were thawed and shaken with one volume of Freon no. 113. This treatment decreased the lipid content of the sera and enhanced the stability of the conjugate. The aqueous portion was removed and fractionated with ammonium sulfate. Immunoelectrophoresis analysis of these sera showed a strong γ-globulin line and a faint albumin line when an agar-glass slide method was used. The albumin line was very faint when the analysis was done with Seraphore III paper. The conjugation was done by a modification of the method described by Marshall (16). With this method, 20 μg of FITC was used as a powder with each milligram of protein. The fluorescein-protein ratios of each of the conjugates prepared in this laboratory and reported in this paper ranged between 13.5 and 15.5. The final protein concentration ranged between 0.9 to 1.1%.

Manual FTA-ABS test. The standard procedure used as a base line in this investigation was that described by the Venereal Disease Branch of the NCDC (21). Tests were performed utilizing both our reagents and those standards supplied by the NCDC. The unknown sera for the tests were furnished primarily by the San Bernardino County Department of Health, San Bernardino, Calif.

RESULTS

Determination of the sources of nonspecific fluorescence. When it was determined that the wash solutions and the 2% Tween 80 in phosphate-buffered saline (PBS) that was used to dilute the conjugate did not contribute to nonspecific fluorescence under the experimental conditions, attention was turned toward the contribution of the antigen, the sorbent, the conjugate, and the serum, either alone or in combination. In separate tests, it was shown that the reagents, when processed individually by the NCDC method, did not show nonspecific staining. Furthermore, when the sorbent was filtered through a 0.22-μm filter (Millipore Corp., Bedford, Mass.) it did not contribute to background, either alone or when used with the other reagents.

The results obtained when two or more of the reagents were used simultaneously in the NCDC procedure are shown in Table 1. The sorbent, conjugate, and wash solutions used in this test were membrane filtered (Millipore Corp.). The antigen suspension for this test had been centrifuged at 27,700 × *g* for 90 min. The supernatant fluid was membrane filtered (Millipore Corp.) and was used in the test as the antigen substitute. The sedimented *T. pallidum* organisms were suspended in saline (*p*H 7.2) and were centrifuged at 275 × *g* for 20 min. We found that the *T. pallidum* supernatant fluid, on incubation with antisera and conjugate, contributed a minor portion of the total nonspecific fluorescence. In each of the preliminary studies, it was apparent that the nonspecific fluorescence was of two distinct types: (i) an overall "glow" and (ii) fine pinpoint ("salt and pepper" effect) particles which were smaller than most bacteria. The results of these experiments indicated that the chief source of nonspecific fluorescence was the reaction of the conjugate and the serum. The patient's serum was the reagent in the test over which it was most difficult to exercise control. Although all sera produced background, it was known through previous experimentation that lipid or hemolytic sera produced excessive background which made accurate assessment extremely difficult. Experiments were designed in an effort to obtain more information concerning the source of the material in the serum which caused nonspecific fluorescence. Thus, fluorescein-labeled antisera to human β-lipoprotein, human albumin, and human 7*S* γ-globulin were obtained and were tested as shown in Table 2. The conjugated anti-human β-lipoprotein gave very heavy overall nonspecific fluorescent background, with particles, at dilutions as great as 1:80. The conjugated anti-human albumin gave background equal to the anti-β-lipoprotein at the low dilutions but it diluted out more rapidly. The background due to

TABLE 1. *Sources of nonspecific fluorescence*[a]

Antigen (washed)	Antigen (supernatant fluid)	Standard 4+ antisera	Conjugate	Fluorescence	
				Specific reactivity	Nonspecific background
	X		X	—[b]	None
	X	X		—	None
		X	X	—	Moderate
X		X		—	None
X			X	—	None
X	X		X	—	None
X	X	X		—	None
	X	X	X	—	Light
X	X	X	X	3+	Heavy
X		X	X	3+	Heavy

[a] NCDC procedure, using standard 4+ NCDC reactive serum, lot 2 antigen, and lot 9A conjugate prepared in this laboratory. Lot 9A had a fluorescein-protein ratio of 14.7 and had 0.94% protein/ml.

[b] Absence of fluorescing treponemes.

TABLE 2. *Nonspecific fluorescence contributed by components of human serum*[a]

Conjugate[b]	Fluorescence	
	Specific reactivity	Nonspecific background
Anti-human β-lipoprotein		
Diluted 1:10	Trace	Heavy with particles
Diluted 1:40	—[c]	Heavy with particles
Diluted 1:80	—	Heavy with particles
Anti-human albumin		
Diluted 1:10	—	Heavy with particles
Diluted 1:40	—	Heavy with particles
Diluted 1:80	—	Fair with particles
Anti-human 7S γ-globulin		
Diluted 1:10	1-2+	Moderate
Diluted 1:40	2+	Light
Diluted 1:80	2+	Light
Diluted 1:160	—	Light
Anti-human γ-globulin		
Diluted 1:80	4+	Very light
Diluted 1:160	4+	Very light

[a] All the reagents used in this test were perpared in this laboratory, except the first three conjugates in the table. The serum was a 4+ sample from San Bernardino County Health Department, San Bernardino, Calif. Regular NCDC procedure.

[b] Anti-human β-lipoprotein had a florescein-protein ratio of 17.4 and 1.8% protein/ml. Anti-human albumin had a florescein-protein ratio of 6.3 and 2.2% protein/ml. Anti-human 7S γ-globulin had a florescein-protein ratio of 6.3 and 1.4% protein/ml. Anti-human γ-globulin had a florescein-protein ratio of 14.0 and 0.94% protein/ml.

[c] Degree of reaction to treponemes.

anti-7S γ-globulin was relatively light as compared to the other two serum components. Thus, the results indicated that β-lipoprotein and albumin were the components of human serum which contributed greatly to background fluorescence.

Attempts to remove nonspecific fluorescent background. Using methods including those used by Deacon (3) and Fife (5), with borate, carbonate, tris(hydroxymethyl)aminomethane, and phosphate buffers at various molarities and *p*H values, we were unable to remove completely the nonspecific staining background. Some background removal was obtained with solutions in the higher *p*H and temperature ranges. The results with phosphate buffers at *p*H 9.2 and *p*H 10 in the first and second wash at 48 C for 10 min followed by a quick, water rinse to remove salt crystals are shown in Table 3. Despite reduction in background fluorescence at the higher *p*H values, a level of nonspecificity remained which rendered impractical an adaptation to automation.

Effect of trypsin on the nonspecific fluorescent background. The above experiments indicated that a nonspecific serum component(s) attached to the glass slide so tenaciously that the washing, as performed in the NCDC test, did not remove it, thereby resulting in its appearance after conjugate application. This material appeared to be either β-lipoprotein, albumin, or possibly some other minor constituent of sera. A study of clearing methods indicated that a method based on digestion would probably be the easiest to automate.

Preliminary experiments utilizing lipase (wheat germ) and lysozyme under a variety of conditions either failed to materially reduce the background or considerably affected the stainability of the treponemes. However, a nearly complete removal of the background with no appreciable effect on treponeme stainability was obtained by employing, in the first wash, a 1:10 dilution of a 2.5% solution of trypsin in PBS at *p*H 7.2 and by utilizing an incubation temperature of 48 C for 10 min.

In an attempt to optimize the interaction, we investigated (i) the effect of various concentrations of trypsin, various pH values, and various temperatures on background removal, treponeme retention, and stainability, and (ii) that step in the procedure during which the introduction of trypsin would provide the best practical results. Experiments were carried out under the following conditions: the dilution of 2.5% trypsin ranged from 1:5 to 1:120, the *p*H ranged from 7.2 to 8.5, and the temperatures varied from room temperature to 48 C. The best results were obtained when trypsin was incorporated into the PBS employed in the first or the second or both washes. As shown in Table 4, the *p*H range for satisfactory trypsin activity was between 7.2 and 8.2, with temperature ranging from room temperature to 48 C and the incubation period lasting for 1 to 10 min. The amount of trypsin in any procedure could not exceed a certain total amount without removal of most of the *T. pallidum* organisms from the slide, the amount being regulated by the temperature, *p*H, and the concentration of the trypsin solution.

TABLE 3. *Effect of temperature and pH of wash solutions on nonspecific staining of T. pallidum*[a]

Method	First wash[b]	Second wash[c]	Fluorescence	
			Specific reactivity	Nonspecific background
1	PBS, *p*H 4.0; RT, 10 min	PBS, *p*H 7.2; RT, 10 min	3–4+	Moderate
2	PBS, *p*H 5.0; RT, 10 min	PBS, *p*H 7.2; RT, 10 min	3–4+	Moderate
3	PBS, *p*H 6.0; RT, 10 min	PBS, *p*H 7.2; RT, 10 min	3+	Moderate
4	PBS, *p*H 7.2; RT, 10 min	PBS, *p*H 7.2; RT, 10 min	3+	Moderate
5	PBS, *p*H 8.2; RT, 10 min	PBS, *p*H 7.2; RT, 10 min	3+	Moderate
6	PBS, *p*H 9.2; RT, 10 min	PBS, *p*H 7.2; RT, 10 min	3–4+	Light
7	PBS, *p*H 9.2; 48 C, 10 min	PBS, *p*H 9.2; 48 C, 10 min	4+	Very light
8	PBS, *p*H 10.0; 48 C, 10 min	PBS, *p*H 10.0; 48 C, 10 min	4+	Very light
9	PBS, *p*H 11.0; 48 C, 10 min	PBS, *p*H 11.0; 48 C, 10 min	2–3+	Heavy
10	PBS, *p*H 9.2; 48 C, 10 min	PBS, *p*H 7.2; RT, 10 min	1+	Heavy

[a] In these tests the same antigen, sorbent, conjugate, and 4+ antisera were used.
[b] After treponeme-antibody-sorbent incubation. Method 1 indicates a wash in PBS at *p*H 4.0 at room temperature (RT) for 10 min.
[c] After conjugate incubation.

TABLE 4. *Use of trypsin in the NCDC procedure for staining of T. pallidum*

Method[a]	First wash[b]	Second wash[c]	Fluorescence	
			Specific reactivity	Nonspecific background
1	1:40 trypsin, *p*H 8.0; 48 C, 1 min	PBS, *p*H 8.5; 48 C, 5 min	4+	Very light
2	1:40 trypsin, *p*H 8.0; RT, 3 min	PBS, *p*H 8.5; 48 C, 5 min	4+	Very light
3	1:20 trypsin, *p*H 8.2; 48 C, 2 min	PBS, *p*H 9.2; 48 C, 10 min	4+	Very light
4	1:40 trypsin, *p*H 8.2; 48 C, 2 min	PBS, *p*H 9.2; 48 C, 10 min	4+	Very light
5	1:60 trypsin, *p*H 8.2; 48 C, 10 min	PBS, *p*H 9.2; 48 C, 10 min	4+	Very light
6	1:20 trypsin, *p*H 8.2; 48 C, 10 min	PBS, *p*H 7.2; RT, 10 min	4+	Very light
7	1:40 trypsin, *p*H 8.2; 48 C, 1 min	1:40 trypsin, *p*H 8.2; 48 C, 1 min	4+	Very light
8	PBS, *p*H 8.2; 48 C, 1 min	1:10 trypsin, *p*H 7.6; RT, 10 min	4+	Very light
9	1:20 trypsin, *p*H 9.2; 48 C, 10 min	PBS, *p*H 7.2; RT, 10 min	2+	Moderate
10	1:80 trypsin, *p*H 8.2; 48 C, 10 min	PBS, *p*H 9.2; 48 C, 10 min	3+	Light

[a] The antigen was lot 2, the 4+ antisera were from the San Bernardino County Health Department, San Bernardino, Calif., and the 9A conjugate was prepared in this laboratory.
[b] The trypsin for method 1 was a 1:40 dilution of a 2.5% concentration prepared in PBS at *p*H 8.0. The slide and solution were immersed in a water bath at 48 C for 1 min and were then rinsed in distilled water for 20 sec. RT, room temperature.
[c] After incubation with conjugate, the slide in method 1 was put in a container containing PBS at *p*H 8.5, immersed in a water bath at 48 C for 5 min and then rinsed in distilled water for 20 sec.

Attempts to add the trypsin solution (i) directly to the antigen solution, (ii) to the antigen after fixation, or (iii) to the antisera-sorbent solution gave less than satisfactory as well as inconsistent results under the varied conditions of concentration, temperature, and *p*H previously described.

Effect of antigen washing. The appearance and pattern of the nonspecific fluorescent background remaining after trypsin digestion at room temperature led us to believe that the problem was inadequate washing rather than inadequate digestion.

The modified FTA-ABS test at this stage of development required an acetone-fixed antigen to which the antisera-sorbent mixture was applied. In a series of washing experiments in which the antigen was washed before application of the serum-sorbent solution, water was used for up to 90 sec followed by rapid drying. This proved to be the most crucial of the washes. The "glow" portion of the nonspecific fluorescence was reduced in various serum samples. It was so effective that the second wash could be reduced to a 1-min distilled-water bath rinse, followed by

TABLE 5. *Comparison of nonspecific fluorescence by NCDC procedure and combined antigen wash-trypsin digestion method*

Reported result[a]	Result determined by			
	NCDC method		Modified FTA-ABS method[b]	
	Specific reactivity	Nonspecific background	Specific reactivity	Nonspecific background
4+	3+	Heavy	3+	Light
3+	2+	Heavy	3+	Light
±	±	Heavy	±	Moderate
−	−	Heavy	−	Moderate

[a] Reported titer from San Bernardino County Health Department, San Bernardino, Calif. NCDC methods were used.

[b] The *T. pallidum* antigen was washed for 60 sec in water; the first wash was with a 1:30 dilution of 2.5% trypsin (*p*H 7.2; room temperature, 10 min), followed by a 1-min water wash. The second wash was with distilled water for 1 min.

rapid drying of the slide. An example of one such test is shown in Table 5.

Effect of antigen preparation methods. The use of the washed antigen with trypsin digestion eliminated most of the haze portion of the nonspecific background. However, it was found that the "salt and pepper" effect previously mentioned still remained as part of the background. Since this effect was present in most of the antigen preparations, especially in concentrates, it was assumed that the particles were mammalian cellular debris from the rabbit testes.

To obtain more information on the origin of these particles, we harvested antigens from a series of rabbits using the same standard method for treponeme production but varying the harvest procedures in order to determine whether the particles would separate by fractional centrifugation. The results of one comparative test are shown in Table 6. One set of slides was tested by the NCDC procedure and the other set was tested by the trypsin digestion procedure. We found that the particles were primarily associated with certain *T. pallidum* concentrates and that these particles were more obvious by the trypsin method than by the NCDC procedure.

It was thought that these particles and *T. pallidum* cells might possess different affinities for the fluorochrome. In a test in which the fluorescein-labeled anti-human γ-globulin was substituted with FITC and fluorescein amine, we found that the low concentration of isothiocyanate caused fluorescence of these particles. A portion of the conjugate was passed through a Sephadex G25 column to remove additional free dye and decomposition products. In comparative tests, it was shown that the Sephadex-purified product had less affinity for the particles; i.e., they were not as brightly stained but were still visible.

Adopted trypsin digestion method (FTA-ABS-T). As a result of the above experiments, the following procedure was adopted as a reference standard for the evaluation of sera with known and unknown titers.

Glass slides etched with a 0.25-cm circle are washed in methanol and flame-dried. A standard loopful of *T. pallidum* antigen is then put on each circle and air-dried. The antigen is then fixed on the slide by immersion in 10% methanol for 10 to 20 sec followed by rapid drying. A solution consisting of one part serum and four parts sorbent is put on the antigen spot and the slide is incubated at 37 C in a humidified chamber. The

TABLE 6. *Comparison of various antigen preparations with respect to background*

Antigen	NCDC procedure		Trypsin digestion method	
	Specific reactivity	Nonspecific background	Specific reactivity	Nonspecific background
6713-NCDC[a]	3+	Moderate	3-4+	Light
Lot 1[b]	3+	Moderate	4+	Light
101067D	3-4+	Heavy	3+	Very light with particles
101067D-1	3+	Heavy	3-4+	Very light
101067D-2	3+	Heavy	3-4+	Very light
101067D-3	± to +	Very heavy	2-3+	Very light with particles
131067D-1	3-4+	Moderate	4+	Very light
131067D-2	3-4+	Moderate	4+	Very light with particles
131067D-3	3-4+	Moderate	3-4+	Very light with particles
510672W-1	4+	Moderate	4+	Very light with few particles

[a] The 6713 antigen was from NCDC.

[b] Lot 1 antigen was prepared in this laboratory. The remainder was furnished by J. N. Miller of UCLA. The dash and figure after the lot number indicate differences in the method of extraction.

slide is then rinsed in distilled water and digested for 10 min with a 1:30 dilution of a 2.5% solution (final concentration, 0.083%) of trypsin prepared in PBS at *p*H 7.2. After digestion, at room temperature the trypsin is removed by thorough rinsing in distilled water followed by a 1-min bath rinse in distilled water. The slide is dried in warm air and the reaction spot is treated with conjugate. After incubation at 37 C in a humidified chamber for 30 min, the slide is rinsed in distilled water and is then placed in a distilled water bath for 1 min. The slide is dried and the antigen spot is treated with a drop of 90% glycerine over which a cover slip is placed. The reaction is observed at 400 × under ultraviolet with the darkfield optics of a Leitz microscope equipped with a Schott BG 12 exciter filter and an OG 1 barrier filter.

Comparative results between the NCDC method (FTA-ABS) and the (FTA-ABS-T). Good correlation of results was obtained on 20 unknown serum samples provided as part of a monthly evaluation survey by the NCDC (Table 7). Further, in contrast to the standard FTA-ABS method, the trypsin digestion slides showed no glow and relatively few fluorescent particles. The absence of background glow in the FTA-ABS-T procedure may account for the nonreactive results obtained on two sera which exhibited variable or borderline reactivity in the FTA-ABS test. In many comparative tests, it has been found that the titers of some sera which were variable or borderline by the NCDC procedure remain borderline in the trypsin digestion procedure. In others, as those in Table 7, the digestion reduced the titer to normal or negative. The trypsin digestion method may give further or better differentiation in this borderline group.

TABLE 7. *Comparative NCDC and trypsin digestion results on 20 unknown serum samples*[a]

Serum no.	NCDC method	Trypsin digestion method
101	3+	3+
102	±	N[b]
103	4+	4+
104	±	N
105	4+	4+
106	3-4+	3+
107	1-2+	1+
108	4+	4+
109	2-3+	2+
110	3+	3+
111	± to 1 +	± to 1 +
112	1-2+	1-2+
113	2+	2-3+
114	3+	3+
115	4+	4+
116	1+	1+
117	3+	2-3+
118	2-3+	2+
119	4+	4+
120	4+	4+

[a] All reagents were prepared in the Aerojet-General laboratory. The results reported were obtained in comparative tests in this laboratory.

[b] Nonreactive.

DISCUSSION

The automation of the FTA-ABS test is dependent, to a large extent, upon the elimination of nonspecific background fluorescence which interferes with instrumented readout. It has been shown that most of this background fluorescence emanates, in the form of a glowing ring, from a reaction between the conjugate, the patient's serum, and the treponemal suspension. Furthermore, it has been demonstrated that (i) β-lipoprotein and albumin are the important contributing factors in human serum which controls this nonspecificity and (ii) the nonspecific components become firmly bound to the glass slide and cannot be removed by washing procedures.

Efforts by other investigators to remove the nonspecific fluorescent background have been unsuccessful. However, these investigators have concentrated on treatment either of the conjugate or of the reaction obtained after the conjugate was added to the antigen-antibody reaction. Among the methods which have been used are (i) washing of the total reaction (1, 3, 5–7, 12, 15), (ii) absorption of the conjugate with various dry animal powders (4, 8, 11, 13, 14), and (iii) use of a high-titered conjugate (9, 11).

Although a number of agents and procedures were employed in our laboratory to reduce the background problem, only the use of dilutions of trypsin in buffered saline under proper incubation and temperature conditions proved successful, provided that the trypsin was properly incorporated into the first or second or both washes. The judicious use of trypsin in this manner resulted in a specific staining of antibody-coated *T. pallidum* after application of conjugate. It appeared that the trypsin selectively removed nonspecificity not related to antigen-antibody reactions, since the background appeared in reactions with sera other than human and conjugated anti-human γ-globulin prepared with various animal species. This also seemed reasonable since we have shown in our laboratory that FITC and certain FITC conjugates are nonspecifically absorbed by a host of bacterial antigens in the absence of antibody. Pittman et al. (18) made a distinction between autofluorescence and nonspecific staining due to fluorescent components of a conjugate and the specific heterologous staining of serologically

selected antigens. It is possible that the staining of mammalian sera with conjugate from various sources represented heterologous staining; however, if this was the case, the heterologous antigen-antibody complex appeared so weak as to be dissociated by the trypsin, leaving the specific antigen-antibody to be stained when conjugate was added.

The failure of the trypsin digestion to eliminate the so-called "salt and pepper" background effect has made it difficult to completely automate the process. However, it does allow for adaptation of the method to a semiautomated procedure in which the readout is performed visually. Studies designed to eliminate the "salt and pepper" effect are underway.

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