STUDIES ON TWO STRAINS OF ESCHERICHIA COLI ISOLATED FROM A HEN SEROLOGICALLY PULLORUM POSITIVE, CULTURALLY PULLORUM NEGATIVE

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by

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TABLE OF CONTENTS

STUDIES ON TWO STRAINS OF *ESCHERICHIA COLI* ISOLATED FROM A HEN SEROLOGICALLY PULLORUM POSITIVE, CULTURALLY PULLORUM NEGATIVE

Introduction

A diseased hen was brought into the poultry clinic of Michigan State College by a poultryman who reported that there was a heavy loss of chicks in his breeder flocks due to what had been diagnosed as pullorum disease by a commercial laboratory. Adult birds gave doubtful reactions to the stained antigen, whole blood plate pullorum test.

The diseased hen was tested by Dr. Saul Narotsky, Instructor in Bacteriology, in charge of the poultry clinic, with the plate method as well as the standard tube agglutination method. The results were as follows: With the plate test, using polyvalent Redigen, Canadian Redigen and T. G. antigen, it gave a three plus reaction; with the tube agglutination test it gave a suspicious reaction in the 25 dilution.

Later this hen was slaughtered and autopsied. The ovaries were still functioning. Cecal and round worms were found. The liver was somewhat enlarged. A bacteriological examination of the ovaries and liver was made by Dr. Narotsky. Two relatively similar organisms were isolated and tentatively identified as *Escherichia coli*. *Salmonella pullorum* was not recovered in a single instance from any tissue. This presented the question:

Were the positive serological reactions to the pullorum disease test, given by this hen, due to these non-specific organisms?

Since the first application of serological methods to the study of Salmonella, it has occasionally been observed that organisms which belong to the Escherichia and Aerobacter groups were agglutinated by Salmonella immune sera. This was reported by Schiff et al (13) who also reported 5 strains of coliform organisms which contained antigenic components similar to those of the Salmonella. For a number of years, Sander and Pomeroy (14) have made cultures from tissues of turkeys reacting positively to the agglutination test for pullorum disease. They observed that in many instances, micro-organisms other than S. pullorum were isolated. This observation suggested the possibility that these organisms might cross-agglutinate with S. pullorum and thus be responsible for the agglutination titers with pullorum antigens.

In 1942, Bunyea and MacDonald (1) reported that some strains of Aerobacter aerogenes and Escherichia acidi lactici are pathogenic for turkeys and poults and possess some cross agglutinating ability in the presence of pullorum antiserum and vice versa.

Considering the literature dealing with the cross-aglutination reactions which certain organisms give with Salmonella sera, and the possibility that the non-specific organisms isolated from this diseased hen might have caused the cross-agglutination reaction with pullorum

antigen, it seemed necessary to make further studies on the cultural characteristics, biochemical properties, serological reaction, and pathogenicity of these organisms.

Materials and Methods

Two different strains of the organism isolated on S. S. agar from the diseased hen, were obtained from Dr. Narotsky and were designated as strains 302-B and 302-C.

In order to study the cultural and biochemical characteristics of the two strains, S. S. agar, Kligler's agar, nutrient agar, blood agar, eosin methylene blue agar, bismuth sulfite agar, lactose motility agar, nutrient broth, nitrate broth, indole test broth, gelatin and different kinds of fermentation broth were used.

For the purpose of ascertaining their serological reactions, the following materials were prepared and used:

Stained pullorum antigen, including T. G. antigen, Redigen, and Canadian strain Redigen were used for rapid whole blood tests.

Pullorum antigen, used for the tube agglutination test, was prepared in the poultry clinic. The turbidity of the antigen corresponded to that of tube No. 0.7-1.0 of the McFarland nephelometer. The hydrogen-ion concentration was adjusted to pH 8.2-8.5 by the addition of N/1 sodium hydroxide solution.

Antigens of strains 302-B and 302-C used for the rapid whole-blood plate test, were prepared separately. Large test tubes, containing nutrient agar medium, were used for producing the antigens. After a 48-hour incubation period, the growth was washed off

with 0.85 per cent saline solution containing 0.5 per cent phenol to produce a very concentrated suspension. This suspension was filtered through sterile absorbent cotton into sterile glass-stoppered bottles. The turbidity of these antigens was adjusted to 50 times that of tube No. 0.75 of McFarland's nephelometer.

Pullorum positive sera, used to test for cross-agglutination with strains 302-B and 302-C were prepared in the poultry clinic.

Bacterial suspensions for immunizing chickens were prepared from S. pullorum and strains 302-B and 302-C. These were grown separately on tryptose agar. After 24-hours incubation, the growth of each agar slant was suspended in sterile saline (10 ml. of Saline to 1 agar slant). These bacterial suspensions were filtered through sterile absorbent cotton, then heated in the water bath at 60° C. for one hour.

Experimental Procedures and Results

The organisms were inoculated into the different media mentioned above and were incubated at 37° C. for 24 hours or longer, after which their morphological, cultural and biochemical characteristics were observed.

Morphological and cultural characteristics: The two strains were morphologically similar. They were non-spore-forming, non-capsulated, motile, short rods, about 0.4 micron to 0.5 micron by 0.9 micron to 2.7 microns. They were readily stained with aniline dyes and were gram negative. They were arranged singly or in pairs.

They were aerobic and facultatively anaerobic and grew luxuriantly in ordinary media. On nutrient agar plates, the colonies formed by strains 302-B and 302-C were white to yellowish-white, entire to undulate, moist, glistening, opaque, homogeneous and spreading.

On eosin methylene blue agar, the colonies formed by strains 302-B and 302-C during 24 hours incubation had blackish center and a metallic sheen, very much like that of indelible ink. On S. S. agar, strain 302-B formed large pink colonies which spread; strain 302-C formed comparatively small colonies with round, entire margins. Both organisms failed to grow on bismuth sulfite agar and Simmon's citrate agar and failed to show hemolysis on blood agar plates. In broth, the two strains produced uniform turbidity with scant, dirty, slimy sediment; later pellicles were formed.

Biochemical reaction: In testing the biochemical reactions of these organisms, gelatin liquefaction cultures were cultivated at room temperature for 48 days; fermentation cultures were incubated at 37° C. for 24 hours and then left at room temperature for 47 days before making final readings. Strains 302-B and 302-C produced acid and gas from dextrose, lactose, mannite, maltose, mannose, sorbitol, dulcitol, d-levulose, dextrine, trehalose, xylose, arabinose and rhamnose, but did not ferment adonitol, salicin, inositol and inuline. In addition, strain 302-B also produced acid from sucrose, but strain 302-C failed to ferment sucrose.

Moreover, these two strains first produced acid from dulcitol and sorbitol, later turning alkaline. The fermentation reactions are shown in Table II.

They did not liquefy gelatin nor did they form hydrogen sulfide, but both reduced nitrate to nitrite. They were Methyl Red positive, Voges-Proskauer negative, and indol positive.

They produced a slight amount of acid in litmus milk which turned whitish-pink in color and which was coagulated by strain 302-C after 13 days incubation, but not by strain 302-B. The biochemical reactions are shown in Tables I and II.

Table I. Biochemical Reactions

Strains	Indol	H_2S	Nitrate	M.R.	V.P.	Gelatin	Litmus Milk
302-B	+	-	+	+	-	-	+
302-C	+	-	+	+	-	-	+

Table II. Fermentation of Carbohydrates

Carbohydrate	Strain 302-B		Strain 302-C	
	acid	gas	acid	gas
Dextrose	+ (1)	+ (1)	+ (1)	+ (1)
Lactose	+ (1)	+ (1)	+ (1)	+ (1)
Sucrose	+ (3)	-(47)	-(47)	-(47)
Mannite	+ (1)	+ (1)	+ (1)	+ (1)
Maltose	+ (1)	+ (1)	+ (1)	+ (1)
Inuline	-(47)	-(47)	-(47)	-(47)
Mannose	+ (1)	+ (1)	+ (1)	+ (1)
Sorbitol	+ (1)	+ (1)	+ (1)	+ (1)
D-levulose	+ (1)	+ (1)	+ (1)	+ (1)
Ducitol	+ (3)	+ (3)	+ (3)	+ (3)
Dextrine	+(13)	+(13)	+(14)	+(14)
Trehalose	+ (1)	+ (1)	+ (1)	+ (2)
Xylose	+ (1)	+ (1)	+ (1)	+ (1)
Inositol	-(47)	-(47)	-(47)	-(47)
Salicin	-(47)	-(47)	-(47)	-(47)
Rhamnose	+ (1)	+ (1)	+ (1)	+ (2)
Arabinose	+ (1)	+ (1)	+ (1)	+ (2)
Adonitol	-(47)	-(47)	-(47)	-(47)

Serological reactions. Concentrated antigens were made of the two strains to be used in the rapid serum test with known pullorum positive serums. The antigen was measured with a medicine dropper. One drop corresponded to 0.05 cc. A wire loop was used to measure the serum. A loopful of serum contained approximately 0.02 cc. A loopful of pullorum positive serum was mixed separately with one drop of each kind of antigen (strains 302-B and 302-C) which had been placed on a glass plate. Meanwhile, the Polyvalent Redigen, Redigen and T. G. Formula were used for control. The results are given in Table III.

Table III. Cross-agglutination tests with pullorum-positive serum and non-specific organisms. Plate test.

Antigen / Antiserum	T. G. Formula	Redigen	Polyvalent Redigen	302-B	302-C
302-B	-	-	-	++++	+++
302-C	±	-	±	++++	++++
Pullorum #318	++++	++++	++++	±	±
Pullorum #331	++++	++++	++++	±	±
Pullorum #2854	+++	++	++	-	+

In order to make further study of serological reactions, the heat-killed suspensions of the two coliform organisms and S. pullorum, were respectively injected intravenously into 3 chickens whose blood serums had been found to be negative to the pullorum plate test and the standard tube agglutination test prior to injection. These chickens were injected four times at two-day intervals. The first dose injected was 0.2 cc., then the doses were increased as follows: 0.5 cc., 1.0 cc., 2.0 cc. Five days after the fourth injection the immunized chickens were tested with the plate test. The results are given in Table IV.

Table IV. Cross-agglutination tests with coli-immune chicken serum and stained pullorum antigen.

Stained Antigens / Serums	T.G. Formula	Redigen	Polyvalent Redigen
Strain 302-B Antiserum	-	-	±
Strain 302-C Antiserum	±	-	-
Pullorum Antiserum	++++	++++	++++

Seven days after the fourth injection, the immunized chickens were bled and serum samples prepared for the standard tube agglutination test. Strains 302-B and 302-C and S. pullorum were used as antigens for serological tests. The results are shown in Table V.

Absorption tests were also made in order to obtain further information concerning their antigenic relationships. In the absorption test, the antiserums were diluted to 1:10 with phenolated (0.5%) saline solution. The following serum-antigen mixtures were made: 1.5 cc. of diluted immune serum and 1.5 cc. of heavy suspension of heterologous antigen; 1.5 cc. of diluted serum in 1.5 cc. of heavy suspension of homologous antigen. The tubes were well shaken and incubated for two hours at 37° C., then for 24 hours in the refrigerator after which they were centrifuged for 5 minutes at 2000 R.P.M. The supernatant fluids were drawn off and used in place of immune serums against their homologous antigens.

The specific antibodies were completely absorbed with the homologous antigens. However, no satisfactory evidence was obtained to show the presence of minor (group) antibodies for S. pullorum in strain 302-B and 302-C antigens. The preliminary homologous titers are given in Table V, and the results of the retitration of antiserum following absorption are shown in Table VI, A., B., C.

Moreover, cross-agglutination and absorption tests with immune serums of 302-B and 302-C and their antigens were also made. The tests show that the two strains are related antigenically. The results of the cross-agglutination tests are given in Table VII and the results of the retitration of 302-B and 302-C antiserums following absorption are shown in Table VIII.

Table V. Seralogical reactions with serums from immunized chickens (Tube test).

Reaction after 24 hours at 37° C.										
Anti-Serum	Anti-gen	1:25	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	Control
302-B	Pull.	-	-	-	-	-	-	-	-	-
302-C	Pull.	±	-	-	-	-	-	-	-	-
Pull.	Pull.	++++	++++	++++	++++	++++	++++	++++	±	-
Pull.	302-B	-	-	-	-	-	-	-	-	-
Pull.	302-C	+	-	-	-	-	-	-	-	-
Pull. #318	302-B	-	-	-	-	-	-	-	-	-
Pull. #318	302-C	+	±	-	-	-	-	-	-	-
Pull. #318	Pull.	++++	++++	++++	++++	++++	++	-	-	-
Pull. #2854	302-B	-	-	-	-	-	-	-	-	-
Pull. #2854	302-C	±	-	-	-	-	-	-	-	-
Pull. #2854	Pull.	++++	++++	++++	++++	++	+	-	-	-
302-B	302-B	++++	++++	++++	++++	++++	++++	+	-	-
302-C	302-C	++++	++++	++++	++++	++++	++++	+	-	-

A. Table VI. Retitration of antiserum following absorption

	Supernatant fluid from												
	302-B Antiserum + Pullorum Antigen							302-C Antiserum + Pullorum Antigen					
Antigens	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:50	1:100	1:200	1:400	1:800	1:1600
302-B	++++	++++	++++	++++	++++	+	-						
302-C								++++	++++	++++	++++	++++	-

Preliminary titer of 302-B antiserum 1:800 Preliminary titer of 302-C antiserum 1:800

B.

	Supernatant fluid from												
	Pullorum Antiserum + 302-C Antigen						Pullorum Antiserum + 302-B Antigen						
Antigens	1:50	1:100	1:200	1:400	1:800	1:1600	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200
Pull.	++++	++++	++++	++++	++++	++++							
Pull.							++++	++++	++++	++++	++++	++++	-

Preliminary titer of Pullorum antiserum 1:1600

Preliminary titer of pullorum antiserum 1:1600

C.

Antigens	Supernatant fluid from											
	302-B Antiserum + 302-B Antigen				302-C Antiserum + 302-C Antigen				Pull. Antiserum + Pull. Antigen			
	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400
302-B	-	-	-	-								
302-C					-	-	-	-				
Pull.									-	-	-	-

(1) Preliminary titer of 302-B antiserum 1:800

(2) Preliminary titer of 302-C antiserum 1:800

(3) Preliminary titer of pullorum antiserum 1:1600

Table VII. Cross-agglutination test with immune serums and antigens of 302-B and 302-C (tube test).

Antigens	Antiserums											
	302-B						302-C					
	1:25	1:50	1:100	1:200	1:400	1:800	1:25	1:50	1:100	1:200	1:400	1:800
302-C	++++	++++	++++	++++	++++	++						
302-B							++++	++++	++++	++++	+	-

Table VIII. Retitration of Antiserum Following Absorption

An	Supernatant fluid from													
	302-B Antiserum + 302-C Antigen							302-C Antiserum + 302-B Antigen						
Antigens	1:25	1:50	1:100	1:200	1:400	1:800	1:1600	1:25	1:50	1:100	1:200	1:400	1:800	1:1600
302-B	++++	++	±	-	-	-	-							
302-C								++++	++++	++++	±	-	-	-

Preliminary titer of 302-B antiserum 1:800

Preliminary titer of 302-C antiserum 1:800

In addition to the biochemical, differential characteristics, the two strains, listed in the above tables, showed antigenic relationships as follows:

1. Antigen of strain 302-B agglutinated with 302-C antiserum; 302-B antibodies were absorbed by 302-C antigen showing a reduction in titer from 1:800 to 1:50.

2. Antigen of strain 302-C agglutinated in 302-B antiserum in high titers; 302-C antibodies were absorbed by 302-B antigen showing a reduction in titer from 1:800 to 1:100.

Pathogenicity tests. Suspensions of living organisms were prepared for feeding to chicks. Strains 302-B and 302-C were grown separately on tryptose agar slants. After 24 hours incubation, the growth of each agar slant was suspended in sterile saline. The turbidity of the suspensions was approximately equal to that of tube 1.5 of McFarland's nephelometer. Pathogenicity tests on these organisms were made as follows:

Experiment I. Three groups of two-day-old chicks, four in each, were set up for pathogenicity tests. Group 1 was given strain 302-B; group 2, strain 302-C; and group 3 was used for control. Each chick in these groups, except group 3, was given one cc. of suspension of living organisms orally by means of a pipette.

The dosed chicks were properly brooded and fed, and appeared normal for 24 hours after exposure. On the second day, a number of the chicks in groups 1 and 2 were affected with diarrhea and somewhat droopy. On

the fourth day, one of the chicks in group 2 was dead. On the sixth day, one of the chicks in group 1 was also dead. Thereafter the chicks were observed for 25 days and the diarrhea, which occurred in some of the exposed chicks, had stopped. The control chicks from the same hatch, brooded and fed in an identical manner, experienced no morbidity during the first 31 days of life.

The autopsy of the dead chicks revealed enteritis and congestion of the liver. The heart sac contained some blood-stained, fluid exudate. Organisms identical with strains 302-C and 302-B were recovered from the intestine, heart and liver of dead chicks in the respective groups. On the thirty-seventh day, one living chick from each group, including the control chicks, was slaughtered and autopsied. There were no lesions to be found in any abdominal organs. However, organisms identical with strains 302-B and 302-C were recovered only from the intestines of the chicks taken from groups 1 and 2; no such organisms were isolated from the intestines of the control chicks.

Experiment II. Two groups of 5-day-old chicks, eight in each, were set up. Group 1 was given strain 302-B and group 2, strain 302-C. The bacterial suspensions were prepared as in Experiment 1. Two chicks in each group received orally 0.5 cc., two received 1.0 cc., two received 1.5 cc. of the respective suspensions and two were left as controls. On the fourth day after inoculation two infected chicks in group 2 died; on the fifth

day after inoculation one infected chick in group 1 died. The autopsy of the chicks revealed enteritis and congestion of the liver; the heart sac contained fluid exudate and the crop, gizzard and intestine were almost empty. Organisms identical with those administered were recovered from the intestines and liver. The rest of the experimental chicks, observed for 23 days following inoculation, remained normal.

Experiment III. Three groups of two chickens, approximately 110 days old, were set up for intravenous and intraperitoneal injection. The bacterial suspensions used were prepared as in Experiement 1. One bird in group 1 was given 0.2 cc. of strain 302-B intravenously and one received 0.6 cc. intraperitoneally. Group 2 was given strain 302-C, administered as in group 1. Group 3 was used as controls. Twenty-four hours after the intravenous injection, cultures were made from the blood of the infected chickens and no organisms were recovered. All these inoculated chickens were watched daily for a period of 22 days, and they showed no clinical symptoms.

Experiment IV. Strain 302-C was fed to chickens in living suspension corresponding in turbidity to that of tube 3 of McFarland's nephelometer. Four vigorous chickens approximately 110 days old, nos. 3448, 3449, 3450 and 3451, were selected. Chicken no. 3448 was given 2.0 cc. orally, chicken no. 3449, 3.0 cc., and chicken no. 3450, 4.0 cc. Chicken no. 3451 was used for

control. The infected chickens were watched daily for a period of 30 days. On the twenty-fifth day chicken no. 3450 developed paralysis and the rest showed no clinical symptoms.

Experiment V. Four vigorous chickens approximately 100 days old, nos. 3472, 3473, 3474 and 3475, were infected intravenously with strain 302-C, using different doses. The blood of these experimental chickens was found to be free from bacteria before inoculation. The turbidity of the bacterial suspension used was the same as in Experiment IV. Chicken no. 3472 was injected intravenously with 0.4 cc. of the bacterial suspension. Chicken no. 3473, received 0.8 cc. and chicken no. 3474, 1.0 cc. Chicken no. 3475 was used for control.

Four hours after the injection the organisms were recovered by blood culture except from chicken no. 3472. Chicken no. 3474 died six hours after infection. At autopsy the chicken showed hydropericardium, discoloration of the cardiac muscle and congestion of the liver. An organism identical with 302-C was recovered from the liver and heart-blood. One day later chicken no. 3473 showed diarrhea, partial loss of appetite and paralysis of the legs. It could not walk on its feet but occasionally walked on its hocks. On the third day the organisms were no longer recovered from the blood; the symptom of paralysis persisted for a period of 15 days and then the bird partially recovered but remained unthrifty. Chicken no. 3472 at first showed depression and poor appetite, one week later this chicken had also recovered.

DISCUSSION

The organisms described here form a rather homogenous group morphologically, culturally and biochemically. They differ from the typical E. Coli only in that both fermented dextrine; strain 302-C did not ferment sucrose; strain 302-B fermented sucrose slowly.

That atypical strains of E. Coli may give serological cross reactions with Salmonella has already been reported by Habs and Arjona (5). Schiff, Bornstein and Saphra (13) also pointed out that serological relationship to Salmonella is more common among non- or slow-lactose fermenters and non-indol producers of the coliform organisms than among those which ferment lactose and produce indol. However, the two strains of E. Coli employed in this study did not give marked serological reactions with S. pullorum serum. Strain 302-C gave a one plus serological reaction with S. pullorum serum in the 25 dilution but this is insignificant.

According to Hadley et al (6), E. Coli was the causative agent of a cholera-like disease of chickens. Twisselmann (15) observed an acute infectious disease of pullets in California which he attributed to E. Coli communis. Osborne, Witter and Hitchner (10) reported that chickens inoculated with the genus Escherichia developed generalized paralysis; other acute and chronic forms of disease in poultry, caused by the genus Escherichia, have been described. In this study, strains 302-B and 302-C

were shown to be capable of causing subacute disease in young chicks characterized by enteritis. One chicken, subjected to intravenous injection and one, fed with strain 302-C, developed paralysis. Twenty chicks were fed a suspension of E. Coli and five (25%) of them died. This does not prove that the loss, which the poultry man concerned reported in his breeder flocks, was due to infection with E. Coli, although such a possibility does exist.

SUMMARY

I. Two strains of E. Coli were isolated from a diseased hen, serologically pullorum positive, but culturally pullorum negative at postmortem.

II. No significant cross reactions were obtained with S. pullorum serum and E. Coli antigen, nor with E. Coli serum and S. pullorum antigen.

III. Strains 302-B and 302-C are closely related antigenically as shown by the fact that pronounced cross-agglutination reactions were obtained.

IV. Strains 302-B and 302-C produced subacute enteritis in chicks and strain 302-C produced paralysis in chickens.

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