A STUDY OF VIBRIOS ISOLATED FROM CATTLE AND SHEEP

by

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INTRODUCTION

The term Vibriosis, in its widest sense, is applied to an infectious bacterial disease which affects cattle and sheep. The etiologic organism is Vibrio fetus. In cattle the symptoms are enzootic sterility and sporadic abortions, in sheep the symptoms are enzootic abortions.

Although infections with other species of the genus Vibrio occur both in cattle, in other animal species and in human beings these infections are not included in the term Vibriosis.

Moreover, in a more narrow and common sense, the term Vibriosis is limited to the venereal V. fetus infection in cattle which is predominantly characterized by a markedly reduced conception rate but also by abortions at the later stages of pregnancy. To these manifestations of vibriosis the term Bovine Genital Vibriosis has been applied and it is this disease that has been and still is the subject of the greatest interest and most extensive investigations in all civilized parts of the world. The reasons for this are the great economic losses in reduced recruiting livestock, reduced milk production, prolonged intervals between successive parturitions and all expenses due to consultation of veterinarians and treatment. In Denmark the losses caused by the disease were estimated to be 30 million Danish kroner ($435,000) in 1951 (64). In Germany Mitscherlich and Forschner in 1956 (33)

estimated the average expenses per animal in infected herds to be 330 DM ($82.50). According to a F.A.O. report of 1954 (67) 40% of all sterility among cattle in the U.S.A. was attributed to Vibriosis and the economic losses were estimated to $138 million. Although venereal vibriosis has been combatted in many countries the disease still represents a great problem where natural breeding is extensively used. Therefore, it is of paramount importance to develop and establish reliable diagnostic methods; not only methods to detect vibrio organisms but also methods for differentiation between the true pathogen Vibrio fetus and nonpathogenic vibrios.

The purposes of this investigation were as follows:

1. To study and compare the biochemical and serological properties of strains of vibrios of bovine and ovine origin which had been isolated in this country. The biochemical tests applied were the conventional ones which will be described later in detail. The serological test was the complement fixation test (later referred to as the CFT) which in Europe has been found to be the most reliable method for differentiation between vibrio strains of various origin. The intention was to see whether sera prepared against the six types of vibrio established by the CFT in Europe could be used to test field strains of vibrio isolated in the United States. Moreover, the author wanted to see which of the methods, the biochemical

or the serological, might serve as the most reliable means of differentiation.

2. The second purpose was to label the six type specific sera with fluorescein isothiocyanate (later referred to as FITC), examine stained smears of the field strains under the fluorescence microscope, and correlate the results with those obtained by means of the CFT and the biochemical tests. As far as vibriosis is concerned the fluorescent antibody technique (later referred to as the FAT) has only been used to detect V. fetus in the semen and preputial washings of bulls. By introducing the method as a means of differentiation the author thereby wanted to see whether it could be applied as an easy and rapid diagnostic tool.

REVIEW OF LITERATURE

The history of vibriosis may naturally be divided into three periods:

1. 1909-1949, a period during which V. fetus was only associated with abortions in cattle and sheep.
2. 1949-1956, a period during which V. fetus, in addition to abortions, became associated with enzootic sterility in cattle.
3. 1956 and onwards, a period during which most attention has been focused on the possible pathogenic role played by various strains of vibrio isolated from cattle and sheep. All the intensive efforts which have been made in this area have aimed at establishing cheap but reliable laboratory tests for differentiation and thereby avoid expensive and time-consuming pathogenicity tests.

Although the problems of differentiating between various strains of vibrio arose long before 1956 this review will be primarily concerned with the last period mentioned.

V. fetus was first isolated from aborted ovine fetuses by McFadyean and Stockman in England in 1909 (29). In 1911 the same workers isolated the organism from aborted bovine fetuses in Ireland and in Wales.

During the period 1917-23, Smith (56, 57, 58, 59) and Smith, et al., (60, 62) in the United States isolated a

spirillum-like organism from 40 cases of bovine abortions altogether in which infection with Brucella abortus was precluded. Smith reproduced the disease by intravenous and subcutaneous inoculations of pure cultures of the organism and it was reisolated either from aborted fetuses or from diseased placentas.

Smith and Taylor in 1919 (62) gave a detailed and elaborate description of the cellular and the growth characteristics of the vibrios they had isolated and established the name Vibrio fetus.

Smith describes as early as in 1918, in his first publication concerning the new abortifacient agent (59), preliminary serological investigations. The agglutination test showed the fetal vibrio strains to be closely related.

Then Smith and Taylor in 1919 (62) tested 22 fetal strains and 2 calf strains. One of the calf strains was isolated from the spleen and one from the liver. Sera were prepared against 3 of the fetal strains and one of the calf strains and antigens were prepared from whole cells of 24 strains. All of the fetal strains and one of the calf strains proved to belong to the same serological group but one calf strain differed slightly from the fetal strains. The second calf strain was completely unrelated to the other strains.

Smith and Orcutt in 1927 (61) applied the agglutination test to 5 bovine fetal strains and to 2 strains isolated from

the spleen of calves suffering from diarrhea. They found that at least 4 types of antigenic factors existed and that any one strain could possess 3 of these factors.

The factors were as follows:

1. A common vibrio factor.
2. Individual strain factors.
3. A calf group factor.
4. A fetal group factor.

Factors 1 and 2 were heat labile while factors 3 and 4 were heat stable. The authors concluded that there was close relationship, but not identity, between the calf strains and the fetal strains. Moreover, they emphasized that vibrios are of complex antigenic structure.

Blakemore and Gledhill in 1946 (6) studied 4 ovine strains from different outbreaks of abortion. They performed OH- and O- agglutination and found that there was a certain relationship between the H- antigens. In contrast, all the O-antigens were heterologous although there was a slight relationship between 2 of them. The conclusion was that routine diagnostic tests carried out against a single laboratory strain were of little value.

Plastridge, et al. in 1947 (41) were the first ones to suggest that V. fetus infection might interfere with conception and/or cause an early embryonic death. This suggestion was later supported by the observations by Plastridge, et al.

in 1951 (39), that there was a strong correlation between a low conception rate and presence of V. fetus antibodies in the serum. Moreover, in the publication of 1947 (41), Plastridge, et al., on the basis of the serum agglutination test, tentatively concluded that "bulls must be regarded as susceptible to V. fetus infection".

However, stronger evidence for V. fetus to cause enzootic sterility in cattle was submitted by the Dutch workers Sjollema, et al. (55) at the 14th International Veterinary Congress in London in 1949. Their conclusions were based on the following observations:

1. Cows bred naturally or artificially by certain bulls showed a very low conception rate (6.7) while cows bred with healthy bulls showed a normal conception rate (1.8).
2. Vibrios were isolated from the semen from 2 bulls causing enzootic sterility and from the vaginal mucus from cows affected with a low conception rate.
3. Seventy per cent of the cows with a low conception rate were positive to the vaginal mucus agglutination test to V. fetus while cows with normal conception rate were negative to this test.

Levi in 1950 (27) worked with 5 strains isolated from ovine fetuses and found that agglutinin titers in sheep sera varied with the antigen used. Experimentally produced rabbit

sera gave higher titers against homologous than against heterologous strains and the author therefore recommended that more than one antigen should be used in serodiagnostic tests.

Gallut in 1952 (17) studied 4 V. fetus strains of human origin and 6 strains of animal origin. At first he applied the agglutination test and discovered considerable divergence among the strains. Each of the 10 antisera agglutinated at least 2 other strains in addition to the homologous one. Then Gallut (18) proceeded to the precipitation test, preparing antigen fractions by extracting cells with a 95% phenol solution. He obtained one phenol soluble and one phenol insoluble fraction. On the basis of qualitative biochemical tests it was concluded that the first fraction was of protein nature and the second fraction of carbohydrate nature. In the precipitation test the phenol soluble fraction reacted with 9 of the 10 sera prepared against the fractions but not with sera prepared against whole cells. The phenol insoluble fraction gave high titers with homologous strains but inconstant titers with heterologous strains. Gallut concluded that the phenol extractable fraction was the antigenic component specific for the species V. fetus. However, Biberstein (4) maintains that Gallut's phenol extractable fraction "rather points to its being an extraction artifact".

Marsh and Firehammer in 1953 (28) tested 23 ovine and

3 bovine strains by means of the agglutination test and they could distinguish between 5 serological types. The ovine strains were of 4 types and the bovine strains of a fifth type. The majority of ovine strains belonged to one type which was sharply differentiated from the other 4 types. The bovine strains showed relationship to 2 of the less common ovine types, types 2 and 5. Although the bovine strains constituted one group their cross-agglutination titers were not uniform.

In their general study of vibriosis in 1953, Plastridge, et al. (40) experienced on the basis of agglutination titrations that bovine strains belonged to one antigenic type while ovine strains belonged to several different types. They emphasized that strains used for antigen preparation should be selected carefully with regard to specificity, sensitivity and stability.

Price, et al. in 1953 (42) studied 34 vibrio strains from the following sources:

Group I: 20 strains from bovine fetuses.

Group II: 2 strains from ovine fetuses.

Group III: 5 strains from bovine vaginal mucus.

Group IV: 3 strains from bull semen.

Group V: 1 strain from bovine uterus.

Group VI: 1 strain from a pig with dysentery.

Group VII: 2 bovine strains of unknown origin. (The

location of the strains in the two animals was not known.)

Twenty-five antisera were prepared.

Results:

Group I: Most of the 20 bovine fetal strains were closely related. However, variations in titers occurred and some failed to agglutinate as many as six of the antisera of this group.

Group II: The 2 ovine strains were closely related to the bovine fetal strains.

Group III: 4 of the bovine vaginal mucus strains were closely related to group I but one (homologous titer 1:9600) did not react with any other sera.

Group V: The single strain from a bovine uterus was not agglutinated by any sera.

Group VI: The pig strain reacted only with its homologous serum.

Group VII: The bovine strains of unknown origin were related to group I.

The authors concluded that strains used for antigen production should be tested against a great number of sera in order to select those with the broadest serological spectrum within their own group. In the material of the authors, only 10 of the 20 bovine fetal strains satisfied this requirement, i.e., they reacted with all the 20 antisera.

Juhler in 1955 (23) studied 21 V. fetus isolated from bulls used for artificial insemination in Denmark. OH-agglutination revealed that 18 of the strains belonged to one group while 3 other strains belonged to one group each. He then performed pure O- agglutination by means of 3 antigens of the main group and antigens of the 3 different strains. His conclusion was that the O- antigen is group specific.

The serological investigations done so far had shown that V. fetus apparently is a heterogenous organism as far as antigenic structure is concerned. However, whether all these studies were done on the true V. fetus or also on other vibrios will always remain an open question. Simple, practical and reliable methods for distinguishing between various types of vibrios were still missing. An important step towards the solution of this problem was made in 1955 when Bryner and Frank (9) reported on the present well-known biochemical tests for differentiation between pathogenic and non-pathogenic vibrios. These workers examined 164 strains isolated from aborted bovine fetuses, vaginal mucus, semen and preputial samples, and the reproductive tract of slaughtered cattle. On the basis of source, pathogenicity, growth characteristics and biochemical properties the strains were divided into two groups:

Group I consisted of strains isolated either from aborted fetuses or from the reproductive tract of animals which had

shown a definite history of infertility. These strains produced catalase, did not produce H_2S and did not grow in deep stab culture. Group II consisted of strains which were only isolated from the reproductive tract of animals which showed no history of infertility. These strains were catalase-negative, H_2S-positive and grew well in deep stab culture.

No cross-agglutination occurred between strains of the two groups.

In 1956 the findings of Bryner and Frank were confirmed by Hubrig and Wohanka (21) who worked with a material of 107 strains.

Frank, et al. (16) extended the studies on the two groups of vibrios to pathogenicity tests. They found that catalase-positive vibrios were not isolated from virgin heifers or from bulls which had never bred. Moreover, this type of vibrio was transmitted from infected bulls to heifers and cows, in the genitalia of which it persisted for a long period of time and caused a low conception rate.

On the other hand catalase-negative vibrios were frequently isolated from bulls which had never bred. This vibrio type also remained only for a short period of time in the genitalia when it was transmitted to heifers. The conception rates of infected females were normal.

In 1955, Wiidik and Hlidar (68) described for the first time a capsular or K-antigen of V. fetus. They performed

extensive agglutination tests on 5 strains and the presence of a K-antigen was indicated by the following observations:

1. The inagglutinability of living or formalinized cultures by homologous O-serum and their agglutinability in OKH-serum, prepared by the use of living or formalinized cultures.
2. A positive Neufeld's quellung reaction.
3. A positive capsular staining reaction after the method of Hiss and Olt.

In most cases the K-antigen was destroyed by heating the cultures to 100 C for 2 hours but in a few cases it was not destroyed unless the cultures were autoclaved at 120 C for 2 hours. The authors concluded that the K-antigen was located on the cell surface and that it varied in quantity from one strain to another. Qualitatively it appeared to be identical in all strains.

The third period in the history of vibriosis started in 1956 with the discovery of a new type of vibrio which would cause much concern during the years to come. Before proceeding to review the publications concerning this particular era it seems natural to present the necessary comments on the investigations hitherto done to characterize various vibrios.

Biberstein in 1956 (5), working with 5 V. fetus strains studied the relationship between bacterial morphology and antigenic sensitivity. As the incubation time of the

cultures used for antigen production was increased the organisms underwent a change from the spirillar to the cocccid form. No distinct correlation was found between morphological changes and changes in sensitivity of the antigens. Nor did the sensitivity decrease markedly by aging of the cultures but the prezone phenomena became more pronounced.

Kuzdas and Morse in 1956 (25) performed extensive biochemical studies on 66 vibrio strains of the most diverse origin. In addition to confirming the results previously obtained by other workers with the catalase- and the H_2S-test they introduced a new useful test. While catalase-positive vibrios did not grow, catalase-negative vibrios did grow in the presence of 3.5% sodium chloride.

Bond in 1957 (7) studied the colonial, biochemical and serological characteristics of 6 bovine, 2 ovine and 2 human strains. Nine of them were catalase-positive and H_2S-negative; one (from bull semen) was catalase-negative and H_2S-positive. Three colonial forms, one smooth (S), one rough (R), and one mucoid (M) occurred on solid medium. While the S- and the M-form remained in a stable suspension in saline, the R-form precipitated easily. The S- and the R-form were catalase-positive and H_2S-negative but the M-form was catalase-negative and H_2S-positive. Agglutination-absorption tests revealed that the S-antigens were more specific than the R-antigens. Conclusively, the author emphasized the importance

of a careful examination of vibrio colonies, since a catalase-negative and H_2S-positive vibrio may be due to its occurrence in the M-form.

Lecce in 1958 (26), working with a material of 18 bovine, 6 ovine and 3 porcine strains studied the oxidative capacity of these strains on 30 different substrates. All the strains were found to possess the same qualitative oxidative capacity, utilizing only formate, pyruvate, ketoglutarate and succinate as electron donors. Lecce also found that the growth of 11 out of 12 catalase-positive bovine strains were inhibited by 0.8% glycine while 5 out of 6 ovine strains were not inhibited. He thereby introduced the glycine test; a test which later would be used to differentiate between vibrios of bovine and ovine origin.

Ristic, et al. in 1956 (49) studied the colonial and antigenic variations of 5 bovine fetal, 3 ovine fetal and 2 human strains. The cultures could be divided into one smooth and 4 nonsmooth colonies with correlated cell morphology. The nonsmooth colonies had a mucoid (M), cut glass (CG), rough (R) or very rough (VR) character. The nonsmooth forms were easily autoagglutinable and they were also agglutinated nonspecifically by normal bovine serum. Cross-agglutination tests showed that the S-forms were specific while cross-reactions occurred among rough variants of homologous and heterologous strains.

Ristic, et al. in 1957 (53) studied the effect of heat on the agglutinability of 5 catalase-positive strains and one catalase-negative strain. Serological heterogeneity and in-agglutinability were demonstrated between smooth and rough variants within the parent culture and among the variants of the five strains.

This heterogeneity was minimized when heat-treated antigen (boiling for 2 hours) was used. The greatest number of cross-reactions occurred when rough heated antigens were agglutinated by sera against rough heated cells. On the basis of these results the authors recommended the additional use of heated antigen in serodiagnostic tests for vibriosis.

Ristic, et al. in 1958 (52) divided 19 strains of bovine and ovine origin into 6 groups on the basis of cellular and colonial characteristics and dissociation pattern in successive generations. Two different colonial forms of V. fetus were observed, one smooth and one cut-glass form. These were accompanied by cellular and antigenic characteristics. Catalase-negative bovine vibrios could be distinguished from V. fetus on the basis of their colony characteristics.

In a series of publications during the period 1959-61 Ristic and different co-workers (47, 48, 50, 51) made extensive basic studies on the antigenic structure of V. fetus.

At first, Ristic and Brandly (47) isolated from a V. fetus strain and defined chemically a heat-stable and

water-soluble fraction which was designated the HS-fraction. It produced agglutinating antibodies in rabbits and was active in the gel precipitation test. Moreover, it was capable of inhibiting specific agglutination of whole cells by their homologous antibodies.

In a subsequent paper Ristic and Brandly (48) reported on the development of an indirect haemagglutination test by sensitizing sheep erythrocytes with the HS-fraction. Sensitized cells were agglutinated by specific sera produced in rabbits and by sera of naturally infected bulls. The specificity of the haemagglutination reaction was shown by its inhibition by the free HS-fraction of polysaccharide nature. This fraction appeared to represent a type-specific O-antigen shared by a number of individual strains of bovine and ovine but not of human origin.

In a third paper Ristic and Walker (51) described a haemolytic test for the detection of V. fetus antibodies. Using complement and sheep erythrocytes sensitized with the HS-fraction the cells were lysed by 21 sera prepared against whole cells of vibrio strains of bovine, ovine and human origin. With regard to sensitivity the hemolytic test proved to be superior to the tube agglutination test.

In the fourth and the last publication Ristic and Murty (50) described the development of a rapid slide gel diffusion test for the detection of V. fetus antibodies. Precipitation

reactions of 4 HS-antigens and 19 specific rabbit sera prepared against whole cells were studied. By mixing the HS-antigens of 2 bovine fetal strains, reactions were obtained with sera against all the bovine fetal strains, two ovine fetal strains and two human strains but with none of the bovine and ovine nonfetal strains. Thus, it appeared to be possible to distinguish between pathogenic and nonpathogenic vibrios by means of the gel precipitation test.

DiLiello, et al. in 1959 (13) performed comprehensive biochemical and serological studies on 68 vibrio strains of bovine, ovine, human, porcine and avian origin.

V. fetus strains of bovine, ovine and human origin appeared to be (with one exception) the same organism serologically, with minor biochemical differences. These organisms were catalase-positive, H_2S-negative and salt sensitive.

Vibrios from nonfetal sources were serologically unrelated to V. fetus and were catalase-negative, H_2S-positive and salt tolerant.

The swine vibrios were biochemically and serologically heterogenous and the authors therefore indicated the possibility of this animal species being carriers for V. fetus infection in cattle, sheep, goats, and, possibly, in man.

Morgan in 1959 (35) performed extensive agglutination and agglutination-absorption tests on a great number of bovine and ovine fetal vibrio strains. He distinguished between 2

O-groups and found both bovine and ovine strains in each group. H-sera were prepared by adsorbing O-antigens with OH-sera. Pure H-agglutination revealed that nearly all strains contained a common H-component. Neither the O-sera, nor the H-sera agglutinated nonpathogenic catalase-negative vibrios.

Bryans and Smith in 1960 (8) studied 55 strains isolated from cattle, sheep and chickens. The last ones were isolated from the liver or bile of chickens suffering from hepatitis. By and large only two biochemical reactions proved to be of value as a basis for differentiating between pathogenic and nonpathogenic vibrios. Pathogenic strains were catalase-positive and salt-sensitive while nonpathogenic strains were catalase-negative and salt-tolerant. No consistency existed between pathogenicity and the ability to produce H_2S. Moreover, the ability to grow in the presence of 1% bile proved to be a useful test for differentiating between bovine strains. Only catalase-positive bovine strains tolerated 1% bile. This test did not give any consistent results with ovine and avian strains.

Roberstad and Morrison in 1961 (54) prepared saline (S) and phenol (P) extracts from 16 fetal strains of bovine, ovine and human strains. The extracts were tested against whole cell antisera in the Oakley-Fulthorpe precipitation test and in the Ouchterlony agar diffusion test. The 16 strains were divided into 3 groups. No significant differences were

observed between the phenol and saline extracts used as antigens and no antigenic pattern was found associated with the source of isolation.

Reich, et al. in 1961 (44) studied 5 bovine fetal strains by means of reciprocal adsorption and cross-agglutination tests. Eleven antigens and five haptens were discovered.

Winter and Dunne in 1962 (70) made an antigenic analysis of 26 catalase-positive strains and 7 catalase-negative strains. Fractions were prepared by phenol extraction and by sonification. The phenol fraction proved to be a toxic substance which was able to kill mice. The ultrasonic extracts contained both heat labile flagellar and heat stable somatic agglutinogens and several precipitinogens. Using the ultrasonic extracts in agar precipitation and indirect hemagglutination tests antigenic relationship was found between all the V. fetus strains and between V. fetus and some of the catalase-negative, saprophytic strains.

Winter (69) continued these studies and he also prepared extracts by means of trichloracetic acid. All these extracts contained one heat stable fraction, probably the polysaccharide moiety of an endotoxin. The heat labile fraction was separated into subfractions by means of ion exchange chromatography and tested in the indirect hemagglutination test and the gel precipitation test. The subfractions were also treated by various enzymes followed by precipitation reactions.

It was concluded that the subfractions, apart from one flagellar component, were of protein nature.

Ogg in 1962 (38) studied the coccoid form of 4 ovine fetal strains. Two of the strains were both catalase- and H_2S-positive while the 2 other strains were catalase-positive but H_2S-negative. The two groups were serologically unrelated but cross-agglutination occurred between the primary spiral and the secondary coccoid form within each group. The coccoid form showed lower antigenicity than the spiral form and Ogg hypothesized that it might represent the carrier state in the host organism.

Nagaswararao and Blobel in 1963 (36) prepared by means of acid precipitation antigenic substances from cell-free filtrates of 22 V. fetus strains. This acid precipitable material (APM) was compared with lysed cell extracts with regard to chemical composition and antigenic properties. Precipitation reactions in the double-diffusion technique of Ouchterlony and immunelectrophoresis revealed that there was a difference both in antigenic and chemical composition between APM and the homologous lysed cell extract. The conclusion was that the major part of APM did not constitute the cellular components freed by lysis of vibrio cells.

As to phage typing of V. fetus and related vibrios there is at present time only one report. Fletcher and Hess in 1964 (14) tested the filtrates of bovine, porcine, avian and human

feces for antivibrio phages. The test strains were 20 microaerophilic vibrios which according to the authors were distributed as follows:

6 strains of V. fetus, type I

4 strains of V. fetus, type II

2 strains of V. fetus, type III

2 strains of V. bubulus

3 strains of V. coli

3 strains isolated from cases of avian hepatitis

Phages of human feces had low lytic activity on V. fetus, types I and II. Phages of avian and porcine feces lysed V. fetus, type I and V. coli. No lysis was observed with strains of V. bubulus. Thus it seemed to be possible to distinguish by means of phage typing between V. fetus on the one hand and V. bubulus on the other hand, using any of the phages. Moreover, it seemed to be possible to distinguish between V. fetus, type I and type II, using phages of porcine or avian origin.

The problem of distinguishing between pathogenic and non-pathogenic vibrios seemed to be definitely solved when Bryner and Frank introduced the catalase- and the H_2S-test in 1955. Bulls and cows infected with catalase-positive vibrios were considered to be infected with the true V. fetus causing enzootic sterility. Such animals were either submitted to various kinds of more or less expensive treatment or slaughtered. However, one should soon discover a new type of vibrio which

in some respects was related to and in other respects was not related to the true V. fetus. Akkermans, et al. in 1956 (1) distinguished between 2 groups of pathogenic vibrios. Strains of group I were isolated from the semen and prepuce of bulls and from the genital tract of cows in outbreaks of infertility. These strains were catalase-positive and H_2S-negative. Strains of group II were isolated only from aborted fetuses and were not found in cases of sterility, nor in bulls. These strains were both catalase-positive and H_2S-positive. Out of 20 strains isolated from aborted fetuses, 5 belonged to group I and 15 to group II.

Mitscherlich and Forschner, 1956 (33), isolated 2 catalase- and H_2S-positive strains from bovine fetuses, 2 from the genital tract of cows and 12 from semen or preputium of bulls. None of these strains were associated with infertility and the workers emphasized the importance of distinguishing between the tyo types of catalase-positive vibrios. The prohibition of using bulls infected with the new vibrio type for natural or artificial breeding would cause serious economic losses and much trouble both to farmers and the A.I. centers.

In 1959 Hubrig and Wohanka (22) reported 2 more isolations from bulls of the new vibrio type which they designated "Art III" in addition to the true V. fetus and the catalase-negative, nonpathogenic V. bubulus; a term which had now come into general use.

Florent in 1959 (15) studied more thoroughly the relationship between the original V. fetus and the new abortifacient agent. He made the following statements:

1. Biochemically the new type, in contrast to V. fetus, produced H_2S in an appropriate medium (containing 0.02% cysteine) under precise conditions (incubation for 5 days). Moreover, it grew in the presence of 1% glycine, while V. fetus did not.
2. Although antigenically related to V. fetus the new type possessed specific enough components in order to be distinguished from V. fetus by means of the agglutination test.
3. The predilection site of V. fetus was the cervix and vagina of the nonpregnant cow and the fetal membranes of the pregnant cow. The predilection site of the new type was the intestine of cattle, sheep and swine.
4. The original V. fetus caused enzootic sterility and sporadic abortions in cattle. The new type caused sporadic abortions in cattle and enzootic abortions in sheep.

In conclusion, Florent distinguished between two types of pathogenic, fetal vibrios:

1. Vibrio fetus venerialis
2. V. fetus intestinalis (Art III, Hubrig and Wohanka).

Having reviewed to some extent the unsuccessful attempts to classify different types of vibrio by means of the agglutination test, Mitscherlich and Liess in 1958 (34) resorted to the complement fixation test (CFT) as a possible means of differentiation.

So far, only a few reports had been published concerning the application of the CFT to vibriosis. Amell and Stockton in 1956 (2) used the test to detect antibodies in vaccinated cattle and found that it by and large gave results consistent with the serum agglutination test. Trilenko (65) developed in 1956 a slow CFT for detection of antibodies in infected cattle. The slow CFT, which consisted in incubating the specific system overnight at 4 C, was superior to the ordinary CFT with regard to sensitivity. Trilenko, et al. (66) and Zyabkin and Ruchii (71) further increased the sensitivity of the test by using concentrated antigen. Biberstein in 1956 (4) attempted by means of the CFT to distinguish between 56 strains of bovine and ovine origin, using as antigens whole cells and extracts prepared by various physical and chemical treatments. Generally, the antigens were too weak or too unspecific to warrant a basis for differentiation.

Mitscherlich and Liess used as antigen an extract prepared by boiling whole cells in phenolum liquidum followed by dialysis to remove the phenol. Thirteen

catalase-positive and negative strains of bovine and ovine origin were tested and divided into three groups on the basis of the CFT:

Group I. Vibrios of this group were catalase-positive and H_2S-negative and were designated *V. fetus, type 1.*

Group II. Vibrios of this group were also catalase-positive and H_2S-negative and were designated *V. fetus, type 2.*

Group III. Vibrios of this group were catalase-negative and H_2S-positive and were considered to be the common *V. bubulus.*

The results obtained by means of the CFT were then compared with those obtained by means of the agglutination test. The last test proved to be satisfactory as far as the distinction between catalase-positive and catalase-negative vibrios was concerned. But only the CFT could be used to distinguish between the two groups of catalase-positive vibrios.

Kamel in 1960 (24) continued the previous work and studied the cultural and serological properties of 160 strains, 155 of bovine origin and 4 of ovine origin and one of porcine origin. By means of the CFT these strains were divided into 7 groups on the basis of the antigen factors 1, 2, 3, 4, 5, 6, and 7. Based on their source of isolation these 7 types were given the names as follows:

Vibrio fetus, type 1

Vibrio fetus, type 2

Vibrio fetus, type 7

Vibrio bubulus, type 3

Vibrio bubulus, type 4

Vibrio bubulus, type 5

Vibrio suis, type 6

Some strains of types 1, 2 and 7 were isolated from aborted fetuses and were therefore considered to be pathogenic.

Types 3, 4 and 5 were not isolated from aborted fetuses and were therefore considered to be nonpathogenic.

No conclusions were drawn with regard to the pathogenicity of the single strain of type 6.

This author found the CFT to form a more reliable basis for differentiation than tests based on cultural and biochemical characteristics. Of the 67 strains which according to the CFT proved to be V. fetus, type 1, 5 strains or 7.5% were catalase-negative.

Söderlind, in 1961 (63) continued the work initiated by Mitscherlich, Liess and Kamel. He tested, by means of the agglutination test, 51 bovine and ovine strains which by the CFT had been identified as follows:

V. fetus, type 1: 20 strains

V. fetus, type 2: 27 strains

V. fetus, type 7: 4 strains

The following results were obtained:

1. Of the 20 strains, previously shown by the CFT to be V. fetus, type 1, 5 strains showed in the agglutination test identical titers with serum against V. fetus, type 1 and V. fetus, type 2.
2. Of the 27 strains previously shown by the CFT to be V. fetus, type 2, 12 strains showed identical titers with serum against V. fetus, type 1 and V. fetus, type 2.
3. The 4 strains previously shown by the CFT to be V. fetus, type 7, did not react significantly with any of their homologous or heterologous sera in the agglutination test.

Thus the CFT proved to be a more reliable and specific test than the agglutination test in differentiating between vibrios pathogenic for cattle and sheep. Moreover, one of the pathogenic types, V. fetus, type 7 could only be identified by means of the CFT.

In a report at the 4th International Congress on Animal Reproduction held in The Hague in 1961 (32), Mitscherlich presented the results of biochemical and serological investigations of an accumulated material of 204 strains of ovine and bovine origin. The common biochemical tests, production of catalase and H_2S and growth in the presence of 3.5% sodium chloride, proved to be useful to the extent of distinguishing

between the pathogenic types, V. fetus, types 1, 2 and 7 on the one hand, and the nonpathogenic types, V. bubulus, types 3, 4 and 5, on the other hand. Further differentiation had to be done by means of serological tests and comprehensive titrations had shown that the CFT was superior to the agglutination test in this regard.

In this report it was also concluded that V. fetus, type 1 was identical with Florent's V. fetus venerialis and that V. fetus, type 2 was identical with Florent's V. fetus intestinalis.

In connection with the investigations reported above, a publication by Melrose, et al. in 1962 (31) is of interest. These workers infected heifers successfully with strains which according to the biochemical tests were V. fetus intestinalis, and the organisms remained in vagina for more than 12 months. The conclusion was therefore that this type of vibrio may also cause enzootic sterility in cattle. Later Mitscherlich[1] tested some of these strains by means of the CFT. He found that the strains in quest were V. fetus, type 1 or venereal vibrio, thus indicating a stronger correlation between pathogenicity and the CFT than between pathogenicity and the biochemical tests.

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[1]E. Mitscherlich, Tierärztliches Institute der Universität Göttingen, Göttingen, Germany. Differentiation of strains of vibrio. Private communication. April 1st, 1964.

In line with the investigations in Europe just described there are two recent reports from the United States. Bryner, et al. in 1962 (10) studied the cellular and colonial characteristics and the biochemical properties of 87 strains isolated from bovine fetuses and from bovine genital tracts.

The following scheme was established:

	Vibrio fetus			Vibrio bubulus
	Type 1	Subtype 1	Type 2	
Catalase	+	+	+	-
H_2S after 24 hours	-	-	-	++++
H_2S after 5 days	-	+++	+++	++++
Salt tolerance (3.5%)	-	-	-	+
Bile tolerance (2%)	+	+	+	-
Glycine tolerance (1%)	-	-	+	+

The biochemical activity of the colonial variants did not differ from their smooth parent cultures.

Ringen and Frank in 1963 (46) modified the commonly used biochemical test media for differentiation between venereal, intestinal and saprophytic vibrios, as they found these media, when used in their conventional form, gave too much overlapping. One tenth of one per cent $FeSO_4 \cdot 7\ H_2O$ was added to the media and the results obtained were as follows:

	H_2S-production	Growth in 1% glycine
V. fetus var. intestinalis (31 ovine strains, 1 bovine strain)	-	+
V. fetus var. venerialis (6 bovine strains)	-	-
V. bubulus (6 various sources)	+	+

As to fluorescent antibody studies on *V. fetus* and related vibrios only 2 reports are available when this thesis is being written. A third report commented on in this review has not been published.

In order to detect *V. fetus* infection in bulls Herschler in 1962 (20) labeled the globulin fraction of 4 *V. fetus* antisera with fluorescein isocyanate. Semen and preputial secretions from artificially and naturally infected bulls were stained with the conjugates and *V. fetus* was successfully detected. Heifers were inseminated with semen and preputial secretions from bulls. *V. fetus* was isolated in pure culture from heifers mated by bulls which were found positive to the fluorescence antibody test.

Mellick, *et al.* in 1964 (30) also reported on successful attempts to diagnose *V. fetus* infection in bulls by means of the FAT.

O'Berry in 1964 (37) compared three methods in the preparation of *V. fetus* fluorescent antibody conjugates.

Lapine and bovine hyperimmune sera were fractionated with

a. ammonium sulfate,

b. ethanol, and

c. ethodin.

The various fractions were labeled with fluorescein isothiocyanate (FITC) and smears and suspensions of V. fetus cells were stained with the different conjugates. Fractionation with ammonium sulfate proved to be superior to ethodin and ethanol fractionation.

EXPERIMENTAL INVESTIGATIONS

Material

Type strains

Vibrio type strains which had been identified by means of the complement fixation test were received from Dr. K. Dräger at Behringwerke Aktiengesellschaft, 355 Marburg/Lahn, West-Germany.

These strains were as follows:

		Source
V. fetus, type 1:	Strain no. 177	Bovine fetus
V. fetus, type 2:	Strain no. 9	Bovine vagina
V. fetus, type 7:	Strain no. 72	Bovine fetus
V. bubulus, type 3:	Strain no. 193	Bovine prepuce
V. bubulus, type 4:	Strain no. 42	Bovine vagina
V. bubulus, type 5:	Strain no. 45	Bovine vagina

The strains were received in a lyophilized state and were resuspended in one ml. of thioglycollate medium (Difco).[1] Five tenths ml. was then transferred to each of two 15x150 mm. screw-cap tubes containing 5 ml. thioglycollate medium. The cultures were incubated aerobically at 37 C for 4-5 days before they were subcultured.

Field strains

The field strains were, with one exception (strain no.

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[1]Difco Laboratories, Detroit, Michigan.

7212), received from Dr. A. H. Frank at The National Animal Disease Laboratories, Ames, Iowa. Strain no. 7212 was received from Dr. J. E. Lovell at the Veterinary Medical Research Institute, Ames, Iowa.

These strains were as follows:

Strain no.	Source
436	Bovine fetus
852	Bovine fetus
5	Bovine vagina
8	Bovine vagina
250	Bovine vagina
482	Bovine vagina
671	Bovine vagina
728	Bovine vagina
758	Bovine vagina
676	Bovine semen
739	Bovine semen
818	Bovine semen
643	Bovine prepuce
7212	Bovine prepuce
831	Bovine bile
105	Ovine fetus
652	Ovine fetus
653	Ovine fetus
657	Ovine fetus
658	Ovine fetus
664	Ovine fetus
665	Ovine fetus
836	Ovine bile
871	Ovine feces
872	Ovine feces

Apart from strain no. 7212 which had been kept in culture since its isolation in June, 1962, these strains had been stored in deep frozen state in thioglycollate medium. After thawing at room temperature they were subcultured on blood agar and on thioglycollate medium. The blood agar plates were incubated at 37 C for 4-6 days in an atmosphere containing 5% oxygen, 10% carbon dioxide and 85% nitrogen.

If growth did not appear directly on thioglycollate medium it usually appeared on the plates and colonies could be transferred to thioglycollate medium on which the author received the strains.

Both the type strains and the field strains were subcultured every third week on thiol medium (Difco) and on thioglycollate medium. During the first four days after subculturing they were incubated at 37 C. Later they were stored at room temperature.

Methods

Biochemical tests

These tests were performed as described by Bryner and Frank in 1955 (9) and by Bryner, et al. in 1962 (10).

The catalase test was performed after macroscopically distinct growth had been obtained on thiol medium after 3 days incubation. The cultures were checked for purity and motility. During the period of time the experiment was underway some of the type strains, V. fetus, type 7 and V. bubulus, types 3 and 4, which had been lyophilized, grew well and type 3 even abundantly but they never became motile.

The medium used for the H_2S-test had the following composition:

Beef extract (Difco)	5 grams
Bacto-peptone (Difco)	10 grams
Sodium chloride	5 grams

L-cysteine hydrochloride[1]	0.275 grams
Bacto-agar (Difco)	1 gram
Distilled water	1000 ml.

pH was adjusted to 7.2.

A 5x80 mm. sterile strip of lead acetate paper was inserted into each inoculated tube and into one uninoculated control tube.

For the salt tolerance test thiol medium was used with the addition of 3.5% sodium chloride.

For the glycine and the bile tolerance tests thioglycollate medium was used with the addition of 1% glycine[2] and 2% oxgall (Difco) respectively.

About 5 ml. of the test media were inoculated with 0.5 ml. of vibrio cultures which had been grown for 4 days on thioglycollate medium. Purity and motility was checked prior to inoculation. In order to perform these tests under maximally standardized conditions 15x150 mm. test tubes with metal caps were used. All tubes except those used for the H_2S-test were incubated aerobically at 37 C for 5 days. The tubes used for the H_2S-test were incubated at 37 C for 5 days in the same atmosphere as that one previously described for the blood agar plates.

Upon reading of the results a positive H_2S-reaction was

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[1]Nutritional Biochemicals Corporation, Cleveland, Ohio.

[2]Arthur H. Thomas, Co., Philadelphia, Pennsylvania.

interpreted as a distinct blackening of the lead acetate paper. With regard to the tolerance tests the appearance of the characteristic sub-surface layer of vibrio growth was considered a positive result. Uninoculated control tubes were used for comparison and in cases where more diffuse growth occurred microscopical examinations were performed. In order to avoid false negative results comparison was made with inoculated tubes which did not contain the inhibitory substances.

The complement fixation test

Preparation of antigens The medium used for antigen production was thiol agar prepared by adding 2% Bacto-agar to thiol broth (Difco). As the type strains grew better on thioglycollate medium these strains were seeded on thioglycollate agar. Fifteen Roux flasks were seeded, each with about 5 ml. of a 4 day old vibrio culture on thiol medium (thioglycollate medium was used for the type strains). The flasks were incubated at 37 C in the same atmosphere as that one used for the blood agar plates and the H_2S-test (5% oxygen, 10% carbon dioxide and 85% nitrogen). The incubator used for this purpose was an old autoclave which was equipped with a heating element, a fan and a thermostat. This incubator had a capacity of 30 Roux flasks. After 2 days incubation at 37 C the flasks were taken out and the surface of the agar flooded with the inoculum. Then the flasks were incubated for another 2 days. For harvesting about 50 ml. 0.5% phenolized saline and glass

beads were added to each flask. The growth was washed off by gentle shaking and the contents poured separately into 50 ml. tubes. Purity of growth was checked by examining Gram-stained smears from each tube. The bacterial suspension was then filtered through 4 layers of cheese cloth and centrifuged at 1500 r.p.m. for 10 minutes in order to remove extraneous material. The supernatant was centrifuged at 3000 r.p.m. for 30 minutes and the sedimented cells were washed twice in phenol saline at the same speed and for the same period of time. Finally, the sediment was transferred to 10 ml. graduated centrifuge tubes which were centrifuged at 2000 r.p.m. for two hours.

The extraction of the antigen fraction to be used in the complement fixation test was done as described by Mitscherlich and Liess (34). To one ml. of packed cells was added 10 ml. phenolum liquidum. This was prepared by melting 90 grams of crystalline phenolum and the melted substance was added to 10 ml. distilled water. The cells resuspended in phenol were then placed in a gently boiling water bath and stirred continuously until the solution became clear. After cooling, the pH was adjusted to 7.2 by means of a 10% sodium hydroxide solution. In order to remove the phenol the extract was transferred to dialysing collodion[1] bags. These were fitted

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[1]Membranfiltergesellschaft, Sartoriuswerke Aktiengesellschaft, Göttingen, West-Germany. Distributed in the U.S.A. by Carl Schleicher & Schuell, Co., Keene, New Hampshire.

to the opening of inverted ordinary test tubes. By means of cloth pegs the tubes were kept suspended in a rack and the collodion bags immersed in continuously flowing tap water. After dialysing for 24 hours 3 ml. sterile saline was added to each bag and the dialysis continued for another 24 hours. Traces of phenol in the remaining contents of the bags were detected by means of Millon's reagent (3). This was prepared by letting 1 part (weight) of mercury react with 2 parts (weight) of concentrated nitrous acid and then adding 2 volumes of distilled water. A 1:10,000 solution of phenol was prepared and Millon's reagent was added dropwise under boiling of the solution. A deep red color indicated a positive reaction. Millon's reagent together with distilled water did not produce any red color. In order to economize with the limited amounts of the extracted antigens 0.5 ml. extract of each antigen was mixed with 4.5 ml. distilled water and Millon's reagent was added. If any red color occurred the dialysis was continued until no phenol was left. When the dialysis was finished merthiolate was added at a concentration of 1:10,000. One drop of a 1% neutral red solution[1] was added as an indicator and neutralization was performed by means of a 0.1% hydrochloric acid or a 0.1% sodium hydroxide solution. A neutral reaction was indicated by a slightly pink color. Finally the extracts were centrifuged at 2000 r.p.m. for 15 minutes in order to

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[1]Hartman-Leddon Co., Philadelphia, Pennsylvania.

remove coarse particles. They were then ready for titration in the complement fixation test.

Preparation of hyperimmune sera Antigens used for the production of type specific hyperimmune sera were seeded and harvested in the manner just described. The only difference was that sterile saline was used for washing the cells. After the final washing the packed cells were resuspended in 0.3% formalinized saline and stored as a concentrated stock suspension. Sterility tests were performed by inoculating tryptose phosphate broth (Difco) and thioglycollate medium with one drop of the suspension. The tubes were incubated aerobically at 37 C for one week. A suspension corresponding to the density of tube no. 5 (1,500 million cells per ml.) of McFarland's nephalometer was used for immunization. Rabbits were inoculated intravenously 6 times at 3 or 4 day intervals and the doses were 0.5; 1.0; 1.5; 2.0; 2.0 and 2.0 ml. One week after the last inoculation 40 ml. blood was collected from each rabbit by cardiac puncture. The serum was separated by centrifugation and immediately frozen at -20 C. Two rabbits, one inoculated with V. fetus, type 1 and one with V. fetus, type 7, died after the first bleeding. After a resting period of one week the four remaining rabbits received a seventh inoculation of 2 ml. followed by a second bleeding one week later.

Originally 12 rabbits were acquired for the experiment. The intention was to prepare antisera specific for the antigen

extract of each of the vibrio type strains and for this purpose preliminary immunization was made with V. fetus, type 1 and V. bubulus, types 3 and 4. Two rabbits were used for type 1 and type 3 and one rabbit for type 4. As preliminary titrations of these antisera after 3 weeks immunization gave rather low titers (maximum 1:80) in the complement fixation test, one proceeded to whole cell immunization. Prior to this, unsuccessful attempts had been made to increase the serum titers by using antigen extracts mixed with Freund's adjuvant (Difco). As sterility tests showed that one of the whole cell suspensions was contaminated, the rabbit used for immunization with this strain was, of course, excluded from the experiment. The 6 remaining rabbits were therefore used for whole cell immunization with one rabbit for each type strain.

Elaboration and performance of the complement fixation test As the system applied by Mitscherlich and Liess (34) was to some extent modified, the system used in this investigation had to be elaborated and established independently of their system. Lyophilized complement and glycerinated hemolysin were used and both were acquired commercially from the Difco Laboratories. The advantage of using commercially available reagents, prepared from one lot of guinea pig serum and one lot of anti-sheep red cell serum respectively, was the stability of these reagents. Having once determined the titers of the complement and the hemolysin the reagents could

be used in the same dilution throughout the whole titration procedure.

Instead of saline a modified barbital buffer described by Campbell, et al. (11) was used. This buffer contained magnesium and calcium ions and was used for dilution of all reagents. The concentrated stock solution of the buffer was kept sterile at 4 C. Diluted buffer for titration was freshly prepared either on the day of titration or on the day before by mixing one part of concentrated buffer with four parts of distilled water to which 0.04% Bacto-gelatin (Difco) had been added. The gelatin was dissolved in part of the distilled water by gentle heating in a water bath. The sera were inactivated in a water-bath at 56 C for 30 minutes.

Sheep blood was collected in Alsever's solution (11) at a ratio of 1:1. Immediately before titration 5 ml. of sedimented sheep erythrocytes were washed three times in about 40 ml. of the buffer by centrifugating the mixture at 2000 r.p.m. for 15 minutes. A fourth centrifugation at the same speed and for the same period of time was done in 10 ml. graduated centrifuge tubes. The supernatant was carefully removed by means of a Pasteur pipette and the required volume of packed cells was pipetted off to make up a 2% suspension.

The hemolytic system was prepared by mixing equal volumes of diluted hemolysin and the 2% suspension of sheep erythrocytes. In order to sensitize the erythrocytes the

mixture was left in a water-bath at 37 C for 10-15 minutes.

Before use all reagents were stored at 4 C and resuspended complement was immediately frozen after the required amount had been removed for the current titration. All pipettes and tubes used for titrations and dilutions were cleaned in chromium-sulphuric acid and rinsed, first in three changes of tap water and then in three changes of distilled water. As to titration of hemolysin and complement and determination of anticomplementary effects, see Tables 1, 2 and 3 respectively.

Determination of antigenic dose In order to determine the optimal antigenic dose of the type antigens some preliminary "block" titrations were performed in which several dilutions of antigen were concurrently titrated against successive dilutions of serum. These titrations were performed with antigens of V. fetus, types 1 and 2 and their homologous sera. However, the complement fixing activity did not increase with decreasing concentrations of antigen. For further titrations of their homologous sera the type antigens were therefore used in concentrations corresponding to their anticomplementary value.

The next logical step after the titers of the type sera had been determined was to evaluate the specificity of these sera by cross-titrations. Prior to these cross-titrations the titers of the type antigens were determined. This was

Table 1. Titration of hemolysin

											Control of non-specific lytic effect		
											Comp.	He-mol.	Buf-fer
Tube no.	1	2	3	4	5	6	7	8	9	10	11	12	13
Buffer, ml.	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	--	---	1.0
Hemolysin,[a] ml.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	--	1.0	--
Dilution of hemolysin	1/1000	1/2000	1/3000	1/4000	1/5000	1/6000	1/7000	1/8000	1/9000	1/10000	--	1/1000	--
2% Sheep R.B.C., ml.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Complement (1:20 dil.), ml.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	1.0	---	---

Mix and incubate in a water bath at 37 C for 30 minutes.

Interpretation: The unit of hemolysin is the highest dilution that gives complete hemolysis.

For further titrations two units of hemolysin were used.

[a]The hemolysin is added separately to the tubes from the corresponding tubes in a previously prepared series of hemolysin dilutions.

Table 2. Titration of complement

									Contr. of hemol.	Contr. of compl.
Tube no.	1	2	3	4	5	6	7	8	9	10
Complement (1:20 dil.), ml.	0.40	0.35	0.30	0.25	0.20	0.15	0.10	0.05	----	0.40
Hemolysin (2 units), ml.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	----
Buffer, ml.	0.40	0.45	0.50	0.55	0.60	0.65	0.70	0.75	0.80	0.80
2% Sheep R.B.C., ml.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Mix and incubate in a water bath at 37 C for 30 minutes.

Interpretation: The exact unit of complement is the smallest amount that gives complete hemolysis. The full unit is 0.05 ml. more than the exact unit. For further titrations the complement was diluted so that 0.5 ml. contained 2 full units.

Table 3. Determination of anticomplementary effects of antigens and sera

	Anticomplementary effect of serum					Anticomplementary effect of antigen						Contr. of nonspecific lytic effect	
												Serum	Antigen
Tube no.	1	2	3	4	5	6	7	8	9	10	11	12	13
Serum, 0.15 ml.	1:5	1:10	1:20	1:40	1:80	----	----	----	----	----	----	1:5	----
Antigen, 0.15 ml.	----	----	----	----	----	undil.	1:2	1:4	1:8	1:16	1:32	----	undil.
Buffer, ml.	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.85	0.85
Complement, (2 full units), ml.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	----	----
Mix and incubate at 37 C for 30 minutes. Then add the hemolytic system:													
2% Sheep R.B.C., ml.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Hemolysin (2 units), ml.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	---	---
Mix and incubate at 37 C for 30 minutes.													

Interpretations: (a) The anticomplementary value of serum is the lowest dilution which, in the absence of antigen, does not interfere with the activity of the complement.

(b) The anticomplementary value of antigen is the lowest dilution which, in the absence of serum, does not interfere with the activity of the complement.

done because the amount of each antigen was limited. Once the titers of the type sera had been determined, using the anticomplementary value of the homologous antigen, the individual type sera were cross-titrated, using the lowest possible dilution of antigen which still gave a strong positive reaction with its homologous serum. Titrations of serum and antigen are shown in Tables 4 and 5 respectively.

Titration of unknown antigens This titration procedure was performed in three steps:

1. At first the anticomplementary and nonspecific lytic effects, if any, were determined for each antigen. (See Table 3).
2. The initial dilution of the antigens for further titrations was the anticomplementary value. As each antigen had to be titrated against 6 type sera plus normal serum, a preliminary screen testing was performed as follows:

	Serum (2 units)						
	Vibrio fetus			Vibrio bubulus			Normal serum (1:5)
	Type 1	Type 2	Type 7	Type 3	Type 4	Type 5	
Tube no.	1	2	3	4	5	6	7

Row no. 1: Conc. antigen of e.g., strain X in all tubes.

Row no. 2: Antigen, dil. 1:4, of strain X in all tubes.

Row no. 3: Homologous antigen (2 units) added to homologous serum.

Volumes of serum, antigen, complement and hemolytic system were the same as given in Tables 3, 4 and 5.

3. If an antigen reacted in a concentrated condition with one or more of the type sera it was titrated at a dilution of 1:2 against these sera. If an antigen also reacted at a dilution of 1:4 with one or more of the type sera it was titrated to the end point against these sera.

Standards used for reading of titration results These were read after the cells had been allowed to sediment by leaving the racks overnight in the refrigerator. In order to arrive at an exact, visible interpretation of the results the standards were set up as indicated in Table 6.

The fluorescent antibody test

Fractionation of serum globulins and determination of the protein content The globulin fractions of hyperimmunsera and of normal serum were obtained by means of half-saturation with ammonium sulfate. The method used is described in detail by Cherry, et al. (12). Eight ml. of each of the immunsera and 10 ml. of normal serum were fractionated. After the globulin fractions had been precipitated for the third time and redissolved in distilled water, they were transferred to 0.9 inch wide dialysing tubes.[1] Dialysis was then performed under

[1]Visking Company, Division of Union Carbide Corporation, 6733 West 65th Street, Chicago 38, Illinois.

Table 4. Titration of serum

	Suspected or known positive serum									Normal serum			
	Contr. of ag.	Contr. of serum								Contr. of serum			
Tube no.	1	2	3	4	5	6	7	8	9	10	11	12	13
Buffer	0.15	0.15	----	0.15	0.15	0.15	0.15	0.15	0.15	0.15	----	0.15	0.15
Serum[a]	----	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Dil. of serum	----	1:5	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:5	1:10	1:20	1:40
Antigen, (2 units)	0.15	----	0.15	0.15	0.15	0.15	0.15	0.15	0.15	----	0.15	0.15	0.15
Compl.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mix and incubate at 37 C for 30 minutes. Then add the hemolytic system:													
2% Sheep R.B.C.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Hemol.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Mix and incubate at 37 C for 30 minutes.													

[a]After the serum, diluted 1:5, has been added to tube no. 4, the contents of this tube are mixed and 0.15 ml. is transferred successively with intercurrent mixings to the following tubes until tube no. 9 is reached. After the final mixing, 0.15 ml. from tube no. 9 is discarded. The same procedure is applied to the normal serum.

Interpretation: The serum titer is the highest dilution of the serum that causes complete (100%) fixation. This dilution is termed one unit.

Table 5. Titration of antigen

	Suspected or known positive serum									Normal serum			
	Contr. of serum	Contr. of antigen								Contr. of serum			
Tube no.	1	2	3	4	5	6	7	8	9	10	11	12	13
Buffer	0.15	0.15	----	0.15	0.15	0.15	0.15	0.15	0.15	0.15	----	0.15	0.15
Antigen[a]	----	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	----	0.15	0.15	0.15
Dil. of antigen	----	undil.	undil.	1:2	1:4	1:8	1:16	1:32	1:64	----	undil.	1:2	1:4
Serum (2 units)	0.15	----	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Compl.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mix and incubate at 37 C for 30 minutes. Then add the hemolytic system:													
2% Sheep R.B.C.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Hemol.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Mix and incubate at 37 C for 30 minutes.													

[a]After undiluted antigen has been added to tube no. 4, the contents of this tube are mixed and 0.15 ml. is transferred successively with intercurrent mixings to the following tubes until tube no. 9 is reached. After the final mixing 0.15 ml. from tube no. 9 is discarded. The same procedure is applied to the normal serum.

Interpretation: The antigen titer is the highest dilution of the antigen that causes complete (100%) fixation. This dilution is termed one unit. For further titrations of sera 2 units were used.

Table 6. Standards for reading of serum titration and antigen titration

For serum titration:					
Tube no.	1	2	3	4	5
Distilled water, ml.	0.65	0.75	0.85	0.95	1.05
Hemolytic system, ml.	0.4	0.3	0.2	0.1	----
Antigen, ml. (The same dilution as used in the titration proper)	0.15	0.15	0.15	0.15	0.15
Corresponding to degree of lysis	100%	75%	50%	25%	0%
Corresponding to degree of fixation	0% -	25% +	50% ++	75% +++	100% ++++

For antigen titration:					
Tube no.	1	2	3	4	5
Distilled water, ml.	0.65	0.75	0.85	0.95	1.05
Hemolytic system, ml.	0.4	0.3	0.2	0.1	----
Serum, ml. (The same dilution as used in the titration proper)	0.15	0.15	0.15	0.15	0.15
Corresponding to degree of lysis	100%	75%	50%	25%	0%
Corresponding to degree of fixation	0% -	25% +	50% ++	75% +++	100% ++++

continuous stirring at 4 C for 36 hours against four changes of saline. Any trace of ammonium sulfate in the dialysate was detected by mixing equal volumes of the dialysate with equal volumes of a saturated (40%) solution of barium chloride.

The protein content of the globulin solutions was determined by means of the Biuret method described by Gornall, et al. (19). For convenience, commercially available Biuret Reagent Tablets[1] were used. Different dilutions of sheep gamma globulin, fraction II[2] were prepared as a standard, the Biuret reagent was added and the color intensity was determined spectrophotometrically[3] at a wavelength of 540 mμ. A calibration curve was drawn as shown in Figure 1. Then different dilutions of the immune and the normal globulin fractions were prepared, biuret reagent was added and the light transmittance measured. The percentage of light transmitted by the different solutions was plotted on the ordinate of the graph and by means of the calibration curve the corresponding protein concentrations were found on the abscissa. The

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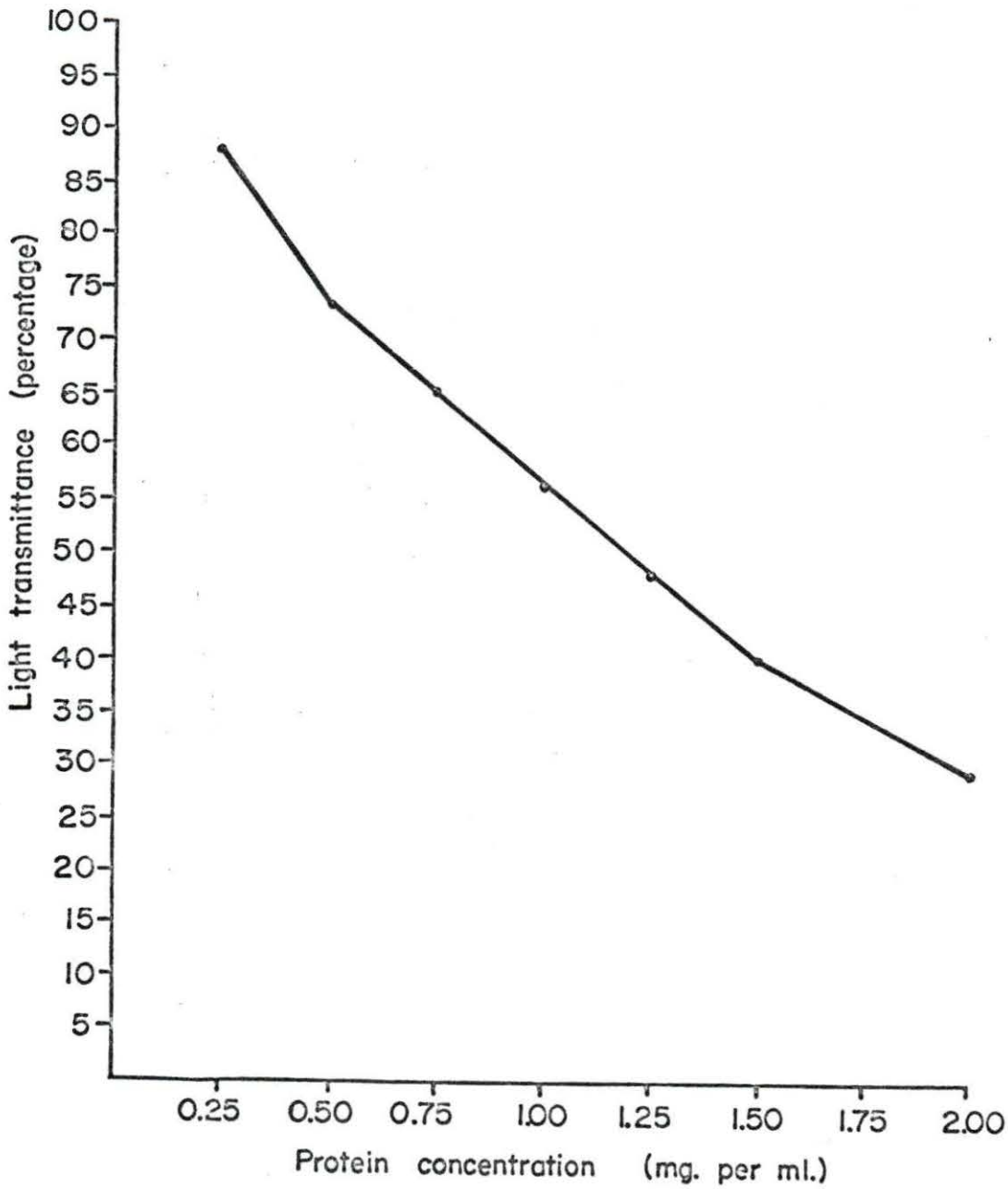
[1]Manufactured by Cambridge Chemical Products, Inc. Distributed by: Scientific Products Division, American Hospital Supply Corporation, General Offices: Evanston, Illinois.

[2]Pentex, Inc., Kankakee, Illinois.

[3]Spectrophotometer used: Spectronic 20, Bausch and Lomb, Inc., Rochester, New York.

Figure 1. Correlation between light transmittance (percentage) and protein content (mg. per ml.) of standard dilutions of sheep gamma globulin.

Light transmittance (percentage)	Protein content (mg./ml.)
88	0.5
74	1.0
65	1.5
56	2.0
48	2.5
40	3.0
29	4.0


Light transmittance (percentage)
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
Protein concentration (mg. per ml.)

protein content of the globulin fractions was then calculated as the average of three measurements of each fraction. Before conjugation the protein content was adjusted to 1% by means of distilled water.

Conjugation of serum globulins All the serum globulins were conjugated with fluorescein isothiocyanate (FITC).[1] The method of Riggs, et al. (45) was used. A mixture, containing 10 ml. saline, 3 ml. carbonate-bicarbonate buffer (see below) and 2 ml. acetone was cooled in a salt-ice bath. Under constant stirring on a magnetic stirrer 10 ml. of the diluted globulin fraction of known protein content (1%) was added dropwise. Then 1.5 ml. acetone containing the required amount of FITC (0.05 mg. dye per mg. of protein) was added in the course of 10 minutes. Stirring was continued overnight at 4 C. The carbonate-bicarbonate buffer (0.5 M) was prepared as follows:

Solution A

Sodium carbonate (Na_2CO_3)	5.3 grams
Distilled water ad	100.0 ml.

Solution B

Sodium bicarbonate ($NaHCO_3$)	4.2 grams
Distilled water ad	100.0 ml.

pH was adjusted to 9 by adding the required amount of solution A to solution B.

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[1]Mann Research Laboratories, Inc., New York 6, New York.

Separation of unconjugated dye This was done by gel filtration through Sephadex[1] gel, grade 25. The gel was prepared by adding 40 grams of Sephadex to a 0.02 M phosphate buffer of the following composition:

Solution A

1 M Na_2HPO_4

Solution B

1 M NaH_2PO_4

Ten ml. of solution A were mixed with 10 ml. of solution B and distilled water added to make up a volume of 1000 ml. The resulting pH was 6.6.

The gel was then washed 5 times by vigorous shaking. Between the washings it was left in the refrigerator until it had settled. Having settled after the final washing most of the clear supernatant was poured off and the viscous gel was transferred to a chromotographic column. When it had settled so that it constituted two thirds of the volume of the column, it was slightly packed by means of positive pressure. A small piece of filter paper was then layered on the surface of the gel and when the overlaying buffer had reached the same level as the filter paper, the conjugate was carefully layered over the gel. Prior to this the conjugate had been equilibrated by dialysis against the same buffer for 5 hours. The column was

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[1]Pharmacia, Uppsala, Sweden.

then left at room temperature until the conjugate had reached the bottom. Now the conjugated globulin molecules were collected by means of a slight positive pressure and the unconjugated dye remained in the column. The conjugates were immediately frozen at -20 C in aliquots of 2 ml.

Staining procedure and testing of conjugates for specificity Vibrios to be tested by the FAT were grown for four days on thiol agar to which 10% defibrinated blood and brilliant green (Difco), 1:40,000 had been added. Plates prepared in this manner were incubated in the same atmosphere as the Roux flasks. The growth was harvested in 0.3% formalinized saline and centrifuged at 1000 r.p.m. for 10 minutes to remove extraneous material. Then the cells were washed three times in saline by centrifuging at 3000 r.p.m. for 15 minutes and resuspended to match a standard consisting of 1.0 ml. of distilled water and 1.5 ml. of tube no. 1 of McFarland's nephalometer. The resulting density was approximately 180 million cells per ml. A loopful of the suspension was placed on slides which had first been cleaned in hydrochloric acid and then in acetone. After drying in the air the smears were fixed by gentle heat. Slides were placed in petri dishes equipped with a moistened piece of filter paper and the smear was covered with one drop of conjugate. After staining at 37 C for 30 minutes the slides were washed for 10 minutes in 0.01 M phosphate buffer with one change of buffer after 5

minutes. The buffer had the following composition:

Solution A

0.1 M Na_2HPO_4

Solution B

0.1 M KH_2PO_4

The diluted buffer for use was prepared by mixing 85.7 ml. of solution A with 14.3 ml. of solution B and adding water to 1000 ml. The resulting pH was 7.4. After washing the slides were mounted in phosphate-buffered glycerol saline (1 part glycerol and 9 parts buffered saline) and examined.

Microscopy A Leitz Labolux microscope with a dark-field condenser, type D, 1.20 A No. 82 was used. The magnifying power of the ocular was 10X and that of the objectives 10X, 45X and 54X respectively. The light source was a High Pressure Mercury Vapor Bulb, HBO 200, and the filters were a "Streuscheibe" filter and a UV-Trans. (2 mm. UB 1) filter.

Controls In order to determine that the fluorescence which was observed was caused by conjugated, specific immunglobulin, the two-step blocking test was performed with each of the type strains and their homologous conjugates. At first, smears of the six type strains were treated individually for 30 minutes with homologous unconjugated antiserum. After washing in buffer the smears were stained for another 30 minutes, washed and examined.

The second control concerned the possible nonspecific

staining of vibrio organisms by conjugated normal globulins. This was controlled by staining smears of type strains with conjugated normal globulin.

Finally the specificity of the conjugates was examined by staining smears of each type strain with the six different immune conjugates.

Staining of field strains Smears of each field strain were stained with the six immune conjugates plus conjugated normal globulin.

RESULTS

Biochemical Reactions

Type strains

The biochemical reactions of the German type strains are shown in Table 7.

It may be seen from this table that the reactions of the fetal strains conform to the classical scheme which is presented in its most complete form by Bryner, et al. in 1962 (10). The fetal strains are all strongly catalase positive, they produce no H_2S, none of them grow in the presence of 3.5% NaCl and with one exception, V. fetus, type 7, they are all bile tolerant.

In contrast, the V. bubulus strains show considerable inconsistency. For the first, V. bubulus, type 3 is bile tolerant, but the most divergent reaction pattern is shown by V. bubulus, type 4. This type is distinctly catalase positive and salt sensitive and the only feature that distinguishes this V. bubulus type from V. fetus, type 7 is the H_2S-reaction; the former being positive and the latter negative. V. bubulus, type 5 conforms entirely to the classical scheme.

In spite of some overlapping in the biochemical tests it seems to be possible by means of these tests to distinguish between the pathogenic V. fetus strains on the one hand and the nonpathogenic V. bubulus strains on the other hand. This is also in agreement with the observations of Mitscherlich (32).

Table 7. Biochemical reactions of the type strains

Type of vibrio	Catalase activity	Prod. of H_2S	Growth in the presence of 3.5% NaCl	Growth in the presence of 1% glycine	Growth in the presence of 2% ox bile
V. fetus, type 1	67 mm.	-	-	-	+
V. fetus, type 2	97 mm.	-	-	+	+
V. fetus, type 7	37 mm.	-	-	+	-
V. bubulus, type 3	0 mm.	+	+	+	+
V. bubulus, type 4	12 mm.	+	-	+	-
V. bubulus, type 5	0 mm.	+	+	+	-

Field strains

The biochemical reactions of the American field strains are shown in Table 8.

Table 8 reveals a most divergent biochemical reaction pattern of the field strains although there is a certain degree of consistency with the classical scheme.

The strains belonging to group I conform completely to V. fetus, type 1 of Bryner, et al. Likewise, the strains of group IIa conform to V. fetus, type 2 of the same workers. However, it is peculiar that the two ovine fetal strains of group IIb are catalase negative, but in other respects are identical with V. fetus, type 2 in the classical scheme.

Group III is composed of 4 strains of different origin. As to the identification of these strains on biochemical basis, the question arises whether they might be a glycine tolerant V. fetus, type 1 or a H_2S-negative V. fetus, type 2.

While the 3 strains of group IVa conform to the typical V. bubulus the single strain of group IVb deviates in one essential respect; it is catalase positive.

The single strain of group Va conforms to V. fetus, subtype 1.

As to the remaining 5 groups with one strain each they share features partly with the two classical V. fetus types and partly with V. bubulus. Whether these strains represent occasional deviations from the common vibrio types or belong

Table 8. Biochemical reactions of the field strains

Group	Strain no.	Source	Catalase activity	Prod. of H_2S	Growth in the presence of 3.5% NaCl	Growth in the presence of 1% glycine	Growth in the presence of 2% ox bile
I	482	Bovine vagina	52 mm.	-	-	-	+
	671	Bovine vagina	95 mm.	-	-	-	+
	728	Bovine vagina	62 mm.	-	-	-	+
	758	Bovine vagina	80 mm.	-	-	-	+
	7212	Bovine prepuce	80 mm.	-	-	-	+
IIa	436	Bovine fetus	108 mm.	+	-	+	+
	653	Ovine fetus	45 mm.	+	-	+	+
	658	Ovine fetus	40 mm.	+	-	+	+
	665	Ovine fetus	79 mm.	+	-	+	+
IIb	652	Ovine fetus	0 mm.	+	-	+	+
	657	Ovine fetus	0 mm.	+	-	+	+
III	852	Bovine fetus	80 mm.	-	-	+	+
	676	Bovine semen	69 mm.	-	-	+	+
	664	Ovine fetus	85 mm.	-	-	+	+
	836	Ovine bile	83 mm.	-	-	+	+
IVa	5	Bovine vagina	0 mm.	+	+	+	-
	8	Bovine vagina	0 mm.	+	+	+	-
	810	Bovine semen	0 mm.	+	+	+	-
IVb	871	Ovine feces	88 mm.	+	+	+	-
Va	643	Bovine prepuce	77 mm.	+	-	-	+
Vb	831	Bovine bile	0 mm.	+	-	-	+
VIa	105	Ovine fetus	42 mm.	+	-	+	-
VIb	872	Ovine fetus	0 mm.	+	-	+	-
VII	250	Bovine vagina	34 mm.	+	-	-	-
VIII	739	Bovine semen	47 mm.	-	-	-	-

to other separate groups is not possible to say at the present time. In order to answer this question many more strains of various origins need to be tested.

Serological Reactions

The complement fixation test

Initial titrations in the CFT gave a hemolysin titer of 1:4,000 in the presence of a 1:20 dilution of guinea pig serum. As indicated before, 2 units of hemolysin were used for further titrations during the investigation. The glycerinated Difco-hemolysin was therefore invariably diluted 1:2,000 immediately before each titration.

The complement titer in the presence of 2 units of hemolysin was 0.10 ml. of a 1:20 dilution of the guinea pig serum. This was the exact unit, and the full unit was 0.15 ml. As indicated before, 2 full units of complement in a volume of 0.5 ml. were used for further titrations. The lyophilized Difco guinea pig serum was therefore diluted 1:33 immediately before each titration after resuspending in the prescribed amount of Kolmer saline.

Type strains The titers of the antigens and the antisera of the type strains are shown in Table 9. Neither the immune sera nor the normal rabbit serum were anticomplementary or caused any nonspecific lytic effect at a dilution of 1:5.

Table 9. Complement fixation titers of the type strains

Type of vibrio	Titer of serum	Titer of antigen
V. fetus, type 1	1:320	1:16
V. fetus, type 2	1:40	1:128
V. fetus, type 7	1:20	Undiluted
V. bubulus, type 3	1:40	Undiluted
V. bubulus, type 4	1:80	1:4
V. bubulus, type 5	1:20	Undiluted

None of the concentrated antigens were nonspecifically lytic. Only the antigens of V. fetus, type 1 and V. fetus, type 2 showed a slight anticomplementary effect; V. fetus, type 1 at a dilution of 1:2 and V. fetus, type 2 in undiluted condition.

With regard to nonspecific reactions with normal serum it was only the antigen of V. fetus, type 2 that reacted slightly (25% fixation) at a dilution of 1:2 with normal serum diluted 1:10. The slight anticomplementary effect of the antigens of V. fetus, types 1 and 2 and the slight nonspecific reaction of the antigen of V. fetus, type 2 were easily eliminated by dilution and caused no problem under the subsequent cross-titrations.

Neither the sera nor the antigens gave the titers one

had hoped they would give. The possible reasons for this will be discussed later. The serum titers ranked from 1:20 to 1:320. In the material of Mitscherlich (32) the serum titers vary from 1:40 to 1:1280. In this experiment the highest antigen titer was 1:128 and 3 of the antigens gave only a distinct reaction when used in undiluted condition. In the material of Mitscherlich the antigen titers vary from 1:4 to 1:128.

The results of the cross-titrations of the type sera are shown in Table 10. It may be seen from this table that the type sera are specific although their homologous titers are rather low. It is only the serum of V. fetus, type 7 that shows any noticeable degree of cross-reactivity as it reacts distinctly at a dilution of 1:10 with antigen of V. fetus, type 1 and V. bubulus, type 4.

Field strains The results of the tirations of antigens prepared from the field strains are presented in Table 11.

The two striking features of Table 11 are the large number of negative results and the considerable number of weak reactions with serum of V. fetus, type 7. Otherwise, anticomplementary effects and nonspecific reactions with normal serum are negligible. However, there are at least 3 strains which react significantly and distinctly enough to be identified by means of the CFT. These strains are strains numbered 436, 658 and 664. Antigens of strain no. 436, isolated from a

Table 10. Cross-titrations of vibrio type sera (the homologous serum titers are included)

Serum	Antigen (2 units)					
	V. fetus, type 1	*V. fetus*, type 2	*V. fetus*, type 7	*V. bubulus*, type 3	*V. bubulus*, type 4	*V. bubulus*, type 5
V. fetus, type 1	++++:1:320	-	-	-	-	-
V. fetus, type 2	-	++++:1:40	-	-	-	-
V. fetus, type 7	++++:1:10	-	++++:1:20	+:1:10	++++:1:10	-
V. bubulus, type 3	-	-	-	++++:1:40	-	-
V. bubulus, type 4	-	-	-	-	++++:1:80	-
V. bubulus, type 5	-	-	-	+:1:5	-	++++:1:20

As mentioned under the experimental procedure the titer of serum or antigen was interpreted as the highest dilution of serum or antigen, respectively, that gave complete fixation of complement. In order to indicate degrees of cross-reactions the following symbols have been used in Tables 10 and 11:

-: No fixation of complement
+: Twenty-five per cent fixation of complement
++: Fifty per cent fixation of complement
+++: Seventy-five per cent fixation of complement
++++: One hundred per cent fixation of complement

Table 11. Antigen titers of the field strains

Strain no.	Source	Serum (2 units) V. fetus, type 1	V. fetus, type 2	V. fetus, type 7	V. bubulus, type 3	V. bubulus, type 4	V. bubulus, type 5	Normal serum[a]	Anticpl. effect[b]
436	Bovine fetus	-	++++: 1:32	++: 1:4	-	-	-	-	+:undil.
852	Bovine fetus	-	-	++++: 1:4	-	-	-	-	-
5	Bovine vagina	-	-	-	-	-	-	-	++:undil.
8	Bovine vagina	-	++:1:2	++++: 1:4	+:1:2	-	++:1:2	-	-
250	Bovine vagina	++++: undil.	-	++++: 1:4	-	-	-	-	-
482	Bovine vagina	++++: undil.	-	++++: 1:4	+: undil.	-	++: undil.	+: undil.	-
671	Bovine vagina	-	-	++++: 1:4	-	-	-	-	-

[a]Nonspecific reaction with normal serum (1:10).

[b]Anticomplementary effect.

Table 11 (Continued)

Strain no.	Source	Serum (2 units) V. fetus, type 1	V. fetus, type 2	V. fetus, type 7	V. bubulus, type 3	V. bubulus, type 4	V. bubulus, type 5	Normal serum[a]	Anticpl. effect[b]
728	Bovine vagina	-	-	++++: 1:2	-	-	-	-	-
758	Bovine vagina	-	-	++++: 1:4	-	-	-	-	-
676	Bovine semen	-	-	++++: undil.	-	-	-	-	-
739	Bovine semen	++++: 1:4	-	+++: 1:8	-	-	+++: undil.	-	-
818	Bovine semen	-	-	++++: undil.	-	-	-	-	-
643	Bovine prepuce	-	-	++++: 1:4	-	-	-	-	-
7212	Bovine prepuce	++++: 1:4	-	++++: 1:4	+: 1:4	-	+:1:4	++:1:2	++: undil.
831	Bovine bile	-	-	++++: 1:2	-	-	-	-	-

Table 11 (Continued)

Strain no.	Source	Serum (2 units)						Normal serum[a]	Anticpl. effect[b]
		V. fetus, type 1	*V. fetus*, type 2	*V. fetus*, type 7	*V. bubulus*, type 3	*V. bubulus*, type 4	*V. bubulus*, type 5		
105	Ovine fetus	-	-	++++: 1:4	-	-	-	-	-
652	Ovine fetus	-	-	++++: 1:4	-	-	-	-	-
653	Ovine fetus	-	-	++++: undil.	-	-	-	-	-
657	Ovine fetus	-	-	++: 1:8	-	-	-	-	-
658	Ovine fetus	-	++++: 1:16	++++: 1:4	-	-	-	-	-
664	Ovine fetus	++++: 1:8	-	-	-	-	-	-	+:1:4
665	Ovine fetus	-	-	-	-	-	-	-	-
836	Ovine bile	+++: 1:4	-	++: 1:4	-	-	+:1:4	+++: 1:2	++++: undil.
871	Ovine feces	-	-	-	-	-	-	-	-

Table 11 (Continued)

Strain no.	Source	Serum (2 units) V. fetus, type 1	V. fetus, type 2	V. fetus, type 7	V. bubulus, type 3	V. bubulus, type 4	V. bubulus, type 5	Normal serum[a]	Anticpl. effect[b]
872	Ovine feces	-	-	-	-	-	-	-	-
Positive control (2 units)		++++	++++	++++	++++	++++	++++	-	-

bovine fetus, and of strain no. 658, isolated from an ovine fetus, react at dilutions of 1:32 and 1:16, respectively with serum against V. fetus, type 2. Antigen of strain no. 664, also isolated from an ovine fetus, reacts at a dilution of 1:8 with serum against V. fetus, type 1. Considering the antigen titers obtained by Mitscherlich, these reactions are significant enough. Although the extensive but low-titered reactions of so many antigens with serum against V. fetus, type 7 apparently are specific in most cases, the author dares not draw any conclusions about the identity of these strains. Both these peculiar reactions on the one hand and the extensive lack of reaction will be discussed later.

The fluorescent antibody test

Type strains When time finally was due for conjugation of the type sera with FITC the author was fully aware that the titers of these sera were not particularly suitable for this purpose. However, as the disposable time was short and the sera had proved to be specific enough in the CFT, conjugation was performed. The results are shown in Table 12. It may be seen from this table that the type strains were stained specifically with homologous FITC conjugated globulin. Moreover, the strains were not stained with conjugated normal globulin. But the fluorescence which was observed was rather poor and unfortunately not suitable for any photography. Moreover, the two-step blocking test was unsuccessful

Table 12. Staining of type strains with fluorescent antibody conjugates

Smear	*V. fetus*, type 1	*V. fetus*, type 2	*V. fetus*, type 7	*V. bubulus*, type 3	*V. bubulus*, type 4	*V. bubulus*, type 5	Conjugate of normal serum
V. fetus, type 1	+	-	-	-	-	-	-
V. fetus, type 2	-	+	-	-	-	-	-
V. fetus, type 7	-	-	+	-	-	-	-
V. bubulus, type 3	-	-	-	+	-	-	-
V. bubulus, type 4	-	-	-	-	+	-	-
V. bubulus, type 5	-	-	-	-	-	+	-

indicating that the unconjugated homologous sera were inadequate in inhibiting the subsequent staining with the homologous conjugates.

Field strains The results obtained by staining the field strains with the 6 type specific conjugates plus conjugated normal globulin are shown in Table 13. It may be seen from Table 13 that none of the 25 field strains were stained nonspecifically with conjugated normal globulin. Eleven of the 25 strains were not stained with any of the type-specific conjugates. The remaining 14 strains stained distinctly and specifically either with conjugate of V. fetus, type 1 or V. fetus, type 2. In two cases (strain no. 643 and 728) where distinct fluorescence was caused by the conjugate of V. fetus, type 1, only a faint fluorescence was caused by the conjugate of V. fetus, type 2. In two cases (strain no. 852 and 7212) distinct fluorescence occurred with the conjugate of V. fetus, type 1 but only a negligible fluorescence with the conjugate of V. fetus, type 7. In addition to these 4 strains mentioned, 8 more strains (nos. 8, 105, 250, 482, 671, 676, 739 and 758) were stained distinctly and specifically with the conjugate of V. fetus, type 1. All these 12 strains showed low-titered reactions with serum against V. fetus, type 7 in the CFT. Thus, the peculiar reactions with serum against V. fetus, type 7 in the CFT were eliminated in the FAT. Noteworthy also is that the two strains (no. 436 and 658) which

Table 13. Staining of field strains with fluorescent antibody conjugates

Smear of strain no.	Source	Conjugates						
		V. fetus, type 1	V. fetus, type 2	V. fetus, type 7	V. bubulus, type 3	V. bubulus, type 4	V. bubulus, type 5	Normal serum
436	Bovine fetus	-	+	-	-	-	-	-
852	Bovine fetus	+	-	(+)	-	-	-	-
5	Bovine vagina	-	-	-	-	-	-	-
8	Bovine vagina	+	-	-	-	-	-	-
250	Bovine vagina	+	-	-	-	-	-	-
482	Bovine vagina	+	-	-	-	-	-	-
671	Bovine vagina	+	-	-	-	-	-	-
728	Bovine vagina	+	(+)	-	-	-	-	-
758	Bovine vagina	+	-	-	-	-	-	-
676	Bovine vagina	+	-	-	-	-	-	-
739	Bovine semen	+	-	-	-	-	-	-
818	Bovine semen	-	-	-	-	-	-	-
643	Bovine prepuce	+	(+)	-	-	-	-	-

Table 13 (Continued)

Smear of strain no.	Source	Conjugates						
		V. fetus, type 1	V. fetus, type 2	V. fetus, type 7	V. bubulus, type 3	V. bubulus, type 4	V. bubulus, type 5	Normal serum
7212	Bovine prepuce	+	-	(+)	-	-	-	-
831	Bovine bile	-	-	-	-	-	-	-
105	Ovine fetus	+	-	-	-	-	-	-
652	Ovine fetus	-	-	-	-	-	-	-
653	Ovine fetus	-	-	-	-	-	-	-
657	Ovine fetus	-	-	-	-	-	-	-
658	Ovine fetus	-	+	-	-	-	-	-
664	Ovine fetus	-	-	-	-	-	-	-
665	Ovine fetus	-	-	-	-	-	-	-
836	Ovine bile	-	-	-	-	-	-	-
871	Ovine feces	-	-	-	-	-	-	-
872	Ovine feces	-	-	-	-	-	-	-

reacted distinctly in the CFT with serum against V. fetus, type 2 were consistently stained with the conjugate of the same vibrio type in the FAT.

Comparison Between the Three Methods Used for Differentiation

The results obtained by means of the three methods are shown in Table 14. As all the low-titered reactions with serum of V. fetus, type 7 in the CFT are not considered to be significant these reactions are recorded as negative in Table 14. It appears from this table that 6 of the 25 strains do not react in any of the serological tests. Nor do these strains show biochemical reactions which allow them to be grouped in the classical scheme. The 19 remaining strains react in one or more of the three methods used. Where reactions occur in two of the methods there is inconsistency in two cases. Strain no. 8 is biochemically V. bubulus (Bryner, et al.) but serologically V. fetus, type 1 in the FAT. Strain no. 643 is biochemically V. fetus, subtype 1, but V. fetus, type 1 in the FAT. Only in two cases are there complete consistency between the three methods. Strains numbered 436 and 658 react distinctly and consistently in all methods and appear to be V. fetus, type 2. Being aware of the very limited material as a basis for conclusions, this all the same indicates that V. fetus, type 2 (Bryner, et al.) is identical with

Table 14. Comparison between the three methods used for differentiation

Strain no.	Source	Biochemical method (Bryner, et al.)	Complement fixation test (Mitscherlich)	Fluorescent antibody test, as based on the complement fixation test
436	Bovine fetus	V. fetus, type 2	V. fetus, type 2	V. fetus, type 2
852	Bovine fetus	?	-	V. fetus, type 1
5	Bovine vagina	V. bubulus	-	-
8	Bovine vagina	V. bubulus	-	V. fetus, type 1
250	Bovine vagina	?	-	V. fetus, type 1
482	Bovine vagina	V. fetus, type 1	-	V. fetus, type 1
671	Bovine vagina	V. fetus, type 1	-	V. fetus, type 1
728	Bovine vagina	V. fetus, type 1	-	V. fetus, type 1
758	Bovine vagina	V. fetus, type 1	-	V. fetus, type 1
676	Bovine vagina	?	-	V. fetus, type 1
739	Bovine semen	?	-	V. fetus, type 1
818	Bovine semen	V. bubulus	-	-
643	Bovine prepuce	V. fetus, subtype 1	-	V. fetus, type 1

Table 14 (Continued)

Strain no.		Biochemical method (Bryner, et al.)	Complement fixation test (Mitscherlich)	Fluorescent antibody test, as based on the complement fixation test
7212	Bovine prepuce	V. fetus, type 1	-	V. fetus, type 1
831	Bovine bile	?	-	-
105	Ovine fetus	?	-	V. fetus, type 1
652	Ovine fetus	?	-	-
653	Ovine fetus	V. fetus, type 2	-	-
657	Ovine fetus	?	-	-
658	Ovine fetus	V. fetus, type 2	V. fetus, type 2	V. fetus, type 2
664	Ovine fetus	?	V. fetus, type 1	-
665	Ovine fetus	V. fetus, type 2	-	-
836	Ovine bile	?	-	-
871	Ovine feces	?	-	-
872	Ovine feces	?	-	-

V. fetus, type 1 (Mitscherlich) or V. fetus intestinalis (Florent). This is also entirely consistent with the sources of isolation of these two strains; strain no. 436 from a bovine fetus and strain no. 658 from an ovine fetus. Moreover, it may be seen that 5 strains, 4 of them isolated from the bovine vagina and one from the bovine prepuce, prove to be V. fetus, type 1 both in the biochemical method and the FAT. Unfortunately none of these strains reacted in the CFT. Provided the reaction in the FAT, which was distinct enough, is significant, this would indicate that V. fetus, type 1 (Bryner, et al.) is identical with V. fetus, type 1 (Mitscherlich) or V. fetus intestinalis (Florent). Five of the strains which were identified as V. fetus, type 1 by the FAT could not be identified by the biochemical method. Since neither of these strains reacted in the CFT, no conclusions can be drawn whether the FAT, as based on the CFT, is a more reliable method for differentiation than the biochemical method.

DISCUSSION

Introduction

It should be emphasized that the author is fully aware that the results to be discussed are rather inadequate and it is regrettable that the results did not prove to be more conclusive.

Observations

Biochemical tests

It appears that three of the type strains and 12 of the field strains cannot be classified according to the conventional biochemical test scheme. These discrepancies give rise to three alternative questions:

1. Do these strains represent distinct groups per se, separable by means of individual well-defined biochemical reaction patterns?
2. Are they occasional variants that diverge from the common well-defined biochemical types?
3. Have their physiological characteristics undergone changes during prolonged maintenance and repeated subcultures on artificial media?

In order to decide whether these aberrant strains represent biochemically distinct groups many more strains of various origin need to be tested.

If the strains are atypical variants of the more common

biochemical types, then it must be concluded that the conventional biochemical tests do not serve as a reliable method for differentiation. As mentioned in the literature review there are varying degrees of confidence in the biochemical tests. Bryner and Frank (9, 10) and Ringen and Frank (46) readily managed to classify vibrios of bovine and ovine origin as one of the three types which are now generally recognized, namely V. fetus venerialis, V. fetus intestinalis or V. bubulus. On the other hand, Kamel (24) and Mitscherlich (32) did not obtain uniform results with the biochemical tests. An intermediate position is occupied by Bryans and Smith (8) who maintain that only the catalase test and the salt tolerance test are of value as criteria of pathogenicity of a vibrio strain.

As to the possibility of vibrios undergoing changes during prolonged maintenance in an artificial milieu opinions also differ. Bond (7) maintains that vibrios grown on semisolid media easily undergo colonial, biochemical and serological changes while they remain stable on solid media. When the original smooth (S) form changes to the mucoid (M) form it becomes catalase negative and H_2S-positive. If this commonly occurs it may explain at least some of the discrepancies in the author's results because the strains were maintained on semisolid thiol and thioglycollate medium. However, the more recent observations of Bryner, et al. (10), who worked with a considerably larger material (87 strains)

than Bond (10 strains), give evidence that the biochemical activity of colonial variants do not differ from their smooth parent cultures.

The complement fixation test

As far as the type strains are concerned it may be speculated why the sera, prepared against whole cells, did not give higher titers. These varied from 1:20 to 1:320. It is a well-known fact that the CFT is not a particularly sensitive test. The worker who introduced this test for the purpose of differentiating between various vibrios did not operate with any particular high-titered antisera. With a few exceptions they varied from 1:40 to 1:640. This may be due to the fact which several workers have experienced, that it is far more difficult to produce sera of high titers against Vibrio sp. than against Brucella sp. However, the sera which were produced in this case were, with one exception, specific. This combination of rather poorly but specific immunsera is in agreement with the general concept of antibody production. According to Raffel (43) the physicochemical properties of antibodies produced in an experimental animal depend on four factors:

1. The antigen used.
2. The animal species used for immunization.
3. The route of administration.
4. The duration of the vaccination period.

The relatively poor antigenicity of vibrio organisms has just been mentioned. Whether a different animal species than the rabbit and a different inoculation route than the intravenous one, might better have stimulated the antibody production, remain to be investigated. What might have influenced the results in this experiment is the rather short vaccination period. The rabbits were bled only 3 weeks after the first inoculation. It is known that during the early stages of an immunization period there are predominantly produced rather specific but poorly reactive antibody molecules. These molecules belong to the 19S or $gamma_1$ globulins which have a molecular weight of about 900,000. Later in the immunization process there are produced less specific but more reactive antibody molecules. These molecules belong to the 7S or $gamma_2$ globulins which have a molecular weight of about 150,000. Both the difference in specificity and reactivity may be attributed to the fact that some of the 19S antibodies are univalent and the 7S antibodies multivalent; i.e., the multivalent antibodies possess multiple combining sites. This may explain why the antisera produced in this case were rather specific but poorly reactive.

Another factor to be considered is the great individual variations in the ability of experimental animals to produce antibodies. Sera should therefore be pooled from several rabbits immunized with each bacterial strain. Since some of

the rabbits in this experiment were used for preliminary immunization with antigen extracts only one rabbit was left for immunization with each of the 6 type strains.

With regard to the field strains two things are striking:

1. 20 of the 25 strains react, although at rather low titers, distinctly with serum against V. fetus, type 7, and,
2. only three strains react specifically at significant titers with serum against one of the other type strains.

It would be most peculiar if 20 of 25 or 80% of the unknown strains should be identified as V. fetus, type 7. Kamel, who was the first one to identify this vibrio type, found only 4 strains or 2.5% of V. fetus, type 7 out of a material of 160 strains (24). That all the low-titered reactions are non-specific and therefore have been recorded as negative are strongly supported by two observations. The serum against V. fetus, type 7 was the only one which showed any degree of cross-reaction; it reacted both with V. fetus, type 1 and V. bubulus, type 4. Moreover, the same degree of reaction with this particular serum occurred in the two cases (strains numbered 436 and 658) where significant reaction occurred with serum against V. fetus, type 2. If the reactions with V. fetus, type 7 serum on this basis are considered insignificant and negative, then the following question arises; how

shall all the negative reactions be interpreted? Apart from significant reaction with type 2 serum in two cases and with type 1 serum in one case the remaining 22 strains did not react specifically with any of the type sera. Does this indicate that the majority of the 25 field strains are antigenically unrelated to the 6 different field strains, or in other words; are indigenous bovine and ovine vibrio strains, isolated in one country, serologically unrelated to indigenous bovine and ovine strains isolated in a different country? This possibility is hardly probable. Although there are not many reports on international exchange of vibrio strains, there are at least two which indicate that strains isolated in various countries are serologically identical or related. Morgan (35) worked with a great number of bovine and ovine fetal strains collected from England, Denmark and U.S.A. Only 2 O-groups were established by means of the agglutination test and there was no consistency between the groups and country of isolation. Wiidik and Hlidar (68) found the capsular or K-antigen to be qualitatively the same in 5 V. fetus strains, 3 of which were isolated in Denmark, one in Sweden and one in Holland. Therefore, it is more probable that the great number of negative results in this investigation is due to poorly reactive antigens and/or sera. As to the sensitivity of the antigens there are several reports (5, 7, 49, 53) dealing with loss of specificity and sensitivity as the

cultures undergo variation during prolonged growth in artificial media. Whether this could be a current factor in the present investigation is not known. With particular regard to this question Mitscherlich was consulted. According to his experiences repeated subcultures of strains in semi-liquid media had never influenced the results observed by means of the CFT.[1] Moreover, if the antigens had developed a broader reaction pattern they should also react unspecifically with other type sera than the serum against V. fetus, type 7.

The fluorescent antibody test

While only 3 of the field strains reacted in the CFT, 14 strains reacted in the FAT. Only one strain (no. 664) reacted in the CFT but not in the FAT. As no reactions with conjugated normal globulin occurred this indicates that the FAT is far more sensitive than the CFT. In other words, only a small number of FITC-tagged antibody molecules are needed to give a visible reaction in the FAT.

Comparison between the three methods used for differentiation

The first purpose of this investigation was to compare the results obtained by means of the biochemical method and the CFT and thereby assess which of the methods is the most reliable one. Unfortunately this is not possible because only

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[1]E. Mitscherlich, Tierärztliches Institut der Universität, Göttingen, Germany. Sensitivity of vibrio antigens in the CFT. Private communication. September 26, 1964.

3 of the field strains react distinctly and significantly in the CFT. Two of these strains (no. 436 and 658) prove to be the same type of vibrio when tested by both methods. On this basis the tentative conclusion might be drawn that the two methods are equally reliable. However, the third strain (no. 664) is, according to the CFT, V. fetus, type 1 but does not fit consistently into the biochemical test scheme. It seems strange that an ovine fetal strain should be V. fetus, type 1. However, this type of vibrio fetus has been isolated from the ovine fetus (32). Pathogenicity tests would be needed to decide whether strain no. 664 is V. fetus, type 1 or not, and thereby make it clear whether this strain can only be identified by means of the CFT.

The second purpose of this investigation was to see whether the FAT, when based on the CFT might serve as a more easy and rapid diagnostic tool than the CFT itself. Unfortunately, this question can not be answered positively because the field strains, with the two exceptions mentioned, either do not react in any of the two tests or in only one of them.

With regard to future prospects the author is of the opinion that there is still much work to be done. Yet there is no generally accepted method of differentiation of various types of vibrio organisms. Considering the great problem vibrio infection still represents in many countries the need for such

a method is obvious. Imagine only a case in which a first prize bull is found infected with V. fetus venerialis. The use of the bull is, of course, prohibited resulting in serious economic losses, and much trouble both to farmers and to the owner of the bull. Then imagine that the diagnosis, V. fetus venerialis, was false because the diagnostic methods were not reliable enough. Not only for academic reasons but also for practical, economic and forensic reasons reliable differential diagnostic methods are seriously needed. Moreover, vibrio strains, isolated from various sources in different countries, should be exchanged and examined in order to establish their homogeneity or heterogeneity. Although the results of the author's investigations were more frustrating than promising, the conviction still exists that the pertinent problem ought to be solved and that it can be solved. Satisfactory V. fetus fluorescent antibody conjugates have been prepared for the detection of V. fetus infection in bulls (20, 30). With careful selection of properly antigenic type strains it should be also possible to prepare type specific conjugates, satisfactory for differentiation between unknown strains.

CONCLUSIONS

1. Thirteen out of 25 vibrio strains of bovine and ovine origin could be identified by means of the biochemical test scheme of Bryner, et al.

2. Only 3 out of the 25 strains could be identified by means of the complement fixation test of Mitscherlich and Liess.

3. Fourteen out of the 25 strains could be identified by means of fluorescent antibody conjugates prepared against strains which had previously been identified by means of the complement fixation test. Therefore, the fluorescent antibody test proved to be a more sensitive method than the complement fixation test.

4. Two strains were consistently identified by means of all three methods. Because of the large number of negative results in the complement fixation test and in the fluorescent antibody test no conclusions could be drawn as to the relative reliability of the three methods employed.

5. The negative serological results are attributed to the rather poorly reactive antigens and antisera employed. Careful selection of strongly antigenic strains for production of immune sera, and more potent antigens might possibly improve the results.

SUMMARY

Whole cell antisera were prepared against 6 German vibrio strains which, according to the complement fixation test of Mitscherlich and Liess (34) had been identified as V. fetus, types 1, 2 and 7, and V. bubulus, types 3, 4 and 5, respectively. Twenty-five American field strains of bovine and ovine origin were tested by means of three methods:

1. The biochemical tests established by Bryner, et al. (10).
2. The complement fixation test of Mitscherlich and Liess.
3. The fluorescent antibody test introduced by the author. This test was a supplement to the CFT as the sera prepared against the German type strains were conjugated with fluorescein isothiocyanate (FITC). The purpose was to see whether this test could be used as an easier and more rapid diagnostic tool than the CFT itself.

Thirteen out of the 25 field strains could be identified by means of the biochemical tests. Only 3 strains could be identified by means of the CFT. Fourteen strains could be identified by means of the FAT. Thus, the FAT appeared to be a more sensitive test than the CFT itself. Only two of the 25 strains, one from a bovine fetus and one from an ovine fetus, were consistently identified as V. fetus, type 2 by

means of all three methods. Because of the large number of negative results in the CFT and in the FAT no conclusions could be drawn as to the relative reliability of the three methods employed. However, it is hoped that the production of more potent sera and antigens may improve the results in future work.

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