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STUDIES IN BOVINE VIBRIOSIS

A thesis submitted for the degree of
Master of Science in the University of Glasgow

by

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INTRODUCTION

INTRODUCTION

Vibriosis is a bacterial disease of cattle and sheep which gives rise to abortion and infertility. The causal organism is Vibrio fetus and although many different species of animals can be infected most work has been done on the cattle infection.

In cattle, there are now thought to be two different forms of the disease, both causing reproductive disorders. One type is venereal, the organism V.fetus venerealis carried by the bull, is transmitted to the cow either at natural service or by artificial insemination. The venereal type of infection has not been reported in sheep. The other type of the disease is non-venereal the organism is V.fetus intestinalis; it causes sporadic abortion in both cattle and sheep, and when this type occurs it is considered unlikely that the infection could be transmitted to the other animals in the herd. It was suggested in the FAO pamphlet on Bovine Vibriosis edited by Laing (1960) that sheep and pigs might possibly act as reservoirs of infection.

In recent years the most serious aspect of the venereal disease has been its potential spread through the technique of Artificial Insemination.

It is essential that semen used in this technique should be vibrio-free as cows receiving infected semen could possibly develop vibriosis. These animals at return to service, (if artificial insemination was not continued) could infect a local bull, and this

animal could then become a carrier, transmitting infection to other cows.

Because of the wide-spread use of artificial insemination it is important to diagnose infection in a bull. Cultural techniques although greatly improved are slow and the organism is not always isolated.

The fluorescent antibody technique (FAT) has been developed as a rapid diagnostic test for identifying many other species of pathogenic bacteria, and this technique is now being used in detecting vibriosis in bulls. Fluorescent antibody techniques can be used in a direct or indirect test to examine for bacteria. With V.fetus at present only the direct method has been used and reported.

I have developed an indirect fluorescent antibody test which could be used in laboratories which lack the facilities for carrying out the technical procedures required to produce a reliable clean conjugated antiserum.

The work done in this thesis compared the efficiency of this indirect fluorescent antibody test with a) the direct fluorescent antibody test and b) cultural methods. This was done by examining preputial washings to which I added known numbers of V.fetus cells. I also inseminated heifers with known numbers of V.fetus cells and cultured the vaginal mucus, the inseminating material being examined by the indirect and direct fluorescent antibody test and culture.

Two groups of bulls were examined, one by the direct fluorescent antibody technique, the other by both the direct FAT and Millipore culture.

LITERATURE

LITERATURE

MacFadyean and Stockman in 1913, described abortions in sheep caused by a vibrio-like organism. They did not give their isolate any precise nomenclature. By using the same type of media which was described by Bang (1897) for the culture of Brucella abortus, they succeeded in isolating this vibrio. (Bang's medium was agar, gelatin and serum in deep layer culture tubes). They attempted to infect 7 pregnant cows with exudate from the aborting ewes, either on its own or together with a pure culture of a previously isolated strain of vibrio. Two cows were infected by mouth and 5 by intravenous injection. Only 2 out of 7 cows aborted and from only one was a vibrio isolated.

Smith (1918) in U.S.A. reported isolating a vibrio organism from bovine abortions. He referred to the above work of MacFadyean and Stockman saying that it was impossible to determine whether or not the vibrio isolated from the sheep was identical to the one isolated by him from cattle. There was nothing in the description of the ovine isolates to conflict with his findings with the bovine vibrios. Smith also drew attention to a statement in MacFadyean and Stockman's paper that in 1911 in Ireland and Wales vibrios were isolated from cases of bovine abortion. Unfortunately no details were given. Smith suggested that both the vibrio and Brucella abortus interfered with placental circulation. He also noted that the vibrio was found in the foetal membranes and especially the chorion.

In 1919 Smith & Taylor named Vibrio fetus as the causal organism of bovine vibrionic abortion, the morphological characters were described as follows:- "The smallest forms appear as minute curved S-shaped rods; the longest may stretch nearly across the field of the microscope". The dimensions are "probably not over 0.2 to 0.3μ the shortest form about 1.5 to 2μ long", when stained in alkaline methylene blue. "A common size in the fetal fluids consists of about two complete turns and measures 4 to 5μ in length .... the diameter of the spiral .... averages 0.5μ". They also noted "A flagellum is found attached to one or both poles of the spirillum". Growth had been obtained on agar slants by adding a bit of tissue ($\frac{1}{8}$ gram) or the equivalent amount of material from the foetal stomach or intestinal contents of other fluids. A small quantity of water of condensation was made by adding bouillon. The tubes were then sealed with sealing wax.

In 1927 in Germany Lerche did an extensive bacteriological survey on bovine abortions. He examined 1565 cases of bovine abortion, the materials he examined being uterine discharge, the foetus and the foetal membranes. He made stained preparations from all cases and cultured V.fetus from 15. He did this by growing the material on agar slopes which had a drop of blood added over the surface. The culture tubes were incubated in a tin along with E.coli cultures; this was a technique to reduce oxygen tension. The material from which V.fetus was not isolated had been diagnosed as positive by staining the aborted material. The organism was described as bent rods,

S-shapes and spirals. He reported 1.4% of the abortions due to V.fetus and 56.6% due to Brucella abortus.

In South Africa in 1931 Snyman reported 13 bovine abortions in a herd of 160. All animals were free from Brucella abortus. He succeeded in isolating V.fetus from one case of abortion. The foetal stomach contents were cultured on serum agar slants to which serum broth and defibrinated horse blood had been added. The tubes were set up in duplicate one set being incubated after sealing the tubes, the other being incubated in an increased carbon dioxide atmosphere. He did V.fetus serum agglutination tests on these animals that had aborted but the titres were inconclusive (ranging from 1:20 to 1:160).

McEwan (1940) in Great Britain reported 5 cases of bovine vibriosis. He examined 247 foetuses, culturing foetal stomach contents, liver and spleen on to liver agar slants, blood agar slants or agar slants with a few drops of sterile defibrinated horse blood added. One set of cultures was grown in air, the other in 10% CO_2 in air. The five positive cases were from 3 herds. He said in his summary that "V.fetus gives sporadic abortions and is of little importance." He also stated that the source of infection was obscure.

Plastridge and Williams (1943) cultured V.fetus on soft agar medium composed of liver infusion broth with 1% peptone and 0.3% agar. The cultures were incubated at 37°C in an atmosphere of 10% carbon dioxide. In 1947 Plastridge, Williams and Petrie isolated V.fetus from 20 out of 72 aborted foetuses. Stegenga (1950) isolated V.fetus from semen

of infected bulls and classified bovine vibriosis as a venereal disease.

Since V.fetus could be isolated from aborted foetuses the next stage was the attempted isolation of V.fetus from living animals. As vaginal mucus was found to contain fewer contaminating organisms than semen this was the sample of choice until the incorporation of antibiotics into the isolation media made isolation of V.fetus from semen and preputial washings a worthwhile diagnostic test. Kiggins and Plastridge (1956) isolated V.fetus from bulls by using blood agar plates which were incubated in the gas mixture 10% CO_2, 85% N_2 and 5% O_2. They cultured 544 semen samples from 149 bulls and succeeded in isolating V.fetus from 145 (27%) of the samples. Preputial washings were collected from 12 carriers bulls (the semen had already been shown to be positive). 39% of the samples gave growth of V.fetus. Plastridge, Koths and Williams (1961) used blood agar plates with antibiotics added to inhibit the growth of the many contaminating organisms found in semen. They found that V.fetus and V.bubulus grew well on this medium containing 2 units of bacitracin and 2 µg of novobiocin per millilitre (ml) of medium. Shepler, Plumer and Faber (1963) incorporated antibiotics into the blood agar medium and also used millipore filtration to separate the vibrios from the many contaminating organisms present in preputial fluid. They used 1 unit of polymixin B, 5 µg of novobiocin and 15 units of bacitracin per ml of medium.

Dufty (1967) added 20 µg per ml of cycloheximide to the antibiotics

used by Shepler et al (1963) for the isolation of V.fetus. Of 100 samples of preputial secretions from 5 known carriers bulls 78% were positive. Winter, Samuelson and Elkana (1967) added 300 units of nystatin per ml of antibiotic medium used by Plastridge et al in 1961 for the isolation of V.fetus.

In most of the papers from 1965 onwards, cultural means of diagnosing vibriosis are compared with the fluorescent antibody technique which is discussed later (page 36). One problem that bacteriologists faced in vibrio infection concerned the identity of the actual organisms isolated from clinical specimens. Although microscopic examination of material from infected animals could show the presence of vibrio organisms a precise identification of the bacterium could only be made by culture. The early workers named their isolates Vibrio fetus but later workers using more complex biochemical tests in identification found strains of vibrio with different cultural, biochemical and serological characteristics. Whether these vibrios are different species is still today undecided. Attempts have been made to link the differentiation of those vibrios with the type of field infection that is caused i.e., venereal or non-venereal. There is also the problem of isolating and differentiating commensal vibrios. The next section deals with the species variety differentiation of V.fetus. This is essential before any consideration can be given to methods of diagnosis of infection.

THE DIFFERENTIATION OF V.FETUS VENEREALIS, V.FETUS INTESTINALIS AND V.BUBULUS.

Table 1 shows the biochemical characteristics of various vibrio isolations and the names used by the authors to differentiate the different types found. The infected animal species is shown and when stated by the author the isolation site. It will be seen that from 1953 to 1958 V.fetus was not differentiated into varieties but a commensal vibrio, now named V.bubulus by Florent (1953) with a different hydrogen sulphide, catalase and glycine pattern had been isolated.

Florent (1959) proposed to call the organism which spread vibriosis venereally and caused infertility V.fetus venerealis. The other organism which was responsible for sporadic abortions, was not transmitted by the bull, but invaded the pregnant uterus from the intestines, was to be called V.fetus intestinalis. Although Florent proposed this splitting of V.fetus it will be seen from the table 1 that the only difference he used was a weak hydrogen sulphide production by V.fetus intestinalis. This suggestion of Florent's however was confirmed by further work which used better media for observing hydrogen sulphide production by V.fetus intestinalis and also the ability to grow in a medium containing glycine. On these criteria two different behavioural forms of V.fetus were found, one called V.fetus venerealis was hydrogen sulphide negative and gave no growth in a medium containing 1% glycine. The other V.fetus intestinalis, did produce hydrogen sulphide and grew in a medium

TABLE 1

Summary of biochemical characteristics of V.fetus isolates

Author	Date	Hydrogen Sulphide In Insensitive Medium	Production In Cysteine Medium	Growth in 1% Glycine	Catalase	Group	Host Species	Name
Florent	1953	+	ND	ND	-	GP A	Bull & Cow	V.bubulus
Bryner and Frank	1955	-	ND	ND	+	ND	Bull & Cow (2)	V.fetus
		+	ND	ND	-	ND	Bull & Cow (2)	V.bubulus
Aldermans, Terpstra and van Waveren	1956	-	ND	ND	+	GP I	Bull & Cow (2)	V.fetus
		+	ND	ND	-	GP II	Bulls (Mostly) (2)	V.bubulus
		Weak +	ND	ND	+	GPIII	Cow (4)	V.fetus
Mitscherlich and Leiss	1958	-	ND	ND	+	GP I = AKK I	Cattle (2)	V.fetus
		Weak +	ND	ND	+	GP 2 = AKK III	Sheep & Cow (2)	V.fetus
Lecce	1958	Said H_2S too tenuous a characteristic to differentiate species		(0.8% Gly)	Also too tenuous			
				+	+		Sheep (2)	V.fetus
				-	+		Cattle(2)	V.fetus
Florent	1959	-	ND	+	+	Type I = AKK I	Cattle (2)	V.fetus venerealis
		Weak +	ND	+	+	Type II = AKK III	Sheep (3) Cattle(2) & (3) & Pigs	V.fetus intestinalis
Di Liello Poelma and Faber	1959	-	-	ND	+	GP I	Cattle	V.fetus
		ND	Weak +	ND	ND	GP 3&4	Sheep & Humans	V.fetus
		+	ND	ND	-			V.bubulus
Laing (Ed)'	1960	-	-	-	+	ND	Cattle (2)	V.fetus venerealis
		-	+	+	+	ND	Cattle (2) & (3)	V.fetus intestinalis
		+	ND	ND	-	ND	Cattle (2)	V.bubulus

1 Extended on Table 14

ND = Not done

Sites (2) Genital tracts
(3) Intestines
(4) Aborted material

AKK = Aldermans et al (1956)

containing 1% glycine. Florent (1959) went back through the literature and renamed Akkermans, Terpstra and Van Waveren's (1956) types I, II and III, V.fetus venerealis, V.bubulus and V.fetus intestinalis respectively.

Today differentiation of V.fetus venerealis from V.fetus intestinalis still relies mainly on the two tests i.e., the ability of the organism to produce hydrogen sulphide in a medium containing a substance which when metabolised would give hydrogen sulphide and its ability to grow in a medium containing 1% glycine.

These were carried out as follows:-

1. Hydrogen sulphide production by growing the bacteria in a semi-solid medium containing 0.02% cysteine. Hydrogen sulphide was detected by blackening of a lead acetate paper which was inserted in the neck of the tube containing the inoculated medium.

2. The ability of the isolate to grow in a medium containing 1% glycine. Different basal media which gave good growth of the bacteria in the absence of glycine were essential. If no growth resulted this indicated inhibition or no tolerance to the glycine.

Hydrogen Sulphide production.

Lecce (1958) thought that "Hydrogen sulphide production was too tenuous a characteristic to differentiate species", while Bryans and Smith (1960) also considered hydrogen sulphide production too variable. Few authors stated clearly how they performed the tests to determine whether hydrogen sulphide was produced or not. They rarely stated the length of incubation time before a final reading

was taken. In the Food and Agricultural Organisation publication "Vibrio fetus infection in cattle" (1960) hydrogen sulphide production was read after overnight incubation in 10% carbon dioxide atmosphere, the growth taking place in broth medium with a lead acetate paper inserted at the neck of container to act as an indicator. The interpretation of their results was slight or no blackening was negative. For a more sensitive medium it was recommended to use semi-solid Martin broth containing 0.02% cystine but the schedule for inoculation, incubation or reading was not stated (unless one assumed it was the same as for the first insensitive medium). Kita, Ogimoto and Suto (1966) read the results of hydrogen sulphide production daily for 7 days. Florent read up to 3 days in 0.02% cystine medium. Park et al. (1962) read up to 7 days. Winter, Burda and Dunn (1965) specified a single colony inoculum into 0.16% brucella semi-solid medium containing 0.02% cystine. Final readings were taken after 5 days incubation. Triple sugar iron (Difco Laboratories) was used for the identification of V.bubulus. This organism produced hydrogen sulphide in this insensitive medium while V.fetus did not. Winter et al. incubated this test for 5 days.

As there was such a variety of methods of observing hydrogen sulphide production and as the period of incubation had an effect on the result, the test could be negative after 24 hours' incubation yet become positive after 48 hours

or longer incubation, it was important that the methods used and the times in this test were specified along with the results.

GLYCINE TOLERANCE

There was also variation in the method used for detecting growth in a medium containing 1% glycine. For example Lecce (1958) used 0.8% and not 1% glycine in his biochemical studies. Park et al (1962) used Albimi brucella broth containing 1% glycine but did state clearly that after 7 days of incubation the reaction was observed; Plastridge, Williams and Trowbridge (1964) used 1% glycine in thiol medium and observed the reaction after 3 days incubation; Philpott (1968) noted his results after 5 days but did not state the medium used in the test. Winter et al (1965) used semi-solid brucella broth containing 1% glycine and read the results after 5 days' incubation.

Some authors incubated their tests in special gas mixtures, others did not specify this requirement.

In table 2 the complete criteria given by the FAO publication on Bovine Vibriosis (1960) to differentiate V.fetus intestinalis and V.fetus venerealis are presented.

The majority of bacteriologists use these tests, some using different gas mixtures and different basal media for hydrogen sulphide production. For comparison I have incorporated the information about V.bubulus, the commensal non pathogenic vibrio first described by Florent (1953).

TABLE 2

DIFFERENTIATION OF VIBRIO SPECIES

The criteria used in the differentiation of *V.fetus venerealis* and *V.fetus intestinalis* is taken from the FAO "Vibrio fetus infection in Cattle" (1960)

	V.fetus venerealis	*V.fetus intestinalis*	*V.bubulus*
Growth atmosphere - 1. 10% Carbon dioxide	+ -	+	
2. 10% Carbon dioxide with oxygen tension reduced to one third	+	+	+
Aerobic growth	-	-	-
Anaerobic growth	-	-	+
Catalase	+	+	-
Hydrogen sulphide production - 1. In semi-fluid Martin broth	-	-	+
11. In semi-fluid Martin broth with 0.02% cystine	-	+	+
Growth in medium containing 3.5% sodium chloride	-	-	+
Growth in medium containing 1% glycine	-	+	(+)
Predilection site	Prepuce of bull, genital tract of cow & heifer	Intestines	Prepuce of bull.
Infected or carrier species	Cattle	Cattle, sheep & pigs	Cattle & sheep
Ability to survive in the bovine vagina	+	-	
Pathogenicity in relation to fertility	Infertility & occasionally abortion in cattle	Abortion in cattle and sheep	Commensal

V.bubulus results added for comparison

In table 3 1961-1965 are listed the strains of V.fetus isolated. The authors in the main used the FAO publication on Bovine Vibriosis (1960) scheme of differentiation.

It would seem in the venereal infection V.fetus venerealis is indeed the causal organism while V.fetus intestinalis gives rise to sporadic abortion; V.bubulus being only a commensal. As with all bacteria, intermediate forms have been isolated but discussion of the significance of these types is out-with this thesis.

Agglutinating antigens

Morgan (1959) did extensive studies on the flagellar and somatic antigens of V.fetus and other vibrios. His isolates were denoted V.fetus if they required an increased concentration of carbon dioxide in the atmosphere for growth on solid media, were catalase positive, did not produce hydrogen sulphide and reduced nitrates to nitrites. He did not subdivide the V.fetus species. All 10 isolates shared one species specific flagellar antigen, and 5 out of the 10 strains also possessed a second common antigen. Using somatic antigens, 5 isolates belonged to one group (A) and the other 5 to a second group (B). He also noted that both cattle and sheep isolates belonged to each group so there was no antigenic host species differentiation.

He also found that catalase negative commensal vibrios were antigenically different from any of his 10 V.fetus isolates.

TABLE 3

Summary of biochemical characteristics of V.fetus isolates

Author	Date	Hydrogen Sulphide Production In Insensitive Medium	In Cysteine Medium	Growth in 1% Glycine	Catalase	Group	Host Species	Name
Hoppe and Ryniewicz	1961	-	ND	-	+		Bull (2)	= V.fetus veneralis
		+	ND	+	+	=GP III AKK	Bull (2)	= V.fetus intestinalis
		+	ND	ND	-		Bull (3)	V.bubulus
Park Munro Melrose and Stevart	1962	-	-	-	+	A	Cattle (2)	V.fetus venerealis
		-	+	+	+	B	Cattle & Sheep	V.fetus intestinalis
		+	+	+	-	ND	Bull (2)	V.bubulus
Mohanty Plumber and Faber	1962	-	ND	-	+	Type 1		V.fetus
		Trace	ND	+	+	" II		V.fetus
		Trace	ND	-	+	" III		V.fetus
		+	ND	ND	-	ND		V.bubulus
			24 hours 5 days					
Bryner Frank and O'Berry	1962	ND	- -	-	+	Type 1	Cattle (2)	V.fetus
		ND	- +	-	+	Sub Type 1	Cattle (2)	V.fetus
		ND	- +	+	+	Type 2	Cattle (2)	V.fetus
		ND	+ +	+	-	ND	Cattle (2)	V.bubulus
Plastridge Williams and Trowbridge	1964	-	ND	-	+	Type 1		V.fetus
		-	ND	+	+	" II		V.fetus
		+	ND	+	+	" III		"
		+	ND	+	-	" IV		V.bubulus
Firehammer	1965	+		+	+	ND	Sheep (3)	V.faecalis
		-	-	-	+	ND	Cattle (2)	V.fetus venerealis
		-	+	+	+	ND	Sheep (2) & (3)	V.fetus intestinalis
		+	+	+	-	ND	Sheep (Ram) (2)	V.bubulus

ND = Not done

Sites (2) Genital tract
(3) Intestines

EPIDEMIOLOGY

Two forms of infection are found in both the bull and the cow, i.e., A. Venereal and B. Non-venereal. These forms will be dealt with separately for each animal.

The role of the bull in bovine vibriosis.

Venereal

Plastridge, Williams and Petri (1947) were the first to suggest that the bull was infected with V.fetus. They found high serum agglutination titres in 5 out of 6 bulls from herds with infertility problems. Rasbech (1951) stated that statistical analysis of VMA results were able to detect which bulls were spreading vibriosis in Denmark. Terpstra and Eisma (1951) isolated V.fetus from 3 carrier bulls. They mated these carrier bulls with a total of 13 heifers and were able to culture V.fetus from vaginal mucus samples from all of them. Most heifers gave positive serum agglutination and vaginal mucus agglutination reactions. They artificially infected the prepuce of a non infected bull with a broth culture of V.fetus. A heifer subsequently inseminated with material from this bull became infected. Also "clean" semen together with a V.fetus culture was inseminated into 7 heifers and all became infected. To

eliminate the possibility of a virus or mycoplasma causing vibriosis, a bacteria free filtrate of V.fetus in "clean" semen was inseminated into 2 heifers. Neither became infected, and only one conceived. Lawson and MacKinnon (1952) also established by a series of experiments that V.fetus was responsible for the bovine venereal disease vibriosis. At this time V.fetus was not sub-divided into V.fetus intestinalis and V.fetus venerealis but only separated from the commensal Vibrio bubulus.

A group of 12 virgin heifers was served by a V.fetus infected bull and a second group of heifers was inseminated with semen from another V.fetus infected bull. Both bulls were known to be infected by their history only, as V.fetus was never isolated from them. At each return to service the heifer was either served by or inseminated with semen from the other infected bull. The following additional procedures were carried out weekly and also at oestrus, on all of the above heifers.

1. By culturing vaginal mucus samples 22 out of 24 heifers gave growth of V.fetus at same time.

2. Using the vaginal mucus agglutination test to detect the presence of vaginal mucus antibodies against V.fetus

again 22 out of 24 heifers gave a positive titre during the period, 2 to 14 weeks after the initial insemination or service.

3. Using the serum agglutination test for the detection of serum antibodies against V.fetus, 17 out of 24 heifers gave a positive titre at some time.

4. The conception rate for each group was determined and this compared with the conception rate of a group of controls. The conception rates (the numbers of inseminations per conception) of the heifers either served or inseminated was 5.2 while that of the control group was 1.6.

The control group of heifers were sampled on 7 occasions and all gave negative results for the tests stated in 1, 2 and 3 above.

A second experiment was carried out by inseminating uninfected semen to which a pure culture of V.fetus had been added to determine whether this would also produce the infertile condition in the

heifer. The results were very similar to these obtained when the heifers were inseminated with naturally infected semen.

The authors also attempted to produce infection 1) *per os* and 2) by the conjunctival route but these methods failed. They also ascertained that the agent responsible for the infertile condition was not capable of passing through a filter which would retain bacteria. In their summary they stated that they found the infertile condition caused by *V.fetus* was not transmitted by ordinary contact in the cow shed, that the serum agglutination test is of little value in diagnosis, but that the vaginal mucus agglutination test is a valuable diagnostic test if the mucus is not collected at oestrus. They also found that the addition of penicillin and streptomycin to the infected semen had little value in preventing the infection in the inseminated heifers.

The bull is thought of as a carrier, the *V.fetus venerealis* organisms living in the prepuce only on the superficial layers of the penis and preputial mucosa. Samuelson and Winter (1966) established the carrier status of the bull by examining infected bulls, in detail. They found no inflammation of the preputial mucosa, nor was there any invasiveness of the tissues. They also established that *V.fetus* was present along the entire

length of the prepuce, with most of the V.fetus organisms being present on the penis and at the fornix of the prepuce. The lumen of the preputial epithelial crypts were found to be the areas where most vibrio cells were present and therefore thought to proliferate. The urethra was not thought to be invaded normally, nor was the semen itself infected except by contact with the prepuce at service. The spermatozoa were normal and there were no abnormalities of the genital organs. No local antibodies were present in the superficial layers of the prepuce nor in the preputial fluid. Unlike the cow there was no qualitative or quantitative alteration in circulating antibodies in the carrier bull, Samuelson and Winter (1966). A carrier bull may therefore infect a cow either at natural service or by artificial insemination.

In the light of the present and above knowledge it is therefore strange that the bull was first incriminated as a source of infection by Plastridge, Williams and Petri (1947) as high serum agglutination titres were found in some known carrier bulls. These bulls were from two herds using natural service. There was considerable variance in titres, any one bull giving different results with different samples. At this

time vibrionic abortions and lowered conception rates were observed in the same herd. The serum agglutination test is not considered a reliable means of diagnosing today due to the fact that many carrier bulls appear to have no circulating agglutinating antibodies. Because of the superficial nature of the position in which V.fetus is located in the prepuce the protective effect of circulating antibodies would be questionable, Samuelson and Winter (1966).

Bulls that have been successfully treated are known to be susceptible to reinfection, although many workers believe that young bulls throw off the infection but older bulls may carry it indefinitely, (Philpott, 1968).

Non Venereal.

In this form of vibriosis which is responsible for sporadic abortions the bull is not thought to be implicated at all. The organism currently thought to be responsible is V.fetus intestinalis. However, V.fetus intestinalis has been reported isolated from the prepuce of bulls by Hoppe and Rycewicz, (1961).

In their experiments they failed to infect heifers with V.fetus intestinalis but did succeed in infecting heifers with V.fetus venerealis.

Park et al (1962) indirectly proved that certain bulls were carrying a strain of vibrio with the biochemical characteristics of V.fetus intestinalis by infecting heifers with semen from the suspect bull. The vaginal mucus from these heifers contained V.fetus intestinalis only (i.e., they grew in a medium containing 1% glycine and produced hydrogen sulphide on a medium containing cystine). From this finding it would appear that certain V.fetus intestinalis strains could be responsible for a venereal type infection. Florent (1963) confirmed these findings.

Role of the cow.

As in the role of the bull this is divided into two types of clinical infection A. Venereal and B. Non Venereal.

Venereal.

As previously stated in the venereal form of the disease the heifer or cow is infected by a carrier bull either by A.I. or natural service. The symptons of

vibriosis are frequent returns to service, sometimes irregular oestrous cycles, and occasionally vaginitis. Diagnosis can only be confirmed by laboratory testssuch as culture of V.fetus from vaginal mucus or by the vaginal mucus agglutination test being positive. (VMA)

Many reproductive disorders show similar clinical features so although a presumptive vibriosis diagnosis can be made on clinical evidence and herd history a laboratory diagnosis is always necessary.

In a herd using natural service an infected cow can transmit the infection to the bull at service and hence start the cycle through which the whole herd could become infected, In a herd using A.I., an infected cow is not thought to spread the disease to other cows.

Lawson and MacKinnon, (1952) found that if a bull were a carrier of V.fetus almost all (22 out of 24) the heifers that were inseminated with its semen or served by it became infected as was demonstrated by lowering of conception rates, the vaginal mucus samples became positive both for culture of V.fetus and for the vaginal mucus agglutination test. They also found that a V.fetus culture, when added to uninfected semen produced the same effect of lowering conception rates, and giving positive titres in the VMA test. The latter

effects could not be produced by inseminating a filtrate of a V.fetus culture along with uninfected semen. They also demonstrated that the addition of streptomycin and penicillin decreased the number of heifers infected within a group. They pointed out that for reliable results in the VMA test, mucus collected at oestrus gave a higher percentage of positive culture results (i.e., 83% positive at oestrus and 27% positive at anoestrus).

Non venereal

In this infection the organism responsible is V.fetus intestinalis. It gives rise to sporadic bovine abortions. Abortion is thought to be brought about by the organism reaching the placenta via the blood stream from the intestines (Florent, 1959). It has been observed that although foetal stomach, lung and lymph nodes are often infected, placental tissue and vaginal mucus from the cow should also always be set up for culture of V.fetus. As the vagina is often infected there seems to be no reason why a bull cannot become infected at natural service. Perhaps V.fetus intestinalis is suited to inhabit the intestinal tract and not the prepuce.

Clinically bovine vibrosis is thought to exist in two distinct forms.

A. Clinical venereal vibriosis

B. Clinical non venereal vibriosis.

A. Clinical venereal vibriosis.

The venereal type of infection is carried by the bull and transmitted to the cow or heifer at natural service or by artificial insemination. V.fetus is not thought to prevent fertilization (Adler, 1960) but causes foetal death about 3 weeks after conception in infected animals and so the female returns to service. After many returns to service the cow may become pregnant and many then calve normally. A cow may harbour V.fetus after calving normally and even, it is thought through 2 gestation periods.

B. Non venereal vibriosis.

The second type of vibrio infection is not thought to be transmitted by the bull, the infection being confined to the cow or heifer. It causes sporadic abortions rather than infertility. The cow or heifer conceives normally, but several months later the organism reaches the placenta

via the blood stream from the intestines and so brings about abortion. V.fetus intestinalis has also been isolated from the intestines of sheep and pigs and some workers think that the latter animals may act as reservoirs of infection where they or their excreta come in contact with cattle.

THE HEIFER MATING TEST

This test was used as a means of isolating V.fetus from bulls as attempts at direct isolation from preputial washings were often unsuccessful.

Rasbech (1951) was the first person to recommend the service of several virgin heifers as a means of diagnosing vibriosis in bulls. He found by statistical analysis of vaginal mucus agglutination results which Artificial Insemination bulls were spreading vibriosis in Denmark. Terpstra and Eisma (1951) performed a series of experiments using heifers (see page 17). They isolated V.fetus from the semen of 3 carrier bulls. One bull was mated with 5 non infected heifers, another with one heifer and the third with 7 heifers. All heifers gave growth of V.fetus from most vaginal mucus samples and most heifers gave positive titres using the vaginal mucus agglutination and serum agglutination tests. Adler, Albertsen, Rasbech and Szabo (1952) diagnosed the carrier state of bulls by inseminating 6 to 12 heifers with semen and preputial washings from one suspect bull. They then collected 6 samples of vaginal biopsy material or vaginal mucus which were set up for the culture of V.fetus during the following 3 weeks. In later experiments Adler (1954) used one heifer

per bull in his heifer mating test. Prior to insemination, vaginal mucus had been collected on 3 occasions and only if all samples were negative using the vaginal mucus agglutination test, and culture of the mucus failed to isolate V.fetus were the heifers used. Corpora lutea were expressed and if the heifer showed signs of oestrus each was inseminated into the uterus with 2 ml of semen. One week later uterine biopsy and vaginal mucus (from the anterior vagina) samples were collected for the isolation of V.fetus. This was done twice weekly until 6 samples were taken. Negative heifers were reused. Using Adler's (1954) basic mating test, Morgan Melrose and Stewart (1957) inseminated 13 heifers with semen from 13 bulls and by culturing V.fetus from the vaginal mucus samples, found all 13 bulls to be vibrio carriers. Another 4 bulls each of which was used to inseminate one heifer, gave growth of V.fetus from only one vaginal mucus sample in each case. They suggested that the bull carrier strain of V.fetus might have been avirulent or that some heifers have a natural resistance to vibrio infection.

Murane, Eales and Monsbourgh (1959) found that a suspect bull from which V.fetus could not be isolated by direct culture of the preputial washings infected 2 out of the 4 heifers inseminated. Another suspect bull served 10 heifers and from vaginal

mucus of 6 of these heifers cultures of V.fetus were obtained. The vaginal mucus agglutination test was performed and 7 of the 10 gave positive titres. Only one heifer became pregnant. Two other bulls served 5 and 6 heifers respectively and V.fetus was cultured from 2 heifers in each group. By inseminating a culture of V.fetus into the vagina, Blobel Simon and McNutt (1957) produced infection in 10 out of 14 heifers. From the 2 latter sets of results it can be seen that the insemination of one heifer per bull would not necessarily have diagnosed vibriosis in a carrier bull.

Infectivity of test-mated heifers must be dependant on several factors i.e., on the number of viable V.fetus organisms present, the type and number of other bacteria present in any sample inseminated, and the virulence of the organism as well as the immunological status of the heifer inseminated. While most ejaculates from most carrier bulls would contain sufficient vibrio organisms to cause infection in all heifers inseminated or served, there must be some bulls whose ejaculates would contain less than an infective dose at some time and so fail to infect this heifer.

None of the previous authors quantitated the number of viable organisms inseminated but Newsam and Peterson (1964) inseminated

150 x 10^6 viable V.fetus cells into the anterior vagina of 42 heifers (25 heifers were in oestrus and 17 were not in oestrus at the time of insemination). V.fetus was cultured from the vaginal mucus samples of 38 out of the 42 heifers. Vaginal mucus agglutination tests gave positive titres from 14 of the 42 heifers. In another experiment using a laboratory adapted strain of V.fetus they inseminated each of 5 heifers with 460 x 10^6 viable vibrios. V.fetus was subsequently isolated from the vaginal mucus of all 5 heifers. They also noted that vulval smearing with viable V.fetus did not produce any evidence of infection in the 7 heifers they tested. From these experiments it would appear that if a sufficiently high number of pathogenic vibrios were inseminated 90% of the heifers would become infected; infection being denoted by the ability of V.fetus to multiply in the bovine vagina.

The strains of V.fetus used by the last 4 authors were thought to be V.fetus venerealis as the bulls used for mating were suspected of having vibriosis due to infertility problems associated with them i.e., the pattern fitted the clinical venereal disease. But Park et al (1962) found that they could only isolate V.fetus intestinalis from heifers inseminated with semen from a bull suspected of being a V.fetus carrier. Vaginal mucus from these heifers was inseminated into vibrio-free heifers and the infection established in them. Infection was denoted by the culture of V.fetus intestinalis from the vaginal mucus and also by the vaginal mucus agglutination test giving positive titres.

Hence the differentiation of V.fetus into V.fetus venerealis which caused infertility and was a venereal disease, and V.fetus intestinalis causing only sporadic abortions, being non-venereal, was in dispute. For this reason Park et al suggested using Bryner and Frank's (1955) classification which only differentiated the pathogenic V.fetus from the non-pathogenic V.bubulus.

The heifer mating test was performed in such a way that a Vibrio fetus free heifer was inseminated with material from a suspect bull on one occasion only and vaginal mucus samples collected which were cultured for the isolation of V.fetus and the V.M.A. test done. So the heifer mating test did not test for infertility induced by insemination of V.fetus along with semen but only tested for the ability of V.fetus if present in the material inseminated, to survive in the bovine vagina after one week up to 4 weeks. The findings of Park et al (1962) confirmed that V.fetus intestinalis could be capable of producing a pattern of infertility normally only associated with V.fetus venerealis. It could be carried by the bull, Hoppe and Ryniewicz (1961). If the bull were not infected venereally, contamination of the prepuce could occur from contaminated bedding. No matter what the route of the infection was, the results were the same as the venereal disease and so it seemed that

there was little purpose in maintaining the division into V.fetus venerealis and V.fetus intestinalis, especially when there was a trend in certain areas to regard V.fetus intestinalis as non pathogenic.

The heifer mating test is still generally considered a good method of detecting vibriosis in the bull and if the vibrio isolated from the heifer is a V.fetus intestinalis, then it should be considered as capable of producing infection. As Park et al (1962) stated "a bull carrying a strain of V.fetus reacting as intestinalis in Florent's biochemical tests should not be regarded as being incapable of giving rise to infectious infertility but should be regarded as infective and treated".

Criticisms of the heifer mating test.

1. It is expensive and time consuming.

A heifer is an expensive animal to buy, house and feed. It must be kept for 6 weeks as it must be established as vibrio free prior to insemination; then starting one week after insemination, 6 vaginal mucous samples are usually collected over the following 3 week period.

2. Infection with V.fetus intestinalis.

If the heifer were carrying a V.fetus intestinalis in its intestines, then it was possible that the vibrio might reach the vaginal mucus and so give a positive culture.

3. Viability.

As the mating test relies on viable organisms, it is dependent on correct storage of the bull semen or preputial washings from the time of collection from the bull until it is inseminated into the test heifer.

4. The stage of the oestrous cycles at which insemination takes place.

It has been experimentally observed in a heifer infected with V.fetus that more positive cultures were obtained from vaginal mucus samples collected at oestrus than at any other stage in the oestrous cycle, Lawson and MacKinnon (1952). Under natural breeding conditions the infection would be transmitted at oestrus. The test animal could be inseminated at oestrus but this could mean a delay of over 3 weeks. The alternatives have been to bring the heifer into an artificial oestrus by an injection of 25 mg of stilboestrol dipropionate in arachis oil, or to express a corpus luteum. If the heifer were in oestrus or given stilboestrol the sample could be inseminated through the cervix. Alternatively heifers have been inseminated at any other stage in the oestrus cycle, the site of inoculation then being the anterior vagina.

5. Naturally resistant animals.

Newsam and Peterson (1964) inseminated 42 heifers with 150×10^6 viable V.fetus venerealis organisms and 38 became positive. This was

shown by the isolation of V.fetus from vaginal mucus and a positive reaction with the VMA test. Blobel, Simon and McNutt (1957) also noted that not all heifers served or inseminated with infected material became infected.

6. Reliability of isolation technique.

Although vaginal mucus was much less contaminated with other organisms than preputial washings or semen from an infected bull, the isolation of V.fetus was greatly increased by incorporating antibiotics into the isolation media. In Great Britain until recently most A.I. bulls were screened yearly by the heifer mating test as it was still considered to be more sensitive than direct culture from preputial washings or semen. The practice of pooling semen from several bulls and inseminating a portion of the pool into one heifer to reduce the number of heifers used was not a satisfactory method. If only one bull carried a Pseudomonas species or some other rapidly growing bacteria were present in large numbers isolation of V.fetus could become difficult or uncertain, even on media containing antibiotics.

Another method of examining bulls for the carrier state of V.fetus is the Fluorescent Antibody Technique.

FLUORESCENT ANTIBODY TECHNIQUE

In 1942 Coons, Creech, Jones and Berliner investigated the possibility of labelling antibodies with a chemical which fluoresced when exposed to ultra violet (UV) light. In this way, sites of antigenic material in the mammalian tissues could be detected. It had been established previously by other workers that certain chemical groupings could be "attached" to antibodies without destroying the specific antibody-antigen reaction. Using impure fluorescein isothiocyanate, conjugated pneumococcal 3 antibodies were prepared. The agglutination titre of this antiserum did not drop after conjugation and rendered pneumococcus 3 antigen fluorescent under U.V. light. The tissues infected with this antigen (pneumococcal 3) were located using the conjugated pneumococcus 3 antibodies. This reaction was found to be specific. Since this original paper many workers have used the fluorescent antibody technique for the detection of specific antigens from the infected material i.e., throat swabs for streptococci. Many diagnostic applications were discussed by Cherry and Moody (1965). It is therefore not surprising that the FAT has been used as a diagnostic tool for the detection of V.fetus from the carrier bulls. In bovine vibriosis the bull acts only as a carrier and therefore does not necessarily have a reliable antibody production against V.fetus either systematically or locally within the prepuce. For this reason, the organism itself must be detected. Anti V.fetus serum is usually prepared and then labelled with a fluorochrome. The conjugated antiserum is then used to detect the presence of V.fetus cells from clinical material

from the bull. The sample material of choice is the preputial washing but semen was used by Taul and Kleckner (1968). Preputial washings are thought by most people to be the most suitable because in the carrier bull, V.fetus organisms are found in greatest numbers in the prepuce, Samuelson and Winter (1966) and only contaminate the semen at ejaculation. However, as it is semen and not PPW that is used in artificial insemination it is possible for a bull to be a carrier yet not infect all the cows inseminated with its semen Murare et al (1959) (see heifer mating section page 29) Herschler (1963) was the first person to use a fluorescent antibody technique to diagnose vibriosis in bulls. O'Berry (1964) prepared hyperimmune antiserum in rabbits and cows using 2 V.fetus venerealis strains. He described the different conjugates obtained and discussed brightness of staining reaction, with the 2 vibrio strains as antigen. Schimmelpfennig and Mitscherlich (1964) used a FAT to differentiate 53 strains of V.fetus, 21 strains of V.bubulus, 3 of V.comma var el tor, and 11 non pathogenic vibrios from water. In 1965 two papers were published which used fluorescein conjugated anti-vibrios fetus serum in the detection of bovine vibriosis. Mellick, Winter & McEntee (1965) prepared their anti-vibrio fetus serum in rabbits, using one V.fetus venerealis strain which had been isolated from an aborted bovine foetus and stored in a lyophilised state until they reconstituted it and

cultured it for antigen production. The anti-vibrio serum so produced was then conjugated with fluorescein isothiocyanate and its specificity established. The FAT was performed on smears prepared from preputial washings from 24 bulls. During the sampling period semen samples were also collected regularly from these bulls and cultured on media containing antibiotics. The semen samples were not collected on the same day as the PPW. In this way they compared the vibrio status of each bull. The results of these studies showed that all the known carrier bulls (11 out of 24) were positive on FAT and on culture. Six of the 24 bulls which were of unknown Vibrio fetus status gave a final result of 4 positive using the FAT. Each of the four bulls which gave these FAT results, also gave at least one positive culture result. The group of negative bulls (7 out of the 24) were negative to both FAT and culture on all occasions samples. The authors concluded that the FAT was "a highly accurate and sensitive method for the detection of V.fetus carrier bulls." By using semen samples for culture and PPW for the FAT they were unable to make the direct comparison of results possible in split sampling. By taking 6 samples they found all animals positive or negative by both methods. Belden and Roberstad (1965) conjugated 4 antisera to V.fetus Montana serotype I, II, III and V. They then used these antisera to ascertain to which serotype each of their 40 vibrio isolates belonged. They compared agglutination cross reactions with FAT cross reactions and found that while

no pattern could be read for the agglutinations, many FAT results did form a pattern. It was finally established by the FAT that there were 3 groups corresponding to Montana Gps. I, II and V. Group III was found to share components with Gp II and V. Belden and Roberstad (1965) brought to notice that there were antigenic differences among V.fetus strain which could render V.fetus antigen non fluorescent when stained with a fluorescent conjugated antiserum prepared against a different strain of V.fetus. They suggested using a pooled conjugate which reacted with all but 2 out of 33 of the vibrios they had serotyped. Two V.fetus isolates and eight saprophytes did not react with any conjugate. Kita, Ogimoto and Suto (1966) classified their 10 Japanese isolates into 3 dist inct groups. Using growth tolerance to 1% glycine and hydrogen sulphide production, all the V.fetus venerealis isolates belonged to one group; all the V.fetus intestinalis belonged to a second, and the third group comprised one intermediate isolate which fell between venerealis and intestinalis in its reactions. Using the FAT, one group comprised all the V.fetus venerealis, the second group comprised most of the V.fetus intestinalis isolates and also the intermediate one. The third group comprised two V.fetus intestinalis isolates. They also found that a pooled conjugate comprising one conjugated antiserum from each of the 3 FAT groups would give good fluorescence with every V.fetus isolate used as antigen. They compared the detection of V.fetus present in the prepuce of

bulls using the FAT with a culture method using a selective medium. The rate of detection was about the same and so they came to the conclusion that "the FA techinque was an effective means of diagnosis of infected bulls." Winter, Samuelson and Elkana (1967) also compared culture and fluorescent antibody methods of detecting bulls carrying V.fetus. They examined preputial washings from 150 bulls, using 269 paired tests. They found that 202 samples were negative with both methods. They diagnosed 64 as V.fetus carriers, 44 being positive with both tests, 12 being positive with the FAT only and 8 by culture only. They concluded from their results that either method was satisfactory for routine detection of carriers but that the combined use of both methods should be used whenever possible. Dufty (1967) also compared culture methods with an immunofluorescent method of detecting V.fetus using 5 carrier bulls and a total of 100 samples. He concluded that the fluorescent antibody technique was the most sensitive of his methods. Philpott (1968 a) estimated that he could detect V.fetus present in PPW containing 100 vibrios per ml of sample. To do this he added dilutions of viable V.fetus culture (on which he performed a viable count) to a negative PPW. In a second paper he compared FAT results with culture performed on the same PPW samples. He examined 142 bulls and of these 17 were positive by both methods, 16 were positive with the FAT only and 10 were positive using culture methods only. He noted

that it was not possible for him to compare the heifer mating test with the FAT but stated that "It is probably of equal sensitivity considerably cheaper and more practicable than the heifer mating test." Samuelson and Winter (1966) used the FAT to determine the sites of proliferation of V.fetus within the prepuce. Their results showed that most vibrios were present in the anterior prepuce around the fornix and in the crypts of the preputial mucosa. Taul and Kleckner (1968) were the first persons to use semen samples successfully for the detection of V.fetus using a FAT. They conjugated their anti-V.fetus serum with fluorescein and also used a counterstain rhodamine by conjugating anti-semen sera with the latter. To perform the FAT they mixed their 2 conjugates together and applied this to the prepared slide. The counterstain dampened any non-specific staining of spermatozoa or cellular debris. Any material still found to be fluorescent could be disregarded if it did not resemble V.fetus morphologically. They reported serological cross reaction between most V.fetus venerealis and V.fetus intestinalis isolates using their FAT. They found one V.fetus venerealis isolate when used as antigen did not fluoresce when stained with conjugated antiserum prepared from a V.fetus intestinalis isolate. They suggested the use of a mixed conjugate that would detect all V.fetus venerealis and V.fetus intestinalis strains for diagnostic purposes. Barnard (1969) using a FAT found that he could not distinguish between V.fetus venerealis and V.fetus intestinalis. He also reported obtaining a

higher proportion of PPW giving positive results using the FAT than using conventional cultural methods. Bingol, Blobel and Scharmann (1969) conjugated antiserum produced using one V.fetus strain as antigen, and found that it reacted with many but not all V.fetus isolates. They also noted that the strains which did give a positive reaction were not always of the same type. V.fetus could always be distinguished from V.bubulus either by FAT, CF test or agglutination.

Many workers from different countries have proved the FAT a sensitive and specific test for the diagnosis of vibriosis in bulls. Some recommend the use of a mixed conjugate to give maximum detection, others advise culture methods as well as the FAT.

In this thesis I give the results of a group of bulls using the FAT only; with another group of bulls I compared my FAT with the best of my culture methods. Also in laboratory experiments I added known numbers of viable V.fetus organisms to negative PPW and compared 2 culture methods of isolation with detection by the FAT.

VAGINAL MUCUS AGGLUTINATION TEST

Stegenga and Terpstra (1949) were first to use a vaginal mucus agglutination (VMA) test for bovine vibrosis. They modelled their test on that developed by Pierce (1946) for Trichomonas foetus. These former authors found that 65% of 37 suspect cows in one herd gave positive titres using their vaginal mucus agglutination test. Blobel, Simon and McNutt (1957) deliberately infected heifers with V.fetus and studied both the VMA and their ability to culture V.fetus from the vaginal mucus. They found that vaginal mucus antibodies took from 4 to 10 weeks to develop after initial infection. They also noted that vaginal mucus should not be collected at oestrus as the result may be falsely negative at this time. The VMA test on cows is mostly used to diagnose the vibrio status of the herd, either all females being sampled and then re-sampled at a later date, or a random sample being taken. Boyd (1955) did a large scale field investigation on 35 swedish herds. In all 3,735 animals were examined and 14,071 vaginal mucus samples collected. He stated that "with certain qualifications the test has been found suitable for herd diagnosis of vibriosis." In the FAO publication "Vibrio fetus infection of cattle." (1960) it is stated that to use the VMA test on its own to examine the vibrio status of a bull, at least 6 heifers should be mated with this bull.

The heifers used must have had negative titres with the VMA test prior to mating. Even if only one of the 6 heifers gave a positive titre with the VMA test the bull is diagnosed as a V.fetus carrier. The VMA reaction is also used together with culture methods in the heifer mating test but the delay of 14 to 105 days between infection and vaginal antibody production renders negative results of little significance. Many heifer mating test animals are only kept for 21 days after infection. The VMA test is still useful as a method of herd diagnosis but today more emphasis is placed in diagnosing vibriosis in the carrier bulls.

The foregoing methods of detection of vibriosis have been used most. The following tests have also been used although they have been less popular.

SERUM AGGLUTINATION

Plastridge and Williams (1943) performed serum agglutination tests on cows from 12 brucellosis free herds. Six of these herds showed evidence of vibrionic abortions. From their serum agglutination results, they concluded that this test was of value as a means of herd diagnosis. Plastridge, Williams and Petrie (1947) noted that V.fetus abortions in cattle were sometimes followed by a lowered conception rate in herds. They also performed serum agglutination reactions on bulls and found 5 gave positive titres and therfore considered them to be infected. Lawson and MacKinnon (1952) found that the serum agglutination test was of little value as a diagnostic test as many cows which

gave a negative serum agglutination reaction gave positive reactions to other methods of diagnosis (i.e., culture or the vaginal mucus agglutination test). Plastridge, Walker, Williams, Stula and Kiggins (1957) stated that it was generally recognised that the bull was the main transmitter of bovine vibriosis and that serological methods of detecting antibody was of little value. Ristic, White, Doty, Herzberg and Sanders (1957) also stated that the serum agglutination test for the detection of circulating antibody in cattle was "not entirely satisfactory."

Other Serological methods.

Haemagglutination test.

Te Punga (1958) performed an indirect haemagglutination (IHA) test on vaginal mucus. He found that he could detect lower levels of antibody and at an earlier stage in the infection than would the vaginal mucus agglutination test. Winter (1963) and also Newsam, Clark and St. George (1967) described an IHA test which gave reliable and specific results.

A complement fixation test (CFT) was used by Kramar and Hoerlein (1969) on both serum and vaginal mucus. Manclark and Pickett (1965) coated latex particles with a soluble antigen extract prepared from V.fetus cells, and detected antibodies using a latex flocculation test.

MATERIALS AND METHODS

MEDIA

A wide range of media was used, as the difficulties in maintaining Vibrio fetus in a viable state were considerable. The organism dies quickly and even freeze drying is not always successful in maintaining viability. This point is agreed upon by bacteriologists working in this field.

MAINTENANCE MEDIUM

This was found to be a good medium for maintaining growth of vibrios and was extensively used in this work.

BATA agar

Blood Agar Base No. 2 (Oxoid)	40 g
Sodium Thioglycollate (BDH)	1 g
Distilled water	1000 ml

This was autoclaved at 15 pounds pressure for 15 minutes. It was then cooled to 50°C and the following added aseptically: Horse blood (oxalated) (Oxoid) 50 ml per litre of medium. Actidione (filter sterilised) (Koch-Light Labs) 0.1 g per litre of medium.

After the addition of the last 2 constituents the plates were poured and they were ready for use.

Vibrio peptone broth (FAO 1960)

This liquid maintenance medium was used as a sub-culturing medium for vibrio species for both general and animal experiments.

Peptone (Evans)	1 g
Sodium chloride	0.5 g
Lemco (Oxoid, Lab-lemco beef extract)	0.5 g
Tap water	100 ml

The medium was adjusted to pH 8. The pressure in the autoclave was raised to 20 pounds pressure and then lowered immediately; after cooling and filtration the pH was adjusted to pH 7.6. The medium was then dispensed and autoclaved at 10 pounds pressure for 15 minutes.

Nutrient agar

This medium was used in roux flasks for growth of the 5 isolates which gave fluorescence with the fluorescein conjugated antiserum.

Nutrient agar (Oxoid)	40 g
Distilled Water	1000 ml

The medium was allowed to soak; then heated in steam for half an hour to melt the agar and then dispensed in 100 ml amounts in 30 roux flasks. These were autoclaved at 15

pounds pressure for 15 minutes. The flasks were removed from the autoclave while still hot and laid flat until the agar solidfied.

SELECTIVE MEDIUM

BAN agar

Direct isolation of vibrio species from preputial washings and vaginal mucus was made on to solid BAN medium, the antibiotics inhibiting many of the contaminating commensal organism present in the specimen. This medium has the same composition as BATA differing only by the addition of the antibiotics:

a) Bacitracin 2 international units per ml of medium (Burroughs Wellcome)

b) Novobiocin 2 micrograms per ml of medium (Glaxo Laboratories, Greenford. This was a complimentary sample)

DILUENT FOR VIABLE COUNTS

Peptone water 0.1%

Peptone (Oxoid) 0.1 g

Tap water 100 ml

The pH was adjusted to pH 7.5. The medium was then dispensed in 100 ml amounts and autoclaved at 15 pounds pressure for 15 minutes. For the counts, 9 ml and 2 ml amounts were aseptically pipetted into sterile universal

containers. Instead of resterilising, the pressure in the autoclave was raised to 15 pounds and then lowered immediately in order to prevent the loss of volume which often occurred on autoclaving for 15 minutes at 15 pounds pressure.

IDENTIFICATION MEDIA

In carrying out the biochemical tests, which were essential for the further identification of Vibrio fetus species, different semi solid basal media were used. By incorporating cysteine hydrochloride 0.02 %, and glycine 1% and 1.5 % respectively into these basal media, hydrogen sulphide production and growth tolerance to glycine could be observed for Vibrio fetus strains.

Hydrogen sulphide production

To 100 ml of each basal medium 0.02 g of l-cysteine hydrochloride (Hopkin & Williams) was added. The medium was then dispensed in 4 ml amounts in bijoux bottles and autoclaved at 10 pounds pressure for 15 minutes. Lead acetate papers (Whatman BDH) were inserted in the neck of the bottle immediately after inoculation.

Tolerance to glycine

To 100 ml of each basal medium 1% and 1.5% respectively of glycine were added. The media were dispensed in 4 ml amounts in bijoux bottles and autoclaved at 10 pounds pressure

for 15 minutes.

These are the basal media used in the tests for identification. All the media were dispensed in 4 ml amounts in bijoux bottles and autoclaved at 10 pounds pressure for 15 minutes.

Basal medium 1

Vibrio Peptone Broth	100 ml
Oxoid Agar No. 3.	0.1 g

Basal medium 2.

Mr. R. M. Gambles, MRCVS, the Ministry of Agriculture Cattle Breeding Centre, Shinfield, Reading, Berkshire, suggested that I tried the following medium which was used routinely by him in identifying vibrio cultures. The additives were the same as the previous media but the composition of the basal medium was different.

Albimi Brucella Broth	1000 ml
Agar (Oxoid No. 3)	2 g
Methylene blue	2 ml of a 0.1% aqueous solution

The constituents in Albimi Brucella Broth are as follows:

Peptone "M"	20 g)	
Dextrose C.P	1 g)	
Yeast autolysate	2 g)	per litre
Sodium chloride	5 g)	
Sodium bisulphite	0.1 g)	

Basal medium 3

Vibrio Peptone Broth	1000 ml
Agar	1 g
Methylene blue	2 ml of an 0.1% aqueous solution

Basal medium 4

Difco Brucella Broth	1000 ml
Agar	1 g

The constituents of Difco brucella broth are as follows:

Bacto	- tryptone	10 g	per litre
Bacto	- peptamin	10 g	
Bacto	- dextrose	1 g	
Bacto	- yeast extract	2 g	
Sodium chloride		5 g	
Sodium bisulphite		0.1 g	

Thiol medium

This medium was used only for growth tolerance to glycine. As the medium contains sulphur, hydrogen sulphide was given off and this medium cannot be used to test for hydrogen sulphide production.

The medium was prepared by dissolving 30 g of thiol medium in 1000 ml of distilled water. It was dispensed and autoclaved as before.

The constituents of Difco thiol medium are as follows:

Proteose peptone No. 3	10 g	per litre
Bacto - yeast extract	5 g	
Bacto - dextrose	1 g	
Sodium chloride	5 g	
Thiol complex	8 g	
Bacto - agar	1 g	
Para-aminobenzoic acid	0.05 g	

Solid glycine medium

A solid medium was also used to study the tolerance of vibrio species to a range of glycine concentrations.

Vibrio Peptone Broth	1000 ml
Agar (Oxoid No. 3)	12 g

This medium was then melted in the autoclave and dispensed into 5 100 ml amounts to which the following concentrations of glycine were added, respectively: 0.6, 0.8, 1, 1.2, and 1.4%. The media were then autoclaved at 10 pounds pressure for 15 minutes, cooled to 56°C and plates were poured for use.

GASEOUS ENVIRONMENT USED FOR GROWTH

Initially culture of Vibrio fetus was attempted on BATA medium in 10% carbon dioxide in air, as in the early literature this gas mixture was regarded as essential, but I found that quite often no growth was obtained. (Topley & Wilson (1964) states that while authors differ in their assessment of an optimal gaseous environment most agree that O_2: 3 - 10%, CO_2: 5 - 10% is desirable. Following these lines the mixture detailed below was used, and with this mixture there was little difficulty in maintaining viable cultures.

Gas mixture

10% carbon dioxide

10% oxygen

80% nitrogen

This mixture was supplied by British Oxygen Company in cylinders.

Containers used to grow vibrio species in the gas mixture

As the vibrio species require to be grown in a gas mixture the containers used for this work were important. McIntosh & Fildes anaerobic jars (BTL) appeared to be the most convenient containers. The hydrogen catalysts were removed from the lid; the anaerobic indicator in the side arm replaced by a carbon dioxide indicator and 2 universals

full of dry Silica jel (to remove moisture) were placed inside, along with the petri dishes, in each anaerobic jar. This was then evacuated and the gas mixture allowed to enter from a balloon: then the jar was evacuated again and the gas mixture allowed to enter for a second time. To check that the jar was properly evacuated and that the concentration of carbon dioxide inside was correct, the indicator tube was shaken. The colour changes for various concentrations of carbon dioxide are given below.

Carbon dioxide indicator.

4 ml of a 0.25% Bromo-thymol blue solution was added to 100 ml of a 0.1% sodium bicarbonate solution. About a 0.5 ml of this mixture was added to the side arm.

COLOUR CHANGE IN CARBON DIOXIDE INDICATOR

Concentration of carbon dioxide	1% ———	10% ———	20%
Colour	blue	green	yellow

STOCK CULTURE OF VIBRIO FETUS

Cultures of Vibrio fetus venerealis and Vibrio fetus intestinalis were obtained from the sources listed in table 4 . The first 10 cultures were used to prepare anti-sera in rabbits. Strains PV, TU and Sh which were obtained

TABLE 4

VIBRIO FETUS CULTURES

Name and type	Origin	Number	Source of isolation
V.fetus venerealis	Central Veterinary Laboratory, Weybridge.	47	Unknown
" "		1980	Bovine source
V.fetus intestinalis	"	GP264	Unknown
" "	"	82	"
" "	"	83	"
" "	"	115	"
" "	"	GP287	"
V.fetus venerealis Serotype I	Dr. Philpott Dept. of Veterinary Medicine, University of Bristol	V33	From cervical mucus of a heifer - from a recent vibriosis outbreak
"		V240	From the vaginal mucus of a heifer - from a Ministry of Agriculture experiment at Reading
V.fetus intestinalis Serotype II	"	C.2	Bovine source
"	"	Sh	From sheep
V.fetus venerealis Serotype I	"	PV	From a carrier bull
"	"	TU	From a carrier bull

* Serotype unknown

at a later date were used as Vibrio fetus antigens in the FAT method to test the spectra of specificity of the antibodies present in the polyvalent antiserum produced against the original 10 strains of Vibrio fetus.

I isolated vibrio species from 2 heifers and 7 bulls and I identified these as far as possible. This identification will be dealt with under the section on biochemical characteristics.

MAINTENANCE OF CULTURES

The ampoules containing the freeze dried cultures, when received, were opened under sterile conditions, reconstituted with vibrio peptone broth and plated out or spotted on to solid BATA medium. The plates, when dry, were then put into a McIntosh & Fildes's anaerobic jar in a gas mixture which initially was 10% carbon dioxide in air, but later the mixture used was 10% carbon dioxide, 10% oxygen and 80% nitrogen, and incubated at 37°C for 5 - 7 days. After this the plates were examined, checked for purity and individual colonies were filmed and stained; then subcultures were made on to BATA medium. This procedure was carried out once a week and this became the routine method of maintaining viable cultures. Later on, in this work, cultures were freeze dried, using the Edward's Speedivac model 5PS centrifugal freeze dryer.

The same procedures were carried out with my isolates from cattle, after the vibrio species had been obtained in pure culture.

INOCULATION OF IDENTIFICATION MEDIA.

The stock cultures and my own isolates of Vibrio fetus were inoculated into the media to check for hydrogen sulphide production and glycine tolerance. One colony was inoculated into a bijou bottle of Vibrio peptone broth and grown in the gas mixture for 2 - 3 days at 37°C. Three to 5 drops of this culture were inoculated aseptically into the identification media: The bacteria were grown in a minimum of 2 different basal media.

a) The Basal media 1 - 4 (page 50)

b) The Basal media 1 - 4 + Cysteine hydrochloride

c) The Basal media 1 - 4 + 1% Glycine

d) The Basal media 1 - 4 + 1.5% Glycine

Lead acetate papers were inserted into the necks of the bottles containing the basal medium and the basal medium + cysteine hydrochloride. The set of 4 bottles was then incubated in air, or in some cases in the gas mixture, at 37°C for 24 hours and they were incubated for a total of 7 days, the results being read daily. The basal medium served as a control: if growth did not occur in this medium but did so in the basal medium + glycine or

in the basal medium + cysteine hydrochloride the whole experiment was repeated. For the basal media, growth and hydrogen sulphide production were recorded. Vibrio bubulus gives hydrogen sulphide production in all the basal media alone. Therefore, recording of hydrogen sulphide production in the basal media signified that Vibrio bubulus, and not Vibrio fetus, had been isolated. Vibrio fetus gives growth but no hydrogen sulphide production in the basal media alone.

MATERIALS USED IN THE PREPARATION OF THE FLUORESCENT CONJUGATED ANTISERUM

Buffers.

Phosphate buffered saline (0.01 molar)

Di-Sodium hydrogen phosphate (anhydrous) 1.2 g/litre of saline. Sodium di-hydrogen phosphate ($2.H_2O$) 1.8 g/litre of saline. The saline was 8.5 g of sodium chloride per litre of distilled water. The 2 solutions were mixed together to give a buffer of pH 7.2. which is 0.01 molar (M). This will be referred to throughout as phosphate buffered saline (PBS). This buffer was used in the sephadex column, and as diluent for the conjugated antiserum.

Phosphate buffered saline 0.15M

Di-sodium hydrogen phosphate 18 g/litre saline. Sodium di-hydrogen phosphate 26 g/litre saline. These solutions were mixed to obtain a buffer of pH 7.2. This buffer was only used for the DEAE cellulose column.

Glycerol/phosphate buffered saline

This buffer was always used as mountant between the slide and cover slip of a fluorescent stained slide preparation.

Glycerol	9 parts
Phosphate buffered saline 0.01 molar, pH 8	1 part

Carbonate/bicarbonate buffer.

This buffer was used for the actual conjugation. It was made up on the day it was to be used as it was unstable.

The constituents were as follows:-

A. Sodium carbonate 16.5 g/litre

B. Sodium bicarbonate 12.6 g/litre

Two ml of solution A. was mixed with 23 ml of solution B. The pH should then be pH 9.

Column chromatography

Sephadex column.

This was used to separate the fluorescent conjugated γ-globulin from the uncombined fluorochrome.

A G25 coarse grain sephadex was used, the column being prepared in PBS (0.01M).

Diethylaminoethyl cellulose column.

This was used to separate the fluorescent conjugated γ-globulin from highly charged high molecular weight proteins.

Whatman's diethylaminoethyl (DEAE) cellulose was used; the column being prepared in PBS (0.15M).

Fluorescent stain

Fluorescein isothiocyanate, purchased from Baltimore Biological Laboratories, U.S.A., was used. 3mg of fluorescein

isothiocyanate was used to conjugate 100 mg of γ-globulin. This fluorochrome stains a bright yellow-green when exposed to ultra violet light.

Dialysis

The vibrio antiserum after precipitation with ammonium sulphide was dialysed against saline to remove ammonium and sulphide ions from the γ-globulin. As the volume of material to be dialysed was small, 0.5 inch diameter dialysis tubing was used.

Concentration of antisera

During purification of the conjugated antiserum it became necessary to concentrate the material. A membrane concentration apparatus was used for this. The material to be concentrated was pipetted into a membrane sac and by using suction, water was withdrawn from the sac. As concentrations took several hours to reduce the volume from 10 ml to less than one ml the apparatus was immersed in an ice bath to maintain the temperature of the conjugated antiserum at around 4°C.

FLUORESCENCE MICROSCOPY

Certain chemicals were found to possess the quality of attaching themselves to certain proteins (antibody) without altering their immunological reactions and to be

capable at the same time of emitting visible light of a different wavelength when irradiated with ultra violet or blue light. (In practice, the ultra violet or blue light has to be filtered out after illuminating the specimen but before it reaches the eyes of the observer.)

Use was made of these facts in the development of the fluorescence microscope. The specific antigen antibody reaction was detected visually by specific fluorescence of the antigen.

MICROSCOPE

A Leitz Labolux microscope was used for the fluorescent antibody work. It has a horizontal movable stage and an angled binocular eyepiece.

A dark field condenser and a mercury vapour lampwere used.

Dark field condenser

This must be correctly adjusted to give even illumination throughout the entire field, using the oil immersion objective (x 100). As each objective, i.e., x 10, x 40, must be centred separately and as the oil immersion objective was in use all the time the correct setting, once obtained for this, was not ordinarily altered.

Mercury vapour lamp

The mercury vapour lamp emits ultra violet blue light of steady intensity. It is enclosed in a specially designed air-cooled housing unit as the mercury arc, in addition to emitting ultra violet blue light, (280-600 millimicrons) gives out a considerable amount of heat. A starter unit provides a transient high voltage. This voltage (approx. 15,000 v) is used to strike the arc, followed by a much lower voltage which is sufficient to maintain it. A few minutes after ignition the light emission remains constant. The average life of the bulb is 200 hours, assuming that the bulb is operating for 2 hour periods. Many bulbs become unsatisfactory before this period due to the intensity diminishing or the intensity varying continually (flickering). It is important that this bulb is fitted and adjusted correctly to give optimal illumination, and that the correct procedure is carried out while in use, as there is some risk of the bulb exploding.

Filters

There is an immovable heat stopping filter incorporated in the Leitz lamp housing, thus preventing the heat emitted reaching the specimen or the microscopist's eyes.

Primary filters.

These are designed to prevent the passage of heat and visible light but to allow certain wavelengths of light to be transmitted. The primary filters used for this were:

a) Heat stopper

b) BG 12 of thickness 0.5 or 1mm

Secondary or barrier filter

This filter is used to prevent harm being caused to the microscopist's eyes by ultra violet light reaching them. In the Leitz microscope the filter K530 is inserted just below the ocular lens but could be placed anywhere between the specimen and the ocular lens.

Objective lens.

A (x 100) apochromat objective was used in this work. In order to reduce the numerical aperture of this lens a funnel stop was inserted in the objective. An oil immersion lens is not necessary for most fluorescent work but in order to identify the fluorescent objects as vibrios it was considered necessary and was used all the time. The fluorescence becomes more dim as the magnification increases.

Ocular lens

A binocular x 12. 5 was used.

A monocular is considered sufficient for fluorescent microscopy as there is less light lost than with the binocular. However, for screening purposes the binocular was much less tiring on the eyes. Probably due to the fact that the mercury vapour lamp was used as light source there was no difficulty in obtaining strong fluorescence, using the above set-up.

Screening procedure

For darkground microscopy glass slides not thicker than 1 mm were necessary. As each slide was prepared in the same way the screening procedure was standardised. Although almost all the fluorescein-labelled slides were screened by myself, on some occasions the number of fields covered in a standard time was more than on other occasions. (For example, due to misting of the oculars). Most of the microscopy was done in a darkened room but while this made the actual detection of the fluorescent vibrio cells easier, it did cause more rapid eye fatigue. The preparation of antigen-antibody mixtures were made on these glass slides, then after staining they were mounted in the glycerol/phosphate buffered saline mountant. (Page 59). Coverslips of size (22 x 40) mm were used to enable the slide to be screened from left to right covering the edges of the

film. A drop of immersion oil was placed on the dark field condenser; on the underside of the slide and over the coverslip. There must be immersion oil between the dark field condenser and the slide and also between the coverslip and the x 100 objective. It was considered important to include the edges of the film in the screened section as it is known to contain more bacteria than the central area. The larger coverslip enables this to be done without mixing immersion oil and Glycerol/PBS mountant. A strict pattern was adhered to, always moving across the film from left to right and when the edge of the film was reached, always moving upwards. This was done for 10 minutes, the number of fluorescent vibrio cells which were seen in that time being recorded. Slides were always screened on the same day as the fluorescent antibody technique was performed. For some experiments, so many slides were made that while all were prepared and fixed on the day of the experiment some were not stained until a week later. During this period the slides were stored in the refrigerator. By carrying out this procedure of storage, there was no loss of fluorescence during storage.

ANTISERUM PRODUCTION

Polyvalent antisera were made by inoculating the Vibrio fetus cultures numbers 1 - 10 in table 4 into 5 rabbits, each rabbit being injected with all 10 strains of bacteria.

Preparation of antigen suspension

Live suspensions of Vibrio fetus were used for rabbit antiserum production. Owing to difficulties in handling a large number of vibrio cultures, only 3 cultures at a time were used in the preparation of material for immunisation. Each culture was plated out on to 10 BATA plates and grown at 37°C in the gas mixture for 3-5 days. Each plate was then harvested into a separate bijou bottle containing 1 ml of vibrio peptone broth, and the suspension filmed and stained by Gram's method. The suspensions which appeared microscopically to be pure, were used as inoculum for large scale culture in roux flasks. The roux flasks contained 100 ml amounts of BATA medium; these flasks being pre-incubated overnight to check that the media were sterile. After using the suspensions of Vibrio fetus for inoculum, 10 ml amounts of vibrio peptone broth were added to each flask to ensure that the entire agar surface was covered. A total of 10 flasks were inoculated per culture, the inoculum for each

flask being derived from the growth scraped from one BATA plate. Once the suspensions had been allowed to soak into the agar surface for a few minutes, the flasks were inverted and the excess liquid allowed to run on to the glass surface below. The flasks were then incubated in the gas mixture at 37°C for 4 - 6 days. Prior to harvesting, the excess liquid was discarded by pouring off aseptically, flaming the neck of the flask and replacing the plug. The surface of the medium was then examined for contaminants. If none was observed the surface was flooded with 10 ml of sterile 0.1% peptone water and then growth scraped off. A sterile scraper was devised to remove the growth from the surface of the roux flasks. Glass beads were not found to be of use for this. The scraper was made by attaching a piece of rubber tubing to a glass pipette, the whole being sterilised (after wrapping in grease proof paper) in the autoclave at 15 pounds pressure for 15 minutes. The suspension was then transferred to a sterile universal container. A second 10 ml of 0.1 peptone water was then flooded over the same surface and the remainder of the growth detached and again transferred to the universal container. This suspension was filmed and stained by Gram's method. If there were no contaminants visible on the stained preparation, the

suspensions from one set of roux flasks were pooled and spun in sterile 100 ml bottles in the refrigerated centrifuge until the supernatant was clear (approximately 4,000 rpm for 30 minutes). The supernatant was discarded and replaced with the same volume of fresh 0.1% peptone water. This process of washing the cells was repeated 3 times, the final spin being at 4,000 rpm for one hour in a graduated centrifuge tube. The supernatant was discarded and the deposit was diluted 1:10 in 0.1% peptone water. To obtain a standard inoculum of this suspension, further dilution (i.e., 1:200) was required before a reading of 30 ± 10 could be made on the Eel Nephelometer. The 1:10 suspension was used in the antiserum production. Equal quantities of vibrio suspenions and Freund's complete adjuvant (Difco) were well mixed by loading and discharging the mixture through a syringe and needle several times. Mixed suspensions were used as inoculum for each of the 5 rabbits. It was not possible to incubate the larger quantity of cultures that would have been required to prepare all 10 strains for inoculation at the same time and so the following schedule was carried out.

TABLE 5

SCHEME OF INOCULATION FOR ANTIBODY PRODUCTION IN RABBITS

Injection number	*Vibrio fetus* strain number	Route	Amounts of suspension and adjuvant injected
First	115,83	I/P	0.5 ml + 0.5 ml Freund's adjuvant
Second	115,83,82* C2, 1980*	I/M	1 ml + 1 ml Freund's
Third	C2,1980,V33	I/M	1 ml + 1 ml Freund's
Fourth	V33, 47, GP264 1980, GP287, 82	I/M	" "
Fifth	GP287, 1980, 82 V33	I/M	" "
Sixth	V33, GP264, 1980 V240	I/M	" "
Seventh	1980, C2, V33 GP264	I/M	1 ml mixture (no adjuvant)

* stored for 1 week at 4°C before injecting

I/P = intraperitoneal injection

I/M = intramuscular injection

Five days after the last injection the rabbits were bled from the marginal ear vein, 20 ml of blood being withdrawn. This was followed by 2 subsequent 20 ml quantities of blood being withdrawn within a further period of 7 days. The titre of the anti-serum so produced was estimated using the agglutination method of

Morgan (1959). The opacity of the Vibrio fetus suspension used for agglutination was adjusted to match Brown's opacity tube no. 1. The agglutination was the flagellar type, the diluent being 0.1% formol saline.

Absorption of antiserum

The pooled vibrio antiserum was used in an indirect or sandwich method of fluorescent antibody staining. The conjugated anti-rabbit γ-globulin was purchased from Sylvana (New York, U.S.A.).

The 10 immunising strains of vibrio (page 55) were used as antigens (page 67). Six slides were prepared (page 88) which were treated with dilutions of (a) my pooled antiserum and (b) the commercially prepared antiserum. Doing this I found that 1:40 dilution of both antisera gave maximum fluorescence (44) with all 10 strains of Vibrio fetus.

The specificity of the staining reaction was checked to ensure that only Vibrio fetus would react with my prepared antibody or with the commercial conjugate. Therefore bacteria which were usually present in semen samples and preputial washings were isolated on BATA plates. The organisms isolated were identified by colonial morphology, microscopic appearance, and some biochemical reactions. The following species were found:

Pseudomonas, Escherichia, Micrococci, Streptococci, Corynebacteria, Proteus and Vibrio bubulus. (Also from stock cultures Brucella abortus and 3 other Vibrio bubulus strains). These bacteria were filmed and stained with the 2 antisera each diluted 1:40 as in the test (which would give maximum fluorescence with Vibrio fetus). Five organisms - a Staphylococcus aureus species, a Streptococcus species and 3 Micrococci species - gave fluorescence at this 1:40 dilution. In some samples of preputial washings a large coccoid organism was present which fluoresced strongly but it had never been isolated. Because of its size and shape it is quite distinct from Vibrio fetus and would not be wrongly identified even as a coccoid Vibrio fetus. Vibrio fetus can become coccoid especially from week-old cultures, and these coccoid forms fluoresce as strongly as the bent rod-like shapes. For this reason it was thought necessary to absorb the antisera with these isolated cocci and thus prevent fluorescence with these cocci. Coccoid vibrios have never been seen on stained slides from fresh clinical material.

Several conjugated anti-rabbit γ-globulin sera were purchased but none was completely satisfactory. Antisera from Burroughs Wellcome, Sylvanna, and Baltimore Biological Laboratories all gave fluorescence with the

5 isolates. Antiserum from Windthrope Laboratories did not give a sufficiently strong fluorescence with Vibrio fetus. At this time a sheep and goat were being immunised against rabbit γ-globulin and the goat antisera was conjugated and tested in the indirect FAT along with the above commercial products. Undiluted the goat antiserum gave good fluorescence against Vibrio fetus and only very poor fluorescence against the 5 isolates. My conjugation of the anti-rabbit γ-globulin was considered to invalidate the convenience of using the indirect fluorescent stain so it was proposed to conjugate the anti-vibrio serum directly and if possible compare the result obtained by using the rabbit anti-vibrio (conjugated by myself in the laboratory) in a direct FAT with the results obtained using the indirect FAT, being Burroughs Wellcome conjugated anti-rabbit γ-globulin. Burroughs Wellcome antisera was chosen because it gave as high a titre as Sylvana, or Baltimore and was more easily ordered and less costly. The unwanted staining reaction of the cocci was first observed while using the indirect fluorescent staining technique. This meant that the cause of the fluorescence was antibodies either in the anti-vibrio serum and/or antibodies in the anti-rabbit serum. Either species might have previously acquired antibodies to these 5 contaminants by a sub clinical infection or the other possibility was

that Vibrio fetus shared antibodies with the micrococci. It was found that both sera had to be absorbed as both appeared to contribute to the fluorescence.

Method of absorption of antisera

Indirect

Both my anti-vibrio serum and the Burroughs Wellcome conjugated anti-rabbit γ-globulin serum were absorbed with the 5 coccoid isolates. These 5 isolates were grown on BATA medium at 37°C, each isolate being inoculated on to about 4 BATA plates. If the growth appeared to be pure it was scraped off the surface of the BATA medium with a strong platinum loop and transferred into a universal container containing 20 ml of 0.1% peptone water, the growth from one plate being inoculated into one universal container. Each 0.1% peptone water suspension was filmed and stained by Gram's staining method and if it appeared to be pure the suspensions were inoculated into roux flasks containing nutrient agar (page 47). The roux flasks were inoculated by pipetting 10 ml of the suspension into each roux flask. About 6 roux flasks were inoculated from each isolate. These were incubated at 37°C for 24 - 48 hours, and harvested in a similar manner to the vibrio species using sterile saline (page 67). The suspension was spun in the refrigerated centrifuge at 4,000 r.p.m., for

20-30 minutes. The supernatant was then discarded and replaced by fresh sterile saline. This process of washing the cells by centrifugation was repeated twice, the final centrifugation being done in a graduated centrifuge tube at 4,000 rpm for 20-30 minutes; then approximately 0.5 ml of the deposit from each isolate was mixed with the vibrio antiserum and incubated at 37°C for 18 hours. Two drops of saturated sodium chloride were added to aid antibody-antigen reaction. After absorption the serum was spun at 27,000 g. for 20 minutes to deposit the cocci. The supernatant was then passed through a millipore filter of 0.22 micron diameter porosity to sterilise it. The filtrate was then used in an agglutination reaction to test if any agglutinating antibodies against the 5 isolates remained. The dilutions were made in formol saline. The antigen suspensions for the agglutinations were prepared in the same way as for absorption. The opacity of each suspension of antigen was standardised. The suspensions were used in the agglutinations at a concentration of 7 times the opacity which gave a nephelometer reading of 90 ± 10. Each antigen was incubated for 18 hours prior to use and if auto-agglutination occurred the supernatant was adjusted to the correct opacity and used as antigen against the antiserum. Equal volumes of antigen and antiserum were incubated for 18 hours and no agglutination occurred with 4 of the 5 antigens. One antigen gave partial agglutination from

serum dilution 1:2 to 1:16. All antigen controls composed of equal volumes of antigen suspensions and saline showed no agglutination. The absorption process was repeated with the antigen which gave agglutination, fresh antigen being used. After centrifugation and millipore sterilisation of the serum the agglutination test was repeated with this antigen suspension only and no agglutination occurred at 1:2.

A serum agglutination titre for Vibrio fetus strain 1980 was also set up and the titre dropped from 1:512 to 1:256 The ability of the antiserum to react with the 5 isolates and so give a positive FAT was examined later when the conjugated anti-rabbit γ-globulin had also been absorbed.

Absorption of conjugated anti-rabbit γ-globulin (B.W.)

The conjugated antiserum was absorbed with the 5 isolates, the same procedure being carried out as with the anti-vibrio serum except that the reaction time of the cocci with the conjugated antiserum was only 4 - 6 hours at 37°C. As before the serum was centrifuged at 27,000 g. at 4°C to deposit the cocci and then sterilised by millipore filtration of the supernatant. To test the efficiency of the absorption of the antibodies to the 5 cocci and to check the titre of the absorbed antisera against the 10 Vibrio fetus strains, the indirect FAT was performed using the 2 absorbed sera (my anti-vibrio and the conjugated anti-rabbit).

Preparation of Vibrio fetus strain antigens to determine the titre of the antisera used in the fluorescent antibody staining reaction

Each of the 10 immunising Vibrio fetus strains were grown on BATA plates for 2 - 3 days. Growth was removed from the surface of the plates and emulsified in saline, and the cells washed by centrifugation at 27,000 g. at 4°C for 15 minutes. The supernatant was discarded and replaced with saline. These films were then made, air dried, fixed in absolute alcohol and air dried and then stained. The results are given in table 6 page 79. From those results the dilutions of each serum to be used in the indirect FAT were 1:40.

Films from the 5 contaminants were prepared in the same way as the vibrio species. All were tested using the vibrio antiserum at 1:40 dilution and the conjugated anti-rabbit at 1:40 dilution. The fluorescence with each was just detectable and no more (±). So absorption had been successful.

Direct

The rabbit anti-vibrio serum was absorbed in the same manner as it had been previously for the indirect FAT. The absorbed serum was then conjugated with fluorescein isothiocyanate following the method used in the Western Infirmary, Glasgow (see conjugation of anti-vibrio serum page 81).

The 10 original Vibrio fetus cultures were titrated against the absorbed conjugated anti-vibrio serum. The

antigenic material was freshly prepared. The results are given in table 7 page 80. The procedures of making and staining the films are described on page 88.

TABLE 6

INDIRECT FLUORESCENT ANTIBODY TITRATION RESULTS

ANTIGEN	ANTISERA		
	A 1:40 B 1:40	A 1:40 B 1:80	A 1:80 B 1:40
47	4+	4+	4+
V33	"	"	"
V240	"	"	"
C2	"	"	"
GP264	"	"	"
115	"	"	"
82	"	3+	"
83	"	4+	"
1980	"	"	"
GP287	"	"	"

A - Own anti-*Vibrio fetus* serum - (pooled, absorbed)

B - Burroughs Wellcome conjugated anti-rabbit γ-globulin (absorbed)

All dilutions were made in phosphate buffered saline.

The following figure code was used throughout this work with both the direct and indirect staining methods.

4+ This means maximal brightness, with cell outline still sharp and discrete.

3+ Brightness slightly less than maximal; cell outline still sharp and discrete.

2+ Brightness less. Cell outline less sharp - a slight haziness

+ Just discernible ± Barely discernible

TABLE 7

DIRECT FLUORESCENT ANTIBODY TITRATION RESULTS.

VIBRIO STRAINS	DILUTIONS OF CONJUGATED ANTI-VIBRIO SERUM		
	1:16	1:32	1:64
1980	4+	4+	3+
GP287	4+	3+	3+
C2	4+	3+	3+
V240	4+	3+	3+
GP264	4+	4+	3+
47	4+	2+	2+
V33	4+	3+	3+
82	4+	3+	2+
115	4+	4+	3+
83	4+	4+	2+
Sh	4+	ND	ND
Serotype II	4+	ND	ND

See foot of table 6 , page 79 , for figure code.

CONJUGATION OF THE VIBRIO ANTISERUM

The polyvalent vibrio antiserum was labelled with fluorescein isothiocyanate. The labelled antibodies were then used in a direct fluorescent antibody technique (FAT) to detect the presence of Vibrio fetus cells, the reaction of specific antibody with antigen being denoted by fluorescent Vibrio fetus cells. Before conjugation γ-globulin was precipitated to prevent labelling of other proteins present in the antiserum. This was followed by conjugation and finally purification of the labelled antiserum by removal of unwanted fluorescent material.

Precipitation of γ-globulin

Ten ml of the absorbed anti-vibrio serum was pipetted into a sterile beaker containing a sterile small metal rod covered in autoclavable polystyrene. The beaker was placed in an ice bath on a magnetic stirrer. The stirrer was adjusted so that it rotated effectively but did not cause frothing of the serum. Five ml of cold (4°C) saturated ammonium sulphate was added dropwise to the antiserum making it 33% saturated. A white precipitate of γ-globulin was formed. This was left to stir for one hour. The precipitated serum was then spun at 6,000 rpm for 15 minutes at 4°C and the supernatant discarded. The precipitate was then resuspended in 33% saturated ammonium sulphate made up in saline (0.15M). This was then stirred for 15 minutes in the ice bath as before and the precipitated γ-globulin

spun at 6,000 rpm for 10 minutes at 4°C. The supernatant was again discarded. The precipitate was resuspended in 0.15M saline (5 ml) and pipetted into a dialysis sac to be dialysed against 0.15M saline to remove the ammonium and sulphate ions from γ-globulin. The dialysate was changed several times and it was tested for the presence of sulphate ions by removing 2 ml and adding to it 4 drops of 0.1N hydrochloric acid and a few drops of barium chloride (2%). If sulphate ions are present a white precipitate will be formed. Complete removal of these ions may take 2 days, so as large a volume of dialysate as possible was used, and changed after several hours. The γ-globulin was then centrifuged at 3,000 rpm for 10 minutes at 4°C and the deposit discarded. The protein content was read in a U.V. spectrophotometer (280 mμ OD = 1 = 1 mg protein per ml).

Conjugation

The fluorescein isothiocyanate which was used was purchased from Baltimore Biological Laboratories, U.S.A., All commercial products (unless the extremely expensive pure crystaline material) contain impurities. For effective conjugation the fluorescein/protein (γ-globulin) ratio is 1:40. 3 mg of fluorescein was weighed out and this was used to conjugate 100 mg of γ-globulin. The globulin should have a volume of 8 ml but if it were less, it would be made up to this volume with 0.15M saline. To the 3 mg of fluorescein 1.5 ml of the carbonate/bicarbonate

buffer was added and the fluorochrome allowed to dissolve. This was then added dropwise to the mixing γ-globulin at 4°C. The mixing was continued in the refrigerator for 18 hours. The pH of the serum to which the buffered fluorescein was added should be pH 9.0. (More buffer could be added to obtain the correct pH).

Conjugation takes place at a high pH and the pH must be reduced to pH 7.2 as soon after conjugation as possible to prevent alteration of the protein-conjugation material, which could occur at a high pH. Gel filtration does this in addition to removing unreacted fluorescein.

Removal of the unreacted fluorochrome

This was done by gel filtration. Ten grams of G.25 coarse grain sephadex was stirred into about 400 ml of phosphate buffer saline pH7.2 (0.01M). After all the large particles had settled, the very fine ones still in suspension were poured off and more PBS poured on to the sephadex and the process repeated. The sephadex was soaked in PBS for 3 hours at room temperature (or overnight at 4°C) to allow adequate swelling of the granules. To pour the column the granules were mixed in the beaker and the slurry poured steadily into the column. The tap at the foot was opened to adjust the rate of flow and the slurry was steadily poured in. There should be no air bubbles in the column nor should there be any ridges caused by the gel being allowed to pack before all the slurry has been poured into the

column. Once the column had packed, PBS was run through it at a steady rate for about 45 minutes. When the level of saline had reached the top of the column the conjugate was pipetted on. When it had just disappeared from the surface of the column, PBS was pipetted on to the top of the column and a saline head (reservoir) put on to effect the separation. The rate was just faster than a drop a second. When the coloured fraction reached the foot of the column, collection of the eluate began. All of the first coloured fraction was collected.

This column retained the unattached fluorescein and eluted the conjugation γ-globulin i.e., material of molecular weight (MW) up to 5,000 will be retained whereas γ-globulin with a MW about 40,000 will be eluted. The eluate was then concentrated to the original 5 ml volume using a membrane concentration method.

Removal of materials causing non specific fluorescence with DEAE cellulose column

A column was prepared using DEAE cellulose. About 6 gms of Whatman's DEAE cellulose was weighed out and mixed into a beaker containing PBS pH 7.2., 0.15 Molar. This was allowed to soak for several hours at room temperature and then the column was prepared in a similar manner to the sephadex one. This column once packed had to be washed overnight with 0.15M PBS or until the pH was 7.2. The 5 ml of conjugated material was passed through the column.

Using this system the large molecular weight proteins were retained and the fluorescein labelled γ-globulin eluted. The eluate this time was more green and usually large in volume if all the coloured material was collected (approximately 30 ml). This was also concentrated using the membrane concentration apparatus and as before it was concentrated to 5 ml. A drop of 2% sodium azide was added to act as preservative. The conjugate was then dispensed in approximately 0.1 ml.

COLLECTION OF PREPUTIAL WASHINGS

The following materials were used:

25-30 ml amounts of sterile saline

Sterile flutter valves with 3ft of rubber tubing attached.

Sterile insemination pipettes

Disinfectant

Rubber gloves

Preputial washings were collected from bulls from various localities by inserting 25-30 ml of sterile saline into the prepuce. This was done by attaching a flutter valve to a universal bottle containing sterile saline. At the same time a sterile insemination pipette was attached to the rubber tubing of the flutter valve. The insemination pipette was inserted into the posterior end of the prepuce and then the saline was run in by raising the level of the universal container and

inverting it. The orifice of the prepuce was kept closed with one hand and with the other hand the saline was massaged inside the prepuce especially at the fornix. The pipette was then drawn forward and the material allowed to run back into the bottle. (If the washing still looked like clear saline then the saline which had run back had never left the rubber tubing and the process was repeated).

Between the collection of each sample the operator's gloved hands were washed in disinfectant and dried.

The maximum time between sampling and dealing with the material in the laboratory was 3 hours.

All the instruments used were rinsed, washed and very well rinsed and then autoclaved at 15 pounds pressure for 15 minutes.

TREATMENT OF PREPUTIAL WASHINGS IN THE LABORATORY

When the preputial washings arrived at the laboratory, they were first mixed for thirty seconds using a vortex mixer. Each sample was transferred to a graduated centrifuge tube and centrifuged at 2000 rpm for 5 minutes to deposit the gross debris. Four ml of the supernatant was transferred to a superspeed centrifuge tube and centrifuged at 27000 g for one hour at $4^{o}C$. Three ml of the supernatant were

carefully withdrawn and discarded. The deposit was then mixed in the remaining one ml for 30 seconds using the vortex mixer. This mixed deposit was then used in 3 ways to detect the presence of V.fetus. Firstly, it was plated out for direct culture of the material on to media containing antibiotics. Secondly, some of the deposit was passed through a millipore filter and the filtrate cultured. Thirdly, slides were prepared for examination by the FAT.

Direct culture from preputial washings

The final spun deposit of the PPW was directly plated out on predried BAN media plates. 0.05 ml was spread out for isolated colonies on each of 3 plates. When the inoculum had dried into the BAN medium plates, they were incubated in jars containing the gas mixture for 7 days at 37°C after which they were examined for the presence of V.fetus.

Millipore filtration.

Millipore filtration was used as a direct means of separating Vibrio fetus from other organisms which could be present in preputial washings. After centrifugation, page 86, the material was drawn into a 2 ml disposable syringe, the needle discarded and replaced by the previously assembled and sterilised swinny adaptor containing a membrane of 0.65 /u

porosity millipore filter. The suspension was then filtered by forcing the syringe plunger down, and the filtrate collected in a sterile bijou bottle. If all the suspension had not left the syringe the swinny adapter and syringe were disconnected and air was sucked into the syringe by lifting the plunger. The swinny adapter was attached to the syringe again and pressure was again put on the plunger. After this procedure, with all specimens, at least 0.6 ml of filtrate was obtained. The filtrate was then spotted over one pre-dried BATA plate and one pre-dried BAN plate. Occasionally it was spread out on another BATA plate to obtain isolated colonies. The inoculum was left to dry at room temperature or 37°C after which the BAN and BATA plates were incubated in the gas mixture in the anaerobic jars at 37°C for 7 days. The plates were then examined for V.fetus.

Preparation of slides to be examined by the FAT.

Smears to be stained by one of the fluorescent antibody techniques were prepared on glass slides; 0·025ml of the material to be examined - either PPW or pure cultures of V.fetus were pipetted onto each of 10 slides - the centres of which were of known area. Different diluents were used. These were 0.1% peptone water, saline and PBS. The smear was allowed to dry in air and then the slide was flooded with

absolute alcohol for 10 minutes. After this time it was again allowed to dry in air, and the slides were ready for staining.

Direct fluorescent antibody technique.

One ampoule of my conjugated antiserum which had a titre of 1:16 was removed from the deep freeze (-20°C). After thawing at room temperature, this dilution was prepared by taking one drop of conjugate and adding to it 15 drops of phosphate buffered saline, 0.01M, pH 7.2 (PBS). A drop of this antiserum dilution was then placed on each prepared slide. In experiments 1, 1A and 1B a negative control slide of the preputial washing pool alone was also treated in the same manner. A positive control slide was also prepared by making a smear from viable culture of Vibrio fetus and after alcohol fixation a drop of the diluted conjugated anti-serum was added to this preparation. It is essential to ensure that the marked area containing the antigen on the slide is completely covered by the conjugate. This was done by using a fine capillary tube with an end sealed in the bunsen to give a fine smooth small sphere of glass. This was used to spread the antiserum, being wiped clean between each slide. The slides were immediately placed horizontally in a perspex dish with a tight fitting lid, the atmosphere having been made moist with hot wet Kleenex tissues. The slides were incubated at

37°C for 15 minutes. Each slide after being taken out of the dish was examined to ensure that no area of the film had dried. If the conjugate dries, it gives rise to a great deal of background fluorescence, making the observation of vibrios difficult and the result of doubtful significance. If drying had occurred the slide was discarded and a repeat slide stained. The slides after this examination were placed on a staining rack and flooded separately with PBS several times. This rinsing process was repeated every 10 minutes for 30 minutes, the slides never being allowed to dry. The surplus PBS was allowed to run off the slide and the slide wiped carefully round the marked area. A drop of mountant (glycerol/PBS) was placed on the film and a coverslip placed over it. The slides were then ready for examination.

A control test to inhibit the fluorescence of the vibrio organisms by the addition of unconjugated vibrio antiserum was not done with each dilution but was done once. Unconjugated anti-vibrio serum diluted 1:40 was added to the film of broth diluted in preputial washings, then after incubating in a moist dish at 37°C for 15 minutes, followed by rinsing in PBS for 20 to 30 minutes, the same method of staining with conjugated antiserum was performed on the same slide. On examination under U. V. light fluorescence of the

vibrio cells was greatly diminished and was just discernible. (The fluorescence of the cells was reduced to + or 2+ from 4+. See page79 for fluorescence code).

Indirect fluorescent antibody technique

The films were prepared and fixed as described. An ampoule of the absorbed anti-vibrio serum and an ampoule of the absorbed conjugated anti-rabbit serum were removed from the deep freeze (-20°C). After thawing the anti-vibrio serum was diluted 1:40 in PBS and a drop of this dilution placed in the films of each preputial washing dilution, in experiments 1, 1A and 1B. The slides were then incubated in a moist perspex dish for 15 minutes at 37°C, the same precautions being taken to ensure that the entire marked area on the slide was covered with the antiserum and that it remained wet during the incubation period. After incubation the slides were removed to a staining rack and each slide flooded separately with PBS. This rinsing process was repeated every 10 minutes for 30 minutes. The surplus PBS was run off the slide and it was wiped dry around the marked area. Without allowing the area inside the marked area to dry a drop of the conjugated anti-rabbit serum diluted 1:40 in PBS was placed on each of the slides. These were then incubated in the same manner for 15 minutes at 37°C. As before the slides were removed from the incubator, and rinsed

in PBS for 30 minutes, prior to mounting in the glycerol/PBS mountant.

A control slide (using a 1:5 broth culture dilution of PPW in experiments 1, 1A and 1B) was treated with only the conjugated anti-rabbit serum, rinsed and mounted. A second control using the same dilution of PPW but using pre-immune or normal rabbit antiserum, instead of anti-rabbit serum was treated in the same way as in the indirect FAT

The foregoing methods were used in all the experimental work. The following 3 experimental sections are treated separately, each containing a description of the experiment followed by results and discussions. In some sections materials and methods are also detailed where they apply only to that section.

The 3 experimental sections are:-

1. Laboratory methods of detecting V.fetus.

In this section V.fetus was deliberately added to preputial washings known to be vibrio free. This is described under experiment 1, and was repeated on 4 occasions, denoted experiments 1A, 1B, 2 and 2A.

The aim of these experiments was to compare the efficiency of the following methods to detect the presence of V.fetus. The 3 methods chosen were:

a) The isolation of V.fetus using a selective solid medium containing antibiotics.

b) The isolation of V.fetus by passing the material through a millipore filter and culturing the filtrate.

c) The detection of V.fetus using a fluorescent antibody technique.

2. Insemination of heifers with laboratory strains of V.fetus venerealis.

In this section known numbers of V.fetus cells were inseminated into heifers and vaginal mucus cultured in order to reisolate V.fetus (experiments 2, 2A, 3 and 3A)

3. Bull survey

In this section some 89 bulls were screened for vibriosis using the FAT. In one group a culture method was also used. Any isolates obtained were identified as far as possible.

Some preliminary experiments were performed in an attempt to establish the best methods of preparing and treating the artificially infected preputial washings. From the results obtained, sample amounts, centrifugation times and the numbers of viable V.fetus cells required to be added to the PPW were determined.

EXPERIMENTS 1, 1A and 1B

EXPERIMENT 1.

Three methods of detecting V.fetus added to preputial washings (PPW) were compared. The PPWs collected from the bulls were negative for the presence of V.fetus. The samples were examined by the direct FAT method and no fluorescing cells were seen. A known number of viable vibrio cells were added initially to the specimen and while this number was possibly reduced by various procedures carried out on the samples there were still organisms present which could be detected by the method under examination. A comparison of the 3 methods showed that the results obtained could be correlated and that the findings had validity. The 3 methods of detection were:

1. FAT (a) Direct method. My polyvalent anti-vibrio serum was conjugated with fluorescein isothiocyanate and used for the examination.

(b) Indirect method. My anti-vibrio serum was used in an indirect technique along with commercially prepared conjugated anti-rabbit γ-globulin.

2. Culture - direct plating of the material.

3. Culture after millipore filtration of the sample.

Viable count

In this experiment, 1 ml amounts of varying dilutions of a viable broth culture of V.fetus were added to 4 ml amount of PPW

It was necessary to know the number of vibrio organisms in the dilutions and this was estimated by performing a viable count. The culture for this count was prepared as follows:

The 10 immunising strains of V.fetus (82, 83, 115, GP 264, GP 287, C2, V 33, V 240, 47 and 1980) were each inoculated from a 4—6 day old plate of BATA medium into a bijou bottle containing vibrio peptone broth. The 10 bottles were then incubated for 18 hours in the gas mixture in anaerobic jars at 37°C. Then 0.5 ml from each bottle was pipetted into one 500 ml of vibrio broth. This gave a total of 5 ml or a 1% inoculum. At 24 hour intervals the broth culture was sub-cultured, always using a 1% inoculum and always incubating in the gas mixture. The reason for incubating for 24 hours was that the organisms were in the logarithmic phase of growth. Six hours incubation and 12 hours incubation gave too few bacteria to be of use in this experiment. I used a 1% subculture to enable me to be certain that only a very small percentage of dead bacteria would be present in the broth and by continuing the 1% subculture for 4 subsequent passages I considered that the number of dead bacteria present would be even less.

Dilution of broth culture for viable count.

The viable count was performed on the last 24 hours broth subculture. The dilutions were made in 0.1% peptone water as follows: 1 ml of the broth culture was added to 9 ml of 0.1% peptone water to make a 1:10 dilution. Further 10 fold or 3 fold dilutions were made by pipetting 1 ml of the appropriate dilution into either

2 ml or 9 ml or 0.1% peptone water. These dilutions were mixed in the vortex mixer for 30 seconds before further dilutions were made (Table 8).

TABLE 8

Schedule of dilutions for viable count.

0.1% peptone water		Broth culture dilution		Final dilution
9 ml	+	1 ml of 1:1	=	1:10
9	+	1 1:10	=	1:100
9	+	1 1:100	=	$1:10^3$
2	+	1 1:100	=	$1:3 \times 10^2$
9	+	1 $1:10^3$	=	$1:10^4$
2	+	1 $1:10^3$	=	$1:3 \times 10^3$
9	+	1 $1:10^4$	=	$1:10^5$
2	+	1 $1:10^4$	=	$1:3 \times 10^4$
9	+	1 $1:10^5$	=	$1:10^6$
2	+	1 $1:10^5$	=	$1:3 \times 10^5$

The following dilutions were used for the viable count: 3×10^3, 10^4, 3×10^4, 10^5, 3×10^5, 10^6. Then 0.1 ml of each dilution was inoculated on to each of 6 plates; the 0.1 ml inoculum was spotted on to the surface of a predried BATA medium plate to cover as evenly as possible the entire surface and so give single cell deposition. The Miles & Misra technique did not give consistent results in my hands.

The plates were incubated in the gas mixture for 7 days.

Dilution of culture for incorporation into preputial washings.

The same broth culture dilutions in 0.1% peptone water (page 96) were used for incorporation into PPW. Only the following dilutions were used: 1:1, 1:10, 1:100, $1:10^3$, $1:3 \times 10^3$, $1:10^4$, $1:3 \times 10^4$. Eight 4 ml samples of pooled PPW were pipetted into sterile universal containers and then 1 ml quantities from the various broth dilutions were added as in table 9.

TABLE 9

Broth culture dilutions in preputial washings

V.fetus broth culture dilutions		Preputial washings		*V.fetus* broth culture final dilution
1 ml of 1.1 broth	+	4 ml	=	1:5
1 ml of 1:10	+	4 ml	=	1:50
1 ml of 1:100	+	4 ml	=	1:500
1 ml of $1:10^3$	+	4 ml	=	$1:5 \times 10^3$
1 ml of $1:3 \times 10^3$	+	4 ml	=	$1:15 \times 10^3$
1 ml of $1:10^4$	+	4 ml	=	$1:5 \times 10^4$
1 ml of $1:3 \times 10^4$	+	4 ml	=	$1:15 \times 10^4$

To prepare these dilutions each broth culture dilution was mixed with the preputial washings in the vortex mixer for 30 seconds. Each was transferred to a graduated centrifuge tube and centrifuged at 2000 rpm for 5 minutes to deposit the gross debris. Exactly 4 ml of the supernatant was transferred to a superspeed centrifuge tube (marked at 1 ml from the foot of the tube). At this stage 0.05 ml of the supernatant was removed and plated out on to BAN media. (This step was taken to compare the effect of high speed centrifugation on the numbers of vibrios present in the dilutions). After centrifugation at 27,000 g for 1 hour the mixed deposit was similarly inoculated on to BAN media. Selective BAN medium was used in plating as PPW contain many commensal bacteria, the majority of which are sensitive to the antibiotics present in this medium. Thus the isolation of *V.fetus* was made easier.

From each PPW broth dilution 0.05 ml was pipetted and deposited as a butt on each of 3 predried BAN medium plates. The butt was allowed to dry into the surface of the medium before being streaked out for isolated colonies. The plates were incubated in anaerobic jars in the gas mixture at 37°C for 5-7 days. The supernatant was then discarded (leaving 1 ml) and the deposit in the tube mixed in the vertex mixer for 3 minutes or until all visible particles were in even suspension.

This suspension was used for:

(A) Culture (second direct culture)

(B) FAT

(C) Millipore filtration followed by culture

(A) Culture.

The suspension was plated out on to plates of BAN medium in the same manner as before.

(B) FAT

0.025 ml of the suspension was pipetted on to slides with a central marked area, 10 slides were prepared from each broth culture dilution in preputial washings (i.e., 1:5, 1:50, 1:500, $1:5 \times 10^3$, $1:15 \times 10^3$, $1:5 \times 10^4$, $1:15 \times 10^4$). To obtain an even spread of the marked area the pipetted suspension was spread out using a straight platinum wire. The slides were air dried, then fixed in absolute alcohol for 10 minutes, and again air dried. These slides were then stored on staining racks in the refrigerator being stained after a period of either a few days or 2 weeks. Four slides from each dilution were stained by the direct FAT and also another 4 slides were stained by the indirect FAT. For a description of the staining techniques see pages 89 and 91 .

(c)

Millipore filtration followed by culture

The remainder of the 1 ml mixed deposit from each PPW dilution was passed through a swinny adaptor containing a membrane of 0.65 μ porosity. Then 0.1 ml amounts of the filtrate were pipetted on to each of 3 BATA plates. The inoculum was evenly spotted over the entire surface of each plate and allowed to dry before incubating at 37°C in the gas mixture for 5-7 days. After incubation, colonies were filmed and examined by Gram's staining method to confirm that they were V.fetus. The number of V.fetus colonies present on each plate were counted and the results recorded.

The procedures outlined in Experiment I were repeated with two other groups of 10 different bulls, these groups are labelled Experiment 1A and Experiment 1B. Thus the preputial washings from 30 different bulls were used as a basis for these comparative experiments.

Using these same bulls a further 2 experiments (Ex 2 and 2A) were performed using the same methods of detection of V.fetus by seeding preputial washings with known numbers of vibrios as was done in Experiment 1. This was done at the same time as the heifer insemination experiments to enable a direct comparison of the heifer experiments with laboratory experiment results, (page 114). The results are given after those of Experiment 1, 1A and 1B as they are comparable.

RESULTS OF EXPERIMENTS 1, 1A and 1B.

Viable counts

The viable count results of the 3 experiments were within 30% of each other. By carrying out the same procedure of 1% sub-cultures every 24 hours for 4 days, the viable count results obtained were sufficiently similar for direct comparison to be made of the results of the 3 experiments. The number of colonies counted on each of the 5 plates at the dilutions which were significant were sufficiently similar to be acceptable in all 3 experiments, see page 107, table 10.

Direct plating result.

There was no difficulty in isolating V.fetus from the preputial washings when the vibrios were present in the sample at the level of 5×10^6 or 5×10^5 viable organisms. As seen from table 11 at dilutions (vibrio broth culture dilutions in PPW) 1:5, 1:50 and 1:500 all but 3 plates from the latter dilution gave growth of V.fetus. As would be expected there were many more isolated V.fetus colonies at the 1:5 dilution, fewer at the 1:50 and fewest at the 1:500 dilution. At the 5×10^3 dilution and higher there was only the occasional isolated V.fetus colony, the other organisms which were not inhibited by the antibiotics in the medium overgrowing the vibrios present. At no time was the BAN medium effective in preventing growth of all the other bacterial species present in the preputial washings, although many were inhibited. There was very little difference between the results of the direct plating of the supernatant after a light centrifugation and those

obtained after a superspeed centrifugation followed by resuspension of the deposit. Escherichia species and Psuedomonas species were often found growing on the BAN medium plates and because of their rapid growth and colony size they did tend to overgrow the vibrio colonies unless the latter were greatly in excess of the former.

The efficiency of direct plating on to BAN medium plates as a means of isolating V.fetus from preputial washings which are known to contain many rapidly growing bacteria seemed to be dependent on the ability of the antibiotic medium to inhibit most other bacteria commonly found present, and also on the relative number of viable V.fetus cells present; the higher the number the easier isolation becomes.

Culture following millipore filtration of the material

Growth of V.fetus was obtained on all plates inoculated in all 3 experiments at dilutions (vibrio broth culture dilutions in PPW) 1:5, 1:50, 1:500 and $1:5 \times 10^3$. At higher dilutions the results varied as can be seen from table 12, p109. It will also be seen that the number of vibrio colonies counted was usually greater from dilutions containing the highest number of vibrios. In experiment 1, all plates inoculated gave growth of V.fetus and although the number of colonies present varied considerably, isolation was made easy in all by the almost complete absence of other bacteria. In experiments 1A and 1B isolation was less successful at

the higher dilutions, and in experiment 1B there seemed to be many fewer colonies of V.fetus at every dilution. There was not the steady gradual drop in the number of colonies present on the plates from the varying dilutions as there was with the viable count. This variation may be due to the amount of filtrate obtained. If the filtrate was small in volume, added pressure was put on the syringe attached to the swinny adapter to force more through the membrane. On many occasions a pure culture of V.fetus was obtained from the filtrate. Even when other organisms were present these were few in number and the colonies small and discrete, except on the very few occasions when an Escherichia species or Psuedomonas species was present. Because the membrane was so effective in retaining the commensal organisms from the preputial washings a very few colonies of V.fetus on a plate were sufficient to make isolation possible.

Millipore filtration followed by subculture was an effective means of separating V.fetus from the majority of other bacteria present in the preputial washings. The larger commensal organisms were retained by the membrane and a proportion of the vibrios passed through in the filtrate. As BATA medium (with no antibiotics) and not BAN medium plates were used to culture the filtrate this emphasised that the millipore filter membrane acted as an effective direct method of separating vibrios from other bacteria.

FAT

The results are summarised in table 13 . There was a gradual decrease in the number of vibrios counted as the dilutions increased. With the 1:5 dilution many vibrios were seen in every field; at the 1:50 dilution a few vibrios were seen in every field; at the 1:500 one vibrio was seen in several fields. With the $1:5 \times 10^3$ dilution the number of vibrios counted in 10 minutes varied from 13 to 28 and so from this dilution also the presence of V.fetus was readily detected. At the $1:15 \times 10^3$ dilution, only a few vibrios were seen in 10 minutes screening but by screening 4 slides of each dilution both this dilution and the next dilution $1:5 \times 10^4$ consistently gave a sufficient number of fluorescing vibrios to make detection certain. At the $1:15 \times 10^4$ perhaps only one vibrio would be seen after screening all 4 slides. These results varied from none to 7 and so detection had become uncertain. For routine work 10 minutes screening is about the time most workers would find they could readily cope with especially if around 6 samples were received at any one time. For practical reasons therefore the dilution in which vibrios were readily able to be detected was the $1:5 \times 10^3$. At this dilution there were on average 60×10^3 viable V.fetus cells in the 5 ml sample which meant that in 0.025 ml without concentration of the sample there should be 300 vibrios on the smear. The average number of vibrios counted was only 21 which is

one fifteenth of the number present. I would estimate that a much higher proportion of the slide was screened than this. A certain amount must also be lost during the staining process, although the procedure was standardised and the PBS for rinsing was always dropped on to the slide from a dropping bottle.

There was no obvious consistent difference between the vibrios counted on the slides stained by the direct and indirect FAT. In both methods the vibrios always stained with maximum brightness. The background fluorescence from debris and clumps of other bacteria was usually slightly stronger with the indirect than with the direct method of staining. Occasionally a large coccus or diplococci were seen staining brightly but because of their size and shape these could not be confused with V.fetus. No smaller cocci were seen fluorescing with anything other than slight fluorescence. There were also small clumps of hard packed debris, the centre of which sometimes fluoresced strongly but with experience these could be ignored. Apart from the above the films contained an even distribution of material. Vibrio cells were never seen in clumps but it was noticeable that a higher number were seen per field around the edge of the film.

Philpott (1968) reported that he could easily detect 100 vibrio organisms per ml and with more careful examination

of the periphery 50 organisms per ml. I estimated that I could easily detect 3000, and with longer screening time 300, but below this the results were not reliable. By screening under the oil immersion lens I would have to screen for much longer to cover the same area in the same time as Philpott screened. He in fact could scan his entire smear in 5 - 10 minutes which I certainly did not manage. The other possibility is that he had many more dead vibrios than he thought from his viable count results. Even with my 4 1% subcultures my total vibrio counts ranged from about double to 4 times the viable count results.

Kita et al (1966) could detect the presence of V.fetus in FPW when he added 10^5 vibrio organisms per ml of preputial washings; they failed to detect 10^4 and 10^3 vibrios per ml by the FAT methods used. Their criterion of detection was to find a vibrio cell within 10 fields (using the x40 objective) so had they screened for a longer period I feel sure they would have been able to detect vibrios present at dilutions with lower numbers of vibrios present. They do not say how their vibrios were subcultured prior to their addition to the PPW but their results were closer to mine than those of Philpott.

Other workers using the FAT as a diagnostic tool did not try to determine the numbers of V.fetus cells present in a sample but rather compared the FAT result obtained by the FAT with other diagnostic means i.e., culture.

TABLE 10

RESULTS OF VIABLE COUNTS IN EXPERIMENTS 1, 1A AND 1B

Broth culture dilution	Amount plated	Average number of colonies counted per plate			Number of colony forming units per plate		
		Ex. 1	Ex. 1A	Ex. 1B	Ex. 1	Ex. 1A	Ex. 1B
$1:10^4$	0.1 ml	>300	>300	>300			
$1:3 \times 10^4$	0.1 ml	198	236	177.4	59×10^6	70.8×10^6	53.2×10^6
$1:10^5$	0.1 ml	53	67.8	52	53×10^6	67.8×10^6	52×10^6
$1:3 \times 10^5$	0.1 ml	29	29	23.6	87×10^6	87×10^6	70.8×10^6

Viable count Ex. 1 59×10^6

Ex. 1A 70.8×10^6

Ex. 1B 53.2×10^6

TABLE 11

RESULTS OF DIRECT PLATING OF VIBRIO FETUS BROTH CULTURE DILUTIONS IN PREPUTIAL WASHINGS IN EXPERIMENTS 1, 1A AND 1B

Vibrio broth culture dilutions in PPW	Number of V. fetus cells present in the 5 ml. sample			Number of BAN plates which gave growth of V. fetus after					
				A. Direct plating of 0.05 ml. of the supernatant			B. Direct plating of 0.05 ml. of the resuspended deposit		
	Ex. 1	Ex. 1A	Ex. 1B	Ex. 1	Ex. 1A	Ex. 1B	Ex. 1	Ex. 1A	Ex. 1B
1:5	$59x10^6$	$71x10^6$	$53x10^6$	3/3	3/3	3/3	3/3	3/3	3/3
1:50	$59x10^5$	$71x10^5$	$53x10^5$	3/3	3/3	3/3	2/3	3/3	3/3
1:500	$59x10^4$	$71x10^4$	$53x10^4$	3/3	3/3	3/3	2/3	0/3	3/3
$1:5 \times 10^3$	$59x10^3$	$71x10^3$	$53x10^3$	0/3	0/3	2/3	0/3	1/3	1/3
$1:15 \times 10^3$	$20x10^3$	$24x10^3$	$18x10^3$	0/3	0/3	0/3	0/3	0/3	1/3
$1:5 \times 10^4$	$59x10^2$	$71x10^2$	$53x10^2$	0/3	0/3	0/3	0/3	0/3	1/3

TABLE 12

RESULTS OF CULTURE FOR VIBRIO FETUS AFTER MILLIPORE FILTRATION

OF THE RESUSPENDED DEPOSIT IN EXPERIMENTS 1, 1A AND 1B

Vibrio broth culture dilution in PPW	Amount plated	Number of V. fetus cells present in the 5 ml. sample			Number of plates from which V. fetus was isolated			Average number of colonies of V. fetus present per plate		
		Ex. 1	Ex. 1A	Ex. 1B	Ex. 1	Ex. 1A	Ex. 1B	Ex. 1	Ex. 1A	Ex. 1B
1:5	0.1 ml	$59x10^6$	$71x10^6$	$53x10^6$	3/3	3/3	3/3	71	>300	350
1:50	0.1 ml	$59x10^5$	$71x10^5$	$53x10^5$	3/3	3/3	3/3	>300	>300	55
1:500	0.1 ml	$59x10^4$	$71x10^4$	$53x10^4$	3/3	3/3	3/3	>300	89	6
$1:5x10^3$	0.1 ml	$59x10^3$	$71x10^3$	$53x10^3$	3/3	3/3	3/3	9	228	10
$1:15x10^3$	0.1 ml	$20x10^3$	$24x10^3$	$18x10^3$	3/3	3/3	0/3	12	3	
$1:5x10^4$	0.1 ml	$59x10^2$	$71x10^2$	$53x10^2$	3/3	1/3	0/3	5	1/3	

TABLE 13

RESULTS OF FLUORESCENT ANTIBODY TECHNIQUE IN EXPERIMENTS 1, 1A AND 1B

Dilutions of broth culture in preputial washings	Direct FAT			Indirect FAT		
	Ex. 1	Ex. 1A	Ex. 1B	Ex. 1	Ex. 1A	Ex. 1B
1:5	+	+	+	+	+	+
1:50	+	+	+	+	+	+
1:500	124	180	110	+	124	+
$1:5 \times 10^3$	25	13	23	28	16	+
$1:15 \times 10^3$	6	28	18	8	16	19
$1:5 \times 10^4$	9	8	5	12	11	7
$1:15 \times 10^4$	0	5	1	2	7	2

Each slide was screened for ten minutes, except those double underlined in which 4 slides were screened for 10 minutes each. The figures denote the number of vibrio cells counted in the stated times. A (+) represents a positive result but the number of vibrios seen were not counted.

RESULTS OF THE LABORATORY DIAGNOSTIC TESTS OF EXPERIMENTS 2 AND 2 A.

Viable count

The viable count of experiment 2 was one half the average results of experiments 1, 1A and 1B. The viable count of experiment 2A was one quarter that of the 3 latter experiments. The drop in results with experiment 2 could have been due to the 1% vibrio broth culture being subcultured for 6 24 hour subcultures instead of the usual 4. Also only one isolate, very recently isolated from an artifically "infected" heifer (A in table 19) was used instead of the 10 V.fetus cultures used in the first 3 experiments (1, 1A and 1B). In experiment 2A, despite the correct 4 subcultures of the vibrio broth culture, the viable count again dropped but this culture was prepared from an isolate obtained from heifers infected in experiment 2. So either animal passage had made the organism grow less well in the broth medium or some of the original 10 cultures used grew better than some of the others and so gave a higher viable count.

Direct plating results

The results of experiments 2 and 2A showed the same trend as experiment 1, 1A and 1B, in that the more vibrios that were present, the more were isolated. The poor results

of experiment 2 were probably due to overgrowth of V.fetus by a large spreading colony which was probably a Pseudomonas species. As before the BAN medium never inhibited all other bacteria and so isolation was dependent on the vibrios being present in larger numbers than any other species.

Culture following millipore filtration of the material.

As with experiments 1, 1A and 2B, the trend was similar, very many vibrio colonies being present at the low dilution and very few colonies at the high dilutions. Also although BATA plates were used there were very few bacterial colonies present other than V.fetus.

FAT

As with the other methods of detection, the FAT results of experiments 2 and 2A gave a similar pattern to those shown in experiments 1, 1A and 1B.

TABLE 14

RESULTS OF VIABLE COUNTS IN EXPERIMENTS 2, 2A, 3 AND 3A

Broth culture dilution	Amount plated	Average number of colonies counted per plate				Number of colony forming units per plate			
		Ex. 2	Ex. 2A	Ex. 3	Ex. 3A	Ex. 2	Ex. 2A	Ex. 3	Ex. 3A
$1:10^4$	0.1 ml	351	139	The same broth culture used for ex. 3 as for ex. 2A	292	3.5×10^7	1.4×10^7	As for Ex. 2A	2.9×10^7
$1:3 \times 10^4$	0.1 ml	119	56		99	3.6×10^7	1.7×10^7		2.9×10^7
$1:10^5$	0.1 ml	40	21		32	3×10^7			
$1:3 \times 10^5$	0.1 ml	15							

Viable count

Experiment 2	3.5×10^7
Experiment 2A	1.4×10^7
Experiment 3	1.4×10^7
Experiment 3A	2.9×10^7

TABLE 15

RESULTS OF DIRECT PLATING OF VIBRIO FETUS BROTH CULTURE DILUTIONS IN PREPUTIAL WASHINGS IN EXPERIMENTS 2 AND 2A

Vibrio broth culture dilutions in PPW	Number of V. fetus cells present in the 5 ml. sample		Number of BAN plates which gave growth of V. fetus after			
			A. Direct plating of 0.05 ml. of the supernatant		B. Direct plating of 0.05 ml. of the resuspended deposit	
	Ex. 2	Ex. 2A	Ex. 2	Ex. 2A	Ex. 2	Ex. 2A
1:5	35×10^6	14×10^6	3/3	3/3	3/3	3/3
1:50	35×10^5	14×10^5	1/3	3/3	0/3	3/3
1:500	35×10^4	14×10^4	0/3	2/3	0/3	0/3
$1:5 \times 10^3$	35×10^3	14×10^3	0/3	0/3	0/3	0/3
$1:15 \times 10^3$	12×10^3	4.7×10^3	0/3	0/3	0/3	0/3
$1:5 \times 10^4$	35×10^2	14×10^2				

TABLE 16

RESULTS OF CULTURE OF VIBRIO FETUS AFTER MILLIPORE FILTRATION

OF THE RESUSPENDED DEPOSIT IN EXPERIMENTS 2 AND 2A

Vibrio broth culture dilution in PPW	Amount plated	Number of V. fetus cells present in the 5 ml. sample		Number of plates from which V. fetus was isolated		Average number of colonies of V. fetus present per plate	
		Ex. 2	Ex. 2A	Ex. 2	Ex. 2A	Ex. 2	Ex. 2A
1:5	0.1 ml	35×10^6	14×10^6	3/3	3/3	>300	>300
1:50	0.1 ml	35×10^5	14×10^5	3/3	3/3	>300	>300
1:500	0.1 ml	35×10^4	14×10^4	3/3	3/3	NC	Approx. 150
$1:5 \times 10^3$	0.1 ml	35×10^3	14×10^3	3/3	3/3	A few	24
$1:15 \times 10^3$	0.1 ml	12×10^3	4.7×10^3	1/3	3/3	*	6
$1:5 \times 10^4$	0.1 ml	35×10^2	14×10^2	0/3	3/3	0	2
$1:15 \times 10^4$	0.1 ml	12×10^2	4.7×10^2	0/3	1/3	0	*

* = One colony only

NC = Not counted

TABLE 17

RESULTS OF FLUORESCENT ANTIBODY TECHNIQUE IN EXPERIMENTS 2 AND 2A

Vibrio broth culture dilutions in PPW	Direct FAT		Indirect FAT	
	Ex. 2	Ex. 2A	Ex. 2	Ex. 2A
1:5	+	+	+	+
1:50	+	+	+	+
1:500	139	113	111	90
$1:5 \times 10^3$	20	9	14	16
$1:15 \times 10^3$	28	14	28	27
$1:5 \times 10^4$	12	4	8	4
$1:15 \times 10^4$	2	3	3	4

+ = many vibrio cells seen fluorescing.

The figures given represent the number of vibrio cells counted in 10 minutes screening.

The figures that are double underlined are the number of vibrio cells counted from 4 slides, each being screened for 10 minutes.

TABLE 18

RESULTS OF VARIOUS READINGS TAKEN OF VIBRIO BROTH CULTURE

USED IN THE LABORATORY AND HEIFER EXPERIMENTS

Experiment number	Viable count per ml	Total number of vibrio organisms inseminated	Total count per ml	Opacity of neat 24 hour broth culture used for viable count
1	6.6×10^7	ND	ND	6
1A	7.5×10^7	ND	ND	6.5
1B	5.5×10^7	ND	ND	2
2	3×10^7	** 6,000	4.2×10^7	2
2A	1.5×10^7	** 3,000	6×10^7	7
3	1.5×10^7	3.75×10^7	6×10^7	7
3A	3×10^7	15×10^7	6.2×10^7	ND
-*	5.7×10^7	ND	7.4×10^7	ND

* = Experiment to compare viable and total counts

** = Estimated approximate number

ND = Not done

DISCUSSION OF RESULTS OF EXPERIMENTS

1. 1A AND 1B

The 3 laboratory methods of detecting V.fetus were compared. Excluding the initial direct culture on to BAN medium plates all 3 methods were performed on the same material. In the second direct culture 0.05 ml was plated out on to BAN medium plates but due to there always being so many other bacteria present this amount was of little importance quantitatively. In practice it never seemed possible to inhibit the growth of all other bacteria present in PPWs by incorporating additional antibiotics in the media without also inhibiting V.fetus and so its isolation depended on V.fetus being present in greater numbers than any other species of bacteria which would also grow on the antibiotic plates. In the portion of the material which was millipore filtered, and 0.3 ml of the filtrate cultured on 3 BATA plates, the number of V.fetus colonies present was counted. This number was compared with the number of vibrio cells seen fluorescing on the prepared slides, but there were discrepancies which could be due to a few factors. If the viable number of V.fetus cells was less than the total number present then the results would favour the FAT which could detect dead vibrios. When viable counts and total counts were done on the same vibrio culture the total count was from twice to almost 4 times the viable result (see page 117). With the

FAT, although 0.025 ml was pipetted on to each slide, the entire film was never screened and so unlike millipore filtrate culture all the organisms present in the measured amount were not observed. In performing the FAT, although the organisms were fixed to the slide and vigorous rinsing was avoided, it was possible for material to be removed from the slide during the staining process. The amount of filtrate obtained after millipore filtration varied from about 0.6 ml to 0.8 ml. Only once did the membrane appear to be punctured and this was very apparent both by the unusually rapid filtration and marked cloudiness of the filtrate. Direct quantitative comparison is not valid for the above reasons but the results can be compared in that for isolation of V.fetus from contaminated material millipore filtration was consistently better than direct culture in all 3 experiments.

In comparing the FAT results with millipore filtrate culture, the FAT was able to detect a smaller number if 4 slides were screened for 10 minutes each. If however, one wished to compare the results after only screening one slide for 10 minutes there was not a great deal of difference in the results. In experiment 1B where millipore filtration was not so successful, a Pseudomonas species was known to be present and the PPW were stored for 2 days before use which may have caused toxic products

to be liberated into the diluent which could have had an adverse effect on the vibrios added later.

Kita et al (1966) were the only workers who compared different methods of detection of V.fetus from artifically infected PPW. Using their methods they found that culture on to an antibiotic medium was comparable to the FAT, while millipore filtrate culture was less successful. The antibiotic medium they used for direct culture was bovine blood agar to which 300 units of mycostatin , 20 µg of novobiocin and 2.5 µg of brilliant green were added per ml of agar base.

Kita et al treated their PPW seeded with V.fetus in two ways, neither of which used high speed centrifugation as I did. In their first method a 10 ml portion was centrifuged at 3000 rpm for 30 minutes. The deposit obtained was resuspended in 0.2 ml and from this smears where prepared in preparation for the fluorescent antibody method of detecting V.fetus. The second method filtered the seeded PPW through filter paper and then used the same treatment as described in their first method. Using the abovemethods and their FAT they could detect vibrios present in PPW to which they had added more than 10^5 vibrio cells per ml of PPW. Using my processing of PPW and FAT I could detect vibrios present when 10^3 vibrio cells were added per ml of PPW. Kita et al also isolated vibrios from the same

material as described above using two types of media. One contained antibiotics and brilliant green (see page 120), the other was a semi-solid thiol medium. Vibrios were cultured when 10^5 vibrios were present per ml of PPW. My results for direct culture on to solid media containing antibiotics were similar.

Kita et al also passed a portion of PPW containing vibrios through a millipore filter of 0.65 u porosity after the initial filtration through filter paper. Vibrios were grown only when 10^5 vibrio cells were present per ml of PPW. I was able to isolate vibrios when 10^3 vibrio cells were present per ml of PPW.

These results suggested that high speed centrifugation of the material deposited a high percentage of the vibrios present in the PPW and that they remained viable. For this reason no doubt, I was able to detect vibrios present in PPW when 10^3 vibrios were added per ml of PPW while Kita et al required 10^5 vibrios to be present per ml of PPW. This was true of both the FAT and millipore filtration followed by culture methods but not for direct plating. My results using direct plating were similar to theirs perhaps because it was impossible to obtain the correct combination of antibiotics which would inhibit all the rapid growing commensal bacteria present in PPW yet allow vibrios to grow freely.

HEIFER INSEMINATION

EXPERIMENTS 2, 2A, 3 AND 3A

INSEMINATION OF HEIFERS WITH LABORATORY STRAINS OF V. FETUS VENEREALIS

The original 10 immunizing strains of V.fetus venerealis and intestinalis had been subcultured on BATA medium once a week for about one and a half years. As the strains had become adapted to artificial medium their virulence for heifers now was unknown. For this reason one heifer (A) was inseminated with V.fetus venerealis and thereafter vaginal mucus samples collected. These samples were cultured to try to reisolate V.fetus venerealis from them. By doing this it was hoped to enhance the virulence of the reioslated inseminated strains of V.fetus. As V.fetus venerealis is generally considered to be the true pathogen, only the 4 V.fetus venerealis strains (47, 1980, V33 and V240) were inseminated. This mixture of strains was used rather than pick one random old laboratory maintained culture. Only one heifer was used in this experiment. This heifer had been a department holding animal and was known to be free of any V. fetus infection. To prepare for the insemination, this heifer was given an intra-muscular injection of 25 mg of stilboestrol dipropionate in arachis oil. This stimulates 2 - 3 days later an oestrous - like flow of mucus from the cervix into the vagina, without ovulation. It also opens the cervix, thus permitting insemination

through the cervix into the uterus. On the day prior to insemination, strains 47, 1980, V33 and V240 were each inoculated (from 4-day old BATA plates) into 4 ml amounts of vibrio peptone broth. These broths were grown overnight in the gas mixture at 37°C. Two ml amounts from each broth culture were mixed together. The material was inseminated through the cervix by attaching a syringe to an insemination pipette. The metal insemination pipette had a piece of rubber tubing attached to one end and after wrapping in greaseproof paper it was sterilized by autoclaving at 15 pounds pressure for 15 minutes. A sterile 5 or 10 ml record syringe was attached to the rubber tubing. The material for insemination was drawn up through the insemination pipette into the syringe and all air expelled from the system before the 2 ml amount was expelled into the uterus. A viable count was not done on the cultures but evidence from later experiments led me to conclude that more than 10^7 viable V.fetus cells were inseminated. Vaginal mucus samples were collected as described on page 124 sampling starting one week after insemination. A total of 14 samples were collected, sampling being done at 3 - 4 day intervals. Each sample was plated out as described on page 127 on to 6 predried BAN plates. After 7 days' incubation at 37°C in the gas mixture the plates were examined. Many colonies were filmed and stained by Gram's method. Only sample No. 1,

No. 2 and No. 7 gave growth of V.fetus venerealis on any of the plates. All other 14 samples were negative. The isolate from sample No. 7 was filmed again and a direct FAT performed on this film to establish that the isolate was a V.fetus. The isolate gave maximum fluorescence. This step of checking the fluorescence of the isolate with the conjugated antiserum was considered desirable as the microscopic appearance of some V.bubulus isolates are very similar to that of V.fetus. This isolate was maintained at room temperature in the gas mixture. As this preliminary experiment showed that it was possible to reisolate the vibrio organisms after insemination further experiments were done using 12 virgin heifers. These animals were obtained from a vibrio-free source, and were divided into 2 groups of six, A and B. Group A (experiment 2) were used in an insemination experiment, using the isolate from heifer A, Group B (experiment 2A) were also used in an insemination experiment using an isolate from one of the group A heifers.

COLLECTION OF VAGINAL MUCUS

The procedures for the collection and culture of vaginal mucus is outlined in this section.

The following materials were used:-

1. Vaginal pipettes

These were made of thick walled glass tubing 15 to 17

inches in length and with a bore of internal diameter 0.25 inch.

To obtain uncontaminated material vaginal pipettes, plugged at one end, were wrapped in greaseproof paper. The wrapping was done carefully to ensure that the pipette, when taken out, could be reinserted into the sterile wrapping "tube".

2. Suckers

These were composed of rubber tubing attached to glass tubing, both of which were approximately 6 inches in length. These were wrapped and sterilised.

3. Kleenex tissues.

4. Disinfectant.

Method of collection of vaginal mucus

The paper wrapping on the vaginal pipette was cut cleanly about 2 inches from the plugged end, the short paper removed and the pipette withdrawn without touching any part of the pipette, except approximately 2 inches from the plugged end. The sterile sucker was then unwrapped and the rubber part was attached to the plugged end of the vaginal pipette. The tail of the heifer was then pulled to one side and held in this position during sampling. (Occasionally if the animal was in a very excitable state the tail was held straight up and back. In this position insertion into the anterior vagina was made more difficult but the pain

inflicted by holding the tail made sudden movement less likely). The lips of the vulva were then wiped clean with several Kleenex tissues. With one hand the lips of the vulva were parted and kept in this position while, with the other hand, the vaginal pipette was inserted. There was less chance of breaking the pipette in the vagina if the rubber part of the sucker was held and not the glass vaginal pipette. The pipette was then inserted into the anterior vagina. If more than approximately 4 inches was still protruding the anterior vagina had not been reached and correct insertion was more easily accomplished withdrawing the pipette and then reinserting it. When the tip of the pipette was at the anterior vagina the operator started sucking. To collect the maximum amount of mucus the vaginal pipette was moved back and forward a few inches, before being withdrawn and only after complete withdrawal did the operator stop suction. The pipette was then replaced in its wrapper "tube" and labelled. The operator's gloved hands were then washed in disinfectant and dried before the next sample was collected.

All but the first 12 samples were collected by myself.

CULTURE OF VAGINAL MUCUS

The following materials were used:

1. Vaginal pipette
2. Metal rod to expel mucus from vaginal pipette
3. BAN media
4. BATA media
5. Glass spreaders
6. Thick platinum loop.

The vaginal mucus was not processed in any way, except that the consistency of the mucus varied considerably and so the method of inoculation of media had to be adapted to ensure adequate sampling.

As vaginal mucus contains many bacteria either as normal flora or from inseminated material, a selective medium was used to isolate V.fetus.

Vaginal mucus samples were always cultured within 3 hours of sampling to prevent loss of viability of any V.fetus present in the mucus. The mucus was expelled directly on to one predried BAN medium plate, using a metal rod, to force the mucus out of the vaginal pipette. In most cases the entire sample was cleanly deposited but if the mucus was stringy and scarce the cotton wool plug itself was drawn along the surface of the media to deposit the maximum amount of sample. From the material collected on this plate 5 others were inoculated, by transferring mucus and spreading it over the entire surface of each plate, either with a thick platinum loop

or a sterilised glass spreader, leaving some mucus on each plate as V.fetus is known to grow along the line of the deposited mucus. If the samples were large in volume and watery in consistency a section of the mucus may be cut off with sterile scissors or burnt off and then spread out on to other plates. Blood stained samples and samples containing pus, were all dealt with in a similar fashion, the aim being to ensure that a representative portion was distributed on to the 6 plates inoculated per sample. These plates were covered by a few spreading colonies of Escherichia species or Pseudomonas species.

None of the 36 samples gave any isolates resembling V.fetus. One sample of vaginal mucus from each heifer was sent to Weybridge for vaginal mucus agglutination tests. All samples were negative.

From both my cultural experiments and the vaginal mucus agglutination test it was shown that the heifers were not carrying V.fetus.

INSEMINATION OF 6 HEIFERS WITH PREPUTIAL WASHINGS CONTAINING V.FETUS CULTURE DILUTIONS.

A broth culture was prepared in the same way as in experiment 1 page 95 except that only one V.fetus culture (the isolate from heifer A, sample No. 7) was used as inoculum. This organism had been isolated from a BAN plate as described previously on page 124 and so the colony used as inoculum for the

broth had been incubated in the gas mixture for 7 days at 37°C followed by 3 days at room temperature. It was then inoculated into the first vibrio peptone broth. Six successive subcultures were made in error instead of the usual 4. Each of the 6 heifers was given one intramuscular injection of 25 mg of stilboestrol dipropionate in arachis oil 2 days prior to insemination. Preputial washings were collected from 10 bulls and each screened for the presence of V.fetus using the direct FAT method. Only these samples found to be negative were used in the pool for insemination into the 6 heifers. Dilutions of the last (6th) 24 hour V.fetus culture were prepared in 0.1% peptone water and a viable count performed as described on page 95 . As well as the insemination experiment, all the laboratory methods of detecting V.fetus used in experiments 1, 1A and 1B were also performed. The tests done were direct culture on to solid media containing antibiotics (BAN), millipore filtration followed by culture and both the direct and indirect fluorescent antibody techniques (see pages 87 to 92). To provide sufficient of the vibrio broth culture dilution in PPW which was inseminated, additional volume of the 1:5 x 10^3 dilution was prepared. This dilution was chosen for insemination into the 6 heifers because this had been found to be the highest dilution from which V.fetus could be isolated by direct plating on to BAN

medium plates (from results of experiments 1, 1A and 1B). At this dilution, ($1:5 \times 10^3$) V.fetus could always be cultured after millipore filtration and the organism was always detected on the slides by both the direct and indirect fluorescent antibody technique. The $1:5 \times 10^3$ PBW dilutions was made up as follows:: 3 ml of the $1: 10^3$ broth dilution in 0:1% peptone water + 12 ml of the preputial washing pool were mixed in the vortex mixer, and it was spun at 2000 rpm for 5 minutes. This was done 3 times so that there was a total of 45 ml. Then 12 ml of the supernatant was withdrawn from each 15 ml amount. The 3 x 12 ml (36 ml) withdrawn was pooled and mixed. This was then used for insemination, 2 ml being given to each heifer; the estimated number of viable V.fetus venerealis inseminated into each heifer was 6000 cells. The same insemination pipette was used to inseminate 2 animals; thus 3 pipettes were used for the insemination of the 6 heifers.

Vaginal mucus was then collected from each heifer twice weekly, commencing one week after insemination. The sampling was continued for 6 weeks, making a total of 12 samples per heifer. The mucus was cultured on 5 BAN plates and one BATA plate per sample (page 127). After 6-7 days' incubation in the anaerobic jars in the gas mixture at 37°C the plates were examined and colonies filmed and stained by Gram's staining method. Any organisms which looked like vibrios were filmed,

fixed in alcohol and a direct FAT performed on them. If the organism gave maximal fluorescence it was called a V.fetus and that sample denoted as positive.

After the 4 heifers had been shown to be negative on culture each animal was again given an injection of stilboestrol. This was done as the natural oestrus cycle of the 6 heifers was not known. Vaginal mucus samples were taken on 3 successive days. It has been shown that mucus collected at oestrus in infected animals gives a much higher percentage of samples containing V.fetus (Lawson and MacKinnon, 1952).

RESULTS OF CULTURE OF VAGINAL MUCUS OF HEIFERS IN EXPERIMENT 2.

Vaginal mucus from 2 out of the 6 heifers gave growth of V.fetus, From both heifers there was only one sample positive. For a summary of results see table 19, p.132.

Heifer No. 2, sample No. 7 was the only sample positive. All 5 BAN plates had several V.fetus colonies per plate. The one BATA plate inoculated was negative due to overgrowth of other bacteria. Most of the BAN plates from the other samples contained only a few bacterial colonies. On one occasion 3 out of 5 BAN plates were overgrown by mould colonies, but the other 2 had only a few staphylococcal colonies.

In heifer No. 1, sample No. 1 was the only sample positive. Of the 5 BAN plates inoculated 3 were positive each having only one or 2 V.fetus colonies per plate. All the negative samples

TABLE 19

RESULTS OF THE CULTURE OF VAGINAL MUCUS FROM HEIFERS IN EXPERIMENTS 2 AND 3

Number of samples	Heifer numbers A	1	2	3	4	5	6
Heifers inseminated with 12,000 *V.fetus* cells 7 days prior to first sample							
1	+	+	-	-	-	-	-
2	+	-	-	-	-	-	-
3	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-
7	+	-	+	-	-	-	-
8	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-
Injected with stilboestrol							
14	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-
Heifers 3-6 reinseminated with 3.75×10^7 *V.fetus* cells							
17	-	-	-	+	-	+	+
18				+	-	+	+
19				-	-	+	+
20				-	-	+	-
21				-	-	-	+
22				-	-	+	+
23				-	-	+	+

For key see foot of table page 134.

had only a few other bacterial colonies on the BAN plates. The BATA plates were nearly always overgrown but on some occasions colonies looking like V.fetus were observed but did not react with the FAT test.

V.fetus was not isolated from the other 4 heifers. For a summary of results see table 19 .

EXPERIMENT 2A (GROUP B)

This experiment was a repeat of Experiment 2, the culture used for insemination being the V.fetus isolate from heifer number 2 sample number 7 in Experiment 2. The 6 heifers forming group B were sampled, prior to insemination, on 6 occasions. The vaginal mucus was cultured in the same way as in Experiment 2 and examined for the presence of V.fetus. As all the samples were negative, the 6 heifers were then inseminated. Owing to the timing of the experiments the re-isolate V.fetus was sub-cultured on BATA medium 3 times before being inoculated into the first of the four 1% broth sub-cultures. Preputial washings were collected as before on the day before insemination from 10 different bulls. In all other respects the same procedures were carried out as in Experiment 2.

RESULTS OF THE CULTURE OF VAGINAL MUCUS OF HEIFERS IN EXPERIMENT 2A

V.fetus was isolated from only 2 of the 6 inseminated heifers.

TABLE 20

RESULTS OF THE CULTURE OF VAGINAL MUCUS FROM HEIFERS IN EXPERIMENTS 2A AND 3A

Number of samples	Heifer numbers					
	7	8	9	10	11	12
Heifers inseminated with 6000 *V.fetus* cells, 7 days prior to first sample						
1	+	+	-	-	-	-
2	+	+	-	-	-	-
3	-	+	-	-	-	-
4	-	+	-	-	-	-
5	-	-	-	-	-	-
6	-	+	-	-	-	-
7			-	-	-	-
Injected with stilboestrol						
8			-	-	-	-
Heifers 9-12 reinseminated with 15×10^7 *V.fetus* cells						
9			+	-	+	-
10			+	-	+	-
11			+	-	-	-
12			-	-	+	-
13				-		+
14						+
15						+

\- is no *V.fetus* isolated

\+ is *V.fetus* isolated

The final sample result in each case is for the culture of *V.fetus* from material collected at slaughter.
Samples were taken at 3-4 day intervals

Heifer No. 8.

Four out of the 5 samples taken from this heifer were positive. From the first sample all 4 BAN plates inoculated gave approximately 80 colonies per plate. From the second sample all 5 BAN plates gave approximately 50 colonies per plate. There were only a few other bacterial colonies present on these plates. From the third sample all 4 BAN plates contained more than 300 V.fetus colonies. The fourth samples also gave very numerous V.fetus colonies on each of the 4 BAN plates inoculated. The fifth sample was negative but only one BAN plate was inoculated from a poor sample.

Heifer No. 7.

Two of the 5 samples taken from this heifer were positive. From the first sample 3 of the 4 BAN plates contained V.fetus colonies. There was an average of 6 colonies per plate. The fourth plate was completely overgrown with bacteria other than V.fetus. All 5 BAN plates inoculated from the second sample were positive, each plate having a few V.fetus colonies. The 3 subsequent samples were negative, some BAN plates having many bacterial colonies other than V.fetus, others having no growth at all on them.

EXPERIMENT 3

Infectivity of the 4 negative heifers.

This experiment was designed to prove that those heifers which failed to become infected (denoted by failure to isolate

V.fetus from any vaginal mucus samples) were capable of being infected with V.fetus provided the number of bacteria inseminated was high enough. As Newsam (1964) had inseminated 150 x 10^6 viable V.fetus organisms into 42 heifers and isolated V.fetus from the vaginal mucus of 38, I aimed at inseminating this number of cells, but, in fact, I used 3.75 x 10^7 viable vibrio cells. The culture used for this was an isolate from one of the 2 positive heifers (No 2) in Experiment 2. The isolate was subcultured as described on page 95 with 4 1 % subcultures in vibrio peptone broth. A viable count and a total count were performed. The 4 heifers were injected with stilboestrol 2 days prior to insemination. This time the broth culture was not diluted before being added to the PPW, i.e. 2 ml of broth culture + 2 ml PPW. Seven days after insemination vaginal mucus samples were collected from all 4 heifers and these were set up for culture on BAN plates as described on page 127 . After 7 days' incubation colonies from each plate were filmed and stained by Gram's staining method. From each heifer 6 samples were collected over a 3 week period. Each sample was dealt with in the same way.

Results of the culture of vaginal mucus

V.fetus was isolated from the vaginal mucus of 3 of the 4 reinoculated heifers. The negative heifer had a prolapsed vagina and the vaginal mucus was contaminated with faecal material from the first sample onwards which may have prevented the establishment of V.fetus in the genital tract or if infection had taken place may have prevented isolation as the plates were

heavily contaminated with many faecal bacteria.

For a summary of the results see table 19 .

Heifer No. 3.

Two of the 6 samples were positive. From the first sample, 3 of the 5 BAN plates inoculated were positive. From the second sample 2 of the 5 BAN plates were positive there being 15 and 10 V.fetus colonies on each plate. All 4 subsequent samples were negative.

Heifer No. 5.

Five of the 6 samples were positive. All the BAN plates inoculated from the first 3 samples gave growth of V.fetus. On all 9 plates there were greater than 300 V.fetus colonies present in pure culture. The fourth and sixth samples were also positive.

Heifer No. 6.

Five of the 6 samples were positive. From sample 1, 2 of the 5 BAN plates had about 5 V.fetus colonies. From the second sample all 4 BAN plates inoculated had about 10 V.fetus colonies. Samples 3, 5 and 6 were also positive.

Heifer No. 4. Never gave growth of V.fetus.

By increasing the number of viable V.fetus cells inseminated, the recovery rate from vaginal mucus was increased. With the first inseminating dose 2 out of 6 heifers were positive, and with the much higher second inseminating dose 3 out of 4 were positive.

All the 6 heifers in group A were slaughtered, the 2 in experiment 2 that had been positive after a single insemination

and the remaining 4 reinseminated in experiment 3, of which 3 had been positive. Samples were taken from the genital tract as described on page 139 and set up for culture.

EXPERIMENT 3A

This experiment was a repeat of experiment 3. Each of the 4 negative heifers from group B was reinseminated with 15×10^7 viable V.fetus cells. This was 4 times the number of organisms inseminated in experiment 3. The culture used for insemination was isolated from one of the positive heifers in experiment 3.

RESULTS OF THE CULTURE OF VAGINAL MUCUS IN EXPERIMENT 3A

Three of the 4 heifers were positive.

Heifer No. 12.

Two of the 5 samples were positive. Samples 1, 2 and 3 were negative, only a few non vibrio colonies growing on most plates. Sample 4 was positive, the V.fetus colonies growing along the line of the mucus left on the plates. Sample 5 was also positive, each of the 6 BAN plates containing a few V.fetus colonies and many colonies of other bacterial species.

Heifer No. 11.

The first 2 of the 3 samples taken were positive. Two samples were taken of sample No. 2, as the first one was poor and this was in fact negative. The second was positive.

Heifer No. 9.

All 3 samples taken were positive. Some plates gave growth of the V.fetus colonies along the mucus left on the plates.

Heifer No. 10.

V.fetus was never isolated from this heifer at any time.

As in experiment 2, by greatly increasing the number of viable V.fetus cells inseminated on the second occasion, the recovery rate of V.fetus from the vaginal mucus was increased. With the first inseminating dose 2 out of 6 heifers were positive, and with the second higher inseminating dose 3 out of 4 were positive.

All the 6 heifers in group B were slaughtered and the material collected from the genital tract cultured for the isolation of V.fetus.

MATERIAL COLLECTED FROM HEIFERS AT SLAUGHTER

The procedures for the collection of material at slaughter are outlined in this section.

After slaughter, the vagina and uterus were collected in a clean pail and brought immediately to the laboratory. The genitalia were deposited on an enamel tray and arranged in such a way that the genital tract was easily discerned. This may entail cutting off loose fat, and other extraneous matter.

Owing to the amount of sampling, it was impossible to have sufficient sterilised instruments available. For this part of the work all instruments had been cleaned thoroughly

then dipped in alcohol and set alight to sterilise and after this stored in a sterile beaker until they were required. After use each instrument was placed in a beaker of water and set under continuously running hot water. They were cleaned again, dipped in alcohol, flammed and were ready for use again. Other sterile instruments used were artery forceps and scissors.

The procedure used for taking non-contaminated samples was as follows:

The surface was swabbed with absolute alcohol. Several layers of muscular tissues were then incised with scalpel and the final incision into the lumen was made with a second sterile scalpel. When the orifice was large enough "throat" swabs were taken of material present in the lumen. (Throat swabs were composed of cotton wool wound firmly round the foot 2 inches of a 6 inch stick. The whole is sterilised with a large plug of cotton wool and covered with silver paper). This material was sometimes very thick in consistency and this seemed the only way to do it. Repeating this technique one half of the vagina and both uterine horns were split open and sampled. With some genitalia, sampling was started at the middle of the vagina (the vulval area was never sampled or split open) and continued until the cervix and finally the 2 uterine horns were exposed. Latterly sampling was started at one uterine horn and continued to the cervix, and then backwards to the other uterine horn then into the vagina. This method was used as I considered there was less chance of

contaminating the vagina from the uterus than vice versa.

Samples were therefore collected from:

1. uterine horns
2. cervix
3. anterior and mid vagina

Material was not collected from around the vulva or posterior vagina as it was often contaminated with faecal material. Samples were collected by inserting sterile "throat" swabs and plating. The mucus was plated on to predried BAN plates and incubated in the gas incubated mixture for 7 days. At all sites the maximum amount of mucus possible was collected; there was usually sufficient in the vagina but there generally was little in the uterine horns.

RESULTS OF CULTURES SET UP FROM MATERIAL COLLECTED AT SLAUGHTER OF ALL 12 HEIFERS

Group A is composed of 6 heifers, 2 in experiment 2 and 4 in experiment 3.

Group B is also composed of 6 heifers, 2 in experiment 2A and 4 in experiment 3A. Experiments 2 and 2A each had the single insemination of vibrio cells while experiments 3 and 3A had in addition a second much larger insemination of V.fetus cells (see table 22 for the estimated number of cells inseminated in these experiments).

Experiment 2.

Heifer Nos. 2 and 6 did not give growth of V.fetus from any site.

Experiment 2A.

The material collected from heifer No. 8 after slaughter was positive. The 2 BAN plates inoculated with vaginal mucus had an average of 14 V.fetus colonies per plate. These colonies were very tiny but as there were also large colonies of an Escherichia-like species present on the plates this may be the reason why the vibrio colonies were abnormally small for the age of the culture. There were 3 V.fetus colonies on the plate from the cervix. The plates from the uterus were negative, only one non-vibrio colony being present on one of these 3 plates.

Heifer No. 7 was negative. The plates from the vagina had many Escherichia-like colonies and many small colonies which were not V.fetus. The plates from the uterus and cervix also contained very numerous small colonies which were not V.fetus.

Experiment 3.

At slaughter 2 of the 4 heifers were negative. Heifer No. 4 was never positive at any time.

Heifer No. 3 had 2 positive samples taken just after re-insemination but no V.fetus was isolated from the material cultured just after slaughter.

Heifer No. 5 gave growth of V.fetus from the vaginal mucus

but not from the cervix after slaughter. There were several V.fetus colonies on each of 3 plates inoculated with vaginal mucus. These colonies were very small but there were also a few large Escherichia-like colonies on each plate. The surface of the plates inoculated with material from the uterus was completely covered with Escherichia or Pseudomonas-like growth.

Heifer No. 6 gave growth of V.fetus on plates inoculated with material from the vagina and from one uterine horn. There were 2 plates inoculated with vaginal mucus and each plate contained 3 Escherichia-like colonies, and one had one Vibrio fetus colony while the other had 2. A plate inoculated with material from one uterine horn contained one V.fetus colony. The other uterine horn and the cervix were negative, there being no bacterial growth on either plate.

Experiment 3 A.

At slaughter 2 of the 4 heifers were negative.

Heifer No. 10 was never positive at any time.

Heifer No. 9 was positive on 3 occasions prior to slaughter but no V.fetus was isolated from any material collected just after slaughter. There was no bacterial growth on any plates inoculated with vaginal mucus. The plates from the cervix contained a few colonies and one uterine horn had very many but none was V.fetus.

Heifer No. 12. Two of the 4 plates inoculated with vaginal mucus contained many V.fetus colonies. The V.fetus could be seen growing along the deposited mucus. The 2 negative plates contained many Escherichia-like colonies.

The plates inoculated with material from the cervix contained several bacterial colonies other than V.fetus, while the plates from the uterine horns were completely negative.

Heifer No. 11. All 5 plates inoculated with vaginal mucus grew almost pure cultures of V.fetus. Each plate had more than 300 V.fetus colonies and on all plates the colonies grew along the mucus deposited on the surface of the plate. Plates from one uterine horn and the cervix contained many non-vibrio colonies, while the other uterine horn plate contained many tiny colonies which were V.fetus.

TABLE 21

RESULTS OF CULTURE FOR *V.FETUS* FROM THE GENITAL TRACT OF HEIFERS COLLECTED AT SLAUGHTER

Heifer number	Culture of *V.fetus* from			
	Vagina	Cervix	Uterine horns	
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	+	ND	-	-
6	+	-	+	-
7	-	-	-	-
8	+	+	-	-
9	-	-	-	-
10	-	-	-	-
11	+	-	+	-
12	+	-	-	-

\+ Culture of *V.fetus*

\- *V.fetus* not isolated

ND Not done

TABLE 22

SUMMARY OF RESULTS OF HEIFER EXPERIMENTS

Heifer number	Samples positive after vibrio insemination	
	First dose: 12000 cells	Second dose: 7.5×10^7 cells
1	1/17	
2	1/17	
3	0/16	2/7
4	0/16	0/7
5	0/16	6/7
6	0/16	6/7
	First dose: 6000 cells	Second dose: 15×10^7 cells
7	2/6	
8	5/6	
9	0/8	3/4
10	0/8	0/5
11	0/8	3/4
12	0/8	3/7

The results are given as the number of vaginal mucus samples from which V.fetus was isolated, out of the total number of samples taken from each heifer.

DISCUSSION

Experiment 2.

Both positive heifers gave cultures of V.fetus from a single vaginal mucus sample. With heifer No. 2 only the seventh sample was positive. There were several colonies of V.fetus on each of the 4 BAN plates that were inoculated and isolation was possibly due to the small number of colonies of other bacterial species present on these plates. At this time for comparison one BATA plate was also inoculated but the surface of this plate was completely covered with bacterial growth, to the extent that there were hardly any isolated colonies, and none of these was V.fetus. Throughout the sampling period almost every BATA plate inoculated was completely overgrown with many species of bacteria other than V.fetus and had this vibrio been present it is unlikely that it would have been isolated from these plates. Each batch of BAN media prepared was inoculated with a V.fetus culture to confirm that the medium was able to support its growth, and so failure to culture V.fetus from the other samples was not due to inhibition of the organism by the antibiotics incorporated into the medium.

Many of the bacteria present in the vaginal mucus may have been introduced from the preputial washings at insemination and this along with the fact that sampling of the vaginal mucus took place twice a week may be responsible for the high number of organisms found in the samples. The preputial washings were

stored overnight at 4°C before being pooled and inseminated and this delay may have caused the release of toxic material from the bacteria which could have had an adverse effect on the V.fetus added later to these preputial washings.

Heifer No. 1 only gave the first sample of vaginal mucus positive, and while some multiplication must have taken place, no subsequent isolation of V.fetus suggested a poor establishment of the organisms in this animal. Isolation was made from the BAN plates, each having only a few V.fetus colonies. As with the previous heifer, the BATA plate was too overgrown with other organisms to be able to isolate V.fetus from it.

Because these 6 heifers were not known to have a regular oestrous cycle, each was injected with the same quantity of stilboestrol dipropionate in arachis oil as before to induce an oestrous-like secretion. Vaginal mucus samples were collected on the 3 following days as it has been observed by some workers that in a V.fetus infected heifer, a higher percentage of positive cultures of V.fetus is obtained from vaginal mucus collected at oestrus than from vaginal mucus collected at any other time in the oestrous cycle. As all these samples were negative this seems to confirm that the infection was not well established or that the stilboestrol does not imitate the natural oestrus in this respect.

Experiment 2 A.

This was a repeat experiment of experiment 2, the differences being that twice the number of viable V.fetus organisms were

inseminated into each heifer and that the isolate of V.fetus used had been passaged through 2 consecutive heifers instead of one for experiment 2. The number of heifers infected was the same (2/6) but the number of colony or plate sample isolations was different. Heifer No. 8 gave growth of V.fetus from 5 out of 6 samples. The negative sample was noted at the time of collection as being very small in volume. This animal was very nervous and moved about a lot and for this reason adequate sampling was often difficult. On this occasion fresh blood was noticed on the very small sample and because of this no repeat sample was taken. There was on this occasion only sufficient material to inoculate one BAN medium plate. On all other BAN medium plates inoculated from every other sample there were many V.fetus colonies. As seen from the results (page 135) there were many more colonies per plate from the third and fourth samples than on the plates from the first and second; and so it would appear that multiplication of the V.fetus had taken place in this animal. The amount of vaginal mucus collected from samples varied but the fourth sample "was noted as very little mucus with slight pus in it," and yet every plate from this sample had very numerous V.fetus colonies on it. The other positive heifer in this group (no. 7) was positive on only 2 occasions, the first and second samples. Some of the BAN medium plates from the subsequent samples were overgrown with organisms other than V.fetus and this was the only heifer in this group that it could be suggested that subsequent

isolation of V.fetus may have been prevented by the abundant growth of other organisms.

In both experiment 2 and experiment 2A, each V.fetus colony isolated from a vaginal mucus sample was filmed, fixed and stained with the conjugated anti-vibrio serum and all the colonies regarded as being vibrios gave strong fluorescence. This FAT was done because colonies resembling V.fetus but growing only on the BATA plates were seen occasionally and some were microscopically like V.fetus but did not fluoresce when stained with the conjugated anti-vibrio serum. These isolates were probably V.bubulus. One isolate gave the biochemical characteristics of V.bubulus but every such isolate was not identified. If an isolate did not fluoresce with the conjugated anti-vibrio serum this was considered sufficient to confirm that it was not V.fetus.

Experiments 3 and 3A

In experiments 3 and 3A, the 4 negative heifers of each group that were reinseminated with the much increased number of V.fetus cells each gave 3 out of the 4 positive. Almost every positive heifer had at least one sample from which V.fetus was not isolated (see page 146). This could be due to inadequate sampling but care was taken to ensure proper insertion of the pipette and that an adequate amount of mucus was withdrawn. The other possibility is that the number of viable V.fetus cells present in the vagina fluctuated greatly. There was one sample (sample No. 2 from heifer No. 8) that was thought to be unsatisfactory and so a repeat was taken with a fresh pipette. The culture results showed that

the first sample was negative while the repeat was positive. In this case the poor sample was thought unsatisfactory because the pipette had possibly not reached the anterior vagina and so it would appear that vaginal mucus collected from some other area of the vagina other than the anterior end could give a false negative result.

General discussion of heifer experiments.

The sampling technique used was satisfactory but the disposable plastic pipettes would have been safer than the glass ones used. Only one breakage occurred with the pipette in the vagina of a heifer. Fortunately the pipette broke into two pieces and both were easily removed before the vagina was injured. Using this type of pipette, although the vulva and mouth of the vagina were always wiped clean before insertion, any material near the posterior end of the vagina was likely to be pushed backwards to the anterior end and might have contributed to the quite high number of organisms often found present in the samples. Seger and Levy (1962) found that there was more contamination in the first 2 - 3 weeks after insemination and also if the samples were collected more than once a week. As my sampling took place twice weekly this may explain the large number of organisms present on most BATA plates inoculated. The BAN medium plates mostly contained very few colonies and from this it would appear that the antibiotics present in the medium were effective in preventing growth of many bacteria present in many of the samples.

There was no delay between sampling and inoculation of the media so failure to isolate V. fetus was not due to the organisms dying before being inoculated on to the culture media. Also because the BAN medium was successful in inhibiting such a high proportion of the other bacteria present I feel confident that if the V.fetus inseminated had multiplied in the genital tract it would have been possible to isolate it.

Material collected from the genital tract cultured at slaughter.

No heifers that were negative while alive grew V.fetus from any part of the genital tract immediately after slaughter. Six of the 10 heifers that gave at least one positive isolation of V.fetus while alive were negative at slaughter. As would be expected from the results of culture of vaginal mucus while the heifers were still alive, most of the animals with the highest number of positive samples were also positive at slaughter. In a few cases, failure to isolate V.fetus may have been due to the presence of other rapidly growing organisms which were present on some BAN medium plates. Failure to culture V.fetus could be due to a very small number being present in the genital tract at the time of slaughter and the method of isolation used not being sensitive enough to detect them. The other straightforward possibility for negative results was that there were no V.fetus organisms present and this would not be surprising with heifers 12 or 10 (see table 20), where only one sample was positive and many were negative.

From these results obtained it would appear that there was

quite a variation in the degree of multiplication or survival of V.fetus among heifers given the same dose of viable organisms. It had been hoped that all animals inseminated with the much larger dose would become infected but 2 of the 8 did not and while the one with the prolapsed vagina and therefore badly contaminated vaginal mucus might explain the failure to culture V.fetus from this heifer, the second negative heifer is less easily explained. A Gram negative rod did invade its genital tract but whether this could have been present at the anterior vagina or cervix early enough to compete with the vibrio is a matter of conjecture. It may have had some natural resistance against vibriosis.

BULL SURVEY

BULL SURVEY

I concluded from experiments 1, 1A and 1B that either the direct or indirect FAT method was the most sensitive of the 3 methods used in detecting the smallest numbers of vibrio cells present in laboratory infected samples. I have already discussed the results obtained by both the direct and indirect FAT method. My antiserum was prepared against 10 laboratory strains of V.fetus; after conjugation this was used in the direct FAT method to detect the presence of these same strains. I now tested the ability of this conjugated antiserum to detect vibrios in naturally occurring infection. As I found the direct FAT method was quicker to do than the indirect method I used this method in examining clinical material from 89 bulls. At the start of the survey I examined clinical material by the FAT method alone, this was done to cope with the flow of samples; but later in the survey I also cultured the samples.

89 bulls were divided into 2 groups of 54 (Group A) and 35 (Group B) bulls respectively. Preputial washings were taken from all animals and treated as follows:

Group A. The preputial washings of the 54 bulls were examined by the FAT method alone. In both groups of bulls any animal giving a positive FAT result was resampled - usually 7 days after the first sampling.

Group B. The washings of the 35 animals were examined by both the direct FAT method and by culture of the filtrate after the

washings were passed through a millipore filter. Although it was found that the FAT test could pick out vibrio cells in clinical and laboratory material, culture of the clinical material was essential to a) compare realistically the efficiency of my conjugate in the FAT method with culture b) identify any isolates found, as precisely as possible.

The majority of specimens were taken from bulls in an area within easy reach of Glasgow. All samples were taken by skilled operators who fully realised the importance of a good sampling technique. A few were taken in the Veterinary School, Garscube, some specimens were sent from Perthshire, and some from more distant areas in Scotland. A very few samples, which were sent by post from the more distant areas could naturally not be examined on the day of sampling as a delay of 3 days usually occurred before the material was received; therefore the FAT method only was used in these cases as V.fetus dies very quickly. All samples for culture were inoculated on the day they were taken.

In the majority of bulls the preputial washings were taken in sterile physiological saline, but an unknown diluent was used for a very few samples. These had been taken by people not connected with the department, and who did not realise the importance of the diluent.

The collection and treatment of preputial washings for a) the FAT method and b) for millipore filtration and culture are stated on pages 85 – 88.

In culturing the isolates, twice the amount of filtrate was cultured on one BAN and one BATA plate compared with experiments 1, 1A and 1B.

RESULTS

Group A.

Of the 54 bulls in this group, 46 were negative on the first sample. These were not re-examined. Seven were positive on the first sample and resampled once again. One bull gave a doubtful result with the FAT test, but this was considered to be positive as this animal had one year previously been positive using the heifer mating test. In the 7 positive bulls the number of fluorescing cells counted in 10 minutes varied from 3 to greater than 300. For individual numbers counted see Table 23.

The 8 positive bulls were treated with streptomycin. Three weeks after this treatment, these 8 bulls were resampled and the FAT test done on the preputial washings. All the samples gave a negative reaction. Almost 4 months after streptomycin treatment, the 8 bulls were resampled and the preputial washings from bulls 1, 5 and 6 were again found to be positive. The number of vibrio cells counted in the 10 minutes screening was 396, 142 and 750 respectively. As this was an unexpected finding, cultures were set up from these 3 positive samples; the material left after preparation of the filmed slides was millipore filtered and the filtrate inoculated

on to 2 or 3 BATA plates. Colonies of cells which after Gram's staining method appeared to be vibrios were again filmed and a FAT test done. All 3 isolates gave a positive reaction so these were obtained in pure culture and the V.fetus identified further. The isolates were found to be V.fetus intestinalis intermediate (Table 24) i.e., no growth in 1.5% glycine. * (These results were confirmed by R. Gambles, Ministry of Agriculture, Shinfield, Reading).

The 3 cultures were streptomycin sensitive, the sensitivity test was done with multidiscs 30/3G - dosage 25 units.

* The use of 1.5% glycine was a modification of the original test and this test is not the accepted method of classification.

TABLE 23

GROUP A

NUMBER OF FLUORESCING V.FETUS CELLS IN PREPUTIAL WASHINGS SEEN DURING 10 MINUTES SCREENING BY THE DIRECT FAT

Bull number	Sample	Date of sample	Number of vibrios counted in 10 minutes	
1	a	18.12.68	45	
1	b	23.12.68	> 300	
2	a	23.12.68	2	(1)
3	a	26.12.68	11	
3	b	3. 1.69	0	
4	a	26.12.68	4	
4	b	3. 1.69	14	
5	a	26.12.68	36	
5	b	30.12.68	25	
6	a	27. 1.69	+	(2)
6	b	10. 2.69	64, 42	
7	a	23.12.68	5	
7	b	26.12.68	3	
7	c	3. 1.69	19	
8	a	4.12.68	+	

+ The number of vibrios seen were not counted.

(1) Previously heifer mating test positive. Both cells seen were atypical.

(2) Two samples collected one immediately after the other

TABLE 24

BIOCHEMICAL RESULTS OF GROUP A BULL ISOLATES

Bull number	Basal medium		1% glycine growth	1.5% glycine growth	Cysteine hydrochloride medium	
	Growth	H_2S production			Growth	H_2S production
1	+	-	+	-	+	+
5	+	-	+	-	+	+
6	+	-	+	-	+	+
7	+	-	+	-	+	+

All these isolates gave the characteristics of V. fetus intestinalis intermediate.

RESULTS.

Group B.

The preputial washings of 25/35 bulls were negative for V.fetus both by the direct FAT and culture; 10 bulls were positive by the direct FAT but only 4 of these 10 animals were positive on culture (Table 26).

TABLE 25

GROUP B

SUMMARY OF RESULTS OF 35 BULLS EXAMINED FOR VIBRIOS BY THE FAT AND MILLIPORE CULTURE METHOD

Total number of bulls sampled	Bulls negative on		Bulls positive on	
	FAT	CULTURE	FAT	CULTURE
35	25/35	31/35	10/10	4/10

TABLE 26

GROUP B

NUMBER OF FLUORESCING V.FETUS CELLS IN PREPUTIAL WASHINGS SEEN DURING 10 MINUTES SCREENING BY THE DIRECT FAT

Bull number	Sample	Date of sample	Number of vibrios counted in 10 minutes	Result of culture
1	a	21.5.69	1	-
	b	27.5.69	0	-
2	a	21.5.69	44	+
	b	27.5.69	36	-
3	a	27.5.69	19	-
	b	4.6.69	70	-
4.	a	27.5.69	11	-
	b	4.6.69	8	-
5	a	11.6.69	35	-
	b	18.6.69	5	-
6	a	18.6.69	6	-
	b	25.6.69	6	-
7.	a	18.6.69	132	-
	b	25.6.69	218	-
8.	a	18.6.69	13	+
	b	25.6.69	50	-
9.	a	25.6.69	30	+
	b	2.7.69	1	-
10.	a	2.7.69	500	-
	b	9.7.69	260	+*

* Preputial washings taken in broth instead of saline

RESULTS OF GROUP B

Bull 2

FAT method

Sample (a) 44 fluorescing vibrio cells were counted in 10 minutes screening.

Sample (b) 36 fluorescing vibrio cells were counted in 10 minutes screening.

Culture method

Sample (a) BATA medium

After incubation this BATA plate contained numerous colonies of several different types. A great many of these were filmed and one colony was found which after staining by Gram's method looked like V.fetus. This isolate was filmed again and a direct FAT performed on the film. Fluorescence was as intense with this preparation as with the control positive slide and so confirmed that the isolate was a V.fetus. The colony was then subcultured on to a plate of BATA medium. Once obtained in pure culture further identification tests were carried out.

BAN medium

The BAN medium plate also contained numerous colonies but no colony appeared to be V.fetus

Sample (b) There were no V.fetus colonies isolated either on

the BATA or BAN media.

Most of the other colonies filmed from both BATA and BAN plates were Gram negative cocco-bacilli. There was also the occasional large Gram negative bent rod, and the occasional Gram positive short rod (possibly Corynebacteria).

Bull 8

FAT method

Sample (a) 13 fluorescing vibrio cells were counted in 10 minutes

Sample (b) 50 fluorescing cells were counted in 10 minutes.

Culture method

Sample (a) BATA medium

The BATA plate contained many colonies. Most of these were rather large and flat for vibrio colonies but one colony which was not pure on the Gram film looked like V.fetus. This was confirmed by performing a FAT on a film from this contaminated colony. The vibrio cells fluoresced strongly. The isolate was subcultured on to BATA medium and obtained in pure culture.

BAN medium

The BAN plate contained only a few colonies, but V.fetus was not present.

Sample (b) No V.fetus isolates were obtained although many colonies were filmed and stained.

Bull 9

FAT method

Sample (a) 30 fluorescing vibrio cells were counted.

Sample (b) One fluorescing vibrio cell was seen.

Culture method

Sample (a) V.fetus was isolated from either the BATA or BAN plate. The vibrio colony was filmed and a FAT performed. The vibrios fluoresced and so confirmed that the isolate was V.fetus. The colony was subcultured for further identification.

Bull 10

FAT method

Sample (a) 500 fluorescing vibrio cells were counted in 10 minutes.

Sample (b) 200 fluorescing vibrio cells were counted in 10 minutes.

Culture method

Sample (a) On both the BATA and BAN platesthere were many isolated colonies but no V.fetus was isolated.

Sample (b) This sample was collected in nutrient broth instead of saline. Both plates contained many vibrio colonies. There were other colonies present but the V.fetus colonies greatly outnumbered the others and were in excess of 300 per plate. A colony was filmed and the FAT performed on it. The vibrio cells fluoresced as strongly as the control. The colony was subcultured and obtained in pure culture.

TABLE 27

BIOCHEMICAL RESULTS OF GROUP B BULL ISOLATES

Bull number	Basal medium		1% glycine growth	1.5% glycine growth	Cysteine hydrochloride medium	
	Growth	H_2S production			Growth	H_2S production
2	+	-	-	-	+	-
8	+	-	-	-	+	+
9	+	-	-	-	+	+
10	+	-	-	-	+	+

Bull (2) gave the reactions of V. fetus venerealis.

Bulls (8), (9) and (10) gave the reactions of V. fetus of intermediate type, fitting none of the known strains.

IDENTIFICATION OF THE ISOLATES

The medium used for further identification was vibrio peptone water made semi-solid and used as the basal medium (see page 50). The method of inoculation and reading of the results is described on page 57 . The results are shown in table 27 .

In summary a total of 89 bulls were screened by the FAT and 17 were positive (19%). 38 bulls were also screened by the millipore filtration culture method and 8 were positive (21%). (All 8 isolates were also FAT positive).

DISCUSSION OF BULL SURVEY

Sampling

In the field the method of taking preputial washings varies, but a most important factor was to ensure that the irrigating fluid was adequately massaged in the prepuce and especially at the fornix. The fornix was known (Winter and Samuelson, 1965) to be the area where the highest number of V.fetus cells were located. It would be comparatively easy to run a volume of liquid into the anterior prepuce and for it to have remained in this site; so it was important to ensure that the sampling liquid was forced back and forward inside the prepuce before allowing it to run back into the collecting container. The amount of liquid used was also of importance as the greater the volume the more concentration of the sample was required. If a very small amount of liquid wereused it

may not reach all the areas within the prepuce. If the bull urinated during sampling the volume collected would have been considerably increased. There were only a few PPW obtained that were obviously contaminated with urine. When this happened I tested the sample as usual but took a repeat sample at a later date. There have been conflicting reports as to the effect of urine on the viability of V.fetus cells. Van de Plassche et al (1963) said that it inhibited their growth, while Dufty (1969) said that it had no deleterious effect. This may depend on the biochemical state of the urine (eg pH), or its bacterial content, both of which will vary both from time to time and from bull to bull. It will be realised that the efficiency of taking the samples will influence the results. Diagnosis of vibriosis depends on the presence of V.fetus cells whether the method of detection is by the FAT or by culture.

In the survey, the time interval between samples for any one bull, varied from 3 to 4 days (with the longest interval of 24 days for sample 8 in Group A). Where very few vibrios were present in the prepuce of the bull it would have been possible to remove the majority of the bacteria with a very thorough irrigation technique. Because 2 samples were not taken from each bull it was only possible for the second sample to be negative. This only occurred with 2 bulls (see tables 23 and 26).

Almost each bull sample had a different appearance, some were very cloudy and a few were quite clear. Any that were very clear were viewed with suspicion as it was possible to run all the saline out of the sampling bottle but for it to be retained in the rubber tubing. If I were present at collection or doing it myself and a sample looked very clear, the collection process was repeated. In fact, most samples were very cloudy.

Fluorescent antibody technique.

The initial light centrifugation deposited much of the gross cellular debris but with some samples there was material present on the prepared slides which fluoresced. In most cases this was of little importance as the fluorescence was of a much lower intensity than the V.fetus cells. Occasionally, there was a mass of debris, the centre of which fluoresced strongly. The vibrios seen on these films were usually seen singly or in pairs and very rarely as a small spiral. For this reason it was supposed that even if the occasional mass of debris did engulf a vibrio and mask its fluorescence, there were others present which would be seen. Spermatozoa were sometimes seen and their broken tails fluoresced but these were never confused with V.fetus cells. On one occasion semen contaminated a saline preputial

washing sample and this gave the smear a rather light matt fluorescent background, probably of seminal plasma. Despite this, several V.fetus cells were seen fluorescing and the positive diagnosis was confirmed on a second semen-free preputial washing sample. A large coccoid cell or pair of cells was seen fluorescing in a few different bull samples. Its fluorescence was about as bright as V.fetus but it could not be confused with it because of its shape. Coccoid vibrios were never observed fluorescing from freshly collected positive preputial washing samples. (Coccoid vibrios from old laboratory cultures did fluoresce.) In the very occasional preputial washing sample, slightly bent rods were seen which fluoresced. This fluorescence was estimated at maximum to be (2+), whereas V.fetus was always (4+), and so such samples were designated as negative. The one V.bubulus isolated gave (+) fluorescence and looked smaller and thinner than the fluorescing V.fetus cells. This apparent difference was not seen when both were stained by Gram's method and observed in the bright field; and so it was assumed that the fluorescing cell wall surface of V.fetus caused this apparent increase in size.

Results.

Group A.

From the results in table 23, it will be seen that 8 out of the 54 bulls were positive. Bull (2) was

treated as positive because it had been a V.fetus carrier in the past, although the 2 fluorescing cells seen were not typical of V.fetus. Two bulls (3) and (8) were positive once and negative once. As bull (3) had only given a count of 10 fluorescing cells in the positive sample, it was not too surprising that a repeat sample was negative. As I did not count the number of vibrios seen with bull (8), I cannot compare the positive finding with respect to numbers with the negative result. Bulls (4) and (7) both gave rather low counts from all samples, while (1), (5) and (6) all gave higher ones. The number of vibrios seen in the second sample from bull (1) was too numerous to count and I expected from this result to find that this bull was a carrier capable of transmitting infection. From the results of Experiments 2 and 2A, when an average of 15 vibrios were counted on the slide, 2 out of 6 heifers became infected from the same artificial sample. Although such a comparison could not be made directly, when very many fold this number were seen on the FAT slides, it did suggest that this bull was a carrier. All these samples were collected in saline prepared and sterilised in my laboratory.

Group B.

For a summary of the results see table 25 .

a) Fluorescent antibody technique.

As was stated for Group A, there was quite a difference in the background fluorescence from sample to sample in Group B. This was not sufficient to prevent V.fetus cells being detected. Many bull samples (8), (3) and (2), contained a number of almost straight fluorescing cells but there were always many curved typical vibrio-like cells fluorescing in greater numbers so there was no doubt about their being positive. Straight fluorescing cells were never seen on their own.

The negative result from the second sample from bull (1) was not unexpected as only one fluorescing vibrio was seen om the first positive sample. There was quite a marked difference between the counts from the 2 samples collected from bull (3), with 19 and 70 vibrios seen, and bull (5) with 35 and 5 counted. This could be due to a variation in the sampling technique, or unexplained multiplication or death of the organisms in the prepuce. Also because the amount of cellular material present could vary and as the sample was processed by first depositing and discarding this gross cellular material, if much were present in one sample then more vibrios could be lost with this one than with another sample. In an experiment set up to estimate this loss, there could be as many as 50% vibrios deposited.

b) Culture.

The results are summarised in table 25 .

It was not surprising that no isolation was made from bull (1). The numbers of vibrio cells counted by the FAT from the samples of bulls (4) and (6) were low and could be the reason for failure to culture V.fetus from them. Bulls (5), (3) and especially (7) were expected to give positive cultures but all 3 were negative. Overgrowth of other bacterial species on the isolation media was not responsible for this. In some cases organisms had passed through the millipore filter membrane, but these were usually few in number. It was possible that the cells seen by the FAT were non viable or rendered so by the effects of being suspended in the preputial washing. Bulls (2), (8), (9) and (10) all gave one of the 2 samples positive by culture also. With bulls (10) and (8) the isolation was made from the sample with the lower count, which was the first sample collected in (8) and the second in (10). As the saline for this group had not been prepared in my laboratory, I was suspicions that it might have had a deleterious effect on the vibrios present and for this reason I requested that the second sample from bull (10) be collected in sterile nutrient broth. The sample resulted in very numerous V.fetus colonies being isolated on the culture medium plates. This one example drew attention to the fact that the FAT was capable of detecting dead or dying vibrios and that for culture isolation a suitable diluent was important.

Biochemical characteristics of V.fetus isolates

Group A.

Some of the bulls in Group A were re-sampled a few months after the initial survey and treatment with antibiotics and the PAT performed. As some were positive the remainder of the processed material was millipore filtered and the filtrate cultured in the same way as with Group B. In this way, 4 isolations were made. All these isolates gave the biochemical characteristics of V.fetus intestinalis intermediates. They produced hydrogen sulphide in the cysteine containing medium, grew in a medium containing 1% glycine but not 1.5% glycine. These isolates seem to be similar to those discussed by Park et al (1962) and Florent (1964) The latter called these V.fetus intestinalis intermediates as they were, unlike the previously described V.fetus intestinalis, able to survive in the bovine vagina from some time. The 3 bulls from which V.fetus was isolated had been positive in the original survey and treated with streptomycin and had been negative subsequently. All the isolates were sensitive to streptomycin so the later "infection" was not due to resistant organisms being induced by treatment. This raises the question was treatment ineffective (treatment may have left a small number of vibrios in the prepuce which given the correct conditions again established themselves in detectable numbers) or were the bulls reinfected? It has been noted that mature bulls are readily "reinfected". There is thought to be no immunity

conferred on a bull by its having been a vibrio carrier. This is probably due to the bull only being a carrier, the vibrios being present as commensals in the prepuce and so there was no immunological response.

The number of vibrios counted on the FAT slides and grown after millipore filtration were very high. It did not seem reasonable that these were faecal strains of V.fetus intestinalis contaminating the sample at collection. The true faecal vibrios are not thought to multiply in the prepuce, nor were their biochemical characteristics that of V.fetus intestinalis. Both the high number of cells counted in the FAT slides and the high number of colonies on the solid media suggested that a high proportion were viable and probably multiplying. Despite the evidence that the vibrios were multiplying and they were V.fetus intestinalis intermediate none of these bulls was giving any evidence of reduced fertility. This could be due to the semen containing too few vibrios to infect a cow or that the antibiotics added routinely to the semen were effective in preventing the growth of V.fetus in the vagina or uterus of the inseminated cow. The strains isolated could be of low virulence and this seemed to be substantiated by the circumstances. One bull had been positive by the heifer mating test a year prior to the FAT survey. The isolate from this was then biochemically V.fetus intestinalis. At that time 1.5% glycine tolerance was not performed by me but it

was retested later and found to be a V.fetus intestinalis intermediate. As the 4 isolates of the latter vibrios were all from bulls on the same premises it did suggest that the bulls found positive in the FAT survey could have been infected from this bull.

It was also possible that for some reason infectivity in cows inseminated with semen from these bulls was not reflected in the information as it was presented. If only a very small percentage of cows were infected and returning to service, but not using AI subsequently, they would eventually become in calf but would then have infected the local bull and in time there would be an infertility problem in the herd. However, this does not appear to be the case at present in any of the areas sampled.

Group B

There were 4 isolates obtained from this group (table 26). One gave biochemical characteristics of V.fetus venerealis while the other 3 did not agree with the reactions of either V.fetus intestinalis or V.fetus venerealis. These isolates did not grow in media containing either 1% or 1.5% glycine, but did produce hydrogen sulphide in a medium containing cysteine hydrochloride. As some of the original strains that I was given as V.fetus venerealis also produced hydrogen sulphide in media containing cysteine and as the methods of reading this test varied it did suggest

that the other 3 isolates were intermediates of *V.fetus venerealis*. The bull carrying the *V.fetus venerealis* had been very recently introduced on to the premises, and as none of these bulls were showing reduced fertility it did suggest that the infection was recently introduced and probably by this bull.

ADDITIONAL RESULTS OF THE BULL SURVEY

Over the 2½ year period since the bull survey was completed some of the bulls were resampled. It will be seen from table 28 that all of the bulls which were initially found to be V.fetus carriers were either again found positive using the FAT or they were not resampled. Many of the bulls that were originally V.fetus carriers were subsequently found positive after treatment and negative results. In addition 10 bulls originally found negative became positive. These findings suggested either failure to eradicate V.fetus from the premises, or reinfection having been reintroduced and spread by the introduction of new bulls.

TABLE 28

RESULTS OF THESE BULLS RETESTED USING THE FAT IN THE 2½ YEAR PERIOD SINCE THE ORIGINAL SURVEY

	Original Group A bulls	Original Group B bulls
The number of Original Carrier bulls	7	10
The number of these positive bulls subsequently found positive	6*	6*
The number of bulls retested which remained negative	14	13
The number of bulls originally negative but became positive	6	4

*One bull from Group A and 4 bulls from Group B were not retested.

GENERAL DISCUSSION

GENERAL DISCUSSION

In the experimental section dealing with the laboratory techniques of diagnosis of V.fetus, 3 methods were examined. In these experiments (1, 1A, 1B, 2 and 2A) known numbers of viable vibrios were added to pooled PPW. After processing in the laboratory these artificially infected PPW were inoculated directly on to a selective medium (BAN page 48). A second portion was passed through a millipore filter of 0.65 μ porosity and the filtrate cultured. From a third portion smears were prepared and stained with conjugated anti V.fetus serum. The results with both the FAT and millipore methods showed that vibrios were detected when 10^3 viable vibrios were added per ml of PPW. With direct culture on to the selective medium the results were not so good, as vibrios were only readily detected when 10^5 vibrios were added per ml of PPW. The direct plating on to selective media results agreed with the findings of Kita et al (1966), while their FAT and millipore filtration methods could only detect vibrios present when 10^5 vibrios were added per ml of sample. Their preparation of the PPW seeded with vibrios, the selective media used, as well as the quantities involved all differed in many details from the ones used here and this might explain the great difference between the results. Philpott (1968b) was able to detect 50 to 100 vibrios per ml of PPW using the FAT, and 200 vibrios per ml using millipore filtration. Mellick et al

(1965), Dufty (1967), Winter et al (1967) and Philpott (1968b) compared FAT and culture methods for the detection of vibriosis in clinical samples from bulls and all found both methods satisfactory. Winter et al (1967) suggested that as their culture and FAT results were to some extent complementary, both should be used for routine diagnosis and thereby increase the chance of making a correct diagnosis. As no selective medium inhibited all other commensal bacteria, isolation methods have depended on the number and species of commensals present in the PPW, as well as the number of vibrio fetus cells present.
In experiments 1, 1A, 1B, 2 and 2A the same vibrios were used as antigen as had been used to prepare the conjugated antiserum and so prevented the possibility of weak or absent fluorescence being due to the antigen belonging to a different serotype from any of the conjugated antibodies. This therefore ensured a positive reaction occurring when V.fetus cells were present. The main discrepancy in this section of the work was between the results of viable count and total count of V.fetus; the latter being 2 to 4 times that of the former. If these results were correct then all the FAT results, in terms of numbers of vibrios counted in 10 minutes screening, in experiments 1, 1A, 1B, 2 and 2A should have been divided by 2 to 4 if they were to be compared directly with the culture results, assuming that the FAT was able to detect these dead cells. Twelve heifers were inseminated with the number of vibrios that was detectable by the least successful of the 3

laboratory diagnostic tests done. In this way it was hoped to compare a heifer mating test with 3 laboratory methods of diagnosing vibriosis in bulls. From only 4 of these 12 animals was V.fetus isolated. The 8 negative heifers were inseminated with approximately the same number of vibrios as Newsam (1964) had used (15×10^7) and again not all became infected. The fact that 6 out of the 8 heifers became positive showed that these 6 were capable of becoming infected and suggested that their first insemination with the smaller number of vibrios was insufficient to cause infection. Murane, Eales and Monsbourgh (1959), Blobel, Simon and McNutt (1957) and Newsam (1964) all failed to infect every heifer inseminated with material containing V.fetus. Of the two that remained negative, one had a prolapsed vagina which was heavily contaminated with faecal bacteria which could have prevented establishment of V.fetus, but for the other no explanation can be offered except perhaps a natural resistance against infection.

Unfortunately it was not possible to inseminate heifers with PPW from a known infected bull. This would have been useful to do as the 3 laboratory methods of diagnosis would also have been simultaneously performed. Until this is done no direct comparison can be made between the efficiency of the heifer mating test and culture or FAT of infected PPW.

Bull survey.

In the original survey preputial washings from 89 bulls were examined, 54 by the FAT only and 35 by the FAT and millipore filtration followed by culture. Seven out of 54 were positive by the FAT. Ten out of 35 were positive by the FAT, with 4 of these 10 positive samples being positive on culture after millipore filtration. At a later date V.fetus was isolated from 4 of the 7 FAT positive bulls. The subsequent retesting of many of these bulls in the original survey revealed that some of the bulls originally found positive became negative after treatment but at a later date became positive again. This raised the question of whether treatment was only greatly reducing the numbers of vibrios present, perhaps below detection level, and after treatment ceased multiplication again took place until the detection level was again reached. The other possibility was that after treatment the bulls were free of vibriosis for a time but were later reinfected. The other interesting fact was that 10 out of 37 bulls originally negative became positive: Unless the first survey failed to detect the presence of vibrios in the PPW of these bulls, this suggested some means of bull to bull infection.

The FAT was the method of choice for its accuracy and speed. From the results obtained in experiments 1, 1A, 1B, 2 and 2A, the FAT and millipore filtration were of comparable sensitivity but in the bull survey the FAT was able to detect more infected bulls than culture following millipore filtration. Isolations were made from

samples with a FAT count of 13 vibrios seen in 10 minutes screening and yet samples with FAT counts of 500, 218 and 132 failed to grow vibrios. Ideally a culture method and a FAT was done on each sample for two reasons; firstly it was desirable if not essential to isolate the vibrio so that it could be further identified and secondly there was no possibility of the FAT failing to detect a V.fetus antigenically different from the strains used to prepare the conjugated antiserum. All isolates gave good fluorescence and on no occasion was V.fetus isolated without vibrios being seen on the FAT slide. The isolates obtained from the survey were 4 V.fetus intestinalis intermediates, one V.fetus venerealis and 3 probably V.fetus venerealis intermediates. All bulls were treated as if they were infected with a pathogenic vibrio although it is not thought that V.fetus intestinalis can survive for a great length of time in the prepuce. Park et al (1962) however, found that V.fetus intestinalis was capable of causing infertility and recommended that it should be treated in the same way as V.fetus venerealis. None of the carrier bulls in the survey were infertile but the very recent introduction of the bull carrying the V.fetus venerealis was thought to be a likely source of infection on the premises, although the other 3 isolates were intermediates. The other 4 bulls were carrying V.fetus intestinalis intermediates. These isolates might have been of reduced virulence but insemination of 2 heifers with semen from one of these carrier bulls gave growth

of V.fetus intestinalis intermediates from vaginal mucus samples. This bull isolate was therefore capable of multiplication in the vagina of these 2 heifers and as such was treated as infected. After treatment this bull gave one doubtful result and then became negative using the FAT method of detection. The heifer inseminations were done prior to the work of this thesis and so no FAT was done on PPW from this bull at the time of infection.

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STUDIES IN BOVINE VIBRIOSIS.

SUMMARY.

Vibrio fetus causes infertility and abortion in cattle, the carrier bull being the main agent of transmission. The work done in this thesis studied different methods of detecting *V.fetus* and where possible compared their results.

In the first section *V.fetus* was added to preputial washings from vibrio-free bulls. After treatment in the laboratory the following procedures were carried out:

1. The material was cultured on to a solid medium containing antibiotics.

2. The material was passed through a millipore filter of 0.65 micron diameter porosity and the filtrate cultured.

3. The material was placed on a slide and treated with a fluorescein conjugated anti-*V.fetus* serum to detect the presence of *V.fetus* cells.

The fluorescent antibody technique (FAT) was found to be specific and of comparable sensitivity to the culture method following millipore filtration. The latter method was found to be more successful than direct culture on to a medium containing antibiotics.

The second section dealt with the insemination of heifers with preputial washings (PPW) containing known numbers of V.fetus cells and the subsequent reisolation of V.fetus from vaginal mucus taken from these animals. It was found that when approximately 10^4 viable vibrios were inseminated, 4 out of 12 heifers gave at least one vaginal mucus sample from which V.fetus was isolated. When approximately 10^8 vibrios were inseminated, 6 out of 8 heifers gave isolates of V.fetus.

The third section was a bull survey in which 89 bulls were examined, 54 using the FAT only and 35 using the FAT and culture following millipore filtration. The results showed that 8 out of the 54 bulls and 10 out of the 35 bulls were vibrio carriers. Only 4 out of the 10 FAT positive bulls gave successful isolation using the millipore filtration culture method. Because of its speed and accuracy the FAT was considered to be a good screening method but isolation was considered essential if further identification of the isolates was required.