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STUDIES ON SALMONELLA PULLORUM ANTIGENS BY MEANS OF INDIRECT HEMAGGLUTINATION TEST

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INTRODUCTION

The serologic means generally available or the detection of Salmonellosis in poultry breeding stocks is the *S. pullorum* agglutination test, but recently bacterial indirect hemagglutination, reported first by Keogh et al(1, 2) and used for the detection of antigens of various bacteria by many workers, have been applied to Salmonellosis in poultry(3, 4).

Gwatkin et al(5, 6, 7), Wright(8, 9) and Byrne(10 reported that there exist no reactors to agglutination test in which standard strain of *S.pullorum* were used as antigen. Finally, Younie(11, 12) was isolated the new strains of *S.pullorum* in Canadian flocks and concluded that antigenic differences existed between the standard strains and cultures which he isolated from chicks.

Since the variant strain of the Canadian investigators occured among recently isolated cultures, it is assumed that smooth-rough variation plays no part in the products of these variants. This suggests that the occurence of the variant be found in form variation. Edwards and Brunner(13) confirmed that the antigenic formula of *S. pullorum* is Ⅸ, Ⅻ, (Ⅻ$_2$), Ⅻ$_1$. In normal cultures, the Ⅻ$_2$ factor is variable. The standard strain contains a small amount of Ⅻ$_2$, while the variant strain contains a large amount of the antigen.

Antigenic variations are of particular importance when they exist in the causative organisms of any disease, the detection or control of which is based on the use of serological methods. The recognition of serological differences among the strains of *S. pullorum* has been stimulated additional interest in the field of antigenic variation.

These serological differentiation of *S. pullorum* strains, involve the use of two antiserums; the Ⅻ$_2$ and Ⅻ$_3$ aggo utinin. The nature of these differences has been variously described by different workers Edwards et. al. (14, 15), Wright(16) and Snoeyenbous et. al. (17, 18) but might not well understood.

The purpose of this studies sets forth the results obtained in serological studies; indirect hemagglutination test, of *S. pullorum* standard and variant strain.

MATERIALS AND METHODS

Test Organism: *S. pullorum* standard and variant strain were used. These strains were kindly supplied by Dr. Edwards, Communicable Disease Center, Chamblee, Georgia, U.S.A. and preserved by subculturing on nutrient stabs at the interval of four week.

Antiserum: By the routine methods of Edwards and Ewing(19), the antisera were produced by rabbit innoculated intravenously, four times during 21 day period, with the *S. pullorum* standard and variant strain separately.

Principles of the Indirect Hemagglutination Test: Hemagglutination test was carried out by the method described by Chun and Park(20) and Chun et. al. (21). Suspensions of 24—48 hours grown cells are employed as crude antigen and followed further modification. Any of the supernatant, containing the liberated erythrocyte sensitizing substance is added to 2 per cent washed erythrocyte suspension and to allow sensitization 2 hours incubated at 37°C with occasional agitation. After incubation, unadsorbed bacterial material is removed from the sensitized erythrocyte by washing three times and then resuspend in 0.85 per cent physiological saline solution equal to the original volume. The treated cell suspension(0.25

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ml) was added to 0.25 ml of immune serums in serial dilutions and to normal serum for control purpose. The results were tentatively read after 2 hours of incubation in water-bath at 37°C according to the method described by Salk(22).

Red Blood Cell: Human O-type, goat and chicken red blood cells were collected aseptically in an equal volume of Alsever's solution. The mixture of red blood cell and Alsever's solution was centrifuged and the erythrocytes were washed three times with 0.85 per cent physiological saline solution. After the last centrifugation at 3,000 r. p. m. for 15 minutes, the supernatant fluid was removed and the packed cells were used. And by the method described by Neter(23), trypsin treatment on red blood cell was carried out as following; 2 per cent suspension of washed red blood cell in the 0.1 M Na_2HPO_4 was mixed with equal volume of 1 : 500 trypsin (Difco 1;250), incubated for 20 minutes at 37°C water-bath incubation, this mixture was washed twice with 0.85 per cent physiological saline solution and resuspended in the original volume with the saline solution.

Ammonium Sulfate Saturation: The treatment with the saturated ammonium sulfate solution was carried out as described by Smolens et. al. (24). Saturated ammonium sulfate(760g/100 ml) was added to the antigens(crude antigen) in concentration of 60 per cent. After replace this mixture at 0°C for an hour, centrifuged at 3,000 r.p.m. for an hour. The precipitate was dissolved in amount of distilled water and then centrifuged at 3,000 r.p.m. for 15—20 minutes. Then, if any sediment was exist, discarded it. Above treatment was done twice more, the supernatants were collected and concentrated in front of a fan. These final supernatants and precipitates were dialysed for running tap water for 48 hours and in dist. water for 24 hours, adjusted the volume(20 mg/ml originally) and then used for antigen.

Trypsin Digestion: Epual volume of 0.1 M Na_2HPO_4 solution was added to antigen: (A) direct trypsinized bacterial cell suspension(20 mg/ml), (B) antigens extracted by hat or acid and its modification(40 mg/ml), and (C) the fractions of ammonium sulfate saturation (40 mg/ml original volume), and then commercial trypsin was added to this to this mixture so as to be the concentration of 1 : 200. After incubated at 37°C for 24 hours, the supernatants were used as antigen.

Precipitation Test: Precipitation test was carried out by ring test, that is by overlaying the antigen of serial dilution in capillary tubes upon the immune serum diluted with normal rabbis serum in 1 : 4. Results were read ay the white ring between the antigen and serum layer after one hour in room temperature.

EXPERIMENTAL RESULTS

1. Effects of Treatment on the Extraction of Antigen:

To determine the effects of various treatments on the liberation of antigens of *S. pullorum* standard and variant strain, the culture suspension in 0.85 per cent physiological saline solution or in pH 2.0 phosphate buffer solution(20 mg/ml) was treated as follows; (A) suspension of cutures in 0.85 per cent physiological saline solution was heated at 100°C for one hour at pH 7.0 and then centrifuged: the supernatant designated as heat extracted antigen(100°C hour at pH 7.0), (B) suspension of cultures in pH 2.0 phosphate buffer solution was heated at 55°C for 24 hours and this suspenstons was centrifuged treatment, adjusted to pH 7.0 with sodium hydroxide: the supernatant designated as acid extracted antigen(55. C 24 hours at pH 2.0) and (C) is the same as original suspensi on of acid extracted antigen, heated at 100°C for one hour and centrifuged and the supernatant was also adjusted the pH to 7.0 : this supernatant designated as acid heat extracted antigen(100°C 1 hour at pH 2.0). As centrifugation, every antigen was centrifuged at 3,000 r.p.m. for one hour. Obtained antigens extracted by various treatments were used as sensitizing antigens for hemagglutination reaction.

As shown in table 1, it is evident that erythrocytes of blood group 0 of human and chicken red blood cells were well modified with both of *S. pullorum* standared and variant strain, but goat cells were negative in standared and positive reaction weakly in variant strain.

Acid extracted and acid heat extracted antigens were negative, These results are coincident with the report of Chun et al (21) who observed negative hemagglutination by *Sh. flexneri* antigens extracted at 55°C for 20 hours at pH 2.0.

From this results, it is evident that 100°C one hour treatment at pH 7.0 is better than any other methods for the extraction of hemagglutinating antigen and there is no evident differences between two strains.

2. Effects of Trypsin Digestion on the Antigen:

Antigens which (A) heat extracted antigen, (B) acid extracted antigen, (C) acid heat extracted antigen and (D) direct trypsinized antigen were used to determine the effects of trypsin on it and to evaluate the difference of the two strain. The results are shown in table 2.

In comparison with the two antigens; such the heat extracted antigen and acid extracted antigen, on the hemagglutination reaction, the later changed to positive reaction in the titer of 640 with human O-type, 1280 with chicken and 40 with goat red blood cell *S. pullorum* variant strain and titer of 40 both of human O-type and chicken red blood cell in *S. pullorum* standard strain, and however the former antigen also revealed positive reaction.

On the direct trypsinized antigen, more or less strong reactions observed but in acid heat extracted antigen, it was also revealed negative results. It is evident from these results that in most instances the trypsin enhanced the antigenecity on hemagglutination reaction but any difference between the two strains could not be noticed by this experiment.

3. Properties of Antigen Obtained by Ammonium Sulfate Saturation:

The fractionation procedure and obtained fractions are outlined in fig. 1, 2 and 3. Heat extracted antigen (100°C 1 hour at pH 7.0) and acid extracted antigen (55° C 24 hours at pH 2.0) of *S. pullorum* standard and variant strain were used and the results are summarized in table 3.

On the treatment of ammonium sulfate saturation, the *S. pullorum* standard and variant strain revealed much differences; on heat extracted antigen, obtained supernatant and precipitate fraction in both of the two strain, however, acid extracted antigen did not obtain the precipitate fraction in the variant strain, otherwise in standard strain, the supernatant and precipitate fraction were also obtained.

In acid extracted treatment, the results in this experiment was coincided with the results of William (25, 26); i.e. an aqueous solution of ammonium salts, containing 33g of the salt per liter, clears *S. pullorum* standard type suspensions following a short period of incubation and centrifugation, has little effect on the turbity of variant type of *S. pullorum* suspension.

As shown in table 3, it can be clearly seen that the supernatant as well as the precipitate of *S. pullorum* variant strain modified the chicken red blood cells excellently, resulting in specific hemagglutination with hemologous antiserum in high titer. By the way, it is doubtful, why the heat extracted antigen lost its hemagglutinability after treated ammonium sulfate treatment. It is evident form this results that the two strains revealed quite different characters in this experiments.

4. Effetcs of Trypsin on the Fraction Elicited by the Ammonium Sulfate Saturation:

To evaluate the effects of trypsin, the antigens which was in the same antigens in experiment were 3 treated with trypsin and carried out the hemagglutination reaction with chicken red blood cell and the results are presented in table 4.

As shown in this results, the more active hemagglutinable substance was the precipitate fraction in *S. pullorum* standard strain and it could be demonstrate after treatment of trypsin and this positive results were only demonstrated the precipitate fraction both of the heat and acid extracted antigens.

The results obtained with the antigens treated with trypsin can be explained by the assumption that the red blood cell modifying antigens was extracted but it was inactive antigenically before treated with trypsin. This results were largely paralleled those of conventional biochemical differentiation described by Williams(25, 26).

5. Precipitation Test:

Ten fold dilution of antigens which were used in this experiments and corresponding antiserum were employed to detect the antigenecity of theantigens and obtained nearly negative results.

DISCUSSION

Since *S.pullorum* is a non-motile and non-flagellated organisms, its serological reactions are due to entirely to somatic antigens. Further, it is not known to heat labile components, hence, in any investigation of variability of its antigenic behavior, it is necessary to consider thoes phenomena of bacterial variation.

In Salmonella group the only variants in which O-antigens are involved the smooth-rough variation of Arkright and the form variation of Kauffmann. All of the experimental work has confirmed by the work of Younie (11, 12) and emphasized the following important

facts; (A) there exist a definete antigenic variation among *S. pullorum* strains, (B) the antigenic variants do not differ from the usual strains in cultural, biochemical, pathogenic or other characteristics, and (C) an antigen ccntaining the variant tyep of the pullorum organisms should be employed to detect variant infection.

Edwards et. al. (13. 14, 15), Wright and Edwards (9) and Snoeyenbous et al (17, 18) have studied to the serological differentiation of *S. pullorum* strains. With these knowledge, about the difference between *S. pullorum* standard and variant strain, this attempt to see what difference, under the variety condition of extraction and further treatment, i.e. trypsin digestion, nd ammonium sulfate saturation etc., would be obtained to the hemagglutination reaction was carried out.

Crude enterobacterial antigens obtained by thd heating of bacterial suspensions have been succesfully used for the indirect hemagglutination test. As shown by Neter et al boiling of bacterial suspension is an excellent methods of treatment for the procurement of erythrocyte modifying antigens. Neter et. al. at first considered the possibility that heat destroys an inhibitors, but as did Neter (27) in a later report, considered it more likely that the effect of heat on bacterial suspension causes release of red blood cell modifying antigen. On the other hand, the treatment of bacteria at 55°C in low pH, is effective in the release of erythrocyte modifying antigen and its difference between the two strains may be used to advantage in further differentiation. By the way, the negative result obtained with the antigen which was not treated with trypsin and after treatment, revealed positive results, can be explained by the assumption that the red blood cell modifying antigens are extracted but by trypsin treatment prove to be antigenecity active in hemagglutination test. The preparation of erythrocyte modifying materials by the heating and other treatment of bacterial suspensions appears to be due to the liberation of polysaccaride (2), protein (28) and O-antigen of E. coli (23) described by many authors and Sieburth (3) also reported that soluble-polysaccharide of *S. pullorum*. The limited erythrocyte modifyng capacity of bacterial polysaccharide has been increased by heat, sodium hydroxide treatment, presumably hydrolysis of lipid to liberate polysaccharide.

Concurrent with antigenic variation of *S. pullorum* cultures, there may occur a change in the susceptibility of the cultures at the sediment effect of the salt ammonium sulfate. An explanation of this difference should provide additional insight into the basic physical or chemical changes that occur in bacterial cells during antigenic variations. Regarding the the relation with the non-serological methods described by Williams (25, 26), the mechanism involved is not clearly understood by this time, but the antigenecity was demonstrated in precipitate fraction of *S. pullorum* standard strain and in presipitate as well as supernatant fraction of variant strain and this was great interest that displayed some difference between the *S. pullorum* standard and variant strain. Further studies are needed to determine the reason for the difference.

SUMMARY

The antigenecity of somatic substances of *S. pullorum* standard strain and variant strain extracted byheat treatment, acid treatment and their modification, ammonium sulfate saturation (60 per cent), trypsin digestion was tested by indirest hemagglutination test and precipitation test and following results were optained.

1. Teatment at 100°C for an hour of the bacteria could extract the antigen of *S. pullorum* standaied strain and variant strain which was demonstrable by hemagglutination reaction with the human O group and chicken red blood cell.

2. Trypsin digestion was more enhanced its antigenecity in acid extracted antigen of *S. pullorum* variant strain compare with the *S. pullorum* standard strain.

3. The extracted antigenic substances of *S. pullorum* standard strain existed chiefly in the elicited fraction of precipitate at the treatment of ammonium sulfate saturation and after trypsin digestion, its antigenecity was demonstrated by hemagglutination.

4. At the treatment of ammonium sulfate treatment, did not occur the precipicate in acid extracted antigens of *S. pullorum* variant strain, however, the heat extracted antigen, potitive reactions were obtained in both of the precipitate and supernatant fraction of the *S. pullorum* variant strain by hemagglutination reaction. After trypsin digestion, these fraction also exhibited positive reactions.

5. Precipitation test also tested dut colud not detect in any sort of the antigens.

血球凝集反應에 依한 Salmonella pullorum 抗原에 對한 硏究

全南大學校農科大學 康 炳 奎

Salmonella pullorum 標準型菌株 및 變異型菌株의 菌體物質을 加熱抽出, 酸性抽出, 酸性加熱抽出, trypsin 消化 及 硫酸 ammonium 飽和등의 方法으로 處理하여 얻은 各 分層의 抗原性을 血球凝集反應 및 沈降反應으로 比較檢討하여 다음과 같은 結果를 얻었다.

1. S. pullorum 菌體抗原은 標準型菌株 및 變異型菌株 다같이 加熱抽出에서만이 人O型血球와 鷄血球에 吸着되어 血球凝集反應의 反應을 나타내었다.

2. Trypsin의 影響은 別로 著明하지는 아니하나 酸性抽出抗原에서 標準型菌株보다 變異型菌株가 血球凝集反應에서 若干 陽性으로 變轉되었다.

3. S. pullorum 標準型菌株의 抗原性物質은 硫安飽和時 液의 그 沈澱分層에 移行되며 이는 同 沈澱分層을 trypsin 消化하므로써 血球凝集反應에 强力한 抗原性을 保有하고 있음을 證明할 수 있었다.

4. 硫安飽和時 S. pullorum 變異型菌株는 酸性抽出抗原에서는 沈澱分層를 얻을 수 없었고 그 上淸에는 抗原性도 없었으나 加熱抽出抗原에 있어서는 上淸 及 沈澱分層 共히 血球凝集反應에 陽性을 나타내었다.

5. 各種抽出抗原分層의 沈降反應에서는 液의 反應을 나타내지 아니하였다.

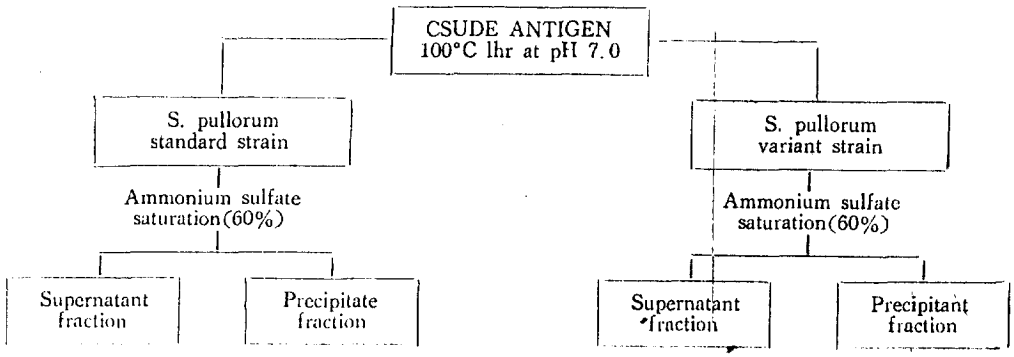

Fig. 1. The fractionation by saturated ammonuim sulfate solution from the heat extracted antigen(100°C 1 hr. at pH 7.0).

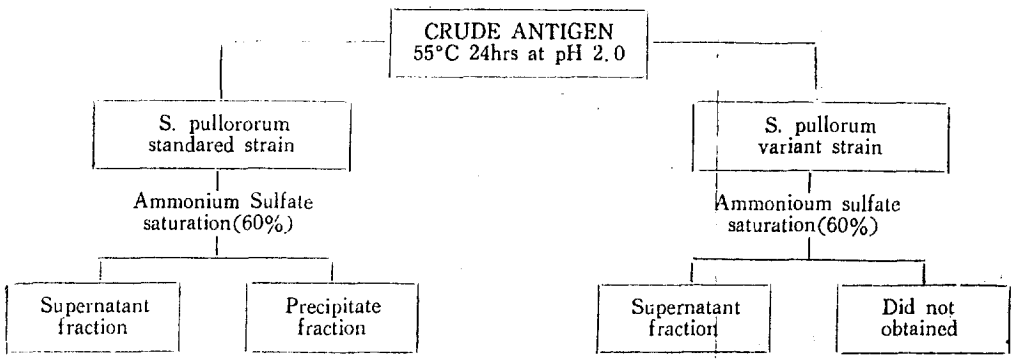

Fig. 2. The fractionation by saturated ammonium sulfate solutior from the acid extracted antigen (55°C 24 hrs. at pH 2.0)

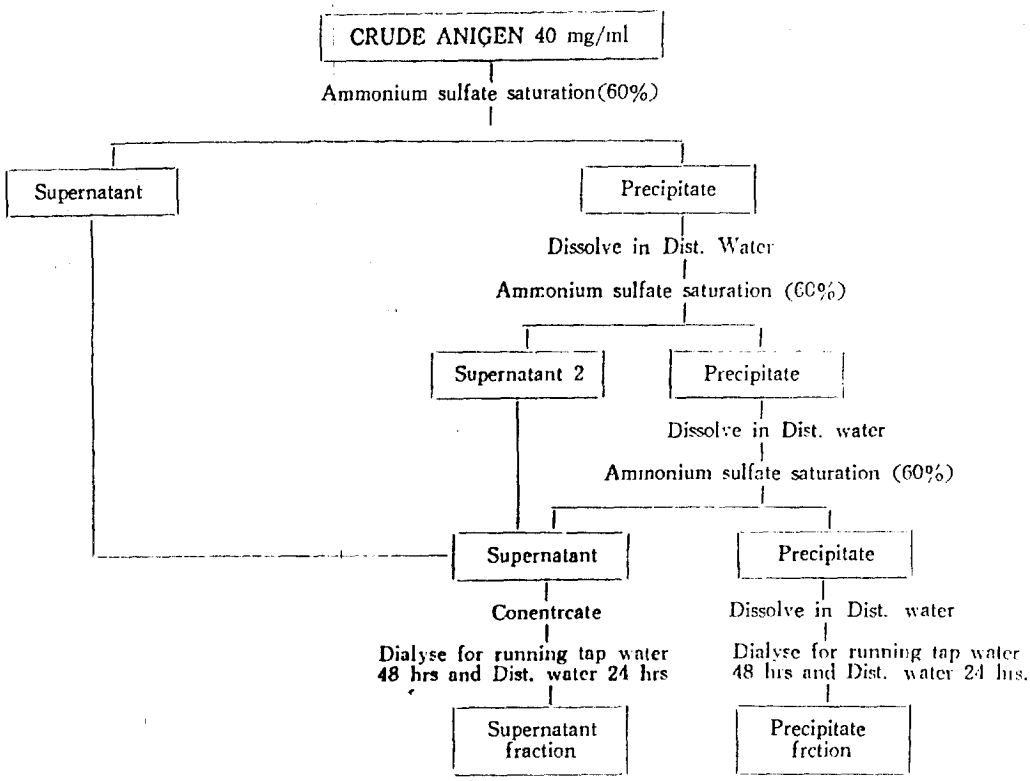

Fig. 3. The pocedure of the treatment of ammonium sulfate saturation

Table 1. Hemagglutination reaction of S. pullorum antigens extracted by various treatment.

ANTICEN EXTRACTION	R.B.C.	TRYPSIN TREATMENT	H.A. TITER	
			S. PULLORUM STNNDARD	S. PULLORUM VARIANT
100°C 1 hr at pH 2.0	human O-type	−	2,560	2,560
		+	320	5,120
	chicken	−	1,280	5,120
		+	1,280	5,120
	goat	−	—	40
		+	—	40
55°C 24hrs at pH 2.0	human O-type	−	—	—
		+	—	—
	chicken	−	—	—
		+	—	—
	goat	−	—	—
		+	—	—
100°C 1 hr at pH 2.0	human O-type	−	—	—
		+	—	—
	chicken	−	—	—
		+	—	—
	goat	−	—	—
		+	—	—

Remark: 1. Trypsin treatments indicated that trypsin treated red blood cell suspensions were used.
2. "—" in titer indicates negative reaction as serum dilution af 1 : 40.
3. Titers were expressed as reciprocl of serum dilutions.

Table 2. T effects of trypsin digestion in indirect hemagglutination tes.

METHODS OF TRYPSIN DIGESTION	R.B.C	H.A. TITER	
		S. PULLORUM STANDARED	S. PULLORUM VARIANT
Direct	human O-type	40	640
trypsinized	chicken	160	2,560
	goat	—	160
Trypsinized with 100°C 1 hr at pH 2.0 antigen	human O-type	1,280	1,280
	chicken	1,280	2,560
	goat	40	160
Trypsinized with 55°C 24 hrs at pH 2.0 antigen	human O-type	40	640
	chiken	40	1,280
	goat	—	40
Trypsinized with 100°C 1 hr at pH 2.0 antigen	human O-type	—	—
	chicken	—	—
	goat	—	—

Remark: Antigen was added to the equal volume of 0.2 M Na_2HPO_4 solution and commercial trypsin was added to these mixture so as to be the concentration of 1 : 200. These were treated at 37°C for 24 hours.

TABLE 3. Properties of antigens of S. pullorum obtained by ammonium sulfate saturation.

ANTIGEN EXTRACTION	AMMONIUM SULFATE SATURATIION	H. A. TITER	
		S. PULLORUM STANDARED	S. PULLORUM VARIANT
100°C 1 hr at pH 2.0	Supernatant	—	10,240
	Precipitate	—	5,120
55°C 24 hrs at pH 2.0	Supernatant	—	—
	Precipitate	—	*

Remark: "*" in titer indicates that did not occured the precipitate fraction at 60 per cent ammonium sulfate saturation.

Table 4. Effect of tyrpsin on the fraction elicited by the ammonium sulfate saturation.

ANTIGEN EXTRACTION	AMMONIUM SULFATE SATURATION	H. A. TITER	
		S. PULLORUM STANDARED	S. PULLORUM VARIANT
100°C 1 hr at pH 2.0	Supernatant	—	5,120
	Precipitate	5,120	5,120
55. C 24 hrs at pH 2.0	Supernatant	—	—
	Precipitate	1,280	*

Remark: "*" in titer indicates that this this fracti was not tested because of did not occur the precitate fraction of 60 percent ammonium sulfate saturation dn shown in Table 3.

REFERENCES

1. Keogh E. V., North E. A. and Warburton M.F.: Nature. 160, 63, 1947.
2. Koegh E. V., North E. A. and Warburton M.F.: Nature. 161, 687—688, 1948.
3. J.M. Sieburth.: Am. Jour. Vet. Res., 72, 729—735, 1958.
4. J.M. Sieburth.: Jour. of Immun., 78, 1957.
5. Gwatkin R.: Canad. Jour. of Comp. Med., 9, 183—191, 1945.
6. Gwatkin R. and Bond E.W.: Canad. Jour. of Publ. Health, 36, 160—166, 1945.
7. Gwatkin R. and Younie A.R.: Canad. Jour. of Comp. Med., 11, 172—178, 1947.
8. Wright M. L.: Proc. th Ann. Meet, Northeast Conf. of Lab. Works In Pulloeum Disease Control, 1944.
9. Wright M.L. and Edwards P.R.: Jour. of Vet. Res., 9, 386—388, 1948.
10. Byrne J.L.: Canad. Jour. of Comp, Med., 7, 227—238, 1943.
11. Younie A.R.: Canad. Jour. of Comp. Med., 5, 164—167, 1941.
12. Younie A.R.: Canad. Jour. of Comp. Med., 6, 172—173, 1942.
13. Edwards P.R. and Bruner D.W.: Cornell Vet., 36, 318—324, 1947.
14. Edwards P.R. and Bruner D.W.: Proc. U.S. Livestocks San. Associa., 130—135, 1946.
15. Edwards P.R. and Bruner D.W.: Cornell Vet., 38, 257—262, 1948.
16. Wright M.L.: Canad. Jour. of Comp. Med., 11, 68—74, 1947.
17. Snoeyenbous G.H. Grotty A.M. and Van Roekel H.: Am. Jour. Vet. Res., 11, 221—226, 1950.
18. Snoeyenbous G.H. Bachman B. and Van Roekel H.: Proc. 23rd Ann. Meet. Northeast Conf. of Lab. Workers in Pullorum Disease Control, 1951.
19. Edwards P.R. and Ewing W.H.: Burges Publ. Co. Minaapolis, Monn. U.S.A.
20. Chun D. and ParkB.: Jour. of Inf. Disease, 98, 82—87, 1956.
21. Chun D., Yang Y. And Park. H.: Jour. of Inf. Disease, 100, 1959.
22. Salk J.E.: Jour. of Immun., 49, 87—98, 1944.
23. Neter E.,F. Bertram and C.E. Arbesmann.: Proc. Socie. Exp. Biol. and Med., 79, 255—257, 1952.
24. Smolens J., Halbert S.P., Mudd S., Doak B.w. and Gozales D.M.: Jour. of Immun., 52, 1956.
25. J.E. Williams: Am. Jour.of Vet. Res., 52, 458—464, 1953.
26. J.E. Williams. Am. Jour. of Vet. Res. 52, 465—470, 1953.
27. Neter E. Gorzynsky E.A. Zalewski N.J., Rachman R. and Gino R.M. Am Jour. of Publ. Health. 44, 49—54, 1948.
28. Boyden S.V. Jour. of Exp. Med. 93, 107—120, 1948.