STUDIES ON BRUCELLA ABORTUS.

by

D.G.B. MATHIAS, B.A.

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CONTENTS.

STUDIES ON BRUCELLA ABORTUS.

I. SOME HISTORICAL CONSIDERATIONS.

With the discovery by Bruce (1) in 1886 of the "Micrococcus melitensis" and his subsequent proof that it was the causal microorganism of Mediterranean, Malta, or undulant fever, there began the study of a genus of bacteria the inertness of which renders its investigation most difficult. The chronicity of the disease usually resulting from infection is another difficult obstacle.

The original nomenclature applied to this genus was misleading. Bang (2), the Danish veterinarian, in 1897 was the first to recognize the rod-like character of a cocco-bacillus which he proved to be the causative agent of "contagious abortion" (Bang's disease) in cattle and which he called "Bacillus abortus".

The two generic names "micrococcus" and "bacillus" misled bacteriological investigations of the human disease and the disease in cattle into separate channels for two decades. In 1918, Evans (3), while engaged in studying the bacterial flora of freshly-drawn milk, recognized the close relationship between Bang's "bacillus" and Bruce's "micrococcus".

Prior to Evans' discovery, Traum (4) in 1914 isolated from an aborted pig foetus a bacillus he considered to be "Bacterium abortus"; in 1916 Good and Smith (5) reported this bacillus as the etiological factor in the production of infectious abortion of swine.

Meyer and Shaw (6) in 1920, and Feusier and Meyer (7), in the same year, suggested the generic name "Brucella" in honour of Sir David

Bruce, a suggestion since widely accepted. Huddleson's (8) studies on the brucella group resulted in his conclusion that there are three distinct species, Brucella melitensis of the goat, Brucella abortus of cattle, and Brucella suis of swine, with other hosts aside from the primary host in each case. Huddleson's nomenclature has been generally adopted and this has freed the literature of misleading terminology.

The term "brucellosis" is used extensively now with reference to the human manifestations of the disease previously called Malta, Mediterranean, or undulant fever. It is also finding acceptance in relation to the animal diseases.

II. INTRODUCTION.

Brucellosis is a public health problem of considerable proportions and one that is becoming more serious. It has spread all over the civilized world and affects persons of all ages and both sexes regardless of occupation. As yet no successful specific or non-specific therapeutic agent has been found for the treatment of the disease.

Brucellosis is a disease communicable to man from the lower animals by direct or indirect contacts or by ingestion of infective animal products.

Because of the high incidence of "contagious abortion" (Bang's disease) in the cattle of the Fraser Valley and the dependence of Greater Vancouver on this source of supply for its dairy products, local cases of human brucellosis might be anticipated.

During the past few years many such cases have been clinically recognized, and verified by laboratory evidence. The increasing interest

shown in the epidemiological and immunological problems raised by these cases of human brucellosis led to the present investigation which was begun in 1937.

At the outset it was deemed desirable to obtain evidence relating to the extent of the risk of contracting human brucellosis, in terms of the degree of contamination with Brucellae of the local raw milk supplies. Such evidence was sought by conducting cultural surveys at intervals on all pooled raw milk distributed in the city of Vancouver. A series of experiments was planned to throw light on various possible means of producing active immunity to Brucellae. After determining the average level of brucella agglutinins in a group of normal healthy students, and of mice, the efficacy of heat-killed suspensions, prepared from locally-isolated strains, in evoking production of specific agglutinins, was investigated. The degree of protection thus afforded against living Brucellae was tested in mice. Finally a few experiments were performed with a view to determining the possible use of "Brucellin" as an immunizing agent, and the feasibility of producing brucella variants of unusual antigenic efficiency.

The investigation has involved study of a number of strains of Brucellae isolated locally. Methods currently available for differentiation show all these strains to have been of the "Brucella abortus" variety.

III. EXPERIMENTAL.

A. Raw Milk Surveys.

Dolman and Hudson (9) presented evidence bearing upon the brucellosis hazard entailed by the consumption of raw milk in and around

Vancouver. Their report was based on a two-year period during which fifteen local cases of clinically-acute brucellosis were shown by serological, cultural, and epidemiological methods, to have been milk-borne and due to Brucella abortus.

Following the above findings, it was thought desirable to undertake a more comprehensive survey which would involve examination of routine samples from all raw milk distributors in the city of Vancouver. Two complete surveys and one daily survey were made.

Routine.

The medium used in culturing milk for Brucella abortus was Huddleson's beef liver infusion agar containing 1:200,000 crystal violet. Plates of this medium were poured 24 hours before they were spread with three or four loopfuls of 24-hour standing cream, centrifuged cream, and centrifuged sediment respectively, obtained from 15 c.c. of milk sample, three plates being used for each fraction. Plates were incubated for six days at 37°C. in an atmosphere containing approximately twelve per cent carbon dioxide. Suspicious colonies were then picked, their identity being confirmed by microscopic examination, their carbon dioxide requirements, their specific agglutinability in the presence of an anti-abortus serum of high titre, and by their susceptibility to fuchsin, thionin, and pyronin.

Results of the Summer Survey.

The first survey was done over the period June 1 to August 10, 1938, during which time two milk samples were examined from every

dairy purveying raw milk in Vancouver. In all, 1,197 plates were spread, of which 161 were overgrown. Of the 1,036 readable plates, nine had typical brucella colonies on them, the total number of brucella colonies on the nine plates being fifteen.

Results of the Fall Survey.

The local health authorities decided that the human brucellosis hazard could be effectively controlled by the elimination of individual "reactors" from every herd supplying raw milk to the city. Three months following the adoption of this policy, a second survey was begun in an endeavour to assess the efficacy of this policy.

The second survey covered the period November and December, 1938. A total of 828 plates were spread of which 173 were overgrown. The 655 readable plates did not yield a single brucella colony.

Concurrently, Miss Hudson of the Provincial Board of Health Laboratories, Vancouver, did a parallel survey. Her results are set forth in a report by Dolman, Hudson, and Mathias (10).

The apparent discrepancy between the two surveys made upon similar samples is not readily explained. All constitutents of the medium were carefully checked and rechecked. The only variables were personal technique and fluctuations in the temperature of incubation. The former factor was dismissed following the obtainment of practically identical results by both technicians concerned, in an experiment carried out under identical conditions. Fluctuations in the electric incubator used in the greater part of this work extended over a range of six degrees from 35°C. to 41°C., the range

depending on the atmospheric temperature.

Results of the Daily Survey on Two Dairies.

To gain information as to the number of days in a month a raw milk supply would contain viable Brucellae, daily samples were cultured from two dairies over a period of one month, one having previously yielded very few positives and one being consistently positive.

Each day of February, 1939, samples from these two dairies were plated. The results obtained in our laboratory were much lower than those obtained concurrently at the Provincial Laboratories. Twelve of the twenty-eight samples from the "positive" dairy yielded Brucellae, the other dairy being consistently negative. The concurrent survey at the Provincial Laboratories yielded, correspondingly, twenty-one positive out of twenty-eight samples and four positive out of twenty-eight.

Conclusions.

Results of the summer survey showed the presence of viable Brucellae in certain raw milk samples. The low incidence of positive cultures obtained must in part be attributed to small inocula being spread on the plates to compensate for the higher total colony counts obtained during the hotter months.

It would be advisable to investigate further the effect of temperature fluctuations on the growth of Brucellae from raw milk.

The apparent discrepancy between results obtained in our labor-

atories and the Provincial Laboratories during the daily survey was in all probability due to the same factor as in the fall survey -- the incubator.

B. The Administration of Vaccine to Humans.

Preliminary Tests on Normal Individuals.

(a) The average agglutinin titre of the blood serum of the normal individual.

Before commencing the investigation of the value of vaccine in inducing a high agglutinin titre in humans, it was necessary to ascertain the average titre of a representative group of apparently normal individuals in this area.

Blood was taken by venepuncture from the median cubital veins of seventy unselected healthy volunteers and agglutination tests done on the serum.

The antigen used was prepared according to Huddleson's method (8) as follows: The surface of the liver infusion agar in Roux flasks was inoculated fairly heavily from a 48-hour culture of Brucella abortus. (The cultures used must be typical strains of Brucella abortus which have shown no tendency to spontaneous agglutination and which have manifested normal agglutinability. Either a monovalent or polyvalent antigen may be employed.)

The antigen bottles were incubated for 48 - 72 hours at

37°C.

The growth was washed off with a phenolized saline solution containing 0.85% sodium chloride (C.P.) and 0.5% phenol crystals (C.P.).

It was put in the icebox and allowed to stand until the microorganisms were killed. The suspension was kept well mixed by shaking and was cultured every 3 - 5 days for sterility.

When sterile, it was filtered aseptically through cotton and gauze. For use, the antigen was diluted with normal saline to MacFarland # 2 standard of turbidity.

The standard practice for agglutination tests was to make doubling dilutions, the initial tube having a final serum concentration of 1:5, the final tube 1:640. Adequate controls were set up with each test.

The tests were incubated for 48 hours in the 37°C. waterbath, removed and left at room temperature for 24 hours. The tests were read at 24, 48, and 72 hours (final reading).

The results obtained from the seventy apparently normal volunteers are set forth in Table I.

TABLE I.

AGGLUTININ TITRES OF SEVENTY APPARENTLY NORMAL INDIVIDUALS.

Highest dilution showing agglutination	Agglutinin titre	No. of individuals	Per cent of individuals
1:5	0	2	2.8
1:5	p	6	8.6
1:5	c	5	7.1
1:10	p	1	1.4
1:10	c	4	5.7
1:20	p	13	18.6
# 1:20	c	1	1.4
1:40	p	16	22.9
# 1:40	c	1	1.4
1:80	p	13	18.6
1:160	p	5	7.1
1:320	p	3	4.3

c = complete agglutination
p = partial "
0 = no "

Complete agglutination in a 1:20 dilution or higher was recorded as a positive result. On this basis, only two individuals (#) or 2.8% showed a positive test. In no instance did tests designated as "partial" show complete agglutination in lower dilutions.

(b) The "Brucellergen" skin test applied to apparently normal individuals.

As an additional check on the apparently normal volunteers, who were subsequently to be given vaccine, the intradermal allergic test as developed by Huddleson (11) was employed. The brucella nucleoprotein solution, designated "Brucellergen", was obtained from his laboratory. The technique for the preparation of this ether-washed antigen is described in "Brucella

Infections in Animals and Man"(8).

The test was performed on fifteen healthy volunteers by injecting intradermally 0.1 c.c. of the nucleoprotein solution in the lateral surface of the forearm. Readings were made in 24 and 48 hours in order to exclude early non-specific reactions. The positive reaction is characterised by a central red area at least 4 X 4 mm. with an outer pinkish area at least 20 X 20 mm., oedema usually being present.

Of the fifteen individuals, five, or one-third, showed a positive reaction.

Administration of Vaccine.

(a) Source of cultures.

Five strains of Brucella abortus, all isolated at the Provincial Laboratories, Vancouver, by blood cultures from five human patients, were used in preparing the polyvalent vaccine.

(b) Preparation of vaccine.

Each of the five strains was seeded on eight beef liver infusion slants. After 72 hours incubation in an atmosphere of approximately 12% carbon dioxide, the growth from all these tubes was washed off with sterile saline, the suspension being divided into two parts, one for parenteral injections, the other for oral administration. Both flasks were heated at 60°C. for

1 hour. Sterility tests were done on both. The suspensions corresponded to a MacFarland # 6 turbidity standard.

(c) Parenteral injection.

Eight volunteers were given six subcutaneous injections at weekly intervals, the dilutions of the doses, of the suspension as prepared in (b) above, being 0.1 c.c. of 1:20, 0.1 c.c. of 1:10, 0.1 c.c. of 1:10, 0.15 c.c. of 1:10, 0.1 c.c. of 1:5, and 0.15 c.c. of 1:5.

Agglutinin titres were done on blood serum obtained from the above volunteers prior to the initial injection and again one week after the final injection. The results are compared in Table II.

TABLE II.

AGGLUTININ TITRES BEFORE AND AFTER PARENTERAL INJECTION OF VACCINE.

Agglutinin titre before injections	Agglutinin titre after injections
c in 1:40	c in 1:320
p in 1:80	c in 1:160
p in 1:80	c in 1:160
p in 1:160	c in 1:160
p in 1:80	p+ in 1:160
p in 1:160	p+ in 1:160
p in 1:40	p in 1:320
p in 1:80	p in 1:80

c = complete agglutination.
p+ = about 50% "
p = partial "

On analysis it is seen that four of the volunteers showed a

marked increase in agglutinin titres, two showed a significant rise, one evidenced a slight rise, whereas one volunteer showed no change.

(d) Oral administration.

Eight healthy volunteers drank six doses of the suspension, prepared as in (b) above, at weekly intervals, the increasing doses being 1 c.c. of a 1:10 dilution, 1 c.c. of 1:1, 2 c.c., 4 c.c., 6.5 c.c. and 10 c.c. Over the six-weeks' period, a total of 23.6 c.c. of a suspension corresponding to a MacFarland # 6 turbidity standard, or the equivalent of over 210 billion Brucellae abortus, was taken by mouth.

None of the volunteers showed any detectable increase in serum agglutinin titre.

Conclusions.

In only one of the fifteen healthy volunteers the brucellergen skin test and the agglutination test indicated a positive reaction. Four individuals gave positive brucellergen skin reactions and negative agglutinin tests, whereas ten individuals were negative to both. This lack of conformation indicated the possibility of the presence of a local hypersensitivity to brucella protein without evidence of detectably increased agglutinins.

Oral administration of a heat-killed polyvalent vaccine was proved to be ineffective in stimulating agglutinin production. Thus, the consumption of pasteurized milk containing dead Brucellae would not induce significant agglutinin titres.

The effectiveness of the heat-killed polyvalent vaccine, when administered parenterally, in inducing an increased agglutinin titre has been shown. Six out of eight persons evidenced significant increases in blood serum agglutinins. The advisability of a much larger experimental group is realized. Only then could a more comprehensive conclusion be drawn on the use of the vaccine.

C. The Value of Vaccine in Protecting Mice Against Active Infection.

The value of increased agglutinin titre in combatting active disease was worthy of investigation.

The agglutinin titre of the normal mouse.

From 0.8 to 1.0 c.c. of blood was obtained by cardiac puncture from each of eight normal mice under ether anaesthesia. The pooled sera gave no indication of any brucella agglutinins. The above result was confirmed by two additional experiments.

The approximate Minimal Lethal Dose for mice.

As the result of preliminary experiments, it was found necessary to give a heavy dose, due to the variability of the response to experimental brucella infection in mice, thus ensuring fairly uniform results.

At four weekly intervals, the * M.L.D. was checked on groups of five mice each. The most consistent results were obtained by the injection of 0.2 c.c. of a (X 10) MacFarland # 2 turbidity standard,

* M.L.D = Minimal Lethal Dose.

of the Huyck strain of Brucella abortus, thirteen of twenty mice dying consistently between 32 and 40 hours. The M.L.D. for practical purposes was, therefore, 0.2 c.c. of a (X 10) MacFarland # 2 turbidity standard injected intraperitoneally.

Parenteral Immunization.

Two groups of mice, comprising thirteen males and thirteen females respectively, were given seven doses of the heat-killed polyvalent vaccine at weekly intervals, intraperitoneally. The suspension was analogous to that used in the human experiments. The doses were 0.05 c.c., 0.1 c.c., 0.1 c.c., 0.15 c.c., 0.2 c.c., 0.2 c.c., and 0.2 c.c.

Three mice per group were sacrificed in order to obtain, by cardiac puncture, a sufficient quantity of pooled blood for agglutination tests. The titre of the male blood was +++ in 1:640, the female +++ in 1:320.

The same day the remaining members of the two groups were each given 0.2 c.c. of a (X 10) MacFarland # 2 turbidity standard, intraperitoneally, of the Huyck strain of Brucella abortus. This was the quantity found by previous experiment to represent 1 M.L.D.

Of the males, one died from the effects of fighting, the nine remaining appeared quite normal. Of the females, one died after 48 hours but the post mortem signs were not of the characteristic type described below. Of twelve normal control mice injected with the same suspension, eight died between 33 and 40 hours. Three of the four remaining died after about 56 hours.

Oral Administration.

Two groups of mice were selected as in the experiment above. They were all fed seven doses, by graduated eye-dropper, of the same bacterial suspension at weekly intervals. These oral doses were approximately 0.125 c.c., 0.25 c.c., 0.125 c.c., 0.125 c.c., 0.125 c.c., 0.2 c.c., and 0.2 c.c.

Three mice per group were sacrificed, as in the former experiment, for blood agglutination tests. The titre of the male blood was + in 1:40, the female + in 1:20.

The remaining members of the groups were each given 1 M.L.D. intraperitoneally. Nine of the ten males died between 46 and 54 hours; all the females died, nine between 33 and 37 hours, one at 97 hours.

The typical post mortem signs in the deaths apparently resulting from the brucella injections were: Hemorrhagic lungs; bloated stomach; pale liver, kidneys and spleen; extensive subcutaneous hemorrhage and engorgement of the peripheral vessels; enlarged adrenals and gall bladder. All the mice were autopsied. Pure cultures of Brucellae were readily obtained at autopsy from the kidney, heart, spleen and liver in twenty-six out of thirty-two attempts.

Conclusions.

Normal mice have no demonstrable agglutinins for Brucella abortus.

The M.L.D. can be ascertained fairly accurately for mice, relatively large numbers of Brucellae being necessary to produce a

fatal termination in approximately 40 hours.

Parenteral injection of a heat-killed polyvalent vaccine produced significant rises in titres of both male and female mice, the males showing the greater response.

The enhanced agglutinin titre apparently protected both the male and female mice against active infection.

Oral administration of the vaccine did not cause a demonstrable rise in agglutinin titre in either the males or females; this treatment did not elicit the response of additional protective forces against active infection. The males lived on the average 12 hours longer than the females.

D. The Production of Brucellin.

The foregoing evidence, relating to the production of agglutinins by injection of killed cultures of Brucellae raised the question as to the efficacy of metabolic by-products of Brucellae in inducing the production of similar or additional antibodies. Huddleson (11) was the first to develop and use a material, analogous to tuberculin in preparation, which he called "Brucellin". The clinical use of this material is still in the early experimental stage. Apparently injection of brucellin evokes a leucocytosis of the neutrophile type, besides increasing the specific phagocytic power of the blood.

Media Employed.

As is generally recognized, the injection of the complex protein solutions in common use for culturing bacteria often has undesirable after-effects. A synthetic medium composed of amino-acids plus

essential growth factors, which would sustain adequate growth of Brucellae, obviously would be advantageous.

Huddleson had employed peptic digest liver broth medium for his preparation of brucellin (12). In the present investigations, the accepted medium for the cultivation of Brucellae, beef liver infusion broth at a pH of 6.8, was one type of medium used. In his recent publication, Huddleson (13) has adopted this medium in the production of brucellin.

It was found in preliminary experiments that a slightly modified form of Gladstone's synthetic medium (14), which he had developed for the cultivation of Staphylococci, would support the growth of three strains of Brucella abortus without the necessity of training the cultures to grow on the medium. Therefore it was used in the production of brucellin. The composition is given below.

Composition per Litre of the Synthetic Medium.

KH_2PO_4		4.5 gm.
Water		500.0 c.c.
N/1 NaOH		26.0 c.c.
*5% amino-acid base		200.0 c.c.
Ferrous ammonium sulphate	M/500 in M/50 HCl	25.0 c.c.
$MgSO_4.7H_2O$	M/60	10.0 c.c.
$NaNO_3$	M/5	10.0 c.c.
Vitamin B_1 (Thiamin)	M/2000 in M/1000 HCl diluted 1/100	20.0 c.c.
Nicotinic acid (amide)	M/200 diluted 1/10	20.0 c.c.
Glucose	M/2	25.0 c.c.
Adjust pH to 7.4 with N/5 NaOH.		
Water to make		1000.0 c.c.
Sterilize by Seitz filtration.		

*The amino-acid base was obtained from proteose-peptone by hydrolyzing 5% proteose-peptone for 40 hours in boiling 2.5 N H_2SO_4. The H_2SO_4 must be exactly neutralized with $Ba(OH)_2$ so that no barium or sulphate ions remain. The resulting precipitate was separated by Buchner filtration.

In addition to the above constituents, Gladstone incorporated other amino-acids in his medium, and used nicotinamide in place of nicotinic acid. The additional amino-acids were omitted as being unnecessary.

Routine.

Two recently isolated smooth strains of Brucella abortus were used in the production of brucellin, the Huyck strain isolated from the blood of a human suffering from brucellosis, and the S 12 strain isolated from raw milk.

Each strain was inoculated into separate 250 c.c. amounts of both the beef liver infusion broth and the synthetic medium. A generous loopful from a 48-hour beef liver infusion agar slant was the inoculum in each instance. The flasks were incubated at 37.5°C. in an atmosphere with an increased carbon dioxide tension of about twelve per cent by volume above that of atmospheric air. If, at the end of five days, growth failed to appear in any flask, it was re-inoculated. Every five days each flask was examined for growth, shaken carefully, and re-incubated under the above conditions.

In twenty-four days all flasks evidenced fairly heavy growth. Each was checked as to the purity of the growth by plating suitable quantities on beef liver infusion agar. No contaminating bacteria appeared on the plates in five days. Each flask of broth culture was now Seitz filtered. Sterility tests were devoid of growth over a seven-day period. Merthiolate, 1/5000, was added to each flask and after standing 2 hours the brucellin was dispensed into sterile vials.

To prove the innocuous nature of the brucellin, 1 c.c. of each of the four preparations was inoculated intraperitoneally into each

of three mice. The brucellins prepared with the synthetic medium gave no evidence of causing discomfort; the beef liver infusion broth brucellins apparently caused a transitory discomfort. All mice remained in normal health.

Use of Brucellin.

The opportunity arose for the trial of brucellin in a human apparently suffering from brucellosis. Because of the origin of the culture, the brucellin produced by the Huyck strain grown on the synthetic medium was selected for the trial.

To test the reaction of the normal human to the injection of this brucellin preparation, two volunteers were each given 0.1 c.c. intramuscularly. No discomfort whatsoever was experienced by either volunteer. Three days later the same two individuals received 0.3 c.c. of the same preparation intramuscularly. No apparent reaction occurred.

The patient (Miss McL.) was diagnosed as suffering from brucellosis on the clinical symptoms of general lassitute, weakness, lack of energy, easy tiring, chills, headaches, leucopenia, and loss of weight, and was supported by the laboratory finding of a blood serum agglutinin titre for Brucellae of +++ in 1:320. She was confined to bed.

Her physician administered the brucellin intramuscularly in consecutive weekly doses of 0.1 c.c., 0.2 c.c., 0.3 c.c., 0.5 c.c. and 0.9 c.c., a total of 2 c.c. The only symptom evidenced from the injections was a slight transitory rise in temperature 24 hours after each injection. Following the fourth injection the patient regained apparently normal health. At the present time, approximately eight

months after treatment, the patient has had no recurrence of symptoms.

Conclusions.

The synthetic medium supported the growth of two strains of Brucella abortus.

Brucellin preparations proved innocuous to mice and the preparation obtained by growth of the Huyck strain on the synthetic medium gave no deleterious effects when injected into two normal human volunteers.

In one clinically typical case of subacute brucellosis, administration of brucellin derived from a synthetic medium seemed to bring about definite improvement.

E. The Effect of pH on the Viability of Brucella abortus.

The results of the raw milk surveys provided ample evidence that many persons in Vancouver daily were consuming thousands of viable Brucellae and yet the number of cases of brucellosis apparently occurring remained small. Consideration was given to the possibility that the acid of normal human gastric juice proved lethal to the greater number of Brucellae and such survivors as there were proved too few to produce disease.

Experiments were devised to ascertain the pH range of tolerance of one strain of Brucella abortus in an adequate medium.

Routine.

In these experiments the "Barons 4" strain of Brucella abortus

was used throughout. The procedure consisted in seeding uniform inocula into 10 c.c. aliquots of beef liver infusion broth, the pH of which had been adjusted to cover a range from approximately 1 to 9. The standard inoculum for each tube was three loopfuls of a five-day beef liver infusion broth culture, which corresponded to a MacFarland #4 turbidity.

Determinations of pH were made with a standard Leeds and Northrup quinhydrone potentiometer. Flasks of the medium were initially adjusted with concentrated NaOH or concentrated HCl to cover the range 1 to 9. These flasks were incubated for 48 hours to allow stabilization of the pH, after which the pH determinations on respective samples were 1.1, 2.3, 2.9, 3.8, 5.2, 6.1, 7.1, 7.9, and 8.7. The standard inoculum was evenly dispersed in each test broth. Subcultures were taken in triplicate from each tube at 15 minutes, 30 minutes, 1, 2, 4, and 16 hours, and plated on beef liver infusion agar at pH 6.8. Control plates were prepared in triplicate with suitable dilutions of the inoculum to prove the viability of the organisms. All plates were incubated for 96 hours at 37°C., under the standard conditions of increased carbon dioxide tension of the atmosphere, to ensure that all viable organisms would form colonies.

Results.

No growth occurred from any subcultures from broths of pH 1.1, 2.3, and 2.9 respectively. Subcultures at 15 minutes, 30 minutes, and 1 hour, from the broth at pH 3.8, showed decreasing amounts of growth; those taken at later intervals were negative. Subcultures from the broths at all other pH levels tested yielded abundant growth.

Three distinct colony types were obtained. Two of these were atypical and were produced from the 15 minute, 30 minute, 1 and 2 hour subcultures. These were much larger (5-10 mm.), one being opaque and a buff colour, the other slightly smaller, translucent, and exhibiting two zones, an inner buff colour and a periphery showing pale green. In every plate showing growth, typical colonies were found. These were small, almost colourless, and translucent. No apparent change occurred in microscopic morphology.

Conclusions.

The limiting pH for the viability of the "Barons 4" strain of Brucella abortus apparently lies between 2.9 and 3.8.

The pH obtaining in the stomach when milk is ingested alone or with other foods is conjectural. Some means of mechanical protection will also be afforded Brucellae by foodstuffs in their passage through the stomach. It is clearly difficult to evaluate the protection afforded by the gastric juice, but, all other circumstances being equal, ingested Brucellae will more than likely survive contact with a gastric juice of abnormally low acidity.

The atypical colonies obtained seemed to be intermediate in form between the typically smooth and those described by several workers as rough. They possibly fall under the "I variant" of Brucellae described by Henry (16).

IV DISCUSSION.

The raw milk surveys have supplied sufficient evidence to show that daily, in Vancouver, many people are ingesting large numbers of viable Brucella abortus in certain raw milks. Despite this, the apparent case incidence is low. The factors in the host or of the microorganism, or both, which determine the onset of the disease in some persons and the resistance of others to the infection, under apparently identical conditions, remain unknown.

The experiments with mice gave evidence that injections of killed vaccine protected the mice against subsequent lethal doses of Brucellae. In human beings, although the injections resulted in an enhanced agglutinin titre, this alone is a poor index of immunity.

The introduction of a synthetic medium for the cultivation of Brucellae has many interesting possibilities. In such a medium, devoid of proteins, when the bacterial cells are removed by Seitz filtration, any proteins in the filtrate will have resulted from the metabolic activities of the microorganisms or the autolysis of the cells themselves.

Huddleson, in his most recent publication "Brucellosis in Man and Animals" (13), reporting on the treatment of 500 cases of brucellosis with brucellin, believes that, of all the specific and non-specific preparations used to date, brucellin is the one in his experience which shows most promise of success. As has been mentioned, his brucellin has been prepared utilizing beef liver infusion broth. He realizes that the beneficial treatment resulting from this complex protein material may result from non-specific protein shock. In the above publication the final sentence concerning the use of brucellin in treatment reads: "Eventually a

preparation of Brucellin will be available that is free from the extraneous constituents that are to be found in the present product". Brucellin prepared from a synthetic medium might prove to be the product sought.

It is unfortunate that circumstances have prevented the use of larger groups of humans and animals.

The experiments with brucellin are not sufficiently extensive to permit definite conclusions. Moreover, it should be emphasized that elaboration of a synthetic medium for production of brucellin is as yet in an early stage. Further experiments are planned involving use of a synthetic medium in which hydrolyzed edestin replaces the amino-acid base obtained from proteose-peptone, and to which selected amino-acids are added. Some recent results obtained with this medium gave promise of a superior product.

V. SUMMARY.

(1) Of 2,110 readable plates obtained during the raw milk surveys in the period from June 1, 1938, to February 28, 1939, 41 had typical brucella colonies.

(2) Oral administration of a killed vaccine proved ineffective in stimulating agglutinin production in humans and in mice.

(3) Parenteral administration of a killed vaccine caused significant increases in blood serum agglutinins in six out of eight persons.

(4) Parenteral injection of a killed vaccine produced significant increases in blood serum agglutinin titres of both male and female mice.

This enhanced agglutinin titre was apparently associated with the protection afforded both sexes against active infection.

(5) A synthetic medium has been developed which supports the growth of Brucella abortus.

(6) A brucellin was prepared utilizing the foregoing synthetic medium.

(7) This brucellin preparation seemed to bring about definite improvement in one clinically diagnosed case of brucellosis.

(8) The limiting pH for the viability of the "Barons 4" strain of Brucella abortus apparently lies between pH 2.9 and 3.8.

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