MONTANA STATE UNIVERSITY

Comparisons of serologic and physiologic groups of Vibrio fetus
by Russell Lowell Berg

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Abstract:
Various serologic and physiologic characteristics of 62 Vibrio fetus isolates were compared.

The Marsh and Firehammer serotypes were determined by agglutination reactions between whole-cell antigens and antisera prepared against specific whole-cell antigens® Somatic serotypes were determined by agglutination reactions between boiled antigens and absorbed whole-cell antisera® Heat-labile antigens were determined by agglutination reactions between whole-cell antigens and antisera which had been absorbed with homologous boiled antigen and also with specific whole-cell antigens® Glycine tolerance was indicated, if growth occurred in medium containing 1% glycine® Hydrogen sulfide production was measured by suspending filter paper strips impregnated with lead acetate over a medium containing 0.02% cysteine.

The Marsh and Firehammer (MF) serotyping system divides V. fetus into four serotypes. Serotype III (MF) consists of V. fetus var. venerealis (glycine intolerant. Serotypes I, II, and V consist of V. fetus var. intestinalis (glycine tolerant).

Three somatic serotypes were demonstrated. Somatic serotype A of Morgan is identical to somatic serotype 1 of Mitscherlich and Liess and contains both serotype III and V (MF) isolates. Somatic serotype B of Morgan is identical to somatic serotype 2 of Mitscherlich and -Liess and consists of serotype II (MF) isolates. Somatic serotype C consists of serotype I (MF) isolates® The glycine tolerance test is reliable for separating the venerealis variety of somatic serotype A isolates from the intestinalis variety of somatic serotype A V. fetus isolates.

Physiologic type 1 (glycine intolerant, H2S negative) consists of serotype III (MF) isolates. Physiologic subtype 1 (glycine intolerant, H2S positive) predominantly consists of serotype III (MF) isolates. Physiologic type 2 (glycine tolerant, H2S positive) consists of serotype I, serotype II and serotype V (MF) isolates.

All serotype I (MF) isolates grew at 45°C, but were the only isolates which grew at this high temperature. All serotype II (MF) isolates grew at 42°C. Some of the serotype V (MF) isolates grew at 42°C, while others failed to grow at 42°C, but grew at 37°C, Isolates which were classified both as serotype III (MF) and as physiologic type 1 grew at 37°C, but hot at 42 or 45°C, All physiologic type 1 isolates grew at 37°C, but failed to grow at 42 or 45°C Physiologic subtype 1 and type 2 isolates grew at all three temperatures.

At least seven heat-labile antigens are present in V. fetus isolates. These antigens may be immunogenically important and may lead to a practical means of diagnosing vibriosis®

COMPARISONS OF SEROLOGIC AND
PHYSIOLOGIC GROUPS OF <u>VIBRIO</u> <u>FETUS</u>

by

RUSSELL LOWELL BERG

A thesis submitted to the Graduate Faculty in partial
fulfillment of the requirements for the degree

of

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in

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ABSTRACT

Various serologic and physiologic characteristics of 62 _Vibrio fetus_ isolates were compared.

The Marsh and Firehammer serotypes were determined by agglutination reactions between whole-cell antigens and antisera prepared against specific whole-cell antigens. Somatic serotypes were determined by agglutination reactions between boiled antigens and absorbed whole-cell antisera. Heat-labile antigens were determined by agglutination reactions between whole-cell antigens and antisera which had been absorbed with homologous boiled antigen and also with specific whole-cell antigens. Glycine tolerance was indicated if growth occurred in medium containing 1% glycine. Hydrogen sulfide production was measured by suspending filter paper strips impregnated with lead acetate over a medium containing 0.02% cysteine.

The Marsh and Firehammer (MF) serotyping system divides _V. fetus_ into four serotypes. Serotype III (MF) consists of _V. fetus_ var. _venerealis_ (glycine intolerant). Serotypes I, II, and V consist of _V. fetus_ var. _intestinalis_ (glycine tolerant).

Three somatic serotypes were demonstrated. Somatic serotype A of Morgan is identical to somatic serotype 1 of Mitscherlich and Liess and contains both serotype III and V (MF) isolates. Somatic serotype B of Morgan is identical to somatic serotype 2 of Mitscherlich and Liess and consists of serotype II (MF) isolates. Somatic serotype C consists of serotype I (MF) isolates. The glycine tolerance test is reliable for separating the _venerealis_ variety of somatic serotype A isolates from the _intestinalis_ variety of somatic serotype A _V. fetus_ isolates.

Physiologic type 1 (glycine intolerant, H_2S negative) consists of serotype III (MF) isolates. Physiologic subtype 1 (glycine intolerant, H_2S positive) predominantly consists of serotype III (MF) isolates. Physiologic type 2 (glycine tolerant, H_2S positive) consists of serotype I, serotype II and serotype V (MF) isolates.

All serotype I (MF) isolates grew at 45 C, but were the only isolates which grew at this high temperature. All serotype II (MF) isolates grew at 42 C. Some of the serotype V (MF) isolates grew at 42 C, while others failed to grow at 42 C, but grew at 37 C. Isolates which were classified both as serotype III (MF) and as physiologic type 1 grew at 37 C, but not at 42 or 45 C. All physiologic type 1 isolates grew at 37 C, but failed to grow at 42 or 45 C. Physiologic subtype 1 and type 2 isolates grew at all three temperatures.

At least seven heat-labile antigens are present in _V. fetus_ isolates. These antigens may be immunogenically important and may lead to a practical means of diagnosing vibriosis.

CHAPTER I

INTRODUCTION

Classification of <u>Vibrio</u> <u>fetus</u> has been confusing because many ser-
ologic and physiologic typing systems were developed by different workers
who did not correlate results.

<u>Vibrio</u> <u>fetus</u> causes important reproductive diseases in cattle and
sheep and occasionally infects man. Recent surveys (Hoerlein et al.,
1964) indicate that vibriosis is rapidly spreading through cattle herds
in the United States. Control and diagnosis of this disease requires
more knowledge of the characteristics of <u>V</u>. <u>fetus</u>. This knowledge may
lead to practical diagnostic procedures and more effective control
through therapeutic agents.

The purpose of the present study was to investigate and compare
serologic and physiologic types of numerous <u>V</u>. <u>fetus</u> isolates.

CHAPTER II

REVIEW OF LITERATURE

Identification

For many years physiologic criteria for identification of $V.$ fetus remained loosely defined and early attempts to identify this species by serologic reactions were unsatisfactory. In recent years more rigid criteria have been adopted which better characterize pathogenic and saprophytic species of Vibrio.

Florent (1953) described a Vibrio isolated from bull semen and cow vaginas which was more anaerobic than $V.$ fetus and produced an abundance of H_2S*. Thouvenot and Florent (1954) proposed that this organism be called Vibrio bubulus.

Bryner and Frank (1955) studied the physiologic characteristics of vibrios isolated from bovine fetuses and reproductive organs of bulls and cows. They concluded that catalase-positive vibrios were $V.$ fetus, while catalase-negative vibrios were not.

Firehammer (1965) described another catalase-positive Vibrio and proposed that this organism, which was isolated from sheep feces, be called Vibrio fecalis. In contrast to $V.$ fetus, this species is H_2S* positive. It resembles $V.$ bubulus in many respects, but differs from it by producing large amounts of catalase.

On the basis of catalase and H_2S tests, these three species can be

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* H_2S production measured by an "insensitive" method such as SIM (Difco Laboratories, Detroit, Michigan) or Triple-Sugar Iron Agar (Difco) or by suspending a strip of filter paper saturated with lead acetate over a medium which does not contain an added source of sulfur.

differentiated as follows: $\underline{V}$. $\underline{bubulus}$ (catalase-negative, H_2S* positive),
$\underline{V}$. $\underline{fetus}$ (catalase-positive, H_2S* negative), $\underline{V}$. $\underline{fecalis}$ (catalase positive,
H_2S* positive). All three species are gram-negative, curved rods. They
are all microaerophilic, and all reduce nitrates to nitrites. Because of
the biochemical inertness of these species, other usual methods of classi-
fication cannot be used except that $\underline{V}$. $\underline{fetus}$ is indicated by lack of act-
ivity. Of these three species $\underline{V}$. $\underline{fetus}$ is the only one considered patho-
genic.

Physiologic groups of $\underline{V}$. $\underline{fetus}$

Akkermans et al. (1956) isolated two varieties of $\underline{V}$. $\underline{fetus}$ from cattle.
One variety was weakly positive for H_2S** and was found only in sporadic
cases of abortion; the second variety was H_2S** negative and was isolated
from the genitals of cows and bulls involved in outbreaks of vibriosis re-
sulting in infertility.

Florent (1959) studied both varieties and suggested that the variety
causing epidemics of infertility be named $\underline{V}$. $\underline{fetus}$ var. $\underline{venerealis}$ and that
the other variety be named $\underline{V}$. $\underline{fetus}$ var. $\underline{intestinalis}$. He was able to
isolate $\underline{V}$. $\underline{fetus}$ var. $\underline{intestinalis}$ from the intestinal tracts of cattle,
sheep, and pigs.

Leece (1958) reported that $\underline{V}$. $\underline{fetus}$ isolates from sheep tolerated 0.8%

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* H_2S measured by an "insensitive" method.
** H_2S measured by a "sensitive" method. A strip of filter paper
saturated with lead acetate is suspended over a growth medium con-
taining an added source of sulfur such as 0.02% cystine.

glycine in the growth medium, while the majority of isolates from cattle did not. Ringen and Frank (1963) found the glycine tolerance test to be the only reliable laboratory method for differentiating V. fetus var. venerealis (glycine intolerant) from V. fetus var. intestinalis (glycine tolerant).

Both Bryner et al. (1962) and Mohanty et al. (1962) reported that V. fetus isolates from cattle could be divided into three groups on the basis of H_2S* production and ability to tolerate 1.0% glycine. Bryner et al. called the three groups type 1 (B)[1], subtype 1 (B) and type 2 (B). Type 1 (B) was H_2S* negative and could not tolerate 1.0% glycine. Subtype 1 (B) differed from type 1 (B) in that it produced H_2S*. Type 2 (B) produced H_2S* and differed from both type 1 (B) and subtype 1 (B) by tolerating 1.0% glycine. Type 2 (B) was identical with V. fetus var intestinalis, while the other two types (B) were considered to be V. fetus var. venerealis. Experimental results (Bryner et al., 1964) indicated that type 1 (B) and subtype 1 (B) were not able to survive a gastrointestinal environment. After oral inoculation of all three types (B) into different lots of cattle, only type 2 (B) isolates could be recovered from the feces and digestive tracts.

It is generally agreed that V. fetus var. venerealis is spread venereally, is the most common cause of vibrionic infertility in cattle and only

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[1] The physiologic types of Bryner et al., (1962).
* Detected only if measured by the "sensitive" method.

occasionally causes bovine abortion. *V. fetus* var. *intestinalis* is undisputedly the only natural cause of vibriosis in sheep. However, there is controversy as to its significance in cattle vibriosis. Most authors agree that this variety can cause sporadic abortions in cattle. Akkermans et al. (1956), Terpstra (1956), Bryner et al. (1964) and Hoppe and Ryniewicz (1961) found that *V. fetus* var. *intestinalis* does not cause infertility. Florent (1959), Hoerlein and Kramer (1963), Bryner et al. (1964) and Wagner et al. (1961) found that *V. fetus* var. *intestinalis* could not survive for long periods in genital tracts of cattle. Therefore, all these authors concluded that this variety did not cause the common, rapidly spreading, venereal type of vibriosis.

Park et al. (1962) found that *V. fetus* var. *intestinalis* (obtained from bulls) could be readily transferred venereally; it could also be transferred by intrauterine inoculation with vaginal mucus or with *Vibrio* cultures obtained from a previously inoculated animal. *V. fetus* var. *intestinalis* could be isolated from many of these animals 12 months after inoculation. Florent (1963) worked with isolates obtained from Park and conceded that Park's work was correct. He felt that these *V. fetus* var. *intestinalis* isolates were somewhat different from those commonly encountered.

Vinzent et al. (1947) published the first report of an abortion in man due to *V. fetus*. Since then *V. fetus* has been recognized as a cause of many human infections of different types (King, 1962). King (1957) reported 11 cases of human vibrionic infections. According to her, *V. fetus* was isolated from seven infections, and four infections were caused by a

"closely related <u>Vibrio</u> species", which she designated as "related vibrios".
In temperature tolerance studies, she found that <u>V</u>. <u>fetus</u> isolates grew
well at 25 C, but failed to grow at 42 C. The "related vibrios" grew well
at 42 C, but failed to grow at 25 C. The "related vibrios" were also anti-
genically different.

Firehammer and Berg (1965) studied the temperature tolerance of 16
serotypes I (MF)[2] isolates, 24 serotype III (MF) isolates, 10 serotype V
(MF) isolates, and four isolates of "related vibrios"[3]. Both serotype III
(MF) and serotype V (MF) isolates were divided into two temperature tol-
erance groups. One group grew at 25, 37, and 42 C; the other group failed
to grow at 42 C. The serotype I (MF) isolates and the "related vibrios"
had the same temperature tolerances. They grew well at 37 and 42 C, but
did not grow at 25 C.

Three of the four "related vibrios" were serotyped according to the
system of Marsh and Firehammer (1953). Of these three, two[4] were serotype
I (MF) and one did not fit any of the types of this system. No signi-
ficant morphologic, biochemical, or physiologic differences were observed
between the "related vibrios" and the serotype I (MF) isolates.

Serologic groups

The discovery by McFadyean and Stockman (1913) that <u>V</u>. <u>fetus</u> was the

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[2] The serologic typing system of Marsh and Firehammer (MF) (1953).
[3] The "related vibrios" were supplied through the courtesy of the late
Miss E. O. King, Communicable Disease Center, Atlanta, Georgia.
[4] One did not agglutinate at serum dilutions greater than 1/50 and was
considered negative in the original paper.

causative agent of epidemic abortions in sheep prompted many workers to investigate the serologic properties of this species. Much of the work was done in attempts to identify the species or in attempts to develop a serologic means of diagnosing vibriosis.

Smith and Taylor (1919) examined 22 Vibrio isolates from cattle fetuses and two from calves. Results of agglutination tests indicated that 21 of the isolates from fetuses were identical, while one deviated slightly. The two isolates from calves were distantly related to the isolates from fetuses. Smith and Orcutt (1927) investigated by the agglutination process five Vibrio cultures isolated from cattle fetuses and two isolated from calves. They concluded that these seven Vibrio cultures had at least four different antigenic factors of which each isolate could possess three.

Blakemore and Gledhill (1946) worked with four V. fetus isolates from sheep and found three somatic serotypes. Levi (1950) found that serum from infected sheep produced high agglutination titers when mixed with antigens prepared with isolates from the same sheep. Plastridge et al. (1951) concluded that V. fetus isolated from cattle belonged in a different serotype than those isolated from sheep. Gallut (1952a) investigated four V. fetus isolates from man and six V. fetus isolates from animals. He found six different antigens and six haptenes.

Gallut (1952b) used a precipitation test to differentiate V. fetus. For each of ten isolates investigated, he obtained both a phenol soluble and a phenol insoluble fraction. The purified phenol soluble fraction appeared to be protein in character and was precipitated by antiserum specific for V. fetus. The phenol insoluble fraction appeared to be

polysaccharide in character. It was precipitated in high titer by homologous V. fetus serum, but only irregularly by heterologous V. fetus serum.

Marsh and Firehammer (1953) performed cross-agglutination studies on three V. fetus isolates from cattle and 23 V. fetus isolates from sheep. They found that the sheep isolates belonged in four serotypes (I (MF), II (MF), IV (MF), and V (MF), while the bovine isolates belonged in a fifth (serotype III (MF)). Serotype I (MF) was distinctly differentiated from the other four types. The other four types, three containing sheep isolates and one containing cattle isolates, cross-reacted with each other.

Price et al. (1955), using the agglutination test, found four somatic serotypes when 14 antisera prepared from 14 boiled antigens were absorbed with different combinations of the boiled antigens. A boiled antigen of ovine origin was serologically unrelated to the isolates of bovine origin.

Bryner and Frank (1955) immunized 25 rabbits with catalase-positive isolates and 12 rabbits with catalase-negative isolates. In an agglutination test, serum which had been obtained through immunization with catalase-negative isolates reacted only with catalase-negative isolates and not with catalase-positive isolates. Serum from rabbits immunized with catalase-positive isolates reacted strongly with catalase-positive isolates and only slightly or not at all with catalase-negative Vibrio.

Wiidik and Hildar (1955) observed that different antigens prepared in the same manner from the same V. fetus isolate produced different agglutination titers with the same homologous serum. They believed that these bacteria had a capsular K-antigen in addition to the thermolabile H-antigen and the thermostabile somatic O-antigen. In their opinion the

differences in agglutination titers could have resulted from quantitative differences in occurrence of the three different antigens.

Biberstein (1956) studied 47 V. fetus isolates and concluded that 44 belonged in one serotype, while each of the other three belonged in different serotypes.

Amell and Stockton (1956), using the complement fixation test, proved that cows vaccinated with V. fetus possessed antibodies specific for V. fetus.

Ristic et al. (1956) worked with both smooth and rough colonial types of V. fetus. Strain specificity was observed among smooth colonial types, while cross reactions were noted among rough colonial variants of homologous and heterologous strains. The rough types had negligible catalase activity. Ristic et al. (1957) demonstrated the presence of a thermolabile superficial antigen on smooth V. fetus cells. Boiling for two hours destroyed or removed the superficial antigen of V. fetus and only partly degraded or removed a superficial antigen of saprophytic Vibrio. Serologic heterogeneity of the rough variants was considerably minimized when heat treated antigens were used for production of antibodies as well as in agglutination tests. Ristic et al. (1958) proposed that colonial types other than rough variants also possessed antigens different from those of smooth colonial types. In addition they found that homologous formalin-killed antigen did not react with antibodies in the serum of a cow that aborted due to V. fetus, while heat-treated, homologous antigen did.

Te Punga (1958) developed an indirect hemagglutination test. He modified sheep erythrocytes by heating them in the presence of material derived

from V. fetus cells. He found this test to be a more sensitive indicator of V. fetus antibodies in rabbit serum than bacterial agglutination tests. He also found that this hemagglutination test could be applied to the detection of antibodies in bovine vaginal mucus, but was concerned as to whether all serotypes causing vibriosis could be detected by this method.

Using 15 V. fetus isolates Mitscherlich and Liess (1958) found two somatic serotypes (1 (Mit)[5] and 2 (Mit)) when the complement fixation test was used to detect reactions between phenol soluble antigens and antisera produced against whole-cell antigens.

Morgan (1959) worked with 25 V. fetus isolates and found two somatic serotypes (A (M)[6] and B (M)) by studying agglutination reactions between boiled antigens and antisera produced against either boiled or whole-cell antigens. Isolates from cattle and sheep were found in both serotypes (M). Absorption with antigens of the homologous group completely removed all agglutinins whereas absorption with antigens of the heterologous group left agglutinins for homologous antigens. Also, by conducting extensive absorptions, Morgan found one common flagellar component and eight specific flagellar components. He reported that there was no correlation between somatic serotypes and the flagellar components.

A heat-stabile, water-soluble substance, termed "HS", apparently partly polysaccharide in nature, was isolated from smooth V. fetus cells by Ristic and Brandly (1959a). This fraction was serologically active in

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[5] The serologic typing system of Mitscherlich and Liess (Mit) (1958).
[6] The serologic typing system of Morgan (1959).

gel precipitation tests and was capable of inhibiting a specific aggluti-
nation reaction. Ristic and Brandly (1959b) agglutinated sheep ery-
throcytes sensitized with the "HS" polysaccharide fraction of V. fetus
with V. fetus-specific rabbit sera and with sera from naturally infected
bulls. They believed this polysaccharide fraction represented a type-
specific O antigen common to a number of individual strains, rather than
a species-wide antigen. Ristic and Walker (1960), using a hemolytic
test, showed that sheep erythrocytes sensitized with the "HS" poly-
saccharide fractions of two V. fetus isolates were lysed by 21 specific
sera. These sera were produced by inoculating rabbits with V. fetus
isolates of bovine, ovine, and human origin. This hemolytic test was
found to be superior to the tube-agglutination test as a tool for de-
tecting small quantities of antibody. Treatment of sheep erythrocytes
with one of the antigens did not block the simultaneous or subsequent ab-
sorption of the second.

Using the rapid slide gel diffusion technique, Ristic and Murty (1961)
performed cross-precipitation reactions between V. fetus polysaccharide
"HS" antigens and V. fetus specific antisera. A cross-precipitation test
between 16 V. fetus antisera and two antigens revealed that the two an-
tigens were different. One reacted with 12 of the antisera, and the other
reacted with the remaining four, as well as with three which had reacted
with the first antigen. In another cross- precipitation test, seven V.
fetus antisera were tested with four "HS" antigens. One of the antigens
reacted with five of the antisera, a second and a third antigen reacted
with four, including the two which did not react with the first. The

fourth antigen reacted only with the antisera which reacted with all three of the other antigens. Different results were obtained if rough variants were used. In addition, these authors found that the first antigen yielded precipitin lines with the sera of 12 cattle artificially infected with _V. fetus_. Specificity of the gel-precipitation technic using this antigen fraction was indicated by the absence of precipitin reactions with control samples of serum from _V. fetus_-free cattle and rabbit antisera specific for _Leptospira_ and _Brucella_. Comparison of the serologic results obtained with the gel-precipitation, hemolysis, and whole-cell agglutination tests indicated that the latter two tests were inferior in accuracy to the gel-precipitation test.

Kamel (1960), using the techniques described by Mitscherlich and Liess (1958), found a third somatic serotype of _V. fetus_ (serotype 7 (Mit). Mitscherlich (1961) verified Kamel's results. In addition he correlated types 1 (Mit) and 2 (Mit) with pathogenicity and compared them with the physiologic varieties (_V. fetus_ var. _intestinalis_ and _V. fetus_ var. _venerealis_) of Florent. He came to the conclusion that serotype 2 (Mit) was responsible for epidemic abortions in sheep and sporadic vibrionic abortions in cattle, and that serotype 1 (Mit) was the causative agent of vibriosis of cattle characterized by both infertility and abortion. He further concluded that serotype 1 (Mit) was identical to _V. fetus_ var. _venerealis_ and serotype 2 (Mit) to _V. fetus_ var. _intestinalis_. Thirty-seven per cent of the serotype 1 (Mit) organisms tolerated glycine. (According to the work of Ringen and Frank (1963), these glycine tolerance results indicate that Mitscherlich's latter conclusion was incorrect.)

Söderlind (1961) compared the agglutination method used by Morgan[7] (1959) with the complement fixation test used by Mitscherlich and Liess (1958). Of 47 isolates belonging to either serotype 1 (Mit) or 2 (Mit), 30 were satisfactorily divided into two serotypes by both methods. The other 17 were clearly split into the two serotypes only by the complement fixation test. Four isolates belonging to the third serotype (Kamel, 1960) could not be differentiated by the agglutination test. Söderlind made no attempt to specifically correlate the type designations of Morgan's somatic serotypes with those of Mitscherlich and Liess.

Winter and Dunne (1962) worked with 26 V. fetus and seven V. bubulus isolates. Phenol and ultrasonic extracts were obtained from several isolates. Ultrasonic extracts from one V. fetus isolate contained antigenic materials which reacted in the indirect hemagglutination test with whole-cell antisera of all the V. fetus isolates and all but one of the V. bubulus isolates. When ultrasonic extracts from one V. fetus isolate was tested against all 33 whole-cell antisera in the agar gel-precipitation test, one line of identity of a heat-labile component was formed between all the V. fetus and two of the V. bubulus antisera; other lines of identity were formed between smaller numbers of antisera. In addition two different lines of identity of heat-stabile components were formed. One was formed between 20 V. fetus antisera (the major V. fetus O group) and the second between three V. fetus antisera (the minor V. fetus O group). Three of the

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[7] Söderlind did not absorb the antisera used in the agglutination test, whereas Morgan did.

V. fetus antisera did not react with this heat-stabile antigen.

Winter (1963), using chromatographic separation, found nine precipitating antigens in one V. fetus isolate. Eight of the antigens were heat-labile, and one of these was considered by Winter to be a species specific antigen. (However, in addition to reacting with all the rabbit anti-V. fetus sera, it reacted with some of the rabbit anti-V. bubulus sera.) The ninth antigen, the heat-stabile antigen responsible for the lines of identity of the major and minor O groups of V. fetus, was found in cell-free suspensions of flagella.

Nageswararao and Blobel (1963) found acid precipitable antigenic materials in the filtrates of 19 broth cultures of V. fetus. When this antigen was injected into rabbits, antibodies were formed. These antibodies and the acid precipitable antigenic materials produced precipitin lines in a double-diffusion precipitin test. They also agglutinated whole-cell and lysed V. fetus antigens. Some heterogeneity between the acid precipitable materials of different isolates was demonstrated.

O'Berry (1964), using ethanol, ammonium sulfate, and ethodin to fractionate antisera demonstrated that fluorescent antibody conjugates capable of producing fluorescence on V. fetus cells can be prepared. The two V. fetus var. venerealis isolates used to immunize cows and rabbits gave homologous agglutination titers of 1:3200 and 1:1600; heterologous titers of 1:100 were demonstrated. Only slight differences were noted between homologous and heterologous fluorescent staining when conjugated ammonium sulfate serum fractions were used. The fluorescent staining of the other two conjugated serum fractions varied with the isolate used for immunization

and the species of animal immunized.

Belden and Robertstad (1965), using cultures representing serotypes I (MF), II (MF), and V (MF) and fluorescent antibody technics, typed 33 V. fetus isolates. They found no cross reaction between serotype I (MF) and the other serotypes. Because reactions did not occur with serotype III (MF) that did not also occur with serotypes II (MF) or V (MF), only reactions occurring with types I (MF), II (MF) and V (MF) were used to establish their serogroups. However, no absorptions were conducted and it is therefore impossible to determine from this study whether serotype III (MF) is significant.

Winter (1966) purified the antigen responsible for the lines of identity of the major and minor O groups of V. fetus (Winter and Dunne, 1962). This heat-stabile polysaccharide endotoxin was comparable to the endotoxins from other species. It had a lethal effect in mice, and produced a biphasic febrile response and the generalized Shwartzman reaction in rabbits. After treatment in a basic solution, the polysaccharide absorbed readily onto red blood cells to serve as an antigen in a passive hemagglutination test. The substance was capable of stimulating formation of precipitating antibody in rabbits. The endotoxin of an isolate of V. fetus var. venerealis and an isolate of V. fetus var. intestinalis formed a line of identity in agar gel-precipitation tests when the antiserum from either isolate was used.

Winter (1965) separated 33 serum samples into two fractions (F1 and F2) by centrifugation in a sucrose gradient. These samples were obtained from cattle ranging in age from two weeks to 12 years. Ten were paired samples

from five heifers drawn before and after experimental infection with V. fetus var. venerealis. Serum from a heifer which aborted in the sixth month of pregnancy was also studied.

Antibodies for the V. fetus polysaccharide endotoxin were found in the majority of sera from normal cattle over seven months of age. The antibody necessary for activity in a passive hemagglutination test was found in the Fl fraction and was associated almost exclusively with the gamma-1 macroglobulin serum component. The antibody necessary for activity in a double diffusion precipitin test was found in the F2 fraction, which contained the characteristic gamma-2 globulin line as well as several other immunoelectrophoretic lines.

Serum from the heifer which aborted due to V. fetus var. intestinalis "unequivocally" contained gamma-2 globulin antibody for V. fetus endotoxin.

Serum drawn from five heifers after experimental infection had developed, contained heat-stabile precipitins (65 C for 30 min), whereas sera drawn prior to the infection did not. Heat lability of the precipitins in the F2 serum fraction of normal cattle was considered characteristic of "nonspecific" agglutinins, (gamma-1 globulin) but not characteristic of "specific" agglutinins (gamma-2 globulin).

The absorption of sera from three normal cattle with V. fetus endotoxin removed all the agglutinins and precipitins for V. fetus endotoxin from these sera. However, absorption with Escherichia coli endotoxin or Salmonella enteriditis endotoxin had little or no effect on hemagglutinating or precipitating activity.

CHAPTER III

MATERIALS AND METHODS

Isolates

The majority of isolates used in this study were furnished by the

Montana Livestock Sanitary Board Diagnostic Laboratory and the Montana

Veterinary Research Laboratory. Dr. E. Mitscherlich[8] supplied seven

isolates each of serotypes 1 (Mit) and 2 (Mit). Dr. B. Morgan[9] supplied

five serotype A (M) and three serotype B (M) isolates. Other isolates

came from Leeds, England[10], Idaho[11], Utah[12], New Zealand[13], the

National Animal Disease Laboratory, Ames, Iowa[14], and the Communicable

Disease Center, Atlanta, Georgia[15]. All isolates were frozen in defi-

brinated bovine blood and stored at -30 C until used.

Media

A basic aqueous medium containing 2.8% Brucella broth[16], 0.5% yeast

extract[17], and 0.1% agar[17] was used for growing inoculum and for all

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[8] Tierärztliches Institut, Universitat Gottingen, Gottingen, Germany.
[9] The Central Veterinary Laboratory, New Haw, Weybridge, Surrey, England.
[10] Courtesy Dr. W. A. Watson, Veterinary Investigation Centre, Ministry of Agriculture, Fisheries and Foods.
[11] Courtesy Dr. F. W. Frank, Dr. D. G. Waldhalm, and Mr. W. A. Minershagen, University of Idaho Branch Experiment Station.
[12] Courtesy Dr. J. Storz, Utah State University, Logan, Utah.
[13] Courtesy Dr. W. A. Te Punga, Wallaceville Animal Research Station, Dept. of Agriculture, Private Bag, Wellington.
[14] Courtesy Mr. J. Bryner and Dr. P. O'Berry.
[15] Courtesy the late Miss E. O. King.
[16] Albimi Laboratories, Flushing, New York.
[17] Difco Laboratories, Detroit, Michigan.

physiologic tests except for the "insensitive" H_2S test. For the glycine tolerance test, 1.0% glycine[18] was added to the above medium; for the "sensitive" H_2S test, 0.02% cysteine[18] was added. The media were dispensed seven ml per tube, in 16 mm screw-cap tubes. All tubes of semisolid medium used in physiologic tests were inoculated with two drops of 48-hour growth. All physiologic tests were conducted in duplicate. The incubation period was five days for all tests except for the temperature tolerance tests at temperatures below 20 C, for which a 10-day incubation period was used. All tests were conducted in an atmosphere containing 3.0% oxygen, 5.0% carbon dioxide, and 92% nitrogen. With the exception of temperature tolerance tests, an incubation temperature of 37 C was used.

Hydrogen sulfide production

Two methods were used to determine H_2S production. The "insensitive" H_2S test consisted of inoculating SIM stabs[19] with a large loop of 48-hour growth. If the stabs became black, the isolate was considered H_2S positive by the "insensitive" method.

The "sensitive" H_2S test was conducted by suspending filter paper strips saturated with lead acetate from tops of tubes containing the medium with cysteine added. If approximately 20 mm of the lower end of the portion of filter paper became black, a 4 + reading was given; isolates which produced approximately 10 mm of blackness received a 3 + reading. Two

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[18] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[19] Difco Laboratories, Detroit, Michigan.

plus (2 +) readings were given for isolates producing approximately 5 mm of blackness. If approximately 2 mm of the paper strip became black, a 1 + reading was given; if the color produced was dark brown instead of black and only at the lower end of the strip, a ± reading was given. Only isolates receiving negative readings were considered H$_2$S negative by the "sensitive" H2S method.

Glycine tolerance test

If growth occurred throughout the entire upper portion of tubes containing the basic medium with glycine added, a 4 + reading was given. Isolates producing less growth were given a 3 + or a 2 + reading, according to amount of growth present. If only a small amount of growth occurred in the center of the tube a 1 + reading was given. (Because the inoculum, two drops of 48-hour growth, was visible, it was occasionally difficult to determine whether growth had occurred or whether the inoculum had spread.) If it could not be determined with certainty that growth had occurred, a ± reading was given. A negative reading was given if no growth was present. Isolates which received a ± or a negative reading were considered glycine negative.

Catalase test

The catalase test consisted of dropping a 3.0% solution of hydrogen peroxide on growth from each isolate. If bubbling occurred, the isolate was considered catalase positive (+). Isolates which did not bubble immediately after hydrogen peroxide was added, but within a two minute period were designated as delayed (D). The amount of growth in each tube

was recorded according to the procedure described previously for the glycine tolerance test.

Temperature tolerance test

Medium was inoculated at room temperature. Immediately after inoculation duplicate tubes were incubated at each of the following temperatures: 45,42,37, and 20 C. Readings were made according to the procedure described previously for the glycine tolerance test. However, because growth readings below 2 + were not observed in the control tubes, growth readings of 1+ were considered questionable and recorded as trace (T). This trace reading of growth may have occurred before the temperature of the medium adjusted to the temperature of the various incubators. Isolates which grew well at 20 C were later tested at 17 C and at temperatures fluctuating from 10 C to 13 C.

Antigen production

Growth for production of antigens was obtained according to the method used by Firehammer and Berg (1966).

For production of boiled antigens, the growth was centrifuged at 4,080 X g for 20 min, the cells were resuspended in distilled water and boiled for two hours under a reflux condenser. After removing the distilled water by centrifugation, the cells were suspended in a 0.4% saline solution in proportions that will be described later.

For production of whole-cell antigens, 3.75 ml of 37% formalin was added to the 1,250 ml of growth and allowed to stand for 12 hours. These formalinized cells were removed from the medium by centrifugation. Both

the boiled cells and the formalinized cells were used in various concentrations. A portion of the packed cells of each antigen to be used in absorption studies was diluted in an equal volume of 0.4% saline solution. For the slide agglutination test, a second portion was diluted with saline until an optical density (O. D.) of 0.8 was obtained on a Lumetron[20]. A third portion was diluted to an O. D. of 0.15 for use with the tube agglutination test. If the antigen was to be used to inoculate rabbits for production of antisera, an additional portion was adjusted to 0.4 O. D. on the Lumetron. All adjustments were made using the red (# 650) Lumetron filter. All antigens were stored at 4 C.

Production of antiserum

Antisera were produced by inoculating rabbits intravenously with 15 ml of antigen. Beginning with 0.5 ml, the amount of inoculum was increased by 0.5 ml until 2.5 ml was given at each inoculation. Inoculations were given at three to four day intervals. The rabbits were exsanguinated five to eight days after the last injection.

Absorptions

Absorptions with both boiled and whole-cell antigens were achieved by mixing antigen with serum at a ratio of one part antigen to four parts serum. The mixture was incubated at 37 C for 3 hours and then placed in a refrigerator at 4 C for several hours. The chilled mixture was centrifuged at 17,300 X g for 15 min. The supernatant was tested for antibodies against the antigen used in absorption by an agglutination process. If a

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[20] Photovolt Corporation, New York, New York.