Domestic Fowl—Source of High Titer *P. tularensis* Serum for the Fluorescent Antibody Technic. (26206)

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Fluorescent antibody technic (FAT) has been offered as a rapid method of identification for many systems. Winter and associates (1) reported on identification of *Pasteurella pestis*. Recently, Smith, Marshall and Eveland(2) have reported on identification of *Listeria monocytogenes*. Most of these workers used hyperimmune rabbit serum prepared by multiple injection technics.

Thomason *et al.*(3) reported nonspecific cross-staining of *Escherichia, Salmonella, Shigella, Proteus* and *Vibrio comma* using heterologous sera. They referred to "natural antibodies" to certain strains of bacteria in rabbit serum. Other authors have reported the same or similar results(4,5). Finkelstein and LaBrec(6) obtained staining of various microorganisms with anti-cholera serum in smears of fecal suspensions from normal animals. In our laboratory, rabbit anti-*P. pestis* fluorescein conjugated globulin stains organisms found in normal pharyngeal washings. Rabbit and monkey anti-*Pasteurella tularensis* globulins (APTG) showed a high degree of nonspecificity at low dilutions and loss of specific staining at higher dilutions. The report of Abramoff and Wolfe(7) on the specificity and high titer of chicken serum to a single antigenic stimulus directed our attention to the domestic fowl in search for a high titered, more specific antiserum.

Materials and methods. Rabbit serum: Twenty-four hour glucose cysteine blood agar (GCBA) cultures of *Pasteurella tularensis* strain SCHU S-4 (PTS4)(8) were harvested in 0.5% formalin, held overnight at 5°C and diluted to a density of 9 x 10^8 organisms/ml (McFarland Nephelometer standard No. 3). Each animal was inoculated intravenously (IV) with 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 and 2.0 ml of this vaccine at 2-day intervals and exsanguinated one week following last injection. *Monkey serum*: Monkeys previously infected by exposure to an aerosol of PTS4 and fully recovered from the disease were bled and serum collected. *Avian serum*: Twenty-four hour GCBA cultures of PTS4 were harvested in saline and adjusted to 5 x 10^7 viable organisms/ml. Eight lb to 10 lb roosters of various breeds were each inoculated IV with 0.1 ml of this suspension, and after 9 days were exsanguinated and serum was collected. *Pseudomonas sp* (isolated from a patient) was cultured on heart infusion agar for 24 hr, harvested and triple washed in saline. A total dose of 10^{10} viable organisms was inoculated IV. Nine days post-inoculation the roosters were exsanguinated and serum collected. All sera were filtered through an EK Seitz pad and stored at –20°C in a mechanical freezer. Agglutination tests were performed to determine the titer of each serum.

Gamma globulin was precipitated by methanol fractionation according to Dubert *et al.*(9), maintaining a temperature of –5°C. The gamma globulin fraction was reconstituted to 5/8 the original volume with physiological saline adjusted with 0.1 N NaOH to pH 8.0. Gamma globulins were conjugated with fluorescein isothiocyanate according to the method of Coons and Kaplan(10) as modified by Riggs(11). Following overnight conjugation at 4°C the mixture was dialyzed seven days against normal saline.

Portions of each serum were adsorbed with whole mouse tissue powder according to Coons and Kaplan(10). Portions of avian and monkey PTS4 globulin were also adsorbed with *Pseudomonas sp* by adding 0.5 ml of packed, washed cells to each milliliter of globulin or globulin conjugate. The combination was placed in a shaker water bath at 37°C for 2 hours and followed by centrifugation at 24,000 G for 30 minutes in the cold (4°C) employing the International PR-2 centrifuge. The supernatant fluid was then

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TABLE I. Fluorescent Cross-Reactions Obtained with Anti-*P. tularensis* Globulin.

Test specimen*	Staining intensity with immune globulin																			
	Avian (1:5120†)								Monkey (1:640)								Rabbit (1:1280)			
	Non-adsorbed				*Pseudomonas*-adsorbed				Non-adsorbed				*Pseudomonas*-adsorbed				Non-adsorbed			
	1:1	1:4	1:16	1:64	1:1	1:4	1:16	1:64	1:1	1:4	1:16	1:64	1:4	1:8	1:16	1:32	1:1	1:8	1:16	1:32
P. tularensis	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+	±	3+	2+	+	−	2+	+	±	−
Pseudomonas sp	2+	2+	+	+	−	−	−	−	2+	2+	2+		−	−	−	−	+	±	−	−
Neisseria sp	±	±	−	−	±	±	−	−	±	±	−	−	±	±	−	−	±	±	−	−
D. pneumoniae	±	±	−	−	±	±	−	−	±	±	−	−	±	±	−	−	±	±	−	−
Herellea sp	±	±	−	−	±	±	−	−	±	±	−	−	±	±	−	−	ND	ND	ND	ND
E. insidiosa	2+	+	+	−	2+	+	+	−	2+	+	+	−	+	+	+	−	ND	ND	ND	ND

* All other organisms examined were negative.
† Agglutination titer before fractionation.
ND = Not done.

filtered through an HA Millipore filter for clarification. Lissamine rhodamine counterstain was added to portions of each globulin according to Smith *et al.*(2).

Bacterial films were prepared from organisms grown on appropriate culture media. Twenty-four hour cultures were suspended in nutrient broth, normal saline and distilled water. A drop of suspension was placed on a slide and if not used immediately the films were stored at −25°C. Pharyngeal washes were collected from laboratory personnel in nutrient broth; organisms were added and mixing was accomplished with a syringe and 23-gauge needle. Smears were prepared with and without addition of PTS4, *Pseudomonas* and a mixture of the following organisms†: *Bacterium anitratum, Corynebacterium diphtheria, Diplococcus pneumoniae*—Types III, VI, XIV, XXIII, *Erysipelothrix insidiosa, Herellea sp, Listeria monocytogenes, Mimae sp, Neisseria perflava, Salmonella paratyphi, Shigella dysenteriae, Shigella flexneri, Staphylococcus pyogenes, Streptococcus pyogenes.*

All films to be examined were fixed in 10% formalin (ACS) (pH adjusted to 7.2-7.4 with 0.1 N NaOH). Period of fixation was 10 minutes, followed by a 10-minute normal saline wash. While still moist, 2 to 3 drops of globulin conjugate (pH 7.2) were spread over the surface of the film. The preparation was placed in a moist chamber and incubated at 37°C for 30 minutes. The slides were then rinsed to remove excess conjugated globulin, placed in flowing normal saline solution for 15 minutes and mounted in phosphate buffered saline-glycerin (pH 7.4-7.6).

A Zeiss fluorescent microscope (Model GF) equipped with an OSRAM HBO 200 lamp was used. Our criteria for positivity required that the particle specifically fluoresce with the Schott filter combinations BG12-GG4 as well as with the BG12-OG4-GG4.

Results. Fluorescent antibody cross-reac-

† Obtained from Division of Immunology, Walter Reed Army Inst. of Research, and Bacteriology, Immunology, and Infectious Disease Branch, Armed Forces Inst. of Pathology, Washington, D. C.

TABLE II. Cross Agglutination Titers of *P. tularensis* and *Pseudomonas sp.*

Avian serum	Agglutinin titers Organisms *P. tularensis*	*Pseudomonas sp*
P. tularensis		
Pre-immune	<1:10	<1:10
Immune	5120	320
Pseudomonas sp		
Pre-immune	<1:10	<1:10
Immune	320	5120

tions of APTG with *Pseudomonas, Neisseria* and *D. pneumoniae* organisms (Table I) occurred with monkey, rabbit and avian globulin. Use of higher dilutions in an effort to eliminate or reduce these cross-reactions was successful only with the avian serum. Normal and pre-immunization serums of all 3 species gave a weak (±) fluorescence with *Neisseria* and *Diplococcus*.

The fractionated and conjugated avian APTG was examined to determine its maximum working dilution with *P. tularensis* and *Pseudomonas*. At a dilution of 1:512 in phosphate buffered saline (pH 7.2) a 3+ reaction to *P. tularensis* and a ± reaction to *Pseudomonas* were obtained. The cross-reaction with *Pseudomonas* was eliminated by specific adsorption with washed, homologous living cells. The working dilution for *P. tularensis* following this adsorption was reduced to 1:128.

Using 1:1, 1:2 and 1:4 dilutions of unadsorbed APTG, cross-reactions occurred with *P. pestis* (non-encapsulated organisms only), *Pseudomonas, Neisseria* and *Diplococcus*. At a 1:64 and 1:128 dilution only the *Pseudomonas* cross-staining persisted. Cross-reactions with other organisms listed above using *Pseudomonas*-adsorbed globulin occurred only with *E. insidiosa* and *Herellea sp* (Table I). These cross-reactions were eliminated at a 1:64 dilution of globulin. Bentonite (12) treatment, mouse tissue powder adsorption and addition of lissamine rhodamine counterstain to non-*Pseudomonas*-adsorbed globulin were studied as a means of reducing cross-reactions, but showed no advantage over dilution of *Pseudomonas*-adsorbed globulin and did not eliminate the *Pseudomonas* or *E. insidiosa* cross-reactions.

Using *Pseudomonas* and *P. tularensis* agglutinating antigens and *P. tularensis* and *Pseudomonas* antisera in agglutination tests, specific cross-reactions were noted (Table II). Further, PTS4 organisms were stained with conjugated avian *Pseudomonas* globulin.

To examine the possible role of diluents, *P. tularensis* and *Pseudomonas* organisms suspended in nutrient broth, distilled water and physiological saline were stained with avian APTG. No difference in intensity of staining and clearness of background was detected.

Slides prepared from normal pharyngeal washes with and without addition of PTS4 and a mixture of other organisms were stained with normal, heterologous immune and *Pseudomonas* adsorbed avian anti-*P. tularensis* globulins. Lissamine rhodamine was added to all APTG. Of 60 coded slides 15 of 16 positive and 43 of 44 negative slides were read correctly.

Discussion. When examining pure cultures, cross-reactions present little or no problem; however, with the FAT for rapid identification, any specific fluorescence may result in a false positive diagnosis unless the particle is morphologically distinguishable or the cross-fluorescence can be eliminated. Past experience in this laboratory with monkey and rabbit serum precluded identification of *P. tularensis* and *P. pestis* in simulated and actual clinical specimens because of our inability to prepare suitable specific antisera. Specific immune monkey and rabbit sera, although possessing high agglutination titers, did not allow sufficiently high fluorescent antibody working dilutions to permit the necessary manipulations. Normal rabbit and normal monkey globulins exhibited fluorescent reactions with a variety of organisms commonly found in clinical specimens.

Abramoff and Wolfe(7) report on the high titered, specific response of the domestic fowl to a single antigenic stimulus, "Although multiple injections are usually effective in enhancing antibody titers . . . such procedures tend to reduce the specificity of anti-

sera." Therefore roosters were given a single IV dose of living *P. tularensis* organisms and an excellent agglutinating response was obtained. This avian globulin conjugate exhibited specific fluorescent antibody staining of *P. tularensis* at a dilution of 1:512.

Although a 4+ staining of *P. tularensis* occurred at a 1:512 dilution of avian globulin, a weak (±) reaction with *Pseudomonas* persisted. At lower dilutions, *Neisseria* and *Diplococcus* exhibited specific fluorescence to APTG of all 3 species but the avian globulin had a sufficiently high, 4+, working dilution to eliminate these cross-reactions. To eliminate the weak cross-reaction of *Pseudomonas*, specific adsorption with living *Pseudomonas* organisms was essential. This adsorption lowered the dilution at which 4+ staining of *P. tularensis* occurred to 1:128 and eliminated the *Pseudomonas* cross-reaction. This suggested a possible common antigen between *P. tularensis* and this strain of *Pseudomonas*. Comparative agglutination studies supported this hypothesis (Table II). Fractionated and conjugated avian *Pseudomonas* globulin also gave specific fluorescence with *P. tularensis* organisms. Specific adsorption with living PTS4 organisms eliminated this cross-staining.

In addition to the *P. tularensis* and *Pseudomonas* antiserums reported above, studies are now in progress using rooster serums prepared against: *P. pestis, Bacillus anthracis,* Rift Valley fever virus, Venezuelan equine encephalomyelitis virus, *Coccidioides immitis* and human, burro, monkey, mouse, rabbit and guinea pig globulins.

Summary. A method for producing a high titer, specific anti-*P. tularensis* globulin is presented. Roosters immunized with living *P. tularensis* organisms produced an antiserum that is more specific and has a higher working dilution than rabbit and monkey sera. The suspending medium for the organisms does not affect degree and intensity of specific staining or working dilution of serum. Cross-reactions obtained suggest that a specific antigenic relationship exists between *P. tularensis* and the *Pseudomonas* used in this study. This cross-reaction was eliminated by specific adsorption with heterologous living organisms.

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Received August 31, 1960. P.S.E.B.M., 1960, v105.