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Detection of Specific Antibody to *Mycobacterium tuberculosis* in Anti-Complementary Human, Rabbit and Bovine Serum by Supplementation with Procomplementary Porcine Serum

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=國文抄錄=

親補體性 돼지혈청의 補强에 의한 抗補體性 사람, 토끼 및 소혈청속의 人結核菌 (*Mycobacterium tuberculosis*)에 대한 特異抗體檢出

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崔 哲 淳·梁 容 泰

보체결합반응은 감염초기의 항체검출에 대한 민감성이 높기 때문에 사람과 동물의 만성질환, 특히 매독, 브루셀라병, 결핵병, 렙토스피라병, 클래미디아감염증, 마이코플라스마감염증, 콕시오이드병, 히스토플라스마병 등의 면역학적진단수기와 예방관리를 위한 전염병학적연구목적에 흔히 사용된다. 그러나 검사혈청이 항보체성 또는 친보체성을 갖는 경우는 특이항체를 검출할 수 없는 단점을 갖고 있다.

그러므로 이 연구에서는 친보체성분을 갖는 돼지혈청을 항보체성 사람, 토끼 및 소혈청에 보강하여 특이항체검출을 위한 민감성과 특이성에 관한 기초실험을 실시하였다. 결핵에 감염된 사람, 토끼 및 소혈청중에 높은 항보체작용이 있는 혈청에 친보체성 돼지혈청을 보강할 때 결핵균(다당체 및 단백질항원)에 대한 특이항체가 검출되었다,

INTRODUCTION

The conventional direct complement fixation test is often applied as an immunodiagnostic method to detect antibody in syphilis, brucellosis, tuberculosis, chlamydial infections, mycoplasma infections, coccidioidomycosis, and histoplamosis in man and animals. The direct CF test is, however, unable to detect antigen-antibody complex when serum is anti-complementary (AC)[1-4].

Various methods have been investigated in attempts to reduce the AC properties of sera. Among these, treatment of serum with HCl [5] kaolin[6], adsorption of serum with a delipidized mouse liver powder[7], adding excess complement[8,9], thermal inactivation[10], adding normal porcine serum[11] and treatment of serum with iodine[12] have been studied.

Of these methods, application of the procomplementary (PC) activity of porcine serum has been proved of value in eliminating AC activity in the serum of many species[11]. From the practical standpoint, the important effects of supplementation unheated or heated normal serum to the coventional CF test system are the increased sensitivity of the test in detecting small amounts of antibody[13], in promoting fixation of complement (C′) by certain antigen-antibody systems that fail to do so in its absence[14-16], or in

reducing anticomplementary activity of heated serum[9,17].

The purpose of this work is to describe the reduction in AC activity of rabbit, human and bovine serum by supplementation with porcine serum in the conventional CF test system.

MATERIALS AND METHODS

1. Complement fixation test

The direct complement fixation (CF) test was adapted from the master volume (0.8 ml) test system of Jeon[15] and the micro technique of Casey[19]. Haemolysisn and complement titrations were performed in the master volume using 0.2ml unit volumes and that was transferred to a microtitration system of 25μl unit volumes. Since the purpose of work was to describe the anticomplementary activity, the 100 per cent lytic unit was employed.

2. Swine sera

Normal swine serum was collected at a slaughter house. The blood was collected in a 4 oz McCartny bottle and allowed to clot for two hours at room temperature and two hours at 4°C before harvesting the serum. Swine serum was dispensed in 1 ml amount and stored at −20°C.

3. Antisera

Sera showing high AC activity were obtained from rabbits, man and cattle with active pulmonary tuberculosis.

4. Antigens

Two antigens : crude poylsaccharide and phenol treated PPD were prepared as described by Choi et al. (1981)[20]. Tuberculopolysaccharide was fractionated essentially according to the method described by Azuma *et al.* (See Fig. 1)[18].

5. Guinea-pig complement (C)

Ten mature guinea-pigs were bled by cardiac puncture. The blood was allowed to clot for about one hour at room temperature and then for approximately two hours at 5°C. The serum was harvested and pooled. Aliquots of 0.5ml were dispensed in one ml ampoules, which were sealed and stored at −20°C.

6. CF buffer

Veronal-NaCl buffer containing 0.1 per cent bovine albumin (Difco, Mich., U.S.A.) was prepared with commercial dehydrated CF buffer tablets (Oxoid, England).

7. Hemolysin

Hemolysin was prepared in rabbit by injection of 10% sheep red cells (lml/kg of body weight) at 1, 2, 3, 4, 6, 8 and 10 days. A 50% glycerinated rabbit hemolysin was made and stored at−20°C refrigerator.

8. Sheep cells

Sheep blood was washed 3 times in saline. For the test, a 2.8 per cent final suspension was prepared by adding 34.7 volumes of VBD to 1 volume of packed cells. These cells were standardized by spectrophotometry as described by the Laboratory Branch Task Force (1965)[19]. The standardized cell suspension was kept at 5°C and used within two days.

9. Titration of haemolysin

Titration of haemolysin was made in 0.1ml volumes of 10^{-3} dilutions from 10^{-3} to 10^{-4} mixed with an equal volume of 2.8 percent sheep erythrocytes(RBC). After sensitization

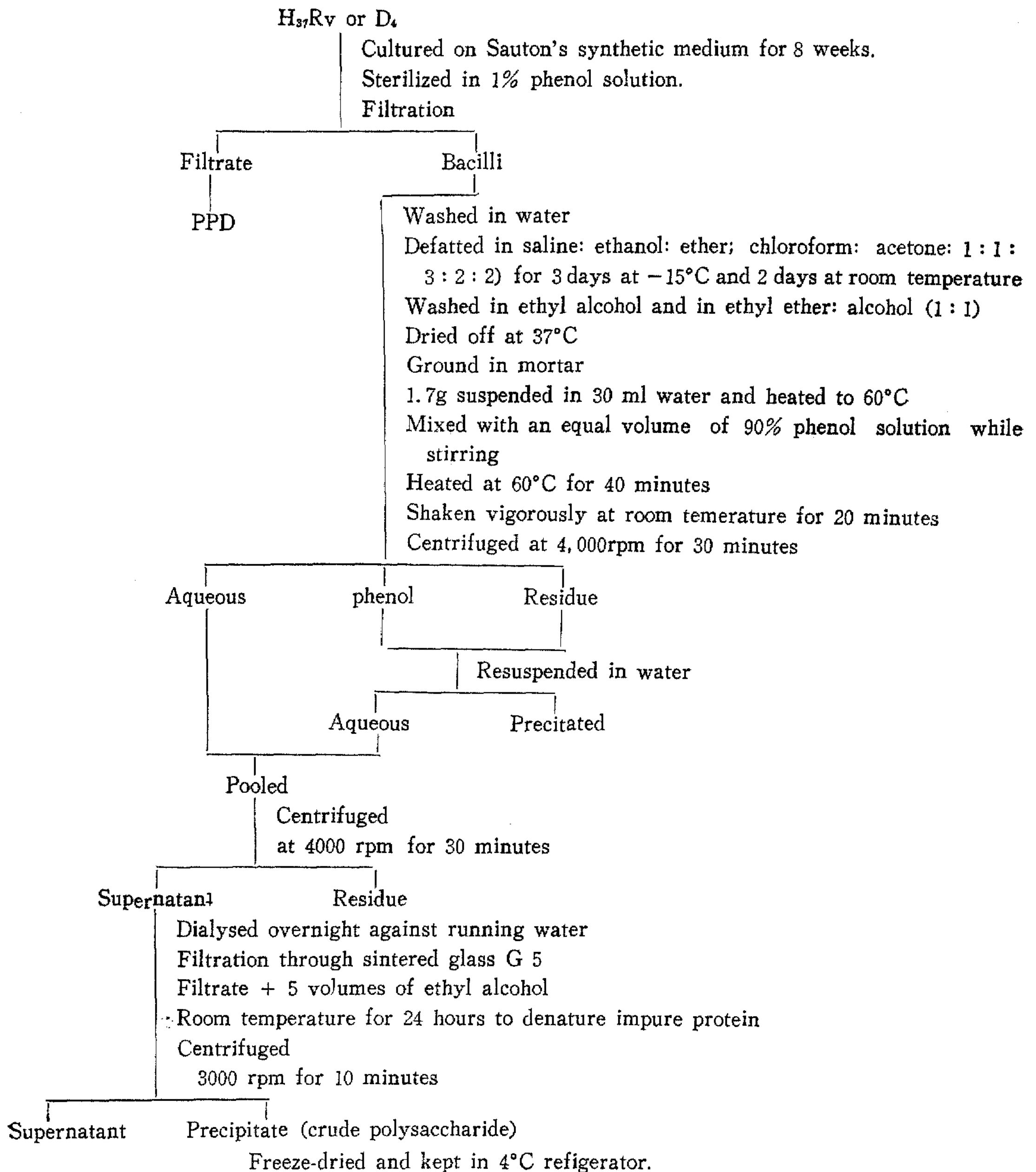

Fig. 1. Fractionation of polysaccharide extracted from human or avian tuberale bacilli

at room temperature (25°C) for 15 minutes, to each dilution 0.2 ml of excess complement (1/40) and 0.4 ml of VBD were added. Readings were made after 30 minutes incubation at 37°C to determine the optimal haemolytic units. The minimum amount of haemolysis which caused complete haemolysis of the RBC was called one unit. To the dilution of haemolysin containing 2 units was added an equal volume of 2.8 per cent RBC. This mixture was used in the test.

10. Titration of Complement

The Complement was diluted 1:40 in VBD,

Table 1. Two-dimensional antigen and antibody titration

Antigen Dilutigon	Antiserum dilution								
	8*	16	32	64	128	256	512	1024	Anitgen control
2*	4 **	4	4	2	1	1	t	t	t
4	4	4	4	4	2	1	1	0	0
8	4	4	4	4	2	1	t	0	0
16	4	4	4	4	4	1	t	t	0
32	4	4	4	3	1	t	0	0	0
64	4	4	4	2	1	t	0	0	0
128	4	4	4	1	t	t	0	0	0
Serum control	3	2	1	0	0	0	0	0	0

* Reciprocal of serum and antigen dilution

** 4=no haemolysis; 3=25% haemolysis; 2=50% haemolysis; 1=75% haemolysis; t=trace of normal cell; 0=100% haemolysis

Table 2. Titration of procomplementary activity of porcine serum with antigen PPD-A(*M. avium*' D_4) and anti-D_4 (*M. avium*) serum

Porcine serum dilution	Antigen	Antiserum Dilution								Porcine Serum control	
		8*	16	32	64	128	256	512	1024	with C	without C
5**	2U	4	4	4	4	4	0	0	0	0	4
	—	0	0	0	0	0	0	0	0	0	4
10	2U	4	4	4	4	4	0	0	0	0	4
	—	0	0	0	0	0	0	0	0	0	4
20	2U	4	4	4	4	4	2	1	0	0	4
	—	0	0	0	0	0	0	0	0	0	4
40	2U	4	4	4	4	4	3	1	0	0	4
	—	2	1	0	0	0	0	0	0	0	4
80	2U	4	4	4	4	4	4	1	0	0	4
	—	4	3	2	0	0	0	0	0	0	4
160	2U	4	4	4	4	4	4	3	1	0	4
	—	4	3	2	1	t	t	0	0	0	4
Contlro	2U	4	4	4	4	4	4	4	2	0	4
	—	4	3	3	2	t	0	0	0	0	4

* Reciprocal dilution of antiserum. ** Porcine serum dilution containing 2 EU of uinea pig C

and the required quantity ranging from 0.02 ml to 0.14 ml at step intervals of 0.02 ml was dispensed using a 0.1 ml Kahn pipette. Cold BVD was added to each tube bringing to volume to 0.6 ml and incubated at 37°C for one hour. To this 0.2 ml of sensitized RBC was added and the mixture incubated at 37°C for one hour. To this 0.2 ml of sensitized RBC was added and the mixture incubated at 37°C for 30 minutes.

The least amount of complement causing complete haemolysis was established as one exact unit (EU). Two EU of complement was used in the test proper.

If 0.06 ml of 1/40 diluted C showed one EU, the quantity of the C which gave two EU was calculated by following equation:

$X=40\times 0.2/2\times 0.08$

Therefore, 0.2 ml of a 1/50 dilution of the complement gave two EU.

11. Titration of antigen and antibody

The two antigens and antisera were each titrated in a two dimensional array using a microtechnique. Antiserum was diluted 1/8 in VBD and inactivated at 56°C for 30 minutes. A 25 μl volume of serial two-fold dilutions of antiserum and antigen were placed in microplate well as indicated in Tabel 1.

A 25 μl volume of BVD was added to antigen and serum control to replace antigen in the serum control and serum in the antigen control and 50 μl VBD to the complement controls. Plates were shaken gently and left for 15 minutes at room temperature. Subsequently, 25, μl of cold complement (2 EU) were added to each well containing both serum and/or antigen. For the Complement controls, 25 μl of complement containing 2 EU, 1 EU and 0.5 EU were added to 50 μl of VBD. Plates were taped and shaken, and reacted at 5°C for 15 to 18 hours.

After the primary reaction, 25μl of sensitized sheep blood cells were added to all wells and plates were retaped, and incubated at 37°C for 30 minutes. Plates were shaken twice at 10 minutes intervals during incubation. Plates were finally left at 5°C for two hours before readings. The minimum concentration of antigen and antiserum which showed complete inhibition of haemolysis without AC effects in any of the controls was desgignated as a unit of antigen and antibody.

12. Titration of procomplementary activity (PC) of porcine sera

Ten samples of porcine sera which lacked haemagglutinating antibody to human(PPD-S) and avian (PPD-A) tuberculin were used. Serum samples were diluted 1:2.5 in VBD and inactivated at 56°C for 30 minutes.

The titration of PC activity was tested as shown in Table 2. Anticomplementary effect, specific complement-fixing antibody, and complementary activity in the absence of guinea pig complement were tested. Twenty-five μl of antigen (2 units) were added to 25 μl of two-fold serial dilution of antiserum. Twenty-five μl of VBD replaced antigen in the control. Plates were allowed to react at room temperature for 15 minutes. To the above were added 25 μl of two-fold serial dilutions of pig serum containing 2 EU of guinea-pig complement The microplates were then left at 5°C for 18 hours, then removed from refrigerator and left at room tempera-trure for 10 minutes.

Subsquently, 25μl of EA were added and the mixture was allowed to react in the incubator at 37°C for 30 minutes.

The least amount of porcine serum reducing AC titre to 4 tube difference(compared with controls) was established as one unit. Two units were used in the test proper.

If 25μl of 1/20 diluted porcine serum showed one unit, the quantity of the porcine serum which give two units was 1/10 (See Table 2).

RESULTS

1. Procomplementary activity of porcine serum

The results in Table 3 show that the AC levels in the rabbit sera were reduced markedly by supplementation with porcine serum. PC activity of 10 porcine ser varied between individuals. As shown in Table 4, of ten porcine sera there was a wide distribution of PC activity, neutralizing AC rabbit serum with a titre of 1:256 to <1:4 ranging

Table 3. Procomplementary activity of porcine sera on anticomplementary rabbit antiserurm

Porcine serum No.	Dilution of porcine serum														
	1 : 5			1 : 10			1 : 20			1 : 40			Control		
	SP	N	HA	SP	N	HA	SP	N	HA	SP	N	HA	SP	N	HA
PS 1	16*	—	16	64	—	4	128	4	—	256	4	—			
PS 2	64	—	64	128	8	—	128	16	—	128	32	—			
PS 3	64	—	64	128	—	16	256	—	—	256	8	—			
PS 4	16	—	16	32	—	32	128	32	32	256	64	4			
PS 5	64	16	64	256	128	16	1024	256	—	1024	512	—	512	256	—
PS 6	32	—	4	64	16	—	256	64	—	256	64	—			
PS 7	16	—	16	64	—	32	128	—	8	128	—	4			
PS 8	126	—	128	128	—	—	256	—	—	256	4	1			
PS 9	16	—	16	64	—	8	128	16	—	128	16	—			
PS 10	32	8	32	128	8	4	512	128	—	512	256	—			

SP=specific serum titre, N=normal serum titre (AC serum titre), HA= specific complement fixing antibody titre showing haemagglutination.

*=Reciprocal of the highest dilution of serum showing 50% haemolysis with specific, 75% haemolysis of normal control C antigens and 25% haemagglutination, respectively. — =negative in 1 : 4 dilution of antiserum.

Table 4. Distribution of procomplementary activity of porcine sera

Porcine serum No. tested	Procomplementary Titre (No.)					
	*<1 : 5	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80
10	2	2	3	2	1	0

* Porcine serum dilution required to reduce AC titre of 1 : 256 to 1 : 4 when 25 μl of AC serum was supplemented with 25 μl of porcine serum.

from 1:5 to 1:40. some porcine sera with high PC activity marked specific antibody as well as AC properties. Only one poricine serum (PS No.5) increased specific and normal comphement-fixing antibody titre.

2. Effect of PC activity to various AC sera

The results in Table 5 show that the AC levels of the 19 rabbit sera were reduced markedly after addition of an equal volume of porcine serum containing 2 PC units. Two units of porcine serum were effective in reducing AC activity for all rabbit sera regardless of both specific and normal complement-fixing antibody titres, and 13 of 23 rabbit sera were freed of AC activity. Spiecificity of mycobacterial antisera against PPD antigen prepared from heat-killed and phenol-killed culture filtrates were not significantly different. However, the Compliement-fixing antibody titre to PPD antigen was higher than that of polysaccharide antigen.

3. Procomplementary activity of porcine serum on human and bovine AC tuberculous sera

Table 6 shows that the AC levels in human and bovine serum varied with the individual. PC activity of porcine serum enhanced in human serum. Low dilution of porcine serum (1/10) completely masked the specific complement-fixing antibody in human tuberculous sera as well as AC serum titre.

Table 5. Effect of procomplementary activity of porcine sera (Two Units) against various species of anticomplementary rabbit anti mycobacterial sera

Antiserum		*M. tuberculosis* (H37Rv) antigen						
Species	Strain	Polysaccharide antigen		Phenol-killed PPD		Heat-killed PPD		Control
		Specific	Normal**	Specific	Normal	Specifice	Normal	
M. bovis	AN_5	16*	—	64	—	32	—	16
	Otto	64	16	256	16	128	16	128
M. tuberculosis	H37Rv	64	—	256	—	256	—	32
	C	16	—	32	—	32	—	32
M. avium	D_4	32	—	256	—	128	—	128
	2437	8	—	32	—	32	—	64
M. intracellulare	Boone	32	16	32	16	32	36	64
	16516	8	8	16	8	16	8	16
M. scrofulaceum	Bridge	8	—	32	—	16	—	8
	Gause	16	8	64	8	64	8	128
M. fortuitum	11997A	32	32	128	32	128	32	128
	12136	8	—	32	—	64	—	64
M. kansasii	Janice	—	—	—	—	8	—	32
	P 16	16	—	32	—	16	—	16
M. marinum	HZ	256	16	256	16	256	16	128
M. gordonae	18635	—	—	—	—	—	—	128
	9738	—	—	32	—	32	—	256
M. terrae	W 995	16	—	64	—	64	—	128
M. smegmatis	Sm	32	—	256	—	256	—	256

* Reciprocal of CF antibody titre — Negative in 1 : 8 dilution of antiserum ** AC titre

Table 6. Procomplementary activity of procine serum on human and bovine anticomplementary sera

Antiserum	Dilution of procine serum									
	*1 : 10		1 : 20		1 : 40		1 : 80		Control	
	SP	N	SP	N	SP	N	SP	N	SP	N
HT 3	—	—	16	—	16	—	16	4	32	16
HT 4	—	—	8	—	16	4	16	8	32	64
HT 5	—	—	8	—	16	—	16	4	16	16
HT 9	8	—	8	—	16	4	16	8	16	32
BT 1	8	—	8	—	16	—	16	4	16	32
BT 2	4	—	8	—	16	4	32	4	64	16

*=Porcine serum dilution SP=specific CF serum titre, N=normal CF serum titre
HT=serum from human tuberculosis BT=serum from bovine tuberculosis

DISCUSSION

Anti- or procomplementary effects are defined as inhibition or enhancement of complement activity by a contatiminating component such as natural hemolysin or additional complement, respectively. Certain bacterias,

yeasts and viruses also interfere with the hemolytic action of complement, and, therefore, gross contamination of serum, antigen or the CF buffered saline solution may lead to AC effects. However, most AC properties of antiserum have been related to both the degree of hyper-gammaglobulinemia (γG) immunoglovlin)and the extent to which sera were heated.[21-24]

Christian[25,26] revealed that sera decomplemented by means other than heat are not anticomplementary and maximum AC activity was induced by heating of sera at temperatures between 52°C to 62°C, and he reported that aggregation of gamma-globulin molecules is responsible for the AC activity of serum. The absorption of guinea-pig serum with solid gamma-globulin resulted in complement component (C1 and C4) distruction that resembled complement inactivation by immune systems[25,26] Ishizaka and Ishizaka[27] demonstrated that heat aggregated gamma-globulin fixes complement in a manner which is indistinguishable from that of antigen-antibody complexes.

Thus, removal of AC activity in human and other animal's serum of specific complement-fixing antibodies has been a problem in the CF test.

AC activity of human serum was first removed by precipitating euglobulin fraction from antibody with diluted HCl.[5] However, this method is not suitable for large scale application because, for the removal of AC activity, it was necessary to adjust the relative concentrations of reagents for each serum.

Removal of AC factors from serum by means of kaolin absorption was reported by Ruge.[6] Later, Rapp et al.[7] attempted to remove AC properties from human serum in the same manner used by Coons et al.[28] to remove nonspecific fluorescent antibody test. The treatment was highly effective in reducing the AC properties in the serum. However, these methods are not suitable for routine tests because they need much time and labour for absorption and recovery of serum.

Wigand[10] attempted to remove AC activity by thermal inactivation at high temperature as in the heat inactivation test used to differentiate nonspecific agglutinin in brucellosis. This proved useful for some sera, but it is not an ideal method for removing AC properties of serum because the heating causes the aggregation of antibody molecules which may give rise to AC activity.[21,22]

Earlier, Davis et al.[22] demonstrated that purified gamma-globulin obtained from normal serum is anticomplementary, and that this AC property could be neutralized by mixing the gamma-globulin with an approximately equal weight of other serum protein fractions such as β-globulin and albumin. The AC property of serum may be removed by adding excess complement. This method has, however, the disadvantage of decreasing or altering the antibody titre.

Akao et al.[29] reported that PC activity of porcine serum was associated with high content of the third cemponent (C3) of complement in the porcine serum and that the PC activity could be effectively eliminated by periodate treatment. Subsequently, Bucca and Alder[11] used porcine serum to neutralize AC activity of sera. However, their method takes 18 hours at 4°C to neutralize AC properties.

In the present work, using a simplified CF test, direct supplementation of porcine serum to serum in the microtest plates reduced the AC activiy of all human, bovine and rabbit serum at least by a four tube

difference and 13 of the 23 rabbit AC sera were freed of AC properties. However, PC activity of porcine sera was enhanced in human sera.

Therefore, the units of PC activity of procine serum must be standardized for supplementation in the conventional direct CF test.

By means of the CF test supplemented with porcine sera, the specific complement-fixing antibody of human, bovine and rabbit anitmycobacterial serum was readily differeniated from AC antibody.

SUMMARY

Adirect complement fixation test supplemented with procomplementary porcine serum was studied using anticomplementary human, rabbit and bovine serum against *Mycobacterium tuberculosis*. Procomplementary activity of porcine serum varied with porcine individual and affected by anticomplementary antiserum. The procomplementary titre of porcine serum against rabbit, human and bovine serum ranged from 1 : 5 to 1 : 40.

By means of complement fixation test supplemented by porcine serum, the specific complement-fixing antibody to both tuberculopolysaccharide and/or tuberculoprotein antigen was readily differentiated from the anticomplementary antibody titre.

REFERENCES

1) Osler, G.G., Strauss, J.H. and Mayer M.M.: *Diagnostic complement fixation method. Am. J. Syph. Gonor. and Ven. Dis. 36 : 140, 1952.*

2) Opferkuch, W., Rother, K. and Schultz, D.R.: *Clinical aspects of the complement system. G. Thieme. 1978.*

3) Mayer, M.M.: *Complement and complement fixation. In Kabat, E.A., Experimental Immunochemistry, 2nd ed., p. 133—240, Springfield, Ill. Charles C. Thomas, 1967.*

4) Lachmann, P.J. and Hobart, M.J.: *Complement technology. In Weir, D.M. (ed.), Handbook of Experimenatl Immunology, 3rd ed., p. 5A1—5A23. Oxford and Melbourne, Blackwell Scientific Publications, 1979.*

5) Sachs, H.: *Ein Hilfs mittel für die Methodik der Wasserman'schen Reaction. Berlin Klin. Wochenschr. 58 : 1075, 1921.*

6) Ruge, H.: *Zur Frage der sog. Eigenhemmungen bei der WaR. Ihre Beswitung durch Kaolin. Z. Immunitaetsforsch. Allergie Klin. Immunol. 108 : 345, 1951.*

7) Rapp, F., Gnesh, G.M., Gordon, I.: *A practical method for removal of anticomplementary properties from human serum. Proc. Soc. Exp. Biol. Med. 90 : 335, 1955.*

8) Lennette, E.H. and Schmidt, N.J.: *Studies on the development and persistence of complement-fixing and neutralizing antibodies in human poliomyelitis. Am. J. Hyg. 65 : 210, 1957.*

9) Brufield, H.P. and Pomeroy, B.S.: *Proc. Soc. Exp. Biol. and Med. 94 : 146, 1957. Cited from Mayer, M.M. Chapt. 4. Complement and complement fixation. In Kabat, E.A. (ed.), Experimental Immunochemistry, 2nd ed., p. 133—240, Springfield, Ill. Charles C. Thomas, 1967.*

10) Wigand, R.: *Eine einfache Methode zur Beseitung der anticomplementären Activatät von Seren. Z. Immunitaetsforsch. Alleggie Klin. Immunol. 124 : 368, 1962.*

11) Bucca, M.A. and Alder, A.O.: *Method for eliminating anticomplementary activity in animals sera. Appl. Microbiol. 18 : 291, 1969.*

12) Polley, M.J.: *Enhancement of haemolytic complement activity by treatment of human serum with iodine. J. Immunol. 107 : 1493, 1971.*

13) Rice, C.E., Konst, H. and Carriere, J.:*Studies of Johne's disease in Canada. X. A more sensitive complement-fixation test. Canad. J.Comp. Med. 25 : 121, 1961.*

14) Boulanger, P. and Bannister, G.L.: *A modified direct complement fixation test for the detection of antibodies in the serum of cattle previously infected with vascular stomatitis virus. J. Immunol. 85 : 368, 1960.*

15) Jeon, Y.S.: *A stuey on the modified complement-fixation test with Bordetella garinarum. A Thesis, The University of Minnesota, U.S. A. 1962.*

16) Boulanger, P., Bannister, G.L., Rucherbauer, G.M. and Corner, A.H.: *Equine infectious anemia: Preliminary investigation of the complent-fixation test for the demonstration of antibodies and antigens. Canad. J. Comp. Med. 33 : 148, 1969.*

17) Rice, C.E.: *Methods of preparing surplementing fractions from fresh unheated bovine sera for use in modified direct complement--fixation tests. Canad, J. Comp. Med. Vet. Sci. 31 : 142, 1967.*

18) Azuma, I., Yamamura, Y. and Fukushi, K.: *Fractionation of mycobacterial cell wall isolation of arabinose mycolate and arabinogalactan from cell wall fraction of Mycobacterium tuberculosis strain Aoyama B. J. Bacteriol. 96 : 1885, 1968.*

19) Laboratory Franch Task Force and Case, H.L.: *Standardized diagnostic complement fixation method and adaptation to microtest.U.S.Public Health Service. Publi. No. 1228, Public Health Monograph No. 74, 1965.*

20) Choi, C.S., Frost, A.J., and Francis, J.: *The specificity of purified protein derivative extracts from cultures of mycobacteria klled by phenol. Res. Vet. Sci. University of Liverpool, 1981 (in press).*

21) Jersild, D.M.: *Ztsch. Klin. Med. 130 : 670, 1936. Cited from Christian, C.L.: Studies of aggregated gamma-globulin. 1. Sedimentation, electrophoretic and anticomplementary properties. J. Immunol, 84 : 112, 1960.*

22) Davis, B.D. Kabat. E.A.: *Harris, A. and Moore, D.: J. Immunol. 49 : 223, 1944. Cited from Christian, C.L.: Studies of aggregated gamma-globulin. 1. Sedimentation, electrophoretic and anticomplementary properties. J. Immunol, 84 : 112, 1960.*

23) Norgaard, O.: *Acta Path. et Microbiol. Scand. 36 : 71, 1955. Cited from Christian, C.L.: Studies of aggreated gammaglobulin. 1. Sedimentation, electrophoretic and anticomplementary properties. J. Immunol. 84 : 112, 1960.*

24) Waldesbuhl, M., Allan, R., Meylan, A. and Isliker, H.: *Anticomplementary activity of γ G-immunoglobulins of their subunits. Immunochemistry 7 : 185, 1970.*

25) Christian, C.L.: *Studies of aggregated γ-globulin. I. Sedimentation, electrophoretic and anticomplementary properties. J. Immunol. 84 : 112, 1960a.*

26) Christian, C.L.: *Studies of aggregated γ-globulin. II. Effect in vivo. J.Immunol. 84 : 117, 1960b.*

27) Ishizaka, T. and Ishizaka, K.: *Proc. Soc. Exp. Biol. and Med. 101 : 845, 1959. Cited from Mayer, M.M. Complement and complement fixation. In Kabat, E.A. (ed.), Experimental Immunochemistry, 2nd ed., p. 133—240. Springfield, Ill. Charles C. Thomas, 1967.*

28) Coons, A.H., Snyder, J.C. Cheever, F.S. and Murray, E.S.: *Localization antigen in tissue cells. IV. Antigens of rickettsiae and mumps virus. J. Exp. Med. 91 : 31, 1950.*

29) Akao, Y. Sano. R., Sugiura, A. and Shimozo, H.: *Procomplementary activity of swine serum and its elimination with periodate treatment. Jap. J. Med Sci. Biol. 15 : 165, 1962.*