FIELD AND LABORATORY STUDIES OF BOVINE GENITAL CAMPYLOBACTERIOSIS WITH PARTICULAR REFERENCE TO DIAGNOSIS

BY

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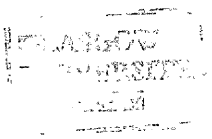

This work is dedicated to my Parents,

The Reverend Wilson Muma Okwaro

and

Mama Grace C. Ondu Muma

Two people who sacrificed much of their own lives so their children could have an education.

and also

To my family for their tolerance.

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SUMMARY

Crude sonicated antigens of Campylobacter fetus have been shown to possess several precipitating antigens of which at least two and at most four, possibly five, were anodally migrating. These antigens reacted with rabbit anti-C. fetus whole cell antisera in the counter-immunoelectrophoresis (CIE) system to produce immunoprecipitates in agarose gels. The reaction appeared to be pH dependent as at pH 8.6, it was less pronounced than at pH 6.6 - 7.6, although it was not possible to classify the antigens using the CIE test. These antigens failed to react with phenol saline extracts of vaginal mucus from cows known to be infected with C. fetus and which reacted to the vaginal mucus agglutination test (VMAT).

Further studies revealed that the agarose type used as support medium was of importance in the use of CIE for immunodiagnosis of C. fetus infection in cattle. Two agarose types, Agarose B (Pharmacia, Sweden) and Litex HSB Agarose (Litex, Denmark) were found suitable and three others unsuitable. The vaginal mucus required to be diluted 1:4 with normal saline, homogenized and centrifuged. The development of directly visible precipitation was favoured by concentrating the sol to 0.5-1.0 ml by dialysis against polyethylene glycol (Carbowax, Sigma Chemicals, U.S.A.) before testing. Despite concentration, the staining of immunoplates increased the chances of detecting positive cases. That the effect of pH was important was further emphasised when bovine mucus samples were found to be most reactive at pH 7.6 and 7.9.

Buffer types tested did not affect the test as long as the gel pH was 7.6 and 7.9. However, the involvement of more than one biotype of C. fetus in field infections led to the realisation that a mixed sonicated antigen comprising one strain of C. fetus subsp fetus and one strain of C. fetus subsp venerealis provided

a wide antigenic spectrum that detected all field infections investigated by the CIE test.

In order to determine the specificity of the test, virgin heifers shown to be free of infection and to be immunologically negative, were infected with C. fetus subsp fetus to study immunoconversion and the type of immunoglobulins reactive in the CIE test. All heifers continued to be immunologically negative until about 3-4 weeks after infection when evidence of immunoconversion was shown by the VMAT. On the 37th day post infection (Dpi), immunoconversion was also detected by CIE in two of the three heifers and in the third heifer on day 113 post infection. From then onwards both VMAT and CIE detected the presence of antibodies, with the period 141-193 days post infection showing the clearest CIE reactions as samples tested over this period from all heifers were positive. While the VMAT tended to be unreliable after about 5 months post infection, positive CIE reactions were recorded over a longer period. In the one heifer which was pregnant even though still harbouring the infection, an anamnestic response to the presence of C. fetus organisms in the cervico-vaginal area was thought to have occurred because positive CIE reaction reappeared 36 days after parturition and persisted till the end of the experimental period at 383 days post infection.

On the basis of studies of the antibodies in the vaginal mucus of the three experimentally infected heifers and field samples, IgG_1 was found to be the predominant antibody associated with CIE reactions. IgA also participated in this reaction but IgM activity was demonstrated in only one sample. IgG_2 apparently did not participate to any level measurable by the absorption method used. Because it was possible that the more abundant antibodies could mask the presence of those present in smaller quantities, it was not possible to draw conclusions regarding the activity of the minor antibodies.

One experimentally infected heifer (24) was repeatedly served, the second one (14) became pregnant and the third (17) was not seen in oestrus after service but was not pregnant. The progesterone profiles and rectal palpation of the heifers 24 and 17 showed both to be cycling during the experimental period. Heifer 24 had an 'abnormal' progesterone profile in her second cycle and may have been pregnant and lost her conceptus. However, this was not conclusively proven and thus the experiment did not fully reveal the possible infertility causing effect of the strain used. However, the experiment showed genital tropism of this strain of C. fetus subsp fetus as two heifers remained infected for over 300 days.

To link suspected field infections with the results of immunological tests (VMAT and CIE), culture of vaginal mucus and bull preputial washings from suspected herds was performed. Two biotypes of C. fetus were found to cause venereally spread infertility in South West Scotland, namely C. fetus subsp fetus and C. fetus subsp venerealis. C. fetus subsp fetus was formerly known as Vibrio fetus var intestinalis and is still regarded by some workers as a cause of sporadic abortions in cattle but not infertility. The biotyping criteria used revealed that C. fetus subsp fetus and C. fetus subsp venerealis could be differentiated by the 0.1% selenite reduction and the 1% glycine tolerance tests, but the reactions to these tests could not be correlated with venereal transmission. Serotyping with monospecific O- antigen antisera revealed that the C. fetus subsp fetus strains and C. fetus subsp venerealis strains tested all belonged to serotype A. Hitherto, only serotype B C. fetus subsp fetus organisms were thought to be associated with venereal transmission in Britain.

The infertility patterns caused by both types of organisms were essentially similar on the basis of herd breeding records. Faecal campylobacters and other allegedly non-pathogenic campylobacters were

also studied to determine if they could interfere with interpretation of genital campylobacter isolates obtained from herds with an infertility problem. They were found to differ from the genital pathogen biochemically and serologically and were considered unlikely to cause diagnostic indecision.

CHAPTER ONE

1:1 General Introduction

The reliance of man on domestic cattle for the production of a substantial part of his food and the competition of these animals with man for available plant food and living space demand that only those animals with the highest productive values at the least cost per animal head be kept. In terms of milk and meat production, the major animal food sources, and in cattle in particular, efficient reproductive capacity may be the most important factor (Salisbury et al 1980). This has led to economic appraisal of the most suitable conditions for efficient cattle raising.

At present it appears that in both dairy and beef production systems a cow must produce one viable calf every year. To achieve this a large proportion of the herd must be served and conceive not longer than 85 days after the previous calving. In the case of heifers mated for the first time a target of about 60% first service conception rate should be aimed for. If more than an average of two services is needed a fertility problem exists.

Several factors acting in combination or separately can frustrate this objective in cattle breeding and may act by affecting fertilization, embryo survival or the maintenance ofpregnancy resulting in abortions or stillbirths. In either case herd milk production is lowered and in beef production where the calf is the profit, the economic basis of farming is endangered. The natural approach to correction of the problem is to "identify the causes and eliminate them" (Salisbury et al 1980).

Among several factors causing this frustration in cattle breeding is Campylobacter fetus infection. The realisation that this organism caused venereally transmitted infertility was a very important finding for the cattle breeding industry. The identification and elimination of

this pathogen from the breeding stock of certain countries, notably Scandinavia (Adler and Lindeggard 1964) has been achieved. This goal has yet to be realised in many parts of the world including Britain, U.S.A., Australia and Africa where its occurrence and adverse economic effects must still be borne by the farming community.

The identification and elimination of a disease condition in a population is in medical terms an attribute of the means of diagnosis and control. Diagnosis is usually based on the clinical symptomatology of the disease, in many cases confirmed by laboratory findings. The major problem of a clinically based diagnosis only is the possible occurrence of other conditions which could cause similar symptoms and thus accurate differential diagnosis is essential. With many infectious diseases laboratory diagnosis depends on the examination of the suspected pathogen for its established morphological features, its biochemical activites and its serological properties. If, as in most infectious diseases, the infection leads to development of an immune reaction the demonstration of specific antibodies against the pathogens will aid diagnosis.

In the case of C. fetus infection in cattle problems of isolation from clinical cases, identification and immunological tests for diagnosis have contributed to the failure of the actual aim of "identify and eliminate". In the case of isolation, the major problems concern the nutritional fastidiousness and the microaerophilic nature of the pathogen. However, recent technical developments have lead to this aspect being less of a problem (Hoffman 1978; George 1978). The accurate identification of the pathogen has probably been the greatest problem mainly because of the complicated classification of the pathogen into certain biovars and serotypes associated with infertility or abortion in cattle (Florent 1959; Berg et al 1971). This may have lead to less attention being paid to certain biovars even when they were isolated in clinical situations akin to infertility and venereal transmission (Park et al 1962). The

obvious contribution of such an unsolved problem to the control of a disease cannot be overemphasised. The confirmation of C. fetus infection in cattle by immunodiagnosis has been based on the use of the vaginal mucus agglutination test (VMAT) (Stegenga and Terpstra 1949). The disadvantages of this diagnostic method are described in Chapter Two along with those of the fluorescent antibody test (FAT) which is an alternative immunodiagnostic method. (Accurate identification of C. fetus organisms is essential for the efficient application of a vaccination based control programme (Clark et al 1976). Therefore an attempt to develop a cheap, rapid and specific immunodiagnostic method for this disease was made. Although such attempts have been undertaken by using haemagglutination methods (Te Punga 1958a, 1958b; Newsam et al 1967b; Newsam and St. George 1967), these methods were technically demanding and have not been widely applied.

The aims of this work were to develop a rapid, sensitive immunodiagnostic method using the counterimmunoelectrophoresis test (CIE) for the diagnosis of C. fetus infection in cattle and to compare its efficiency with that of the VMAT and bacteriological isolation and identification of the pathogen from animals in infected herds. In the course of these studies it became apparent that the problems of identification of the pathogen based on presently accepted methods and classification were also operative in South West Scotland. Because of this, isolation and identification formed the second major part of this thesis. Following the development and application of the CIE, its specificity and the nature of the antibodies involved were also studied by experimental infection of virgin heifers.

1:2 The Sequence Of the Investigation and The Presentation of the Results

The use of the CIE test in diagnostic work requires the availability of a soluble antigen which contains anodally migrating, reactive components which under suitable conditions move towards cathodally directional gammaglobulins specifically produced by an infected animal against the antigens

(Corkill 1977; Poli *et al* 1980).

The occurrence of such antigens in *C. fetus* organisms has been recorded (McCoy *et al* 1975a, 1975b, 1976a, 1976b). However, they demonstrated two such antigens by conventional immunoelectrophoresis procedures. The crossed antigen-antibody immunoelectrophoresis (Laurell 1965) is a much more sensitive technique and should reveal more antigens if they are present in *C. fetus* extracts. This part of the study is presented in Chapter three and was conducted with rabbit anti-*C. fetus* antibodies.

Chapters 4a and 4b describe the conditions found to favour the use of CIE in the immunodiagnosis of *C. fetus* infection in cattle and the application of the test in clinical field infections. Chapter 5 records the specificity of the CIE test on the principles of antigen specificity, local genital antibody reactivity with the soluble antigen used and the demonstration that IgG_1 and IgA to a lesser extent were the precipitins involved. The results of bacteriological isolation and characterisation, the epidemiology of field infections and the involvement of more than one biotype of *C. fetus* on genital infections in South West Scotland are recorded in Chapter 6. Chapter 7 discusses the results obtained in the work with emphasis on rapid diagnosis by CIE, the problems of isolation and characterisation of genital *C. fetus* organisms and the clinical data related to the biotypes isolated and the conclusions arrived at on the basis of this study.

CHAPTER TWO

LITERATURE REVIEW

2:1 Historical Aspects of Bovine Genital Campylobacteriosis and the Causative Organism with Particular Reference to Britain

The first association of gram negative comma shaped bacteria with ovine abortion and experimental bovine abortion is attributed to McFadyean and Stockman (1913). In the United States, Smith (1918) isolated a similar 'sprillum' from aborted calves and considered that this was the same organism which was described earlier by McFadyean and Stockman (1913) in the United Kingdom. Smith and Taylor (1919) named this organism Vibrio fetus.

It was to take twelve years before Mattick (1925) further described the disease in the United Kingdom and another 15 years before McEwen (1940) again described it in connection with aborted bovine foetuses in the South East of England. In the 1950's, following the reports by Sjollema et al (1949) that V. fetus was a venereally spread cause of infertility, it developed national significance (Hignett 1950, 1951, Deas 1950 and Lawson and MacKinnon 1952). The farming community and the veterinary profession expressed their concern on the possible spread of this disease by artificial insemination (AI) by means of parliamentary questions (Anon 1953, 1954). Deas (1950) described the occurrence and epidemiology of vibrionic infertility in Scotland.

Towards the end of the 1950's, perhaps as a result of use of antibiotic treated semen and the use of antibiotics to treat bulls and cows (Edgson and Scarnell 1955; Melrose et al 1957) there was an attitude of success towards the control of this scourge. Thus the opinion expressed that 'in Britain, this infection appears to be of little importance now, though exact figures of incidence are not available....' (Allison 1963) was not however universally accepted.

By the end of the 1960's although the occurrence of the disease was still recorded in AI studs (Phillpot 1968b) it was surprising that at the same time, the general view held was that the 'disease was fast becoming a museum piece' (McLaren 1977). Later, however, MacLaren and Wright (1977) reported that several herds in the South West of Scotland had an infertility problem caused by this organism. The continued occurrence of this infection and its infertility syndrome in Scotland was emphasised even more by the recent report by Roberts (1979) that the organism was involved in several other infertility cases in the Aberdeen area. Therefore the disease could have persisted in the United Kingdom from before 1913 to the present time.

2:2 Present Nomenclature and The Classification of Members of the Genus Associated with and Causing Reproductive Failure

Following their studies on comma to spiral shaped gram negative bacteria, Sebald and Veron (1963) observed that *V. fetus* and some other related organisms differed from the type species of the genus Vibrio- *Vibrio cholerae*. Not only were these inert with respect to glucose fermentation but also the genetic composition of the fermentative group was different from that of *V. fetus* (Véron 1966). Sebald and Veron (1963) proposed that this organism and other non-fermentative vibrios be removed from the genus Vibrio and be placed in a separate genus, Campylobacter. Véron and Chatelain (1973) used this nomenclature in their subsequent study and it has now been fully accepted (Skerman *et al* 1980). However, the classification of Véron and Chatelain (1973) placed the original *V. fetus* (intestinalis)(Florent 1959) as the type species while the classification of Smibert (1974) put the original *V. fetus* subsp *venerealis* (Florent 1959) as the type species. In this work, the nomenclature of Skerman *et al* (1980) is used throughout. *C. fetus* subsp *fetus* was described by Florent (1959) and is believed to cause sporadic abortion in cattle but not infertility (FAO Expert Panel

1960; Hoppe and Ryniewcz 1962) and resides in the intestinal tract of cattle, pigs, swine, birds and humans. Park et al (1962) isolated organisms showing the biochemical behaviour of C. fetus subsp fetus which appeared to cause infertility and to be potentially venereally transmitted. The genital tropism of these organisms was subsequently confirmed by Florent (1963) who described them as 'intermediate group'. He however thought that they tolerated poorly 1% glycine unlike Park et al (1962) who repeatedly demonstrated that these organisms grew in media containing 1% glycine. Elazhary (1968) used this proposal of Florent (1963) and called organisms that could persist in the genitalia of cattle and also survive in the intestinal environment C. fetus subsp intermedius. From the work of Véron and Chatelain (1973), these organisms could not grow in 1% glycine media but produced hydrogen sulphide in semisolid media to which cystine had been added. In this way they resembled similar venereally transmitted organisms isolated by Bryner et al (1962) and considered to be a biovar of C. fetus subsp venerealis (Florent 1959) which is the organism most commonly associated with venereal campylobacteriosis in cattle. Bryner et al (1962) had called their isolates biosubtype I and biotype I, the latter being the same as C. fetus subsp venerealis of Florent (1959). It can therefore be seen that Véron and Chatelain (1973) classified the venereally transmitted C. fetus subsp fetus organisms both as Vibrio (Campylobacter) fetus subsp intestinalis and Vibrio fetus subsp intermedius though the former ought to be 1% glycine tolerant and the latter intolerant.

This would mean that organisms which are venereally transmitted but behave biochemically as C. fetus subsp fetus (intestinalis) such as those found during this work could not be similar to those described by Bryner et al (1962) and Elazhary (1968). Organisms such as the latter could not grow in media with 1% glycine. Berg et al (1971) also described organisms which behaved biochemically as C. fetus subsp fetus

but called them serotype B as they differed from serotype A and C of C. fetus subsp fetus which were not venereally transmitted. Recently Dedie et al (1977) described the isolation of serotype A organisms which could be of significance in genital tract pathology in cattle but behaved biochemically as C. fetus subsp fetus . In the U.S.A., Whitford et al (1977) also reported the isolation of both serotypes A and B of C. fetus subps fetus from aborted foetuses. In the United Kingdom, 1% glycine tolerant C. fetus organisms are being called 'intermediate' (Anon 1978) but are therefore not the same as the C. fetus subsp intermedius of Bryner et al (1962), Elazhary (1968) and Véron and Chatelain (1973). This means that the nomenclature of venereally transmitted C. fetus subsp fetus organisms has not been clarified. Because they tolerate 1% glycine they have been considered as C. fetus subsp fetus in this work.

The remaining members of this genus found in genitalia of cattle have been described as C. sputorum subsp bubulus (Florent 1953; Loesche et al 1965), which is catalase negative, being strongly H_2S positive and growing in 3.5% NaCL and is also serologically distinct and C. fecalis (Firehammer 1965) which is catalase positive but like C. sputorum subsp bubulus is also hydrogen sulphide positive. These last two are considered to have no reproductive pathogenicity in cattle. Other members of the genus occur (see Table 6.1) but do not inhabit the bovine genital tract. The differentiation of C. fetus subsp venerealis into two biovars and C. fetus subsp fetus into ecological and pathogenic types is not recorded by Skerman et al (1980). However for clinical veterinary purposes it is worth noting that campylobacter organisms associated with genital pathogenicity may be tabulated as follows:-

1. C. fetus subsp venerealis biotype I (A-1)(Berg et al 1971)
2. C. fetus subsp venerealis biosubtype I (A-sub I)(also called C. fetus subsp intermedius)(Bryner et al 1962; Elazhary 1968)

3. C. fetus subsp fetus sero A-2 (Dedie et al 1977; Whitford et al 1977)

4. C. fetus subsp fetus sero B (Berg et al 1971)

In addition

5. C. fecalis and

6. C. sputorum subsp bubulus may also be isolated during routine bacteriological examination of specimens from the reproductive tract. The requirement that an isolate be identified as being pathogenic (Morgan 1957)) would therefore include full biotyping and serotyping of all isolates. Then, with the clinical history of the case, a decision can be made as to whether the isolate is possibly pathogenic without the use of animals for pathogenicity studies. The criteria chosen for differentiation have to be correlated with pathogenicity based on clinical observations.

2:3 Epidemiology and Clinical Features of the Disease with Particular Reference to Cattle

During the period 1919-1943 the major pathologic effect of C. fetus in bovine reproduction was considered to be sporadic abortion. However laboratory observations with the serum agglutination test (SAT) led Plastridge and Williams (1943) to suggest that as well as the cow, the bull might also be susceptible to vibrionic infection. Further clinical observations and considerations of the breeding records of some herds made Plastridge and Williams (1947) suggest that apart from abortion, conception failure in infected herds appeared to occur. This view was soon to be supported by Sjollema et al (1949) who also observed poor conception rates and irregularities of the oestrus cycle associated with service by specific bulls and also that both abortion and repeat breeding were associated with inseminations using semen from an infected bull. Thus towards the end of the 1940's and the beginning of the 1950's it was concluded that this infection was

venereally transmitted (Herrick 1949; Stegenga 1950; Rasbech 1951; Lawson and MacKinnon 1952).

Further advance in understanding of the epidemiology of the disease came with the finding that the bull was indeed the carrier of the bacterium (Plastridge et al 1951; Terpstra and Eisma 1951). The bull's health was not affected in anyway nor was the semen quality. It was considered that bulls 5 years old and over were more susceptible to infection (Adler 1957; Wagner et al 1965: Serger et al 1966; Lein et al 1968; Schutte 1969). Part of the explanation of this was thought to be associated with the deeper epithelial crypts in the glans penis and prepuce of older bulls than in younger bulls (Samuelson and Winter 1966). However recently no difference in age susceptibility to infection was found (Dufty et al 1975; Bier et al 1977). Bulls once infected remain so for long periods if not permanently (Laing 1960) but spontaneous recovery has been reported (Vandeplassche et al 1963; Frank et al 1958).

Although infected females develop a clinical immunity and breed normally they may remain vaginal carriers of the organism for varying periods post infection, during gestation, and even for over 200 days after parturition (Vandeplassche et al 1963). Such female animals obviously perpetuate infection in a herd since under commercial conditions calving cows are expected to be served and conceive well within that period. Thus in an infected herd, unless service management changes, infection could be present but not easily noted in the older cows as they may have normal fertility (Boyd 1955). During infection the female cattle may show mild vaginitis and cervicitis (Laing 1960; Vandeplassche et al 1963) perhaps with catarrhal exudate. The major effect appears to be return to service at intervals which are often longer than the normal oestrus cycle length. Repeated returns to oestrus may occur over a period of 3-6 months, after which most animals conceive even though the pathogen

is still found in the anterior vagina (Frank et al 1964). However at this time the organism will have been cleared from the uterus and oviduct by an immune reaction (Winter 1973). Abortion may occur in a few animals and when it does it will be most common at 4-7 months of gestation. It is thought that the return to service at intervals greater than 18-24 days may indicate that fertilisation has occurred but that the conceptus was lost after 17 days either by the direct action of the organism on the embryo or due to the poor uterine environment caused by inflammation which may interfere with early embryonic attachment to the uterine mucosa (Adler 1959).

The clinical symtomatology described was associated with C. fetus subsp venerealis infections. The consensus of veterinary opinion was that C. fetus subps fetus did not cause infertility but occasionally caused abortion when infection was acquired by the oral route, became blood borne, and settled in the placenta of pregnant cows causing placentitis and thus abortion (Smith and Taylor 1919; Simon & McNutt 1957). The pathogenesis has probably been proposed on the basis of a similar mechanism by which the organism infects sheep and causes abortion (Miller et al 1959). Venereal transmission has not been seriously considered. Recently Agumbah and Ogaa (1979) demonstrated that the organism could be venereally transmitted and indeed had some degree of genital pathogenicity as suggested by Park et al (1962) and confirmed by Florent (1963). The importance of this biotype in venereal transmission and the field evidence of infertility is considered in Chapter 6 of this work. These organisms were of serotype A of Morgan (1959) and Berg et al (1971) and thus would corroborate reports of Dedie et al (1977) and Whitford et al (1977) that this serotype as well as serotype B was also venereally transmitted. Therefore the strictness of the bio-classification and of aetiological classification (Florent 1959) in association with infertility may not be strictly adhered to.

2:4 Immunology of Campylobacteriosis in Cattle

Immunity to pathogenic microbial organisms may be mediated via circulating or local antibodies which coat (opsonise) the pathogens so that phagocytes may then ingest and destroy the pathogens (humoral immunity and local mucosal immunity) or in addition, antigen sensitised mononuclear cells may be induced so that the lymphocytes will augment the 'phagocytic' function of macrophages (cell-mediated immunity). Extracellular parasites are frequently eliminated by phagocytosis. Those parasites which are not killed by phagocytosis may remain and can even destroy phagocytes unless in the presence of opsonins, have been termed facultative intracellular organisms. The major immune responses to these facultative intracellular organisms is therefore both humoral and cellular (Collins 1971; Mackaness 1970).

During genital infection with C. fetus it has been shown that little or no systemic humoral immunity is involved as judged by the rare increase in specific antibodies in the sera of infected cattle using the serum agglutination tests (Boyd 1955; Winter 1965, 1979; Larson and Ringen 1967; Van Aert et al 1977; Schurig et al 1978). However, specific immunoglobulins of the classes IgA, IgM, IgG_1 and IgG_2 have been demonstrated in the vaginal mucus of infected heifers (Pedersen et al 1971; Wilkie et al 1972; Duncan et al 1972; Corbeil et al 1974a, Van Aert et al 1977). These antibodies are presumably locally produced since local genital synthesis of these immunoglobulins has been demonstrated (Butler 1973). Further, it has been established that a cellular response to genital infection with C. fetus characterised by an early short lived polymorphonuclear cell infiltration is soon replaced by a moderately long standing (1-3 months) mononuclear cell infiltration in the tissues of the tubular genital tract (Simon and McNutt 1957; Dozsa et al 1960; Frank et al 1962; Vandeplassche et al 1963; Corbeil et al 1975). Corbeil et al (1975) have demonstrated

that polymorphonuclear cells could kill C. fetus in the presence of antibody (opsonins) of the class IgG and complement. Glass adherent cell types however did not need the antibodies. IgA antibodies were unable to mediate this cellular function (Corbeil et al 1975). However, they have been shown to cause immobilisation of motile C. fetus cells singly (Corbeil et al 1974a).

By using live C. fetus cells, formalinised cells, and a soluble sonicated antigen of C. fetus, Corbeil et al (1975) further demonstrated a delayed hypersensitivity reaction in previously immunised animals and histological examination of the areas of injection revealed the presence of mononuclear cells predominantly. It is genereally believed that delayed hypersensitivity is a function of cellular immunity (Collins and Mackaness 1970; Collins 1971). Consideration of these facts would lead to the current view that immunity to this infection in the genitalia of cattle is mediated by a non-specific polymorphonuclear cell function before the production of antibodies. Within the next 30-90 days post-infection, as immunoglublins are synthesised locally, immunoglubulin immunity by opsonisation of the bacterial cells allows the mononuclear cell to kill off the C. fetus organisms by IgG and their immobilisation by IgA leads to the eventual uterine clearance and subsequent vaginal colonisation without repopulation of the uterus. Thus both antibodies and cells co-operate as postulated by Winter (1973).

Finally in consideration of immunity to this pathogen, the nature of antigens which elicit protective immunity has been sought. In recent years antigens designated (a), (b), (c), (d), (e), (f), (g), (0) and (1), (2), and (3) have been described in C. fetus (McCoy et al 1975a, 1975b, 1976a, 1976b; Winter 1966 and Schurig et al 1978). Antigen (a) may also be known as the 'K' antigen (Wiidick and Hlider 1955; Border and Firehammer 1980) and may be similar to antigen (1) (Schurig et al 1978) or the heat labile acid precipitable material (APM) of Nageswararao

and Blobel (1963) and Choudari and Derbyshire (1971) and the post growth broth (PGB) antigen of Myers (1971). It is further known as the anti-phagocytic surface glycoprotein (McCoy et al 1975a). Both antigens (b) and (c) are also heat labile surface antigens. Antigens (d) and (e) are probably flagellar antigens and (f) a heat stable somatic antigen (McCoy et al 1976a). The (O) antigen is the same as the endotoxin (Winter 1965, 1966) and is chemically a lipopolysacharide. The new antigens (2) and (3) have not been fully characterised but it would appear that antigen (3) is immunologically related to antigen (f) (Schurig et al 1978). In recent attempts to determine the protective immunogenic antigen, Border and Firehammer (1980) identified the 'K' antigen (a) as being associated with protective immunity in bovine infection as did Berg et al 1969, Myers et al (1970) in ovine infection. In studies presented in this thesis particular interest rested in those antigens with anodal mobility in electrophoretic systems so that they would be reactive in the counterimmunoelectrophoresis (CIE) tests with the antibodies in the vaginal mucus since Tiselius and Kabat (1939) demonstrated antibody activity to be associated with γ-globulins and Heremans (1959) with what were then called arcs now known to correspond with IgA and IgM (Aalund 1968). These molecules possess cathodal electrophoretic mobility. In this situation, it would appear that antigens (a), (b), and probably (e) would be the most suited as the first two have been shown to have anodal mobility (McCoy et al 1975; McCoy et al 1976a), and (e) has a heterogenic electrophoretic mobility. Antigen (d) could not be demonstrated by immunoelectrophoresis. Thus of the anodally migrating antigens, only antigens (a) and (b) should be most reactive. Antigen (a) is generally considered to be liberated in higher quantities than (b) Schurig et al (1978) and the latter to be slightly faster in electrophoretic mobility (McCoy et al 1975a). Using these markers only, postulation as to the antigens associated with

immune reaction in genital campylobacteriosis is discussed in Chapter 7 of this thesis, with reference to the nature of demonstrated anodal antigens in Chapter 3 and the reactions of the crude soluble antigen with vaginal mucus from infected cattle presented in Chapters 4 and 5.

2:5 The Diagnosis of Bovine Campylobacteriosis.

2:5:0 Clinical Symptoms and Epidemiology as aids to Diagnosis and Differential Considerations Thereof.

The following description is based on the reports by Boyd (1955), Horlein et al (1964), Horlein (1970, 1980), Serger et al (1966) and Laing (1960).

The clinical picture to be seen in a herd infected with C. fetus (venereally transmitted strains) will depend on the immune status of the herd and the breeding system practised. In a herd previously unexposed, introduction of the organism by an infected bull will lead to an acute infertility syndrome in the cows which he served, characterised by repeated return to oestrus at variable intervals, generally greater than 18-24 days. Some animals may return within these intervals. If introduced by an 'infected cow' the infertility will be found in cows or heifers served by the bull after she has been served. The cow which is the source of the outbreak may breed normally. When a bull is used to serve several cows in a day, the majority of those served at first will return to oestrus while those served later may breed normally. This has been attributed to loss of the organism with increased service. This picture will continue for 3-4 months in the majority of infected animals but by 5-6 months most will have become pregnant. Thus when services, service order, inter-service interval and history of the bulls in the herd are considered it is possible to postulate the existance of a venereal infertility and C. fetus infection is the most important one to consider first. In subsequent years, if no action is taken,

this infertility syndrome will be seen only in cattle being bred for the first time (heifers) or previously unexposed cows added to the herd provided the infected bulls are still used. If on the other hand heifers are bred to young unused bulls, their fertility will be normal until they are introduced into the cow herd when they undergo the same acute infertility. Therefore age group consideration and breeding system used may be useful in suggesting venereal infection. In beef herds in which the bulls run freely with the cows for a long time, the finding of a large number of non-pregnant cows long after they were left with bulls is suggestive of C. fetus infection. If pregnancy diagnosis by rectal palpation is not routinely performed, then, since most beef herds are managed to calve within a specified period, the prolongation of the calving season and the finding of calves of varying ages will also be suggestive. These clinical and epidemiological considerations should then lead to confirmation of infection by definitive means (Culture, biotyping and serotyping) or by demonstration of the antibodies to the pathogen in the vaginal mucus of served females by laboratory methods. The symptomatology described above would apply also to bovine genital trichomoniasis. In this disease however, abortions may be more common and in particular there may be reports of cows suffering from endometritis or 'whites' due to pyometra post-service (Bartlet 1949). If rectal palpation for pregnancy diagnosis was routinely performed, some cows may be found which have developed foetal macerations. These are the two major causes of repeat breeding with irregular cycles in which laboratory methods of diagnosis are indicated.

2:5:1 Immunological Techniques Used in the Diagnosis of Bovine Campylobacteriosis

2:5:1:2 Serum Agglutination Test : Application And Limitations

Plastridge and Williams (1943) first used this technique to

determine the occurrence of C. fetus infection in the United States and initially proposed titres of 1:200, 1:100 and 1:50 as positive, suspicious and negative respectively. In subsequent studies, Plastridge and Williams (1947, 1948) modified these to 1:400 or above, 1:200 and $<$1:200 respectively. Subsequently several researchers who tried to use this test found that no significant increase of antibodies occurred in cattle during infections with these organisms (Boyd 1955; Van Aert et al 1977; Schurig et al 1978) and the method has practically been abandoned. It has been shown (Winter 1965) that the agglutinating activity of bovine sera to C. fetus cells may be due to natural antibodies of the endotoxin of C. fetus and will occur in infected as well as non-infected animals. However, it would appear that when performed within a few days after an abortion, the serum agglutinating antibody of C. fetus cells increases and could be useful in diagnosis (Plastridge and Williams 1948).

2:5:1:3 Vaginal Mucus Agglutination Test : Application, Limitations and Interpretation

Following the observation of Pierce (1946) that antibodies to Trichomonas fetus occurred in the vaginal secretions (mucus) of infected cattle, Stegenga and Terpstra (1949) adopted the agglutination method for Tr. foetus (Pierce 1947, 1949) to C. fetus infections and established that antibodies to C. fetus occurred in vaginal mucus of infected cattle. Subsequent work (Lawson and MacKinnon 1952; Hughes, 1953; McEntee et al 1954; Boyd 1955) confirmed the specificity of the test. There after it became the recommended immunological diagnostic test in the United Kingdom (MacKinnon 1954) and elsewhere (Laing 1960) even though other workers found it less reliable (Hunter 1956). This test has since been used with satisfaction provided that its limitations were recognised (Boyd 1955; Boyd and Reed 1960; Kendrick 1967; Clark 1971). The major limitations are 1) the occurrence of false positives whenever the mucus is contaminated with blood elements or serum as during

the short metoestrus period or when the genital tract is injured or due to frequent repeated examinations (Clark et al 1970). 2) False negatives occur at oestrus when the antibodies are diluted by the copious mucus flow (Lawson 1959). 3) Only some of the infected animals form antibodies (Simon and McNutt 1957; Newsam 1960; Schurig et al 1973). 4) Moreover it may take as long as eight weeks or more before agglutinins are found (McEntee et al 1954). Thus unless a considerable number of animals are tested delays in diagnosis may be encountered. 5) By about six months after infection, half or more of animals which developed antibodies may become negative (Clark 1971). Therefore a large number of samples may be needed to demonstrate infection. 6) It would appear that the phenomenon of antigenic variation of the organism (Schurig et al 1973) could be one other factor associated with false negatives when infection is actually still present so that antibody of changing specificity is continually produced. This possibility should be considered by diagnostic laboratories.

2:5:1:4 Complement Fixation Test : Application and Limitations

The possible use of the complement fixation test for the diagnosis of bovine genital campylobacteriosis was described by Trielenko (1956), Trielenko et al (1961) and Zyabkin and Rukii (1961). In a recent evaluation of this technique, Ruckerbauer et al (1971) found it to be useful only in following the immune response in cattle vaccinated against C. fetus but not for diagnostic purposes. This was in agreement with Drapalyuk (1960) who found it non-specific. It is not used in routine diagnostic laboratories except perhaps in Germany (Chaumet 1961). Because of the absence of significant quantities of anti C. fetus antibodies in serum, it is unlikely to be of use. However, the technique may be highly sensitive to detect the very low specific anti C. fetus antibodies which may enter the circulatory system (Winter 1979).

2:5:1:5 Haemagglutination Methods in The Diagnosis of Bovine Genital Campylobacteriosis

The presence of antigens of C. fetus which could be absorbed onto erythrocytes to be used in the antigenic analysis of C. fetus isolates was first described by Biberstein (1956). In 1958, Te punga applied these principles to demonstrate the possibility of developing this technique for the immunodiagnosis of C. fetus using both rabbit anti C. fetus antibody and the vaginal mucus from infected cows (Tepunga 1958a, 1958b). By using this indirect haemagglutination test, he was able to demonstrate an earlier and higher level of immune response in infected animals than could be detected by the vaginal mucus agglutination test. Since the antigen used was heat stable, he speculated on the possible cross-reaction occurring between strains and the probability of missing some infected animals. Although some non-specific agglutination occurred, he found that this problem could be eliminated by freezing mucus at -20^{0}C in the tanned red cell method or including normal rabbit serum in the untanned method. Newsam et al (1967b) and Newsam and St. George (1967) extended these studies to include tanned and untanned sheep erythrocytes for the diagnosis of C. fetus infection in cattle using vaginal mucus from cattle. They found that a low level of false negative diagnosis could be obtained if at least 15 vaginal mucus samples from an infected herd were tested in the tanned method at a dilution of 1:80 or above and at a dilution of at least 1:10 in the untanned cell method. A haemolytic system of haemagglutinating principle was found to be more sensitive earlier than these tanned and untanned cell methods (Ristic and Walker 1960). Although proven to be useful in detecting infection, these haemagglutination methods have not been used routinely in the immunodiagnosis of C. fetus infection in cattle. Two possible reasons may be advanced for this - namely the difficulty of keeping sensitised erythrocytes for long periods in the laboratory without bacterial

contamination or breakdown of the sensitised cells and the technical difficulties of performing the tests especially if attachment of the antigens to erythrocytes required alkali treatment as was found by Newsam and St. George (1967). The first reason could be eliminated if it could be shown that formalinized cells could still be used to attach C. fetus antigens and that such treated cells still reacted with known positive vaginal mucus to a higher degree compared to mucus from non-infected animals. So far this technique has not been attempted. It is proposed that if haemagglutination methods detect infection earlier than VMAT, then they could be an adjunct to the technique studied in this work, namely CIE.

2:5:1:6 Fluorescent Antibody Methods in The Diagnosis of C. Fetus Infection in Cattle

The fluorescent antibody technique (FAT) was first described by Coons et al (1942) but it was Moody et al (1956) who first showed its application in the identification of bacteria in smears. Herschler (1962) first showed its application for the identification of C. fetus in semen and preputial suspensions. However, Mellick et al (1965) extended this principle to the routine diagnosis of C. fetus infection in cattle. In Great Britain Phillpot (1966, 1968a,b) used it for diagnosis of this infection in bulls. This method is currently in use in many laboratories and provided four examinations are undertaken, it is considered sufficiently accurate in identifying infected bulls (Winter et al 1967). Its major disadvantages are (a) that the organism is not available for biotyping and serotyping which tests are believed to be of epidemiological and perhaps of immunological importance (Clark et al 1979) and (b) the expensive equipment and high degree of technical competence required for its successful use.

The principle of FAT has now been applied also in the screening of vaginal mucus of suspected infected cattle with satisfactory results

(Shires and Krammer 1974; Suarez et al 1976). This latter application of FAT would greatly speed up the diagnosis of C. fetus infection in cattle. Unfortunately it is not yet routinely applied in diagnositc laboratories. It would appear that the major problem would be associated with the physical nature of bovine vaginal mucus although Shires and Krammer (1974) have described technical methods by which these could be overcome. Therefore whenever the facilities are available routine application of the vaginal mucus FAT method would be complementary to the bull sheath washing FAT and culture.

2:6:0 Bacteriological Diagnosis of Campylobacter fetus Infection in Cattle

Although the immunological techniques described above are of importance in the identification of C. fetus infected herds, positive diagnosis must depend on isolation of the organism (Hignett 1951; Horlein et al 1964; Horlein 1970; Bearden and Fuquay 1980). Not only must this be done but the isolated organism must be shown to be C. fetus (Morgan 1957; Morgan et al 1957) since campylobacters other than C. fetus may inhabit the genital tract of cattle or be isolated from aborted foetuses and placental tissues (Florent 1953; Bryner and Frank 1955; Firehammer 1965; Neill et al 1978).

Specimens used for the isolation of the pathogen are obtained from

1) The female genital tract (vagina, cervix and uterus) and are essentially mucus or uterine exudate.

2) The foetus and placental contents. These are the foetal stomach contents, ground foetal lung, spleen and liver and, if obtainable, the amniotic fluid.

3) The bull: preputial washings in saline or Phosphate Buffered Solution pH 7.2 or scrapings of the preputial and penile mucosa will be taken or semen samples be obtained for culture.

The ease with which isolation is made will depend on a thorough

understanding of the biology of the organism principally its nutritional fastidiousness and gaseous requirement, and the appreciation of the ubiquitous microbial content of the specimens so that fast growing bacteria which will outgrow and mask the desired pathogen can be inhibited by various technical methods. Therefore for successful isolation of C. fetus attention must be paid to all these. The selected review below represents the best conditions under which isolation may be made to date.

2:6:1 Isolation Methods with Particular Emphasis On Samples, Sample Handling, Media and Gaseous Requirement For Accurate Isolation

To suppress the other bacteria in the vaginal secretions of cattle, preputial washings and semen of bulls, attempts have been made to incorporate chemicals, especially dyes (Ryff and Lee 1945; Terpstra and Eisma 1952; Florent 1956) into the media used for isolation. Success occurred mainly with brilliant green (Florent 1956) but some investigators did not have similar success (Bryans and Shephard 1961). Consequently antibiotics were incorporated into media (Rolle and Mundt 1954; Kawashima et al 1954; Bryans and Shephard 1961). The study of Plastridge and Koths (1961) revealed that the effectiveness of the antibiotics was dependent on the media to which they were added, the superiority of solid blood agar with the antibiotics being noted.

As isolation rates were still not satisfactory other technical methods were tried. Plumer et al (1962) introduced the technique of passing samples through millipore filters before culturing and they obtained satisfactory results. However Shepler et al (1963) combined the use of antibiotics in blood agar with 0.65μm millipore filtration and greatly improved on the rate of isolation of C. fetus from known infected animals. Since then both procedures have been found satisfactory although filtration reduced numbers of organisms seeded (Winter et al 1965; Dufty 1967) but also eliminated Proteus and Bacillus spp both of which contaminate bull genital samples (Kotsche 1980).

To further improve on the isolation rate, especially from bulls, transport media for preputial scrapings which promoted the growth of C. fetus have since been described (Clark et al 1974). It was further found that specimens should be kept cooled at between 4-6°C and should be cultured within 8-10 hours (Clark et al 1972). For preputial washings or scraping in saline, centrifugation of samples to deposit gross debris and perhaps large bacteria has been practised for a long time (Hughes 1956) and is still recommended (Schutte 1969). In using solid blood agar media with antibiotics added, the prior drying of the media at 37°C for about 30 minutes helps to create a lower water content which restricts the swarming of the two major contaminants usually encountered, namely Proteus spp and Pseudomonas spp (Serger et al 1966).

Finally it has been known since the early work with the organism that growth of C. fetus in environments with high oxygen content has been poor (Reich et al 1956). This microaerophilic nature of the organism has contributed to its poor recovery from clinical specimens. The recommendation has been to grow it in an atmosphere to which 10% CO_2 is added. However, studies had shown that the limiting factor was the oxygen concentration in the gaseous mixture (Reich et al 1956). Thus Kiggins and Plastridge (1956) recommended growing this organism at a concentration of not more than 6% O_2 and formulated a gas mixture of 85% N_2, 10% CO_2 and 5% O_2. In a recent study (Hoffman 1978) it was shown that this microaerophilic nature of the organism is aided by the presence of the enzyme super oxide dismutase. By incorporating substances that could destroy the formation of super oxides in media George (1978) could grow some strains of the organism in air. Therefore this problem seems to be near a solution. For clinical work, the superiority of the gas mixture of Kiggins and Pastridge (1956) or anaerobic tablets (Oxoid, England) in jars from which catalyst has been removed has been

documented (Agumbah 1977; Roberts 1979). Furthermore the 10% blood agar plates with the antibiotic formulation of Shepler et al (1963), with mycostatin added, at 300 units/ml (Dunn et al 1965) is also superior (Dufty 1967) although the latter author did not note suppression of growth of Proteus mirabilis. In the isolation of the organism from clinical specimens described in Chapter 6 of this work, due regard to the facts discussed above was given with very satisfactory results. It would therefore be reasonable to recommend that as long as these principles are adhered to, the isolation of C. fetus from clinical specimens can no longer be regarded as difficult. FAT examination in the bull may augment these results of culture. If negative on four weekly samples this should indicate the probability that the animal is not infected but is not absolutely infallible. When samples still are highly suspicious (in very valuable bulls) even after these examinations, perhaps the 'biological culture' technique of Adler (1957) using virgin heifers may be attempted. Otherwise this method is not only tedious, but inapplicable in large bull studs as in A.I. bulls, since the cost would be high even though heifers are re-usable and several samples from different bulls can be screened simultaneously in one heifer.

2:6:2 Typing of Genital Campylobacters as an Aid in Clinical Decision Making and Control Of the Bovine Disease

Because of the occurrence of at least two campylobacters in the genital tract of cattle that are not C. fetus namely C. sputorum subsp bubulus (Florent 1953) and C. fecalis (Firehammer 1965) proof that the isolated organism is infact C. fetus is obligatory (Morgan 1957). This aspect is fulfilled by performing biochemical and tolerance tests on the isolates. Further, epidemiological evidence so far, has implicated two serotypes namely serotype A and serotype B organism of C. fetus subsp venerealis and C. fetus subsp fetus respectively to be the only ones that are venereally transmitted (Berg et al 1971). Therefore complete

typing ought to include both biotyping and serotyping of clinical isolates.

2:6:2:1 Biotyping and Clinical Decision Making in Genital C. fetus Infection in Cattle

In 1955 Bryner and Frank demonstrated that genital Campylobacters could be differentiated on the basis of their catalase content. Frank et al (1958) showed that the catalase positive strains had reproductive pathogenicity while catalase negative strains did not. Earlier, Price (1954) and Price et al (1955) had shown that campylobacters of foetal origin were generally hydrogen sulphide negative while non-foetal ones were both hydrogen sulphide positive and serologically distinct from the foetal ones. In subsequent studies by several workers (Kuzdas and Morse 1956; Reich et al 1956; Akkermans et al 1956; Diliello et al 1959) it became accepted that by using the catalase, the hydrogen sulphide production and the 3.5% NacL tolerance tests (Kuzdas and Morse 1956) cattle campylobacters could now be divided into three groups viz the catalase positive, hydrogen sulphide negative, salt intolerant group which appeared to cause infertility, the catalase negative, hydrogen sulphide positive salt tolerant strains considered non pathogenic and catalase positive, hydrogen sulphide weakly positive and salt intolerant group that appeared to cause primarily abortion in cattle (Akkermans et al 1956).

Later Lecee (1958) reported that ovine and cattle campylobacters could be differentiated on their ability to tolerate 0.8% glycine. Only one out of 12 bovine strains tested grew in media with 0.8% glycine added while all 5 sheep strains grew. Florent (1959) developed this principle and was able to divide catalase positive pathogenic campylobacters into a) C. fetus subsp venerealis which was only venereally transmitted, caused infertility in cattle and could not be found or did not survive in intestinal environments. b) C. fetus subsp fetus

(intestinalis) could survive in intestinal environments of cattle, pigs and sheep but was not venereally transmitted and caused sporadic abortion in cattle, abortion in sheep but not infertility in cattle.

While these divisions have been confirmed by recent investigators (Véron and Chatelain 1973) it was found that among the so called intestinal strains or related vibrios (King 1957), other tolerance markers could be used to differentiate C. coli and C. jejuni. These were the nalidixic acid tolerance tests, growth in 8% glucose media and growth in media with 1:33,000 and 1:100,000 brilliant green added, tolerance to 2,3, 5 triphenyl tetrazolium chloride at 1 mg/ml medium (Véron and Chatelain 1973; Neill et al 1978).

These other campylobacters were also catalase positive, salt intolerant and tolerated 1% glycine and thus the use of these tests was to be corroborative other than individual as in fact had been advised several years before (Kuzdas and Morse 1956). Neill et al (1978) speculated on the possibility of faecal (intestinal) isolates being found in the vaginal mucus of cattle due to faecal contamination. If this were so, then in clinical reproductive work, isolation of campylobacters from vaginal mucus and indeed preputial washings as well, requires that the isolate be further shown not to be of the faecal types. By the application of this array of tests and on finding 1% glycine tolerant strains, the performance of 1.5% glycine tolerance (Bracewell 1974) it is considered that a decision as to the pathogenicity of the isolate could be made based on the biotyping results and the clinical history of the case. These tests have been applied in all isolates obtained during this work and on the basis of the results, the clinical decision on the pathogenicity of the variant isolates obtained was arrived at.

2:6:2:2 Seroclassification : Epidemiological Significance and Application in Disease Surveillance

Pathogenic campylobacters have been shown by Mitscherlich and Liess

(1958a) and Morgan (1959) to belong to serotype 1 and 2 or A and B, serotype I corresponding to A and 2 to B. More recently Berg et al (1971) found out that of the group known as C. fetus subsp fetus (i.e. intestinalis of Florent) a third group, serotype C, could be found. In cattle all three serotypes could perhaps be isolated but in cases of genital disease, only serotype A and B were incriminated. The former was subdivided into two subtypes when serotyping and biochemical typing systems were combined. These authors (Berg et al 1971) thus implied that among venereally transmitted pathogenic campylobacters, three groups were involved viz:

(1) C. fetus subsp venerealis biotype I, serotype A

(2) C. fetus subsp venerealis biosubtype I, serotype A

(3) Serotype B organisms which behaved biochemically as C. fetus subsp fetus (intestinalis). C. fetus subsp fetus serotype A biotype 2 (A-2) was considered as being abortifacient in cattle but they did not associate it with venereal transmission.

Recently, Dedie et al (1977), Whitford et al (1977) have incriminated some strains of A-2 organisms in genital pathology and abortion. Venereal transmission of C. fetus subsp fetus has been described recently (Agumbah and Ogaa 1979) although the serotype was not stated. It would therefore appear that seroclassification of isolates might be of epidemiological significance. It would be possible to follow the spread of a particular serotype in certain situations and by considering the breeding management, it would be possible then to determine the means of spread. Where bulls were involved, the isolation of the same serotype in breeding contacts would be used in the surveillance of the disease. This principle has been applied to determine the means of spread of the variant isolate found in South West Scotland and described in this work.

2:6:3 Problems in Immunodiagnosis and Bacteriological Diagnosis of Campylobacter fetus Infection in Cattle and the Need for the Development of Further Diagnostic Tests

The demand that a diagnosis of C. fetus infection in a suspected herd be confirmed by demonstration of antibodies in the vaginal mucus of cattle by the VMAT and the isolation and characterisation of the pathogen requires laboratory methods. In the review presented so far, the shortcomings of the VMAT have been pointed out. These shortcomings have led other workers to abandon VMAT because of the occurrence of false positive and false negative results (Horlein et al 1964) and to develop reliable cultural methods. False negatives may also be due to development of immune response to certain antigenic determinants on the infecting strain not found on the test antigen strain since the organism has been known to be antigenically heterogenous (Larson and Ringen 1967). It may also be due to oestrus cycle variation with respect to sampling time or in case of 'O' specific anti C. fetus antibodies, the inhibition of agglutinating bacteria if these possess the capsular or 'K' antigen described here as antigen (a) (McCoy et al 1976a, b). The activities of these anti C. fetus antibodies in other biological functions other than agglutination ought to be studied. Perhaps the terminology agglutinins coined since 1949 (Stegenga and Terpstra (1949) may have persisted. It is known (Aalund 1968) that antibodies may be reactive in many other biological functions simultaneously or just in one function alone.

The laboratory differentiation of the genital isolates of C. fetus has been described. The difficulty has been obvious from the review presented, particularly as recent research seem to indicate that even more diverse campylobacters can be isolated not only from the genital tract of cattle but also from the placenta and aborted foetal tissues

as well (Ellis et al 1977; Neill et al 1978, 1979). Thus at the very best, the laboratory confirmation of infection will take at least three days in case of the VMAT or 10 days for full biochemical classification of genital isolates. The disease has commonly spread and become more or less chronic when action is usually requested but an immunological test that would confirm infection within 60 days since introduction of infection with a result available on the same day as the samples were taken would be desirable. Such a method should be less tedious and cheaper than the vaginal mucus agglutination test used today. Above all, it should be at least as specific as the VMAT and ought to eliminate the false negatives associated with oestrus onset. However, if such a test were to be useful, its results must be correlated with actual infection. Thus no matter what immunological test was devised, proof of infection by culture and biotyping and seroclassification of isolates will always be desirable. One such test is the counterimmunoelectrophoresis (CIE) test which utilises the principle of electrophoresing soluble anodally migrating antigen and cathodally migrating antibody in two wells suitably cut into a supporting medium (agar, agarose, cellulose) and placed in electrophoretic alignment so that when both antibody and antigen meet in the intervening space, a precipitation line (visible or invisible) is formed. If this is invisible at first, it can be shown to exist by a variety of methods. Below is presented a short review of the use of this rapid, sensitive diagnostic method in human and veterinary medicine.

2:6:4 The Counterimmunoelectrophoresis Test (CIE) : Application in Human and Veterinary Medicine

The first evidence of the use of the known cathodal electrophoretic mobility of antibody at certain pH values and anodal antigens to produce an immunoprecipitate if electrophoresed in a supporting medium was presented in 1954 (Macheboeuf et al 1954). However, its possible clinical

use in medicine is attributed to Bussard and Huet (1959). Its versatility was further demonstrated by Culliford (1964) in a forensic study of sperm antigens and their specifically produced rabbit antibodies. Kohn (1968) then described it formally. The demonstration that this technique could be used to diagnose 'Farmer's Lung' very rapidly (Jameson 1968) and subsequently to demonstrate the Australian antigen in hepatitis B sufferers (Gocke and Howe 1970) led to its widespread application in human medicine. Thus it has been used to diagnose several bacterial diseases (Dorff et al 1971; Greenwood et al 1971; Feldman and Du Clof 1973; Coonrod and Rytel 1972; Bartram et al 1974; Fossieck et al 1973; Diaz et al 1976, 1978; Shakelford et al 1974) viral diseases (Berlin and Pirojboot 1972), mycotic diseases (Gordon et al 1971; Remington et al 1972; Kleger and Kaufman 1973; Dee 1975; Gumaa and Mahgoub 1975; Picardi et al 1976) and non-infectious diseases (McElborough 1974; Peltier et al 1977). The principles underlying its use and the multiplicity of names by which the method may be known in human medicine has recently been reviewed by Corkill (1977).

The use of this technology for the rapid diagnosis of diseases of veterinary interest was not as dramatic as in human medicine. Perhaps its first use in veterinary medicine may be attributed to the work of Cho and Ingram (1972, 1973) who used it to diagnose Aleutian disease in mink and Bohac and Derbyshire (1975) for enteric viruses in pigs. Muhammed et al (1978) also used it in the diagnosis of Johnes disease in sheep, goats and cattle. However, since the beginning of this work in 1978, several papers have appeared on the use of the method in diagnosis of bacterial and viral diseases of domestic animals (Papp-vid and Dulac 1979; Muhammed et al 1980; Poli et al 1980; Ekern et al 1981 and Carter and Chengappa 1981). The work presented in this thesis is an attempt to determine whether this method would be applicable to the

diagnosis of C. fetus infections in cattle. It is rapid and, when done in agar or agarose as the support medium, it is not only cheap but the technical requirements are also minimal and yet the results can be available on the same day as the samples are taken or at the very latest the following day. This would reduce the laboratory time required to confirm infection with C. fetus from 72 hours (VMAT) to between 4-24 hours.

CHAPTER THREE

3:0:0 A STUDY OF A CRUDE SOLUBLE ANTIGEN OF C.FETUS TO DEMONSTRATE THE PRESENCE OF ANODAL ANTIGENS AS A PRELUDE TO THE APPLICATION OF THE COUNTERIMMUNOELECTROPHORESIS METHOD IN THE DIAGNOSIS OF C.FETUS INFECTION IN CATTLE

3:0:1 Introduction

The literature pertaining to the antigens of C. fetus and their electrophoretic mobility has been reviewed in the previous chapter. There it can be seen that at least three antigens, namely (a), (b) and (e) would have an anodal electrophoretic mobility. Of these only two antigens (a) and (b) possess complete anodal mobility. If these antigens were able to react with specific anti C. fetus antibodies in the vaginal mucus of cattle during counterimmunoelectrophoresis, then this technique would be applicable in the immunodiagnosis of C. fetus infection in cattle. By using varying pH ranges it is even possible to increase or decrease the number of detectable anodal antigens if these are proteins whose directional electrophoretic mobility would be related to their iso-electric points.

Since the organism C. fetus is also known to be antigenically heterogenous, a study of the soluble antigens in as many strains as possible would assist in the selection of the strains which possess the desired antigens in the correct proportion. Individual isolates vary in their diagnostic potential (Boyd 1955) and the use of pooled antigens has been recommended for use in preparing antisera to be used in the FAT method of diagnosis (Belden and Robertstad 1965; Phillpot 1968b). In order to determine which antigens would be appropriate with respect to the CIE, soluble antigens were studied by means of two dimensional electroimmunodiffusion and double immunodiffusion in agarose gel (DIDG).

The anodally migrating antigens once demonstated were further used in the CIE technique to illustrate their seroactivity under varying pH ranges with rabbit antisera prepared against whole organisms. Application of the technique to known vaginal mucus agglutination test (VMAT) positive mucus samples was then tried after it was found to be reactive with rabbit antisera. This chapter records the methodology and results of these studies.

3:1 Materials and Methods

3:1:0 Bacterial Isolates Used in the Study: Their Source, Biochemical and Seroclassification

(1) Antigen C842-72: was isolated by the author in November 1978 from a repeat breeding cow No. 72 in a herd with a fertility problem (Chapter 6, Farm A). The organism was classified as C.fetus subsp fetus (Véron and Chatelain 1973; Skerman et al 1980) and belonged to serotype A (Morgan 1959; Berg et al 1971). This isolate resembled the genitally pathogenic strain of Dedie et al (1977) isolated in Germany, and that isolated from an aborted bovine foetus in the United States, Whitford et al (1977). It was however different serologically but was similar biochemically to the serotype B strains known to be venereally transmitted (Berg et al 1971). The details of biochemical reactions and seroclassification are in Appendix 4 and Chapter 6.

(2) Antigen C842-22: was isolated from a repeat breeder cow No. 22 from the same herd as C842-72 above and both were biochemically and serologically indistinguishable from that isolate.

(3) Antigen C842-16: was also from the same farm as above and isolated from cow No. 16 at the same visit. Again it was biochemically and serologically indistinguishable from antigen C842-72 and C842-22 above.

(4) Antigen C842-148: was isolated from a repeat breeder cow No. 148 in Herd A at a later visit and was biochemically and serologically identical to the previously described isolates.

(5) Antigen NCTC 10354: had been isolated in 1952 from an infertile cow and was obtained from the National Collection of Type Cultures, Colindale, England. This was classified as C. fetus subsp venerealis (Florent 1959; Véron and Chatelain 1973; Skerman et al 1980) and was serotype A (Morgan 1959; Berg et al 1971).

(6) Antigen C377-19 : was isolated by the author in March 1980 from cow no. 19 in Farm G (Chapter 6). It was classified as C. fetus subsp venerealis (Florent 1959; Véron and Chatelain 1973; Skerman et al 1980) and belonged to serotype A (Morgan 1959; Berg et al 1971)

(7) Antigen C849-41 : was isolated by the author in November 1980 from cow no. 41 in Farm M (Chapter 6). It was classified as C. fetus subsp venerealis (Florent 1959; Véron and Chatelain 1973; Skerman et al 1980) and belonged to serotype A (Morgan 1959; Berg et al 1971). This organism and C377-19 appeared to have been introduced into two farms, G and M, by bull CT but the first to be isolated (C377-19) was from a cow served by CT about $1\frac{1}{2}$ years earlier (June 1979).

(8) Antigen NCTC 5850: was obtained from the National Collection of Type Cultures, Colindale, England and had been isolated from an aborted lamb in 1939 and was biochemically classified as C. fetus subsp fetus but was found by the author to belong to serotype B of Morgan (1959) and Berg et al (1971)(see Chapter 6 and Appendix 4).

(9) Belv +C: had been isolated at Irvine General Hospital, Ayrshire, Scotland from a human case of enteritis. It was biochemically classified as C. jejuni (Skerman et al 1980). It did not react with specific A and B antisera prepared by the author in rabbits or to serotype A, B and C antisera donated by Border and Firehammer, Montana State University, U.S.A.

Thus it was serologically unrelated to C. fetus serotypes A, B and C and was therefore recorded as unclassified (Chapter 6 and Appendix 4).

(10) Antigen C383-157: was isolated in Farm H_2, cow no. 157 and was biochemically and serologically similar to the isolates from Farm A. This was the isolate later used in experimental infections (Chapter 5).

3:1:1 Growth and Treatment of Cells Prior to Preparation of the Crude Soluble Antigens

Smooth bacterial cells chosen by the criteria of Bond (1957) were grown on blood agar plates in which antibiotics were incorporated as described by Shepler et al (1963) as were 300 units/ml of mycostatin (Dunn et al 1965). Plates were incubated at 37°C for three days in anaerobic jars without catalyst caps in an atmosphere of 85% N_2, 10% CO_2 and 5% O_2 (Kiggins and Plastridge 1956). After three days all plates were examined visually for growth and apparent purity and those judged pure were washed with 0.25% formol saline solution in groups of four and the washings in each group were pooled into sterile plastic universal containers and labelled accordingly. These pools were then kept at 4°C until the following day when Gram stained films were made from each group and examined for purity. All groups found pure were pooled into large plastic bottles and centrifuged at 3900 r.p.m. for 40 minutes and washed twice with normal sterile saline solution (PSS). The pellet remaining was resuspended in 10-20 ml of 0.25% formol saline solution per 2.0 grams of wet cell weight and stored at 4°C until required for ultrasonication.

3:1:2 Ultrasonication of Cells and Concentration of Soluble Antigens

Films prepared from the stored cells, were again stained by Grams method and checked for purity. If no contamination had occurred during storage the cells were centrifuged at 3,900 r.p.m. for 40 minutes and washed twice in sterile normal saline and finally 450-500 mg wet weight of cells were reconstituted with 5.0 ml of normal sterile saline solution in sterile universal bottles. These were then ultrasonicated

in the 20 KC/S M.S.E. ultrasonicator or M.S.E. Soniprep 150 for 4 bursts each of two minutes or 3 bursts each of three minutes with one minute rests between bursts. The containers were ice cooled throughout the sonication procedure. All sonicates belonging to the same antigen were then pooled and the debris removed by centrifugation at 10,000 r.p.m. for 15 minutes at 4°C in the M.S.E. 18 centrifuge. The translucent supernatant then constituted the crude antigen. This was then freeze dried and reconstituted to give the desired suitable concentration in the case of antigens C842-148, NCTC 10354, C849-41, C383-157. Antigens NCTC 5850, Belv +C and C377-19 were concentrated to the required level by dialysis in visking tubing against moistened carbowax (Polyethylene glycol, Sigma Chemicals, U.S.A.). Prior to concentration by freeze drying or dialysis, 10.0 ml of each antigen in 1.0 ml aliquots were pipetted off and stored at -20°C for protein determination described in Appendix I and to provide crude soluble antigen to be used for CIE in the course of this work.

3:1:3 Preparation of Rabbit Anti C. fetus Antisera

The bacterial cells were grown and stored as described above except that after staining to confirm purity the last pellet was reconstituted in sterile normal saline to give an optical density of 0.55 ± 0.01 at 525 nm absorption in the M.S.E. Spectro-plus spectrophotometer. Rabbits weighing at least 2 kg were inoculated as described by Walsh and White (1968) except that the 4.0 ml i/v injection was given 6 times, 3-4 days apart as detailed in Chapter 6. Antisera were prepared against strains C842-72, NCTC 5850, NCTC 10354, C377-19 and C842-148. Rabbits were exsanguinated 7-10 days after the last injection and the antisera were prepared as described by Garvey et al (1979). All antisera were stored at -40°C until required for use.

3:1:4 Preparation and Use of the Various Gels in the Methods of Double Immunodiffusion in Gel (DIDG) and Electroimmunodiffusion

(1) Double Immunodiffusion in Gels (DIDG)

1% w/v Koch light lab agarose or Gibco agarose gels were made by dissolving and steaming the agaroses in barbitone acetate buffer pH 7.2. The agarose was then cooled to near its gelling point (approximately 56°C) and 15 mls were poured onto 10 x 10 cm glass plates placed on a levelled table (Shandon Southern Ltd). The gels were allowed to solidify and either a pattern of 6 peripheral wells with a central well was punched out with gel punchers (Shandon Southern Ltd.) or a 4 peripheral well with a central well pattern was prepared. The central well was to contain the antiserum under test while the peripheral wells labelled 1-6 clockwise were to contain the antigens being analysed. The peripheral wells were 5mm from the central wells. When the central wells had been filled with the antisserum and the peripheral wells with the desired antigens, the plates were placed on a metal box containing cotton wool moistened with 0.5% formol saline for bacteriostasis. The lid was placed on the box which was left on the bench at room temperature. The gel plates were examined daily for the appearance of precipitation lines. The wells were re-charged if required. When it was considered that no more precipitation lines would form as judged by the appearance of stable lines after at least 3 chargings and waiting for 48 hours after the last charging the plates were then photographed stained or unstained.

(2) Electroimmunodiffusion

Two techniques were used namely (i) the two dimensional crossed electrophoresis and (ii) counterimmunoelectrophoresis.

(i) Two dimensional Crossed Electrophoresis

In this method the protein concentration of the antigens C842-148, C383-157, C849-41, NCTC 10354 and NCTC 5850 were determined by the method of Lowry et al (1951) (See Appendix I) before the protein content was

adjusted to about 10mg/ml by either freeze drying or dialysis against carbowax as previously described. The concentrated antigens were electrophoresed in 1% Litex HSB agarose in barbitone-lactate buffer pH 8.6 (Laurell 1965) at 10 volts/cm in the anodal direction for 3 hours. All the agarose above a horizontal line 2 mm above the well was then removed and replaced with the identical agarose containing 15 µl of the appropriate antiserum per cm^2 of gel. After this gel had set the antigen was again electrophoresed anodally at right angles to the previous direction into the agarose with antiserum mixture. The voltage was reduced to 2.0-2.5 volts/cm of gel and the electrophoresis was performed for at least 18 hours. In all cases the buffer in the chamber reservoir was barbitone buffer pH 8.6 ionic strength (μ) = 0.05, and the wicks for connecting the agarose gels to the buffer chambers consisted of Whatman no. 41 filter paper. At the end of electrophoresis all plates were removed, immersed in 0.85% normal saline overnight, dried and stained with 0.1% w/v Coomassie brilliant blue (BDH Chemicals, England) or they were pressed, washed in tap water, pressed again, dried and stained with Coomassie brilliant blue (BDH Chemicals, England).

(ii) Counterimmunoelectrophoresis (CIE) with Rabbit Antisera and Trials of CIE With Known (VMAT) Positive Samples

In this system two rabbit whole-cell antisera against antigens C842-72 and NCTC 5850 were tested against five sonicated antigens, namely Belv +C, NCTC 5850, C377-19, NCTC 10354 and C842-148 in a pH gradient from pH 6.6-8.6. The agarose gels used were Koch Light agarose and that supplied by Gibco (Grand Island Biological, U.S.A.). A 1% w/v agarose gel in barbitone acetate buffer of the above pH range was used. The various dilutions (1:2 - 1:32) of antisera under test were on an initial trial titrated with dilutions of antigen 148 by the method of Papp-vid and Dulac (1979). On this basis serum dilution of 1:4 and antigen dilution 1:8 were the minimum precipitating dilutions.

For all subsequent CIE tests antigen 148 in 1:4 dilution was chosen to give twice the minimum detectable antigen concentration. Gels were cast on 10 cm^2 glass plates to give a depth of about 1.5 mm when set and 5 mm diameter anodal wells and 3mm diameter cathodal wells, were punched in the gels 4mm apart. The antisera under test were placed in the anodal wells and the antigens in the cathodal wells. Power at the rate of 10 volts/cm was applied across the gels which were connected by Whatman no. 41 filter paper wicks to electrophoresis chambers filled with barbitone buffer pH 8.6 for $2\frac{1}{2}$-3 hours. During electrophoresis checks were made for the precipitation of antigen-antibody in the space between the wells. Seven known VMAT positive phenol saline extracts of vaginal mucus in 0.5% phenol saline were also tested. The second part of the CIE with rabbit antisera was performed using gels of pH 7.4. At this pH (see results below) antiserum NCTC 5850 detected homologous antigen only and antiserum C842-72 detected the organisms related to it (148) and the other antigen, C377-19. The object here was to determine if the method could lead to some grouping of the sonicated antigens tested and to observe the pattern of cross-reactions which occurred. Two other antisera, antiserum to the antigens C377-19 and C842-148 were tested along with antiserum NCTC 5850 and C842-72. Nine sonicated antigens were assessed by this method (See Table 3.1b).

3:2:0 Results

3:2:1 Double Immunodiffusion in Gels (DIDG)

The relationships of various campylobacters revealed by the agarose gel diffusion method is shown in the photographic records Figures 3, 1-3 with antisera NCTC5850 and C842-148. Figure 3-3 shows reaction of C842-148 with its serum. These reactions were obtained after 5-7 days incubation and daily recharging of wells for 4-5 days. In Figure 3-1 the middle line antigen when present was the highest in concentration or fastest in diffusion rate as it was the first to appear. The curved line near

the antigen wells was considered to be due to the 'O' antigen in the crude soluble antigens analysed (Winter et al 1978; Border and Firehammer 1980). The fact that it failed to form when antiserum NCTC 5850 was used against most of the other serotype A organisms is consistent with this observation as NCTC 5850 was found to be serotype B (see Chapter 6). Formation of a similar curved line near antigen NCTC 10354 could not be explained but Winter et al (1978) noted that a similar precipitation line occurred which they attributed to antigen (a) complexed with antigen 'O'.

Figure 3 .1

Central well contains antiserum NCTC 5850

Well No. 1 = Antigen 5850

2 = Antigen 148

3 = Antigen C383-157

4 = Antigen C377-19

5 = Antigen C849-41

6 = Antigen 10354

3:2:3 Two Dimensional Electroimmunodiffusion

The photographic representations Figures 3-4, 3-5 and 3-6 illustrate the patterns of anodal antigens of C842-148, NCTC 5850 and NCTC 10354. The latter did not photograph very well and the line drawings next to it represents the pattern observed by the naked eye. Similar problems of photography could not allow the inclusion of the patterns of antigens C849-41 and C383-157. Both of these, however had two anodal antigen peaks (see Table 3-1) similar in shape to antigens marked C

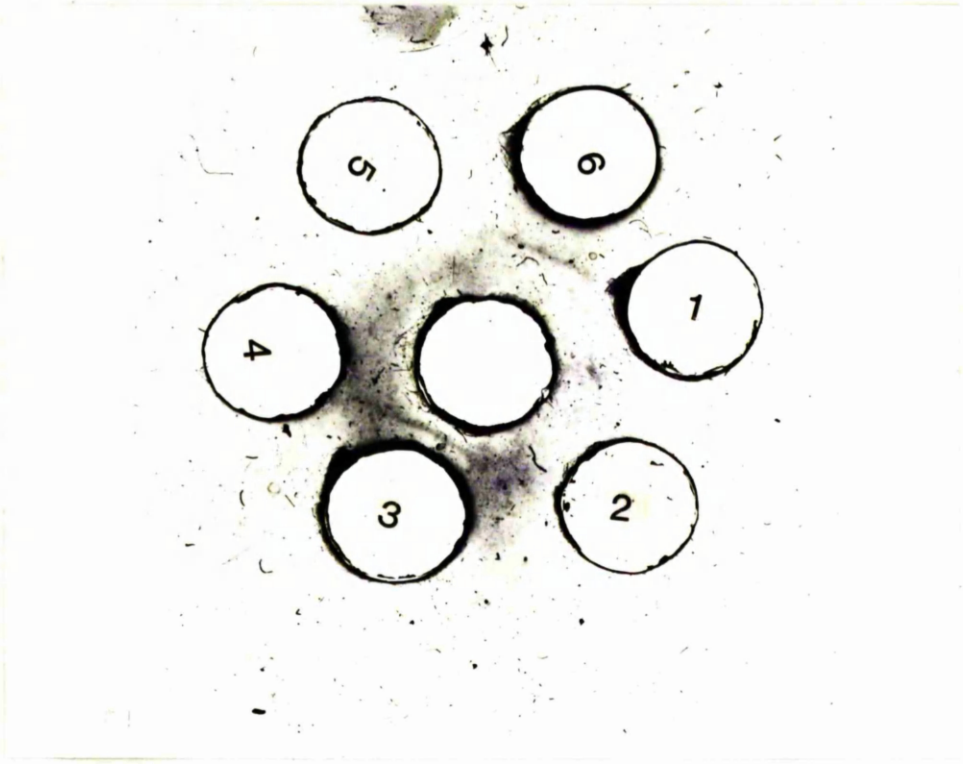


Figure 3-2: Central well contains antiserum to C842-148.

Peripheral wells:
1 = Antigen NCTC 5850
2 = Antigen C377-19
3 = Antigen C842-148
4 = Antigen C848-41
5 = Antigen C383-157
6 = Antigen NCTC 10354.

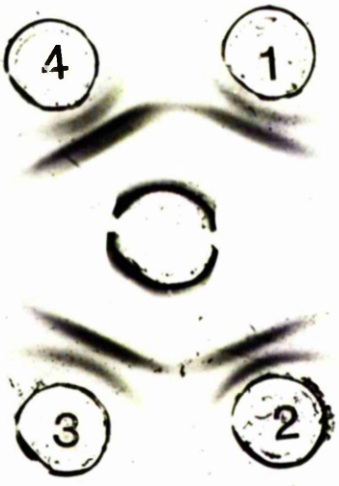


Figure 3-3: Central well contains antiserum to C842-148. All four peripheral wells contain antigen C842-148.

for C148 and 3 for antigen NCTC 5850. Thus a maximum of four or five anodal antigens were demonstrated (antigen C842-148) and a minimum of two (antigens C849-41 and C383-157).

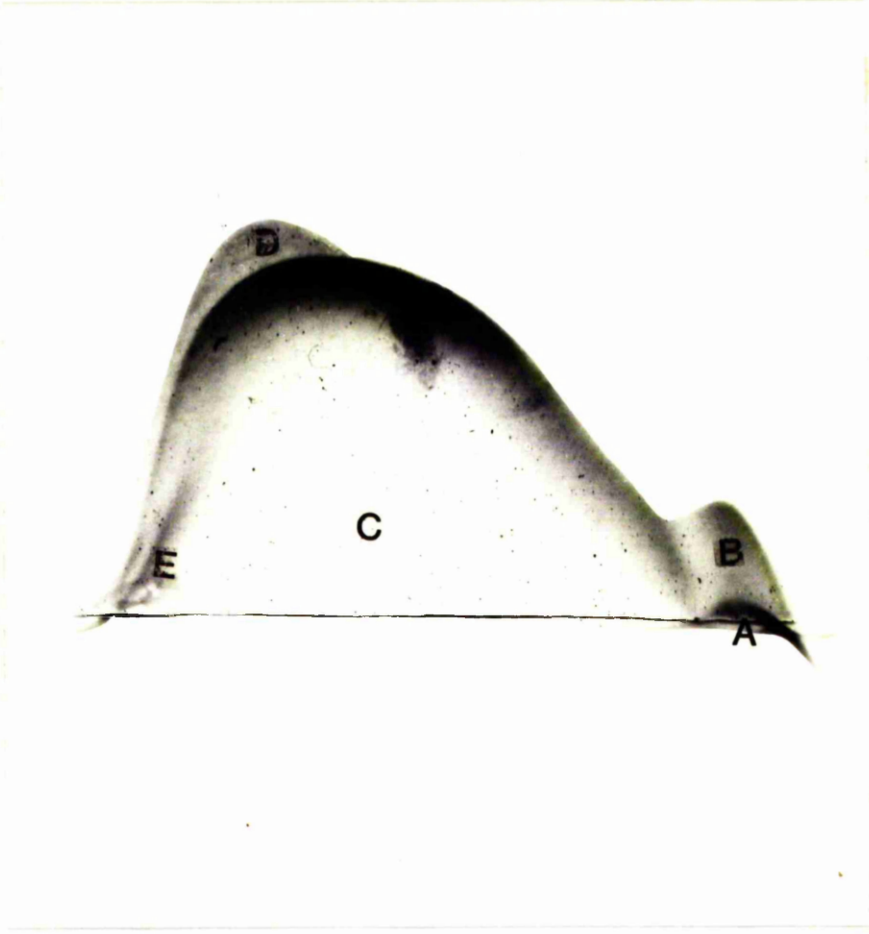


Figure 3-4: Patterns of anodal antigens of C842-148.

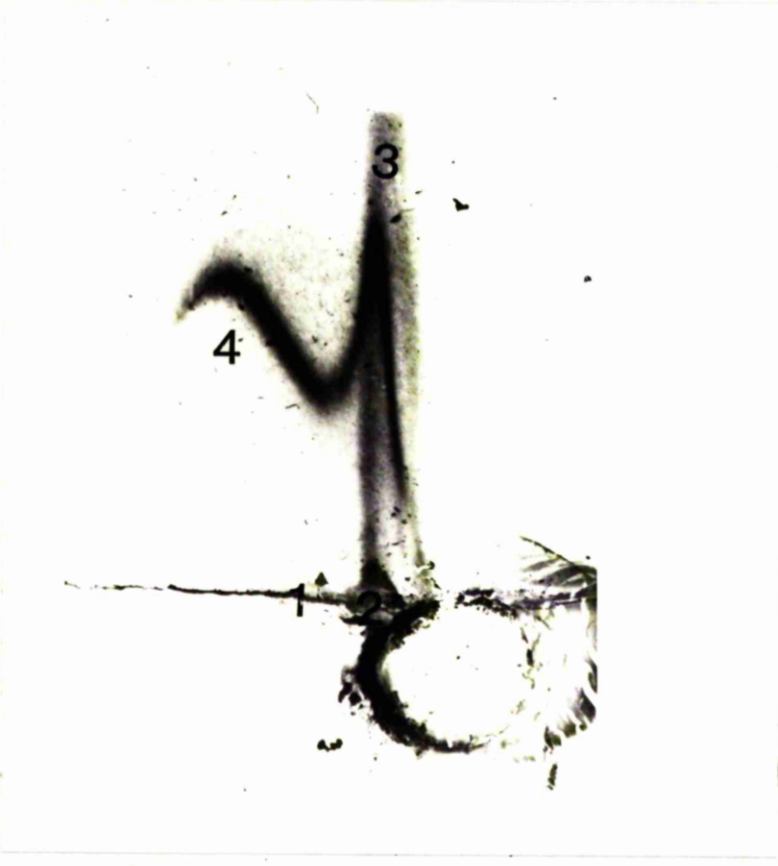


Figure 3-5: Patterns of anodal antigens of NCTC 5850.

(Figure 3-6)

ANODAL ANTIGENS OF NCTC 10354

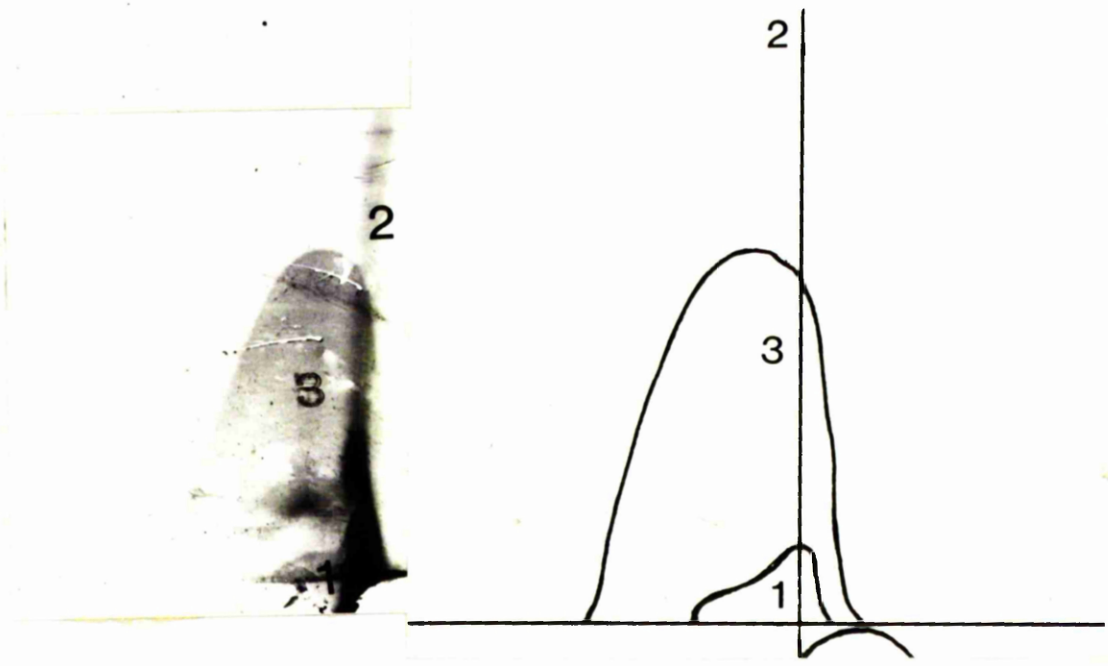


Figure 3-6: Patterns of anodal antigens of NCTC 10354.

Table 3:1

Number of Anodal Antigens in Crude C.fetus Antigens Studied

Isolate	Number of Antigens
C842-148	4(5?)
NCTC 5850	4
NCTC 10354	3
C849-41	2
C383-157	2

(?) Possible presence of an antigen but not conclusively identified

3:2:4 Counterimmunoelectrophoresis With Rabbit Anti C.fetus Sera and Known VMAT Positive Phenol Saline Vaginal Mucus Extracts

Table 3-2a summarises the CIE results with rabbit anti-C. fetus antisera against various sonicated antigens in the pH gradient of 6.6-8.6 in the agarose gels. Table 3-2b summarises the CIE results with other antigens at pH 7.4. Both the agarose types used showed similar activity and thus it was considered that either type could be used in this work with rabbit antisera. The possible influence of pH in CIE tests was noted by the formation of better precipitation lines between pH 6.6-7.6 and weakening ones at pH 8.6. Probably pH 7.4 appeared to favour homologous antigen-antibody reactions as at this pH antiserum NCTC 5850 detected only it own antigen and antiserum C842-72 detected antigens C842-148 but also C377-19. At this juncture it was thought that this pH might separate C. fetus subsp fetus organisms of non-venereal origin from C. fetus subsp fetus organisms of venereal origin and C. fetus subsp venerealis. However when other antisera were included it became apparent that there was no clear cut specificity as antiserum C842-148 detected antigen NCTC 5850 at this pH (7.4)(see Table 3-2b).

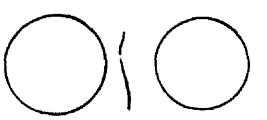
All the seven phenol saline extracts of vaginal mucus were negative in both agarose types used here at the entire pH range 6.6-8.6. As these were from cows known to be infected and which were positive in the VMAT it was not immediately known why they did not precipitate. Even when saline diluted portions of these mucus samples (1:4 dilution) were retested no reactions occurred. The fact that anodal antigens of C. fetus were not only shown to be present in the sonicated antigens but they were reactive in CIE with rabbit antisera meant that such a reaction ought to be obtainable with vaginal mucus of infected cows. Therefore further development studies with vaginal mucus samples were considered necessary and these were carried out and reported in the next chapter.

Figure 3-7 below exemplifies CIE reaction between antigens 22 and 148 and the antiserum C842-148 leading to the formation of immunoprecipitates. Thus anodal antigens of C. fetus could be reactive in CIE tests.

Figure 3-7

COUNTERCURRENT IMMUNOELECTROPHORESIS

148



22

Ag **Ab**

CIE reaction of antiserum 148 and antigen 148 and 22 in agarose gels.

Table 3-2a: Results of Counterimmunoelectrophoresis (CIE) Using Two Rabbit Anti C. fetus Antisera and Five Sonicated C. fetus Antigens in Gels of Varying pH Values

	pH Ranges			
Antiserum 5850 / Sonicated Antigens	6.6	7.4	7.6	8.6
5850	+	+	+	+ (wk)
C842-148	-	-	+	+ (wk)
C377-19	-	-	-	-
NCTC10354	--	-	-	-
Belv +C	-	-	-	-
Antiserum C842-72 / Antigens	6.6	7.4	7.6	8.6
5850	-	-	-	-
C842-148	+	+	+	+ (wk)
C377-19	+	+	+	+ (wk)
NCTC10354	-	-	+ (wk)	+ (wk)
Belv +C	-	-	+	-

wk = Weak positive reaction (precipitation). Was always better developed after overnight saline wash (See ASW in Chapter 5)

Table 3-2b: Restuls of Cross-Reactivity Tests Between Nine Sonicated Antigens With Four Rabbit Anti C. fetus Sera In Gel At pH 7.4

	Antiserum			
Antigens	NCTC 5850	C842-148	C842-72	C377-19
C842-72	ND	+	+	+
C842-22	ND	+	+	ND
C842-16	ND	+	+(AS)	ND
C377-19	-	+	+	+
NCTC 5850	+	+	-	-
C842-148	-	+ (2 lines)	+ (2 lines)	+
Belv +C	-	-	-	-
NCTC 10354	-	+	-	+
C383-157	-	+	+	-

KEY ND = Not done
AS = After staining

3:3 Discussion of Results of Preliminary Study of The Crude Sonicated Antigens of C. fetus

The presence of precipitating antigens in sonicated extracts of C. fetus organisms has been reported (Winter and Dunne 1962; Winter 1963). These antigens generally fell into three categories as judged by positions of the precipitation lines between the serum and antigen wells, namely: lines of group I, nearest the antiserum well, lines of group 2 in the middle portion and a line of group 3 near the antigen well, the latter always crescent-shaped, single and associated with the O-antigen of C. fetus (Winter 1963). The immunodiffusion plates presented in this chapter revealed the same group of antigens in some of the crude C. fetus sonicated antigens studied here, although a crescent shaped line near antigen NCTC 10354 was formed as was the same line with antigen NCTC 5850 when using the antiserum NCTC 5850. The antigen NCTC 5850 belonged to serotype B while antigen NCTC 10354 belonged to serotype A. However some antigens are known to cross-react with anti A and anti B specific sera (Morgan 1959) although as shown in Chapter 6 of this work antigen NCTC 10354 did not show any evidence of cross-reaction with anti B anti-sera. Winter et al (1978) also reported that a crescent shaped line adjacent to the antigen well may be formed with antigen (a) of some isolates of C. fetus when this antigen complexes with 'O' antigen.

Of more interest in this work was the demonstration that all five C. fetus organisms studied by the two dimensional antigen-antibody electrophoresis (Laurell 1965) had anodally migrating antigens. Thus antigen C842-148 had the highest number of anodal antigens (4 or 5). NCTC 5850, also had four. All the remainder had at least two anodal antigens and this agrees with the reports of McCoy et al (1975a, b, 1976a, b). It was found very difficult to demonstrate the antigens of C. fetus subsp venerealis by this technique. In the trials made during this work the

anodal antigens of these strains formed faintly precipitating peaks which were impossible to photograph even though the protein content of these antigens was the same or higher than those of antigen C842-148 which formed very good precipitates (see Figure 3-4). The nearest to a poor reaction is represented by the pattern of antigen NCTC 10354 (Figure 3-6). These anodal antigens of C. fetus appeared to consist of two major antigens which were also electrophoretically fast and at most two others which did not move very far from the wells of application. Of the major antigens, one, named antigen C in the Figure 3-4 and 3 in Figure 3-6 was the most abundant. Because it was the most abundant in the pattern of NCTC 5850, (Figure 3-5) the antigen marked 4 may correspond to those called C and 3 as specified in Figures 3-4 and 3-6 respectively. However, the antigen 4 of NCTC 5850 like that called antigen 1 of NCTC 10354 (Figure 3-6) had similar shapes - appearing as if they were made up of two molecular moieties of varying electrophoretic mobilities. Whatever their nature and identity, the anodal antigens of C. fetus were shown to react with anti C. fetus antibodies in the CIE test. As far as can be ascertained any attempt to react C. fetus antigens with their antibodies under this system has not been reported. The importance of such a reaction is the possible clinical application of the principle in the immuno-diagnosis of C. fetus infection in cattle by the CIE technique. The latter is a rapid test requiring little by way of equipment and could provide the answer to a diagnostic problem within hours and is thus ideal in clinical situations. The demonstration of the reactivity of these antigens with whole cell antisera against C. fetus organisms opened the way for the application of the test to clinical C. fetus infections. The results presented in this work did not lead to a clear cut specificity of reaction between C. fetus subsp fetus and C. fetus subsp venerealis although the latter showed cross-reaction in the CIE with one of the C. fetus subsp fetus

organisms used here (C842-148). C. fetus subsp venerealis did not however cross-react with antigen NCTC 5850 in the CIE although both possessed similar precipitating antigens (see Figure 3-1). This technique has been found to separate groups of mycoplasma organisms (Argaman and Razin 1969) and streptococci (Dajani 1973). It is possible that by studying more biotypes of C. fetus a similar pattern might emerge, although of those studied here, one antiserum C842-148 cross reacted with most other antigens and might indicate a cross-relationship between C. fetus subsp venerealis and C. fetus subsp fetus. In the process of using this method with the sonicated antigens of C. fetus, it appeared that the pH of the gel system was important. Thus at pH 8.6 where most immunoelectrophoresis tests are done, the CIE reactions were poor as evidenced by weak precipitation lines which became more distinct only after overnight washing in normal saline. Moreover antiserum 5850 detected only its homologous antigen at pH 7.4 although this was not found to be true for all antisera tested. The lability of these precipitating antigens to acid pH has been reported (Winter 1963) but no reports could be found to suggest that some might be labile at around 7.0. It is unlikely that this pH dependent detection of particular antigens was due to alkaline lability of some antigens. Rather, as Winter (1963) reported that most of these antigens are proteins, the pH effect could be due to alterations of total change carried by the antigens leading to alterations in their electrophoretic mobility. This is fully discussed in Chapter 4 where further development of the test led to further appreciation of the pH effects.

The failure of phenol saline extracts of vaginal mucus from cows shown by the VMAT to possess C. fetus antibodies, to react with the sonicated C. fetus antigens in the CIE test was at first difficult to understand. This was particularly so as even 1:4 homogenized saline

dilutions of such positive mucus had also failed to elicit the reaction. The only apparent explanation appeared to be the possible unsuitability of the agarose types under use as it was known that agaroses play an important part in CIE tests where a suitable electroendosmotic flow is required (Hibrawi et al 1977). The blind trial of several such agaroses especially those recommended by other workers for this test was performed with satisfactory results. These studies are reported in the next chapter.

CHAPTER FOUR

THE APPLICATION OF COUNTERIMMUNOELECTROPHORESIS (CIE) TO THE DIAGNOSIS OF CAMPYLOBACTER FETUS INFECTION FROM FIELD CASES

4:1:0 Introduction

It has been shown in the previous chapter that Campylobacter fetus (the strains designated as C. fetus subsp venerealis and C. fetus subsp fetus) possess anodal antigens that serologically react with antisera prepared in rabbits against whole organisms. The results further indicate that a range of pH 6.6 to 8.6 was suitable for rabbit antibody activity. When, however, phenol-saline extracts of vaginal mucus samples positive in the VMAT from cattle were tested against the soluble antigen, no reaction was apparent. Several factors could be responsible for this observation amongst which were the pH of the gel system, the type of media (agarose or agar) used, sample dilution and the method of power application. The fact that the anodal antigens of sonicated C. fetus cells formed immunoprecipitates with rabbit antibody suggested that a similar reaction might occur with vaginal mucus antibody. Thus conditions under which anti-C. fetus antibody in vaginal mucus samples reacted with these anodal antigens were investigated.

4a:1:0 Investigation of Conditions Affecting the CIE Test with Bovine Vaginal Mucus Samples : Pilot Tests

Materials and Methods

4a:1:1 Treatment of Samples

Vaginal mucus was collected by sterile glass pipette (Pierce 1946) from cows and served heifers from farms in which a fertility problem characterised by repeat breeding and, in some cases, abortions were the major clinical complaints. C. fetus had been isolated on the first farm, designated Herd I (Table 4a-3/or Farm A, Chapter 6), from some cows and from a bull used in natural service. Each sample was then diluted 1:4 with sterile physiological saline solution (PSS) and homogenized in Griffith

tubes. Prior to centrifugation, 2 ml of the mucus was pipetted into a separate tube to be used for agglutination tests. The homogenized samples were then centrifuged in the MSE bench centrifuge or the MSE GF8 centrifuge at 3900 r.p.m. or 2200 r.p.m. respectively for 40 minutes. The clear supernatant (Sol) obtained was then decanted and retained for testing. The centrifuged samples used in counterimmunoelectrophoresis studies described below are designated 'GM' in the table 4a-3.

4a:1:2 Treatment of Vaginal Mucus with Sputolysin (Cal Biochem, Dithiothreitol, Cleland's Reagent)

Because of the physical difficulty of grinding (homogenizing) several samples, mucolytic agents or enzymes were to be tried for use to aid in solubilizing the vaginal mucus. Hirsch et al (1969) had demonstrated that dithiothreitol was effective in solublizing mucin samples and this compound was chosen for trial. Moreover Meyer et al (1973) had used it to solublize bovine cervical mucus. El-Refaie and Dulake (1975) had found it useful in solublizing human sputum prior to culture or before using the sputum in counterimmunoelectrophoresis tests to diagnose pneumococcal chest infections.

Thus a 1:10 dilution of sputolysin (Calbiochem, U.S.A.) was made in saline (PSS). Equal volumes of vaginal mucus and the diluted sputolysin were mixed in a blender and incubated at $37^{o}C$ for 15 minutes to one hour. At the end of the chosen time the viscosity of the resulting solution was empirically compared with untreated samples by the ease with which it could be pipetted into a pasteur pipette attached to a bulb. The free-flowing mucus was centrifuged as described above and the resulting sol was used in counterimmunoelectrophoresis, immuno-electrophoresis and immunodiffusion tests. In the latter two methods, development of arcs of bovine immunoglobulins were demonstrated using rabbit antibovine IgG_1, IgM and IgA (Miles Research Labs) As a comparison,

serum from a brucellosis negative reactor cow was similarly treated with sputolysin and subjected to immunoelectrophoresis and immunodiffusion tests. The sputolysin treated samples used in the CIE are designated 'SPM' in the table at the end of this section (Table 4a-3).

4a:1:2:2 Extraction of Ground (Griffith Tube Homogenized) Samples

2 ml homogenized vaginal mucus prepared as above was mixed with 2 ml 3% molten Difco agar at 57°C and allowed to set at room temperature (20-22°C), as described by MacKinnon (1954). The set gel was then overlaid with 2 ml 0.5% phenol saline and the system incubated for 18-20 hours at 37°C. The supernatant fluid was pipetted off and used in agglutination tests described below (Chapter 6, see also Appendix 2). Portions of this were retained and examined for precipitating antibodies by counterimmunoelectrophoresis as described below (Chapter 4a). These samples are the phenol extracts designated as 'PN' in the table below. (Table 4a-3).

Performance of the Counterimmunoelectrophoresis Tests

4a:2:1 pH Gradients in Different Gels: Preparation of Buffers and Gels of Varying pHs.

Veronal Buffer of composition:

Sodium barbitone	20.6g/litre
barbitone	4.0g/litre
sodium azide	0.01% (w/v)

was prepared and its pH adjusted by 0.1M HCl to 8.6 or as required. Either agarose B (pharmacia, Sweden) or Noble Agar (Difco) was then added to portions of the buffer to make 1% w/v. Each was steamed for one hour to dissolve the agarose or agar, cooled and dispensed in 15 ml portions in universal bottles. These were stored at 4°C.

To prepare agarose B at pH values 6.6 or 7.9, the required amount of agarose was added to the pH 8.6 stock buffer in a bottle and the pH

was adjusted to either 6.6 or 7.9 with 0.1M HCl while the agarose buffer mixture was being stirred by a magnetic stirrer. The mixtures were then steamed and dispensed as above. Noble agar was not tested at the lower pH values since as reported below, it was found not to be a good medium for the test.

The second buffer was that of Calström" and Liberg (1975) composed of 10.3g sodium barbitone dissolved in 23.5 ml of 0.1M HCl, and made up to 1 litre with distilled water. This buffer was then used to prepare barbitone/HCl-gels of pH 7.9 without dilution. It is claimed that bovine immunoglobulins are better separated at this pH when used in electrophoresis systems. Only gels containing agarose B (Pharmacia, Sweden) at 1% w/v were used in the preliminary tests reported here.

The third buffer was barbitone acetate buffer. This was composed of 10.0g barbitone sodium, 6.5g sodium acetate in 64.4 ml 0.1M HCl as described by Sargent and George (1975). The pH was adjusted to 7.9 with 1.0M HCl and agarose B (Pharmacia, Sweden) at 1% w/v was incorporated.

The gels so made were then melted by boiling in a water bath or by steaming when required and 15 ml poured onto 10 cm^2 glass plates or 9.6 ml onto 8 cm^2. These were placed on a level table to give a depth of about 1.5 mm when gelled. Paired wells 4 mm apart, 5 mm diameter for anodal wells and 3mm diameter for cathodal wells were punched into the gels and the anodal wells filled with ground centrifuged vaginal mucus, sputolysin treated or extracted with phenol saline. Three known negative samples (as revealed by vaginal mucus agglutination tests) were also included. The cathodal wells were filled with 1:4 dilution of sonicated antigen 148 known to possess at least four anodal antigens (reported in Chapter 3). The slides were then placed in the Kohn" U-77 electrophoresis chamber of the Shandon electrophoresis equipment and the chambers were filled with 500 ml of the barbitone buffer of pH

8.6, μ = 0.05 and the plates connected to the buffers by means of Whatman No. 41 filter paper wicks.

4a:2:2 Electrophoresis - Voltage and Current Variations

4a:2:2:1 Application of Constant Voltage and Controlled Time

A constant voltage of 200 volts was applied across the gel using the Shandon Volkam Power pack in the first trials. Then in later trials 10 volts were applied per cm of gel. The effective length of the gel was taken to be 9 cm when using the 10 x 10 cm gels or 7 cm when using the 8 x 8 cm gels, because the wicks were placed to cover 0.5 cm on either side. The electrophoresis was continued and interrupted at one hourly intervals to inspect the development of visible precipitation between antigen and antibody wells. After $2\frac{1}{2}$-3 hours electrophoresis was stopped whether or not visible precipitation lines appeared. The gels were immersed in 0.85% NaCl solution overnight and treated as described below.

4a:2:2:2 Application of Constant Current and Controlled Time

Because Muhammed et al (1978) showed that the constant current method was superior to constant voltage methods, their technique was adopted. After initial trials it became apparent that for 8 cm x 8 cm slides a constant current of 5ma/slide was satisfactory while for the 10 cm x 10 cm slides 7.5 ma/slide was adopted. The slides were treated as above except the constant current was applied for $2\frac{1}{2}$-3 hours. All slides were then removed and immersed in 0.85% saline overnight to remove unwanted protein. The following day the slides were washed in tap water, covered with blotting paper (ensuring that no bubbles were entrapped in the wells) and dried at 37^{o}C for 3-4 hours. All were stained with 0.1% w/v Coomasie Brilliant Blue R250 (B.D.H. Chemicals, England) for periods varying from 1-2 hours and in some cases overnight. The stained plates were then decolourised by flooding in a solution of 450 ml 96% Ethanol,

450 ml distilled water and 100 ml glacial acetic acid. The decolourising was continued until the background was clear, but without decolourising the precipitation lines. When precipitation lines were observed between the antigen and the antibody wells, the vaginal mucus was taken as being positive to the CIE test. The number of precipitation lines formed was noted as was the development of the lines before (BS) or after (AS) staining. When as shown in the results it became clear that the method worked at pH 7.9 in agarose B, all other portions of the stored vaginal mucus were tested at this pH. These results are also included with those of the first few known positives from Farm A which were used to establish the optimal testing conditions.

4a:3 Results of the Samples used to Establish the Test Conditions and from Subsequent Field Cases Suspected of C. fetus Infection

In Noble agar pH 8.6 gel, no directly visible precipitation were formed. Moreover, the precipitation lines seen after staining were faint and appeared very close to the antigen well. Of seven (7) positive samples which were tested five gave this faint precipitation reaction. They could be seen only by oblique lighting of the slide. In agarose B, pH 8.6, three of seven samples which were saline homogenized gave precipitationlines close to the antibody well, one just at the edge of the well and the remaining three gave negative reactions. All were noted after staining (Table 4a-1). In agarose B pH 7.9 with the Calstrom" and Liberg (1975) buffer barbitone and barbitone acetate buffers all seven positives were detected (Table 4a-2). The lines had moved further away from the antibody well. Two of seven samples gave directly visible precipitation at the end of the electrophoresis period. At pH 6.6 in agarose B only five of seven known positives were detected, and in these the precipitation lines were close to the antibody well and had two limbs directed to the antigen well. Only one sample gave a directly visible precipitation line at the end of the electrophoresis

period. Figure 4a-1, illustrates the results of seven positive ground vaginal mucus samples and three negatives included as controls. Figure 4a-2, illustrates the nature of the reaction at pH 6.6 with the limbs of the precipitate being noted in the upper two wells to the left. Again three known negatives to the right did not react to the test.

There was little difference in the formation of precipitation lines whether constant voltage of 200 volts in total was used or 10 volts/cm of gel or with the constant current. Only with 200 volts was there a problem with condensation on the chamber cover which occasionally flooded the wells. The condensation was caused by the heat effect noted with the use of 200 volts. The heat produced was less marked when the 10 volt/cm gel regime or the constant current regime were used.

The vaginal mucus samples which had been extracted in phenol saline did not react with the antigen. All were negative whether or not positive by the VMAT. Thus this form of sample treatment was abandoned. The sputolysin treated mucus gave variable results. When tested after 15 minutes incubation, weak precipitation lines were formed and in one case from a suspicious VMAT sample a broad band of precipitation line between antigen and antibody wells was formed.

When tested after one hour sputolysin incubation, all were negative. Indeed those left overnight after sputolysin treatment did not form immunoprecipitates even in immunodiffusion tests or immunoelectrophoresis tests against rabbit antibovine sera of the antibody classes IgG_1, IgM and IgA. IgM was not demonstrable even in untreated vaginal mucus. These reactions are depicted in Figures 4a-3, 4a-4, 4a-5 and 4a-7.

Figure 4a-1: Seven CIE positive samples detected in agarose B at pH 7.9. Three known negatives (bottom 3 pairs of wells to the right) did not show precipitation.

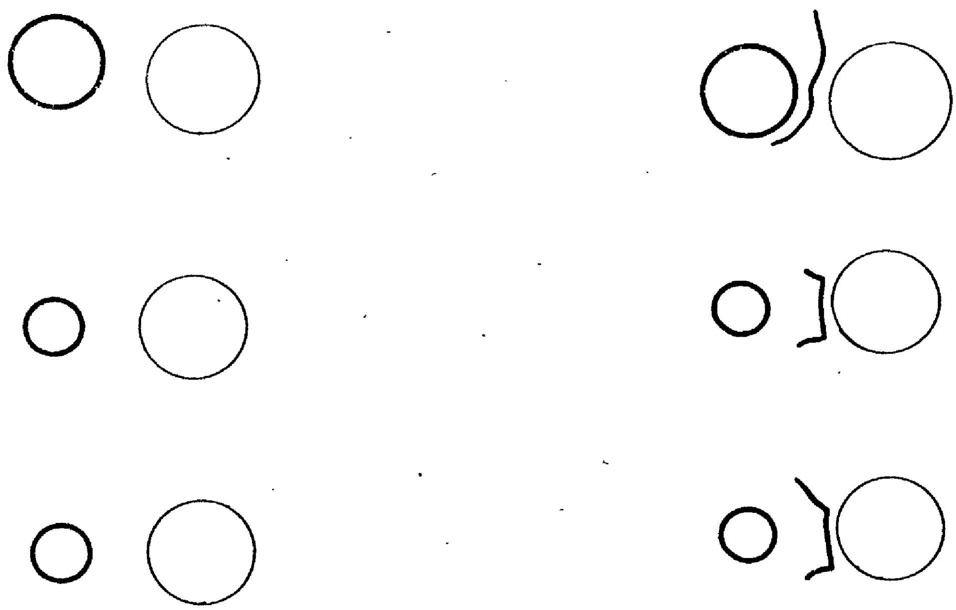


Figure 4a-2: Three CIE positive mucus samples at pH 6.6 in agarose B to illustrate the nature of the precipitation occurring at this pH. Three known negatives also failed to show precipitation.

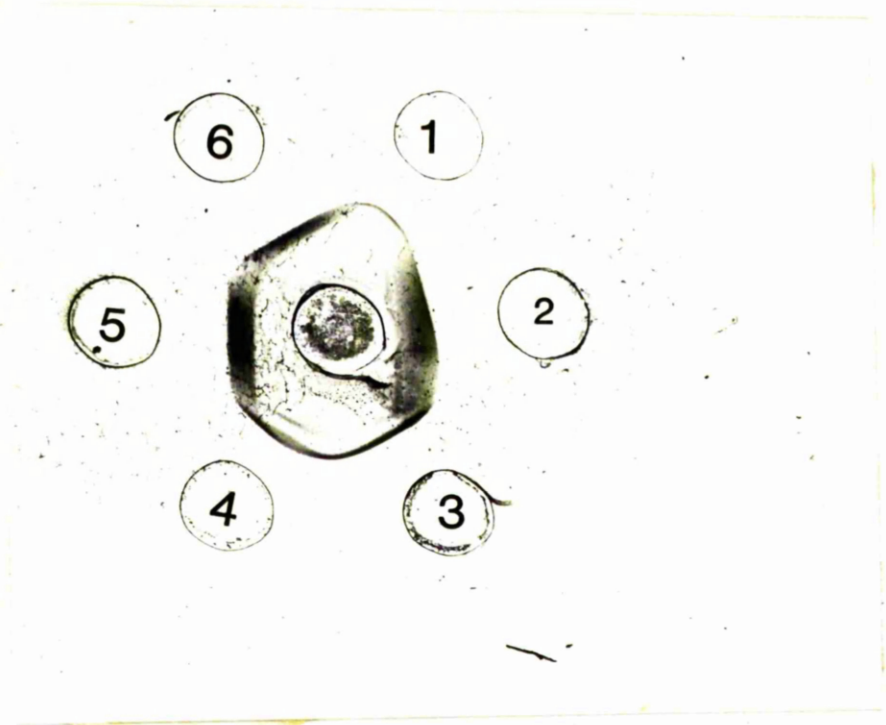


Figure 4a-3: Immunodiffusion test of untreated serum (wells 5 and 6) and sputolysin treated vaginal mucus (wells 1, 2, 3 and 4) and development of lines with RAB IgG_1 serum. Evidence of double line formation in areas of vaginal mucus.

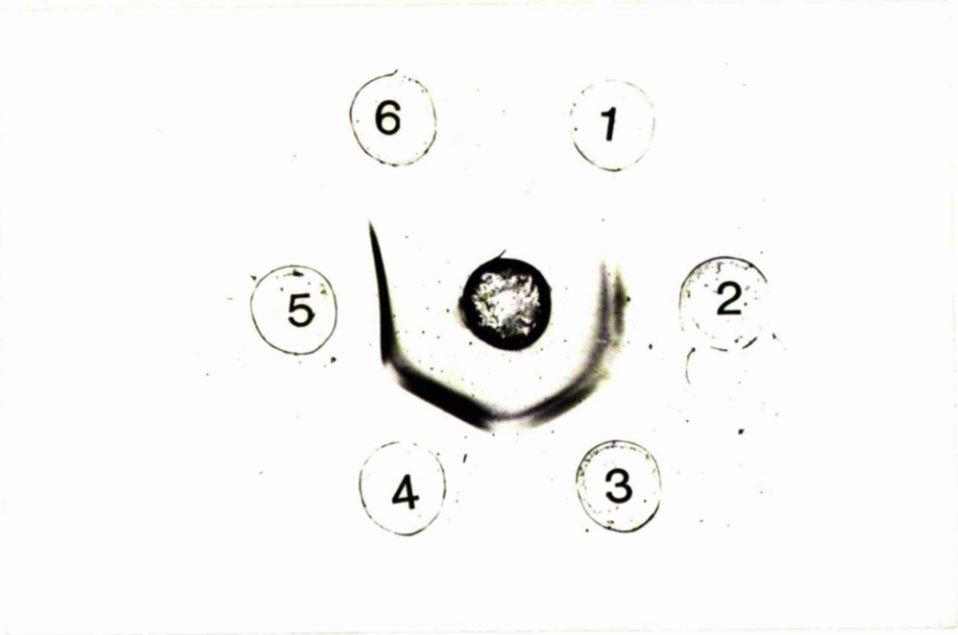


Figure 4a-4: Immunodiffusion test of sputolysin treated vaginal mucus (wells 2 and 3) and untreated bovine vaginal mucus (well no. 4) and untreated bovine serum (well no. 5) and development of the lines with RAB IgA. Well no. 1 contained sputolysin alone, and well no. 6 buffer alone.

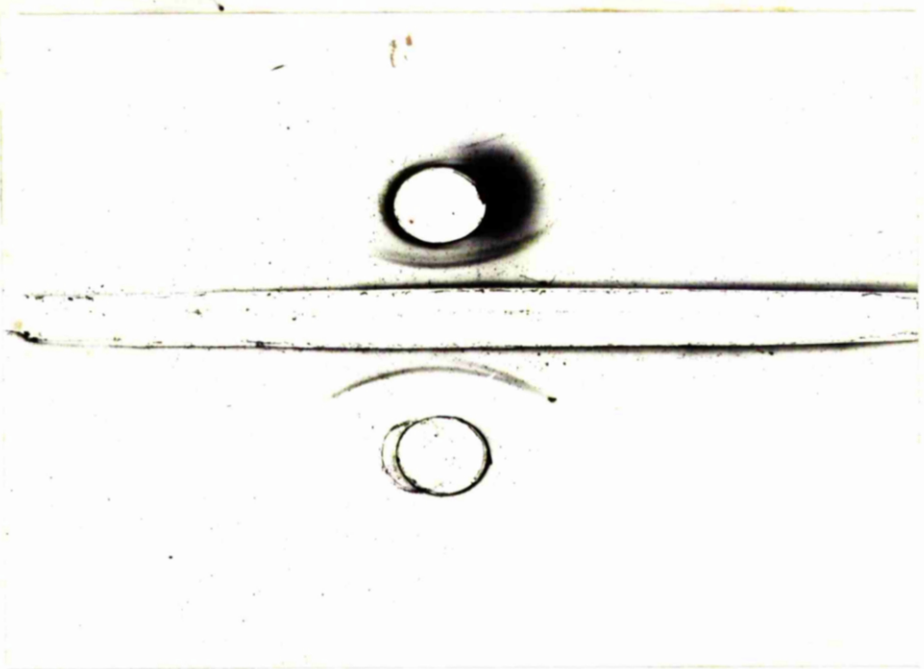


Figure 4a-5: Immunoelectrophoresis of sputolysin treated serum (upper well) and vaginal mucus (lower well) and development of lines with RAB IgA serum.

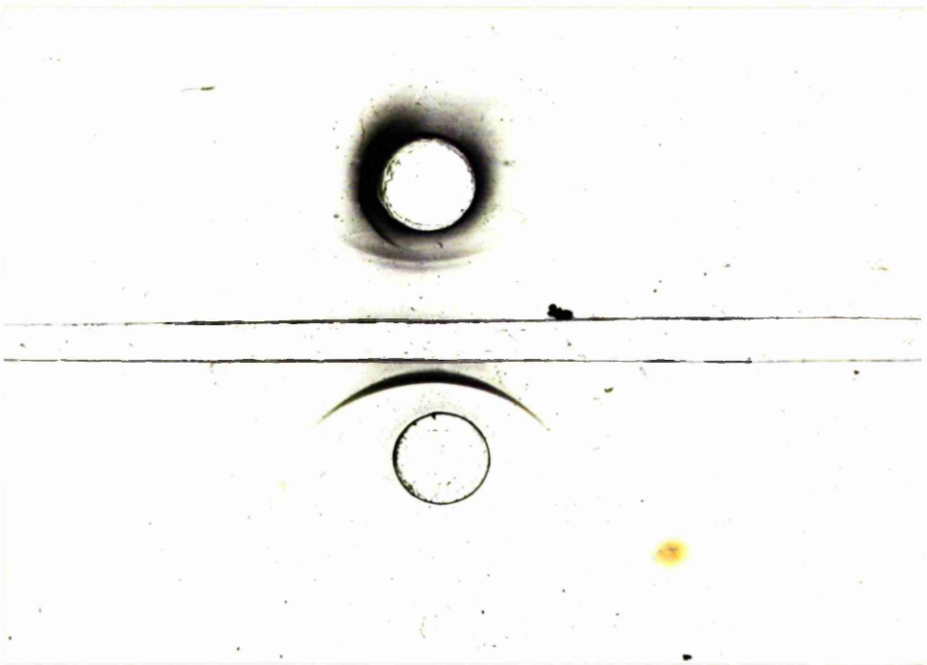


Figure 4a-6: Immunoelectrophoresis of sputolysin treated serum (upper well) and untreated vaginal mucus (lower well) and development of lines with RAB IgA serum.

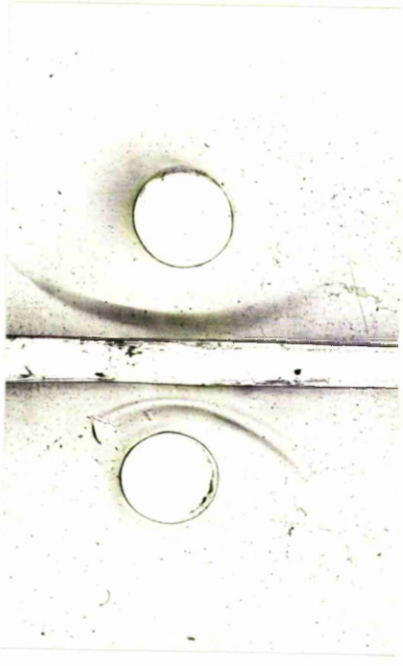


Figure 4a-7: Immunoelectrophoresis of untreated serum (upper well) and treated vaginal mucus (lower well) and development of the lines with RAB IgG_1 serum.

As a result of the observations on CIE reactions occurring in agarose B pH 7.9, all vaginal mucus from field cases were tested in agarose B pH 7.9. The table 4a-3 below lists all such samples examined in this pilot testing. It further shows the reaction obtained with the standard agglutination test with antigen 1980 (Weybridge) as the agglutinating antigen.

Table 4a-1: CIE RESULTS OF KNOWN VMAT POSITIVE MUCUS ON GEL TYPES SHOWING AT LEAST A REACTION

SAMPLE NO.	NOBLE AGAR pH 8.6	AGAROSE B pH 8.6	AGAROSE B pH 6.6	AGAROSE B pH 7.9
70	+ ve (AS) Very Weak	+ve (AS) Near Ab well	+ve (BS)	+ve (BS) off Ab well
71	+ve (AS) Very Weak	+ve (AS) Near Ab well	+ve (AS)	+ve (BS)
205	+ve (AS) Very weak	+ve (AS) Slightly off Ab well	+ve (AS)	+ve (AS)
1698	+ve (AS) Very weak	-ve	+ve (AS) Weak	+ve (AS)
648	-ve	-ve	-ve	+ve (AS)
79	+ve (AS) Very weak	+ve (AS) Off Ab well	+ve (AS)	+ve (AS)
64	-ve	-ve	-ve	+ve (AS)

BS = Before Staining

AS = After Staining

Ab = Antibody

Table 4a-2: CIE RESULTS OF THE SEVEN VMAT POSITIVE SAMPLES IN VARIOUS BUFFERS AT pH 7.9

SAMPLE NO.	BUFFER TYPE USED TO DISSOLVE AGAROSE		
	BARBITONE HCL pH 7.9	BARBITONE pH 7.9	BARBITONE ACETATE pH 7.9
70	+ve (BS)	+ve (BS)	+ve (BS)
71	+ve (BS)	+ve (BS)	+ve (BS)
205	+ve (AS)	+ve (AS)	+ve (AS)
169B	+ve (AS)	+ve (AS)	+ve (AS)
79	+ve (AS)	+ve (AS)	+ve (AS)
64	+ve (AS)	+ve (AS)	+ve (AS)

Table 4a-3: The Results Obtained in Counterimmunoelectrophoresis Testing of Vaginal Mucus and Compared to the Results of VMAT and Cultures from Tested Farms

Herd	Sample No.	Animal No. & Sample Nature	Counterimmunoelectrophoresis Results: Result	No. of PPT Lines	BS or AS	VMAT Results	Farm Status (Culture)	Identified or missed by CIE
1	C589	66GM	-ve	-	-	+ve		
		70GM	+ve	1	BS	+ve		
		70PN	-	-	-	+ve		
		25GM	+ve	1	AS	+ve		
	C749	64GM	+ve	1	AS	+ve	Infected	Identified
		204BGM	+ve	1	AS	+ve		
		71GM	+ve	1	BS	+ve		
		64PN	-ve	-	-	+ve		
		25GM	-ve	-	-	+ve		
		169PN	-ve	-	-	+ve		
	C642	64GM	+ve	1	AS	+ve		
		109BGM	-ve	-	-	Susp.		
		79GM	+ve	1	AS	Susp.		
		169BGM	+ve	1	AS	+ve		
		22PN	-ve	-	-	+ve		
		17PN	-ve	-	-	+ve		
	C749	190BPN	-ve	-	-	+ve		
2	C928	5GM	+ve	1	AS	+ve	Infected	Identified
		46GM	+ve	1	BS	+ve		

Herd	Sample No.	Animal No. & Sample Nature	Counterimmunoelectrophoresis Results: Result	No. of PPT Lines	BS or AS	VMAT Results	Farm Status (Culture)	Identified or missed by CIE
3	C759	69GM	+ve	1	AS	+ve	Infected	Identified
		69PN	–	–	–	+ve		
		41GM	+ve	1	AS	+ve		
4	C581	17GM	–ve	–	–	+ve	Unknown	Unknown
5	C993	22SPM	+ve	1	AS (Broad)	Susp.	Infected	Identified
	C34	103SPM	+ve	1	BS	+ve		
		84GM	–ve	–	–	+ve		
		56GM	–ve	–	–	–ve		
		10GM	–ve	–	–	–ve		
		Unknown GM	–ve	–	–	ND		
		105GM	–ve	–	–	–		
	C250	65GM	+ve	1	BS	–ve		
		Unmkd GM		1	AS	+ve		
			+ve(Wk)	1	AS	–ve		
		163GM	–ve	–	–	–ve		
		193GM	+ve	1	AS	Susp.		
		43GM	+ve	1	AS	+ve		
		106GM	+ve	1	AS	–ve		
		97GM	+ (Wk)	1	AS	+ve		

Herd	Sample No.	Animal No. & Sample Nature	Counterimmunoelectrophoresis Results			VMAT Results	Farm Status (Culture)	Identified or missed by CIE
			Result	No. of PPT Lines	BS or AS			
5 (contd)	C250	14GM	-ve	-	-	-ve		
		69GM	-ve	-	-	-ve		
		91GM	-ve	-	-	ND		
		60GM	-ve	-	-	-ve		
		13GM	+ve		AS	-ve		
6	C175	1GM	-ve	-	-	+ve	Infected	
		2GM	-ve	-	-	+ve		Missed
		3GM	-ve	-	-	-ve		
		4GM	-ve	-	-	Susp.		
7	C263	63GM	-ve	-	-	+ve	Non-infected	Correctly diagnosed
		33GM	-ve	-	-	Susp.		
		102GM	-ve	-	-	-ve		
		13GM	-ve	-	-	-ve		
8	C275	118GM	+ve	1	BS	Susp.	Infected	Identified
		124GM	+ve	2	BS	+ve		
		75573GM	-ve	-	-	Insuff.		
		94979GM	-ve	-	-	+ve		
		123GM	+ve	1	BS	Insuff.		

Herd	Sample No.	Animal No. & Sample Nature	Counterimmunoelectrophoresis Results: Result	No. of PPT Lines	BS or AS	VMAT Results	Farm Status (Culture)	Identified or missed by CIE
8 (contd)	C275	99772GM	-ve	-	-	-ve		
		32494GM	-ve	-	-	+ve		
		74GM	-ve	-	-	Susp.		
		96873GM	-	-		+ve		
		83GM	+ve	1	BS	Insuff.		
		7558GM	-ve	-	-	+ve		
9	C562	85	-ve	-	-	-ve	Non-infected	Correctly diagnosed
		60	-ve	-	-	-ve		
		47	-ve	-	-	-ve		
		17	-ve	-	-	-ve		
		84	-ve	-	-	-ve		
		64	-ve	-	-	-ve		
10	C591	1	-ve	-	-	-ve	Non-infected	Correctly diagnosed
11	C645	11	-ve	-	-	-ve	Non-infected	Correctly diagnosed
		10	-ve	-	-	-ve		
		4	-ve	-	-	-ve		
		7	-ve	-	-	-ve		

Herd	Sample No.	Animal No. & Sample Nature	Counterimmunoelectrophoresis Results: Result	No. of PPT Lines	BS or AS	VMAT Results	Farm Status (Culture)	Identified or missed by CIE
11 (contd)	C645	6	-ve	-	-	-ve		
		3	-ve	-	-	-ve		
		15	-ve	-	-	-ve		
12	C641	1	-ve	-	-	-ve	Non-infected	Correctly diagnosed
		2	-ve	-	-	-ve		
13	C731	7	-ve	-	-	-ve	Non-infected? Unknown status	Unknown
		48	+ve	1	BS	Susp.		
		37	+ve	1	BS	-ve		
		35	-ve	-	-	Susp.		
	CAN.HL	44	+ve	1	BS	-ve?		
	C892	51	+ve	1	BS	Antigen		
		20	+ve	1	AS	Instability		
		45	-ve	-	-	No		
		24	-ve	-	-	valid	Unknown	
		35	-ve	-	-	VMAT		
		54	-ve	-	-	results		
		48	-ve	-	-	in later		
		38	-ve	-	-	samples		

Herd	Sample No.	Animal No. & Sample Nature	Counterimmunoelectrophoresis Results: Result	No. of PPT Lines	BS or AS	VMAT Results	Farm Status (Culture)	Identified or missed by CIE
13 (contd)	C892	4	-ve	-	-	No valid results	Unknown	Unknown
		7	-ve	-	-			
14	C251	0721	-ve	-	-	-ve	Non-infected	Correctly diagnosed
		0722	-ve	-	-	-ve		
		0723	-ve	-	-	Insuff.		
		0724	-ve	-	-	"		
		0725	-ve	-	-	"		
		0726	-ve	-	-	"		
15	C333	135	-ve	-	-	Insuff.	Non-infected	Correctly diagnosed
		100	-ve	-	-	-ve		
		94	-ve	-	-	-ve		
		115	-ve	-	-	-ve		
		112	-ve	-	-	Insuff.		
		110	-ve	-	-	-ve		
16	C355	HI	(±)	-1	Washed off Non specific	-ve	Non-infected	Correctly diagnosed
		H75	-ve	-	-	-ve		
		51	-ve	-	-	Insuff.		

Herd	Sample No.	Animal No. & Sample Nature	Counterimmunoelectrophoresis Results: Result	No. of PPT Lines	BS or AS	VMAT Results	Farm Status (Culture)	Identified or missed by CIE
16 (contd)	C355	52	-ve	-	-	-ve		
		26	-ve	-	-	-ve		
17	C377	40	-	All negative on CIE		+ve		
		99	-		-	+ve	Infected	
		4	-			+ve		
		62	-			+ve		
		91	-			+ve		
		93	-			ND		Temporarily
		86	-			-ve		missed as farm
		44	-			-ve		later (see 4b)
		48	-			-ve		identified when
		102	-			+ve		soluble antigen
		19	-			+ve		from one of the
		1	-			+ve		isolates from
		71	-			+ve		this farm was
		92^{x}_{b}	See 4b			ND		now included in
		84	ND			ND		the test antigen
		54^{x}	See 4b			ND		
18	C437	30	-ve	-	-	-ve		

x = Later shown positive in CIE with mixed antigen

Herd	Sample No.	Animal No. & Sample Nature	Counterimmunoelectrophoresis Results: Result	No. of PPT Lines	BS or AS	VMAT Results	Farm Status (Culture)	Identified or missed by CIE
18 (contd)	C437	Spottie	-ve	-	-	-ve	Non-infected	Correctly diagnosed
		3	-ve	-	-	-ve		
		70	-ve	-	-	-ve		
		39	-ve	-	-	-ve		
		33GM	-ve	-	-	-ve		
		68GM	-ve	-	-	-ve		
		Unknown	-ve	-	-	-ve		
		28GM	-ve	-	-	-ve		
		74GM	-ve	-	-	-ve		
19	C218	171GM	-ve	-	-	-ve	Non-infected	(False positive)
		196GM	-ve	-	-	-ve		
		142GM	-ve	-	-	-ve		
		199GM	-ve	-	-	-ve		
		20GM	+ve	1	AS	-ve		
		27GM	-ve	-	-	-ve		
		240	-ve	-	-	-ve		
20	C871	13GM	+ve	1	BS	Susp.	Infected	Identified
		15GM	-ve	-	-	+ve		

Herd	Sample No.	Animal No. & Sample Nature	Counterimmunoelectrophoresis Results: Result	No. of PPT Lines	BS or AS	VMAT Results	Farm Status (Culture)	Identified or missed by CIE
21	C841	3	+ve	1	BS	+ve		
		11	+ve	1	AS	-ve	Infected	Identified
		9	+ve	1	AS	+ve		
		7	+ve	1	AS	-ve		
		10	+ve	1	AS	-ve		
		6	ND	-	AS	-ve		
		4	+ve	1	BS	+ve		
		8	+ve(7.2AGB)	1	AS	-ve		
		12	ND	-	-	ND		
		1	+ve(wk)	1	AS	+ve		
		2	-ve	-	-			
		55	-ve	-	-	-ve		
	C351	52	-ve	-		-ve	Infected	Identified
		73	+ve	1	AS	+ve		
		11	+ve	1	AS	-ve		
		77	+ve	1	AS	+ve		
		107	-ve	-	-	+ve	Infected	
		113	-ve	-	-	Susp.		
		86	-ve	-	-	+ve		
		Unmkd.		1	-	ND		

Herd	Sample No.	Animal No. & Sample Nature	Counterimmunoelectrophoresis Results: Result	No. of PPT Lines	BS or AS	VMAT Results	Farm Status (Culture)	Identified or missed by CIE
21 (contd)	C351	79	+ve	1	AS	+ve		
		21	-ve	-	-	+ve		
		23	-ve	-	-	+ve		
22	C310	31	-ve	-	-	-ve	Infected	
		61	+ve	1	BS	(Trace)	(See C849	Identified
		151	-ve	-	-		of	
		169	+ve	1	BS	+ve	3/11/80)	
		36	+ve	1	BS	Susp.		
		122	ND		-	-ve		

4a:4 Discussion On the Use of CIE in Field Cases

The details of the samples presented in Table 4a-3 above shows that during the pilot testing period 22 farms in which infection with C. fetus was suspected were either visited for investigation or samples from suspected cases were submitted for vaginal mucus agglutination tests. On the basis of VMAT ten farms could be declared infected. In one other herd (Herd 7, C263) one animal gave a positive VMAT but as discussed in Chapter 6 of this work, this farm was culturally negative for pathogenic C. fetus although two animals yielded a catalase negative C. sputorum subsp bubulus. Of these herds CIE would have missed two cases i.e. samples from the case C377 (Farm G, Herd 7) and C175 (Herd 5). The details presented in Chapters 3, 4b and 6 would partially explain this discrepancy. However the CIE would have classified the farm C218 (Herd 19), as positive since one animal out of seven tested gave a well defined precipitation line after staining of the immunoplates. Although another farm C355 may have been also considered positive immediately after the test since one of five tested animals gave a suspicious reaction (see page 77 of this Chapter), the reaction must have been non-specific as it was washed off on overnight immersion of immunoplates in phosphate buffered saline pH 7.2 which is one way of determining some non-specific reactions (Crowle 1973). If this is considered, these two of ten negative farms could have been wrongly classified as would one that was positive. However, the technique appeared promising and further work on it was thus continued.

The samples which were treated with sputolysin could only be useful in the test if the incubation period was about 15 minutes. When left longer than this it seemed to result in breakdown of immunoglobulins since after 1-3 days not even immunoglobulins in vaginal mucus which was previously positive could be detected by immunodiffusion. The probability of breakdown of immunoglobulins was considered as being

possibly a split-line effect (Skwaril 1960). This would probably have meant a breakdown at the site of pepsin activity on immunoglobulin molecules so that both the F (ab) 2 and the FC piece would precipitate with antigen and rabbit antibovine serum against the different molecules of immunoglobulin classes (Moorehouse and Hugh-Jones 1981) and the subsequent lack of immunological activity would be due to continued breakdown of inter-heavy chain disulphide bonds (James et al 1964).

However it is known (Gunewardena and Cooke 1966) that sputolysin (dithiothreitol) degrades human IgG 'into half molecules rather than light and heavy chains' at high concentrations (Stanworth and Turner 1978). This method of sample treatment was abandoned. None of the phenol saline extracts gave any precipitation with sonicated antigen. The reason for this is not known. In work presented in Chapter 4b below the reactivity of immunoglobulins in bovine vaginal mucus was poor from pH 7.4 downwards particularly in agarose B which was the medium in which all the pilot tests were conducted. Indeed at the pH value of 6.6 several known positive samples (CIE) became negative (see photo-graphic inclusions in 4b below). The pH of phenol-saline used in the extraction of the samples of the mucus was always about 5.8.

In all but one herd in which infection was detected by the CIE (C759) there was at least one sample and at most four (C928 and C275 respectively) giving a directly visible precipitation line at the end of electrophoresis. In the majority of positive samples, evidence of immunoactivity was noted after staining with Coomasie Brilliant Blue (Kenacid, B.D.H., England). Thus it would appear that the direct reaction depends on the concentration of antigen specific immunoglobulin in the sample. This would vary with time after infection and stage of the oestrus cycle when sampled. Dilution of antibodies in the large volume of mucus in samples taken around oestrus is a major cause of reduced

antibody concentration (Lawson 1959). The results from farm C218 (Herd 19) which gave one positive CIE reaction and from farm C371 (Herd 13) which gave five direct CIE positives could not be easily explained. Since the positive reactions did not occur in a well defined state in Noble agar and as reported earlier on two other agarose types, media must in themselves play a role in this test. The results of the pilot test therefore led to the following conclusions:-

(1) Antibodies specific to C. fetus infection in the vaginal mucus of infected cattle can be detected by CIE using a soluble C. fetus antigen, in this case a crude soluble fraction obtained by sonication. The antigenic heterogenicity of the organism may lead to false negative results as in C377 (Herd 17) which as reported later, could be detected by including a soluble antigen obtained from one isolate from an animal in that particular farm.

(2) Media and pH of the buffers in which the gels are dissolved are also important in their own individual capacity. Buffers used at pH 7.9 best demonstrated precipitation. More acidic pH may not be desirable for bovine vaginal mucus samples although with rabbit antibody even pH 6.6 could yield positive reactions. At this pH some bovine vaginal mucus samples which were positive to the CIE became negative.

(3) Neither treatment of mucus with sputolysin nor phenol saline extraction was suitable. In the former case apparent break-down of immunoglublins as judged by the split-line effect appeared to have occurred. In the latter case dilution or pH effect may be incriminated. Both methods were therefore abandoned.

4b The Evaluation of Agarose B and Litex HSB Agarose Over Several pH Ranges to Determine the Most Suitable Gel and pH Combinations For Use in Counterimmunoelectrophoresis As an Immunological Test for the Diagnosis of Campylobacter fetus Infection In Cattle

4b:0 Introduction

In the preceeding section evidence has been presented to show that both the pH and gel type used influenced the occurrence, position and intensity of precipitation lines in C. fetus CIE using bovine mucus. The work of Hibrawi et al (1977) similarly indicated that the type of agarose was important when this technique was applied to the diagnosis of human myoglobinuria. These authors found that of 22 agarose samples from different manufacturers only 6 were suitable. It has been shown in Chapter 4a that agarose B allowed precipitation lines to be demonstrated better than two other agarose preparations (Kochlight Labs. Ltd., England; Gibco, for Grand Island Biologicals, U.S.A.) and Noble agar (Difco). The possibility of others being suitable was then considered. Hibrawi et al (1977) recommended Litex HSB and Litex HSC agarose (Litex, Denmark) for the test. Both types were then used in comparative trials along with agarose B. Only the results with agarose of Litex HSB and Agarose B are presented since Litex HSC agarose was abandoned after initial trials which showed that apparently non-specific lines were formed in it.

4b:1 Materials and Methods

4b:1:1 The pH Range in Two Different Buffers

The composition of the two buffers was as described in section 4a except that in the case of Calström and Liberg buffers of pH value 5.8 to 7.6 the required amount (1g/100 ml) of agarose B or Litex HSB was added to the stock pH 7.9 buffer and the pH was adjusted with 0.1M HCl. With the barbitone acetate buffer molar HCl was used to alter pH as required. The agarose was stored at 4°C in bulk

quantities of 200-500 ml. This when re-melted was used to make gels of depth 1.5 mm on degreased glass plates 10 x 10 cm or 8 x 8 cm. These plates had previously been smeared with a little of the gel to facilitate bonding of the working gel to the glass. Wells were punched into the gels as before with anodal (antibody containing) wells being larger (5 mm diameter) than the antigen wells (3 mm diameter) and being 4 mm apart as before.

4b:2 Performance of the Test With Samples From Suspected Field Infections

Samples were received from practitioners from herds in which infertility characterised by repeat breeding was the predominant clinical symptom. Many of the farms used both AI and natural service. Other premises were visited by the author when practitioners suspected an infectious type of infertility. All samples were diluted 1:4 in 0.85% saline and homogenized by grinding in Griffith's tubes and centrigued as before. Portions of 2.0 ml were saved for agglutination tests. The preliminary tests reported in section 4a showed that sample dilution may have influenced formation of direct (BS) or stained (AS) precipitation, the remainder of the supernatant was then concentrated by dialysing in 0.6 cm diameter visking tubing (Medicell International, England). The dialysis was performed by immersing the tubing in polyethylene glycol of molecular weight 20,000 (Carbowax, Sigma Laboratories, U.S.A.). The rate of fluid withdrawal from the tubing was observed and when 0. 5 ml - 1.0 ml of the fluid remained and still had a pipettable viscosity the concentration was stopped and the contents of the tubing were transferred to labelled small plastic tubes in which they were stored at -20^{o}C unless tested immediately. The cathodal wells were now filled with a mixture of equal parts of antigen 148 or C383-157 and sonicated antigen C377-19. The first two were the variants of C. fetus subsp fetus isolated from clinical cases

C842 and C383 respectively (See Chapter 6). Antigen C377-19 was from the clinical case C377 (Herd 17) which had not been detected when only sonicated antigen 148 was used. The organism was C. fetus subsp venerealis. Both types were however serotype A. The plates were connected to the electrophoresis chamber buffers using wicks consisting of strips of two layers of Whatman No. 41 filter paper. A constant current of 5 ma/ slide for the 8 x 8 cm slides or 7.5 ma/slide for the 10 x 10 slides was passed for 3 hours. At the end all plates were removed and observed for direct precipitation between the anodal and cathodal wells. These were recorded when present. The plates were then immersed in saline overnight and processed for staining as before. Precipitation lines appearing after staining were also recorded. All samples with precipitation were regarded as positive. Moreover since there was no difference in results between the buffers, the table 4b-1 and 4b-2 below detailing the results only report the effect of pH gradient in the test. Samples are listed with clinical case numbers and animal numbers. During all testing known positive samples in the test (C275 No. 118 and 124) and known negative cases were included as controls.

4b:3 Determination of Infection Status Of Tested Herds

4b:3:1 Vaginal Mucus Agglutination Test

Since the development of antibodies (agglutinins) was first described by Stegenga and Terpstra (1949) and the VMAT for this disease was developed, many authors have concluded that with reservations the test is specific on a herd basis and indicates infection (Hughes 1953; Lawson and MacKinnon, 1952; Boyd 1955; Boyd and Reed 1960; Kendrick 1967 and Clark 1971). This test is therefore used as the standard immunological diagnostic method. The technique used is that recommended in the United Kingdom (MacKinnon 1954; Laing 1960; Anon 1978) and the vaginal mucus was collected by aspiration through sterile glass pipettes

(Pierce 1946). For details of the standard VMAT see Appendix 2. Two antigens, strain 1980 and Norman, the standard antigens used at Central Veterinary Laboratory, Weybridge, England, were provided by Mr. K.P. Lander. In addition two other antigens were prepared from organisms isolated during this work. These antigens were reconstituted in 0.25% formol saline to the same optical density as the Weybridge antigen. These were strain 148 and C377-19. Strain 148 was C. fetus subsp fetus (see Chapter 6) and C377-19 was C. fetus subsp venerealis. These were used when stocks of the official Weybridge antigens could not be obtained. The interpretation of the test was as in Appendix 2.

4b:3:2 Cultural Examination of Vaginal Mucus

Since not all infected animals show agglutinins (Newsam and Peterson 1964; Clark 1971) a negative immunological finding is not diagnostic of absence of infection in the individual animal and indeed immunological diagnosis must be used on a herd basis. Moreover in some infected herds the test may be inconclusive (Garcia et al 1979), and thus still agrees with the statement by Plastridge (1947) "that if you can actually isolate vibrio from the fetus then you can say you know definitely the cow has an infection with this germ. If you test a cow and you do not have a reaction you can never be sure you have infection or not". Thus cultural examination of suspected cows, heifers and bulls is necessary. For the cows and heifers the best time for positive diagnosis is generally between 3 weeks to 3 months post infection (Horlein 1970, 1980). In this work therefore, vaginal mucus collected in the sterile glass pipette was heavily smeared onto blood agar plates to which antibiotics had been added (Shepler et al 1963; Dunn et al 1965) with sterile swabs and the plates incubated for 3 to 10 days at 37°C in a gas mixture of 85% N_2, 10% CO_2 and 5% O_2 (Kiggins and Plastridge 1956). Occasionally anaerobic jars with 'gas paks' (Oxoid, England) without the catalyst capsule were used after it had been shown

that this gas mixture also just supported the growth of C. fetus subsp venerealis. All plates were examined as reported by Agumbah and Ogaa (1979) for the presence of colonies resembling those of campylobacters. Smears were prepared from suspected colonies and fixed by heat, then stained with Gram's dilute carbol fuchsin for 3 minutes in order that the characteristic shape of the organism could be demonstrated microscopically. Colonies which contained campylobacter-like organisms were subcultured for biochemical and serological typing as reported in Chapter 6. On the basis of biochemical tests isolates were classified as feto-pathogenic types (C. fetus subps fetus or C. fetus subsp venerealis) or nonpathogenic types (C. sputorum subsp bubulus and C. fecalis). Cultural examination of bulls will be dealt with in Chapter 6 along with biotypes and serotypes of organisms isolated during this work.

4b:4 Combined Results of Counterimmunoelectrophoresis (CIE), Vaginal Mucus Agglutination Tests (VMAT) and Culture to Determine the Efficiency of the CIE as a Diagnostic Test

The results presented in table 4b-1 and 4b-2 present the findings as revealed by the two immunological tests VMAT and CIE and confirmation of infection on a herd basis by cultural examamination. CIE was performed on Litex HSB and Agarose B with a range of pH from 5.8 to 7.9. Of the two samples used in many cases as known positive controls C275 No. 118 and C275 No. 124, sample 118 was suspicious on VMAT, negative on culture and positive always on CIE. Sample C275 No. 124 was positive on vaginal mucus test, negative on culture but also always positive on CIE. Later the sample C340 No. 65 was used as a known positive sample. The vaginal mucus was positive in CIE, negative in VMAT but C. fetus subsp fetus was isolated from the vaginal mucus in profusion on two occasions. The last isolation, C250 No. 65b was

made only two weeks before the animal calved (see Chapter 6 for details). In Litex HSB the control positives formed two lines of precipitation while only one line was formed in Agarose B. Moreover it took about 1½-2 hours for the precipitation lines to appear in Litex HSB agarose and about 2-2½ hours in agarose B. The lines were also weaker in some cases in Agarose B but could be visualised with dark field illumination.

Some samples were positive in one gel but not in the other, for example C841, No. 8 was weakly positive after staining at pH 7.2 in agarose B only and not at all in Litex. Some samples were positive at some pH levels but not others, for example C769, No. 7 was strongly positive at pH 7.6 in Litex HSB but negative at pH 7.9, while C769-157 was positive at pH 7.9 in Litex HSB and negative at pH 7.6.

Others were positive at all pH ranges, for example C275, No. 124 and 118, and C250 No. 65.

In general as pH dropped from 7.4 downwards the number of positive reactions became smaller, for example in C756, only sample No. 6 could be detected with confidence at pH 7.4 in agarose B while at pH 7.6 in agarose B and Litex HSB, 5 of 9 samples gave visible precipitation lines, see figures 4b-7a to 4b-7f.

Detailed information on all the samples can be seen in Tables 4b-1 and 4b-2.

It can be seen in the tables that the great majority of samples which were detected at other pH values were also detected at either pH 7.6 or 7.9 or both. Because the majority of samples were detected at the pH values 7.6 and 7.9, it was felt that when samples were positive at either pH value no further testing was done below pH 7.6. Therefore in subsequent samples only these two pH values were used for tests.

As described in Chapter 3, a mixed sonicated antigen was prepared from isolate C377 no. 19 (*C. fetus* subsp *venerealis*) and one of

either sonicated antigens C842-148 and C383 No. 157 (C. fetus subsp fetus). Using this mixed antigen it was possible to demonstrate lines of precipitation with mucus samples from case C377, (Herd 17) Nos. 54 and 92b. This is shown on Figure 4b-7a, the second well on the left (C377-54). The infecting organisms, C. fetus subsp venerealis was isolated from both of these animals (see Chapter 6). These mucus samples had been previously negative when using sonicated antigen C842-148 (see Chapter 4a). The mixed antigen was used for all subsequent field samples.

Concentration of ground saline extracts of the mucus as previously described improved the direct (BS) detection of lines of precipitation. After concentration there was always at least one sample which gave a strong clear line in most known cases tested except C767. Thus the clinical case C310 (Farm M) gave two positive samples at pH 7.6 in Litex HSB before staining and one after staining when samples had not been concentrated. This farm was thus considered to be infected on this basis. Two months later samples were received from the same farm for further examination by culture. This time fresh mucus samples from cows were cultured and those for CIE concentrated in polyethylene glycol as described. One of the three samples that had been positive previously was still positive at pH 7.9 before staining. Another sample, being tested for the first time was strongly positive before staining at pH 7.9. This was the sample C849 no. 41. The infecting organism was isolated from this animal and also from another one C849 no.151. No CIE was done on this latter case as there was only enough mucus for culture. The organism was C. fetus subsp venerealis and it was later found out that the bull which introduced infection into the herd 17 (C377 or Farm G) came from this farm. That it was now detected by CIE must have been due to the inclusion of C377-19 in the sonicated testing antigen.

The results obtained are detailed in Tables 4b-1 and 4b-2 below.

Nonspecific precipitation in agarose gels has been reported by Crowle (1973) and Hibrawi et al (1977). In this work it was noted that the problem was most severe in agarose B gels at pH 7.2 and lower and a photograph of this effect at pH 6.6 is shown (Figure 4b-1).

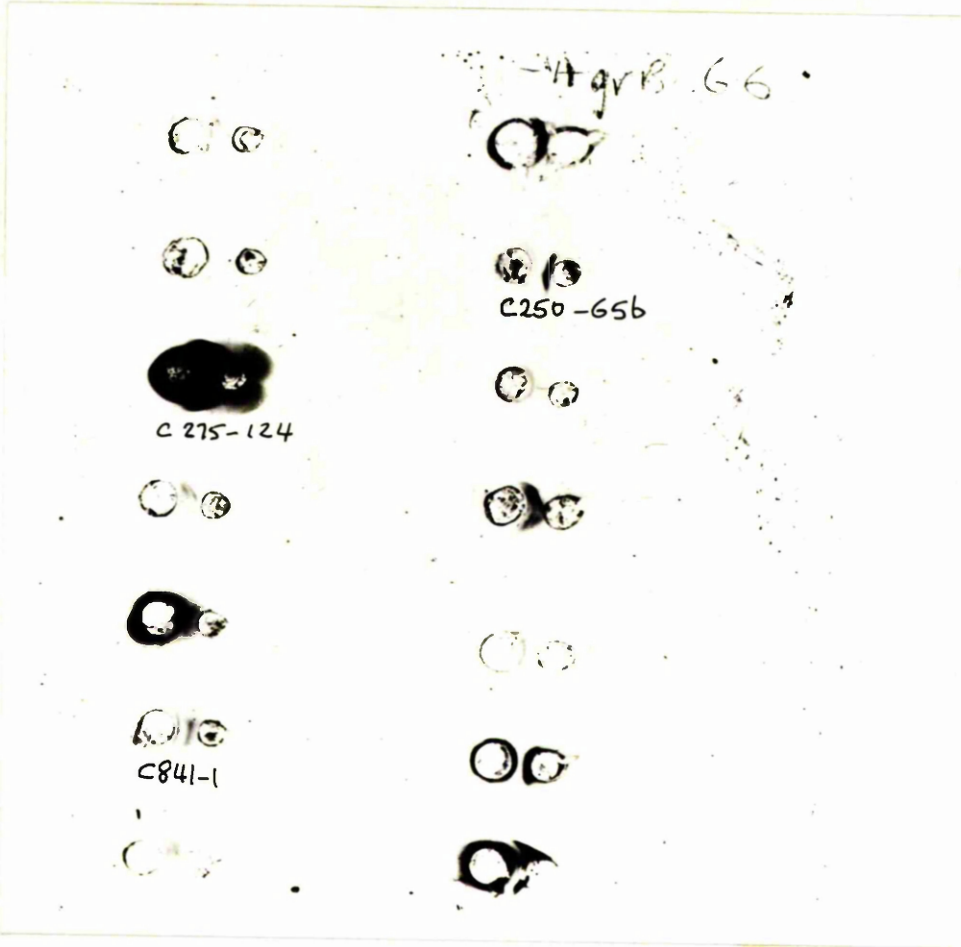


Figure 4b-1: Nonspecific precipitation in agarose B, pH 6.6 barbitone acetate.

In Litex HSB agarose the problem was not evident and Figure 4b-2 shows the reaction of a test sample at pH 6.6 as in agarose B above.

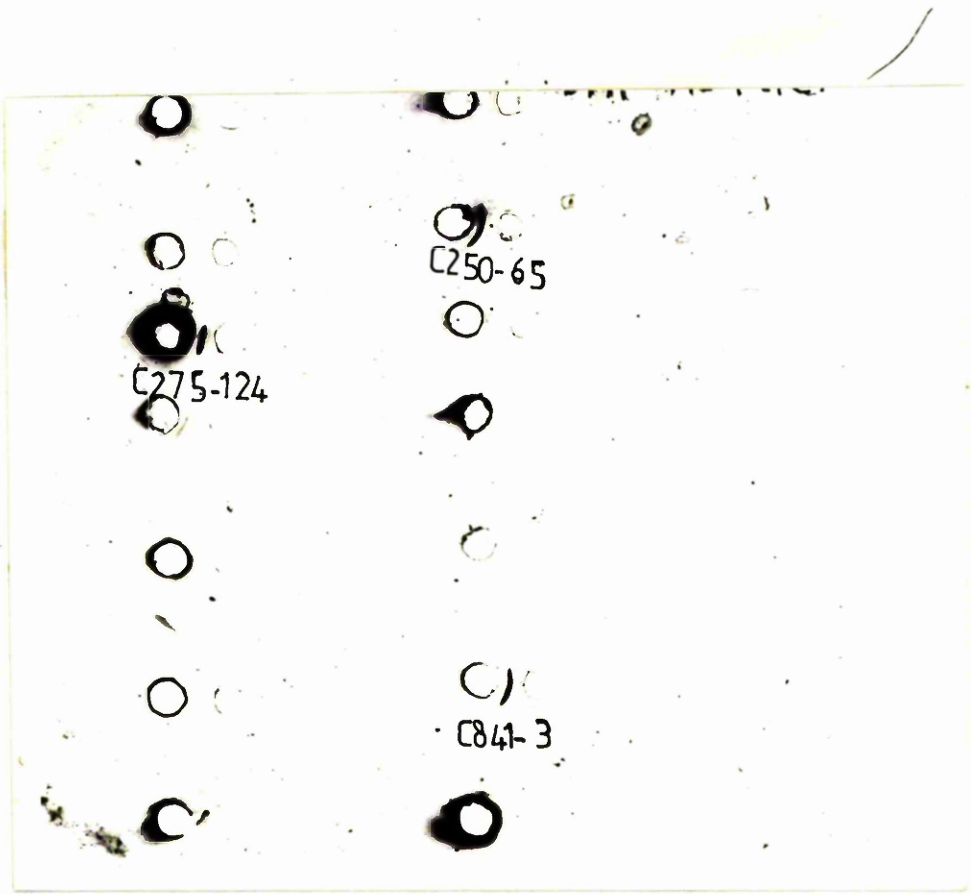
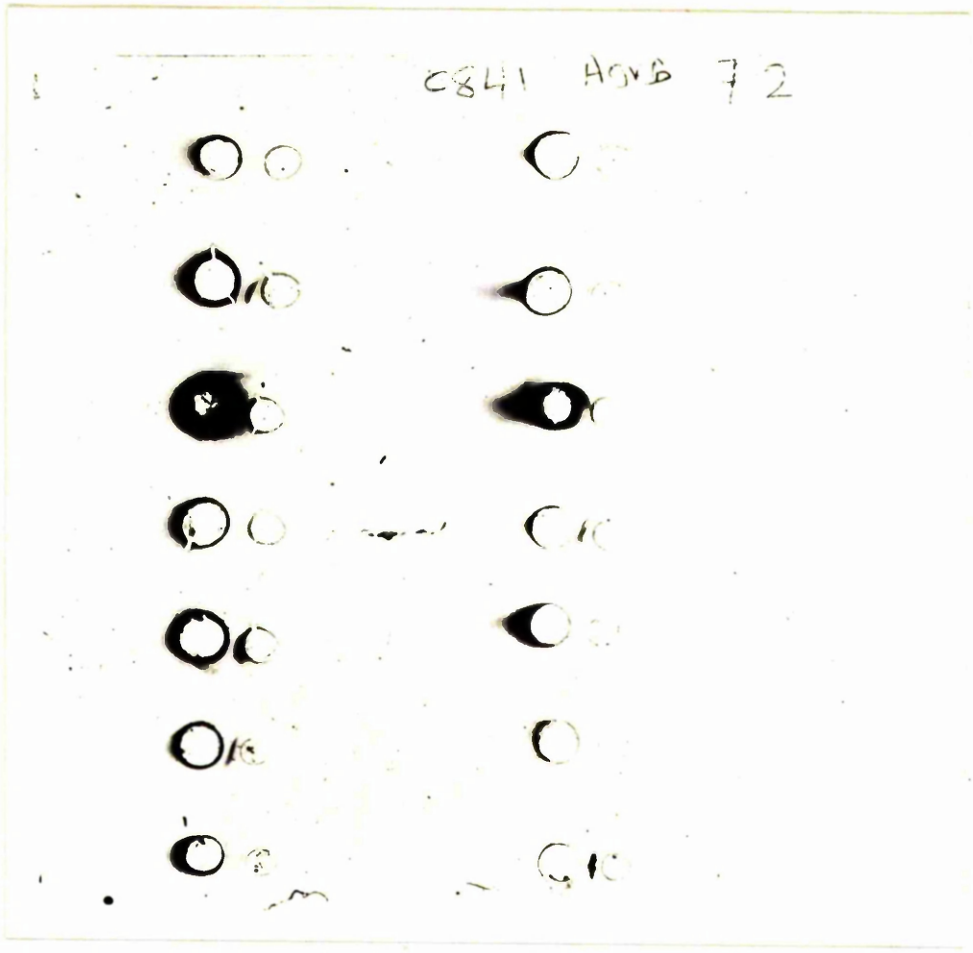


Figure 4b-2: Litex HSB gel, pH 6.6 barbitone acetate. Note the absence of nonspecific reactions seen in Figure 4b-1 above.

One agarose B gel at pH 7.2 is also shown (Figure 4b-3) and



at this pH the problem of non-specific precipitation was less evident. Figures 4b-4 to 4b-7f show the reactions of various test samples at differing pH values at which some samples showed precipitation lines and others did not, and further illustrate the pH effect in CIE. Figure 4b-7g shows total absence of non-specific reaction in agarose B at pH 7.6. No non-specific precipitation was observed above this pH value. By comparing the results from the samples tested at various pH values from pH 5.8 - 7.9 the percentage of samples positive at

each pH value in each gel was calculated (Tables 4b-3 to 4b-4) and from this the histogram of Figure 4b-7h was constructed. The superiority of pH values of 7.6 and 7.9 in both gels is demonstrated.

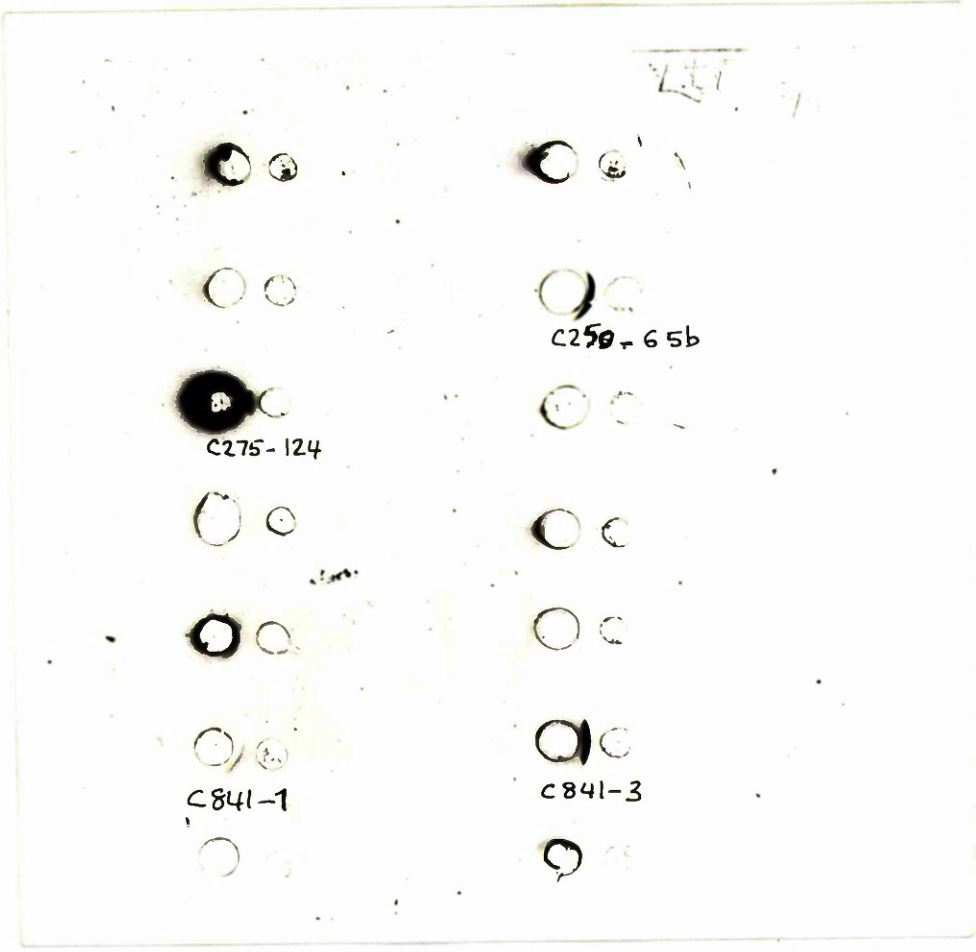


Figure 4b-4: Litex HSB gel, pH 7.4 barbitone acetate. Note formation of directly visible precipitation lines in 3 samples (Nos. C275-124, C250-65 and C841-3), and also a weak stainable precipitation line (No. C841-1). Two wells were not in alignment and thus the precipitation line slants a little as would be expected using this technique.

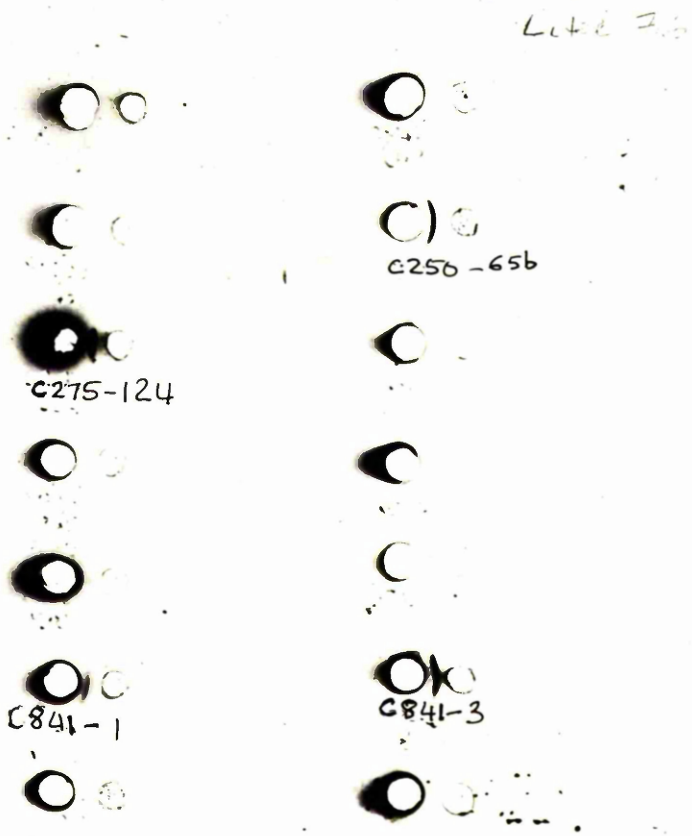


Figure 4b-5: Litex HSB gel, pH 7.6 barbitone acetate. Same samples as for Figure 4b-4. Note shifts in positions of precipitation lines. Also No. 841-1 forms a better precipitation line..

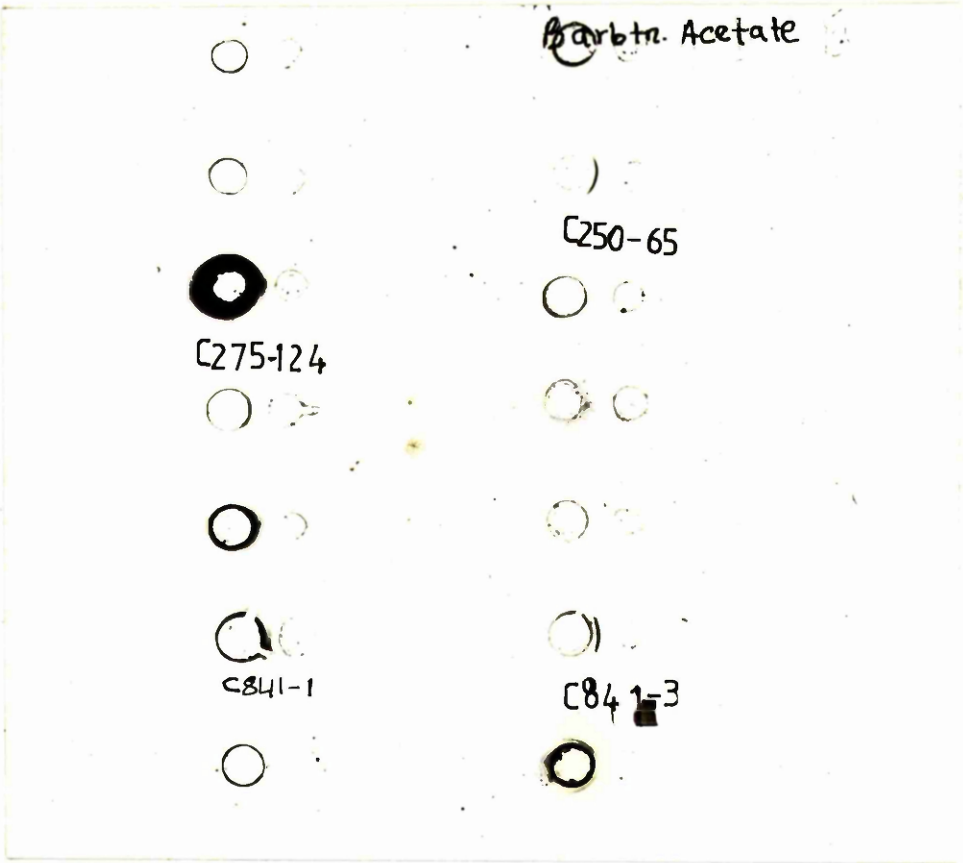


Figure 4b-6: Litex HSB gel, pH 7.9 barbitone acetate. Note finer precipitates at this pH. Well No. 3 shows two parallel precipitation lines, but after staining obscured by the amount of protein material retained by the gel around the well. Sample no. C841-1 shows a weaker precipitin line at this pH which could be difficult to interprete.

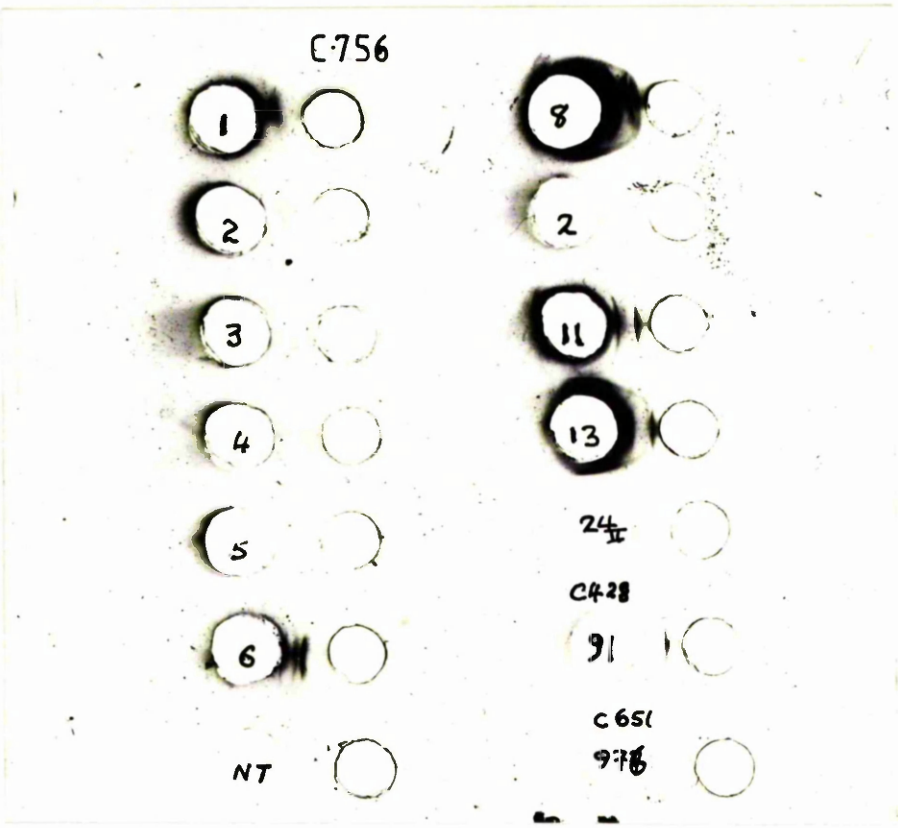


Figure 4b-7a: Litex HSB gel, pH 7.6 barbitone acetate, Sample No. 756. The precipitation lines in 6, 11 and 13 are well defined. No 8 is also detected, but at this pH excess proteinaceous material was retained around the well obscuring the lines of precipitation after staining. C428 No. 91 and C651 976 were included as known +ve and negative controls.

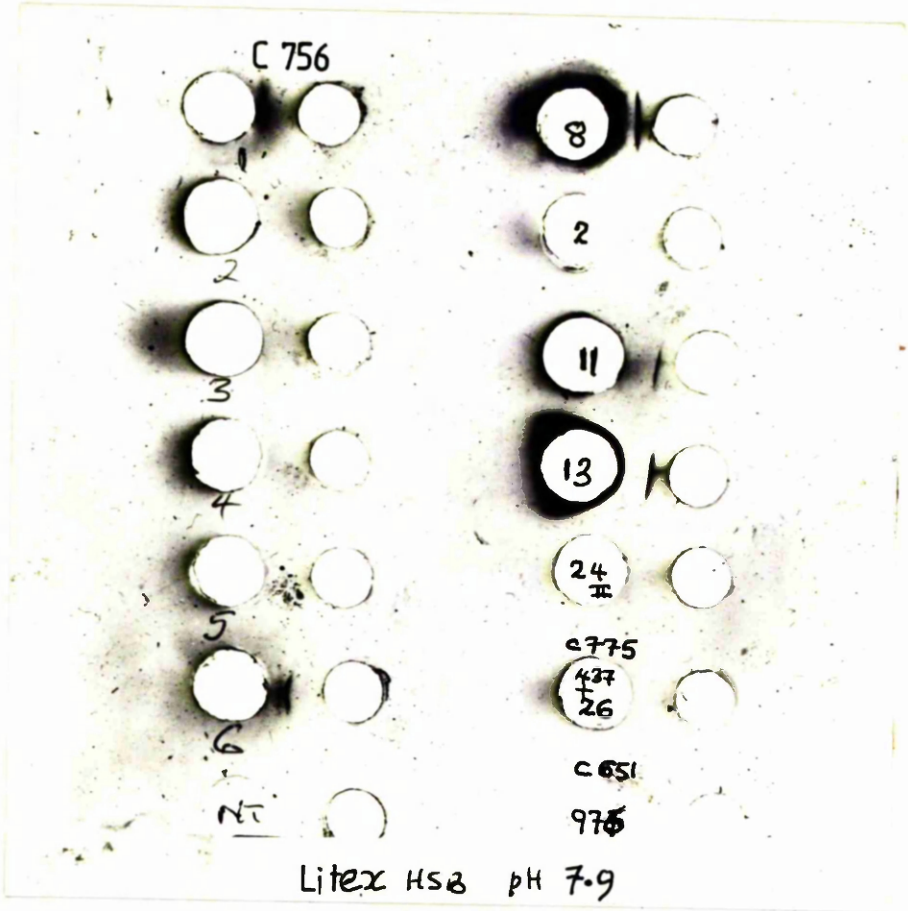


Figure 4b-7b: Litex HSB gel, pH 7.9 barbitone acetate. Note the clearer lines of precipitins in wells no. 8, 11 and 13 which are now single lines.

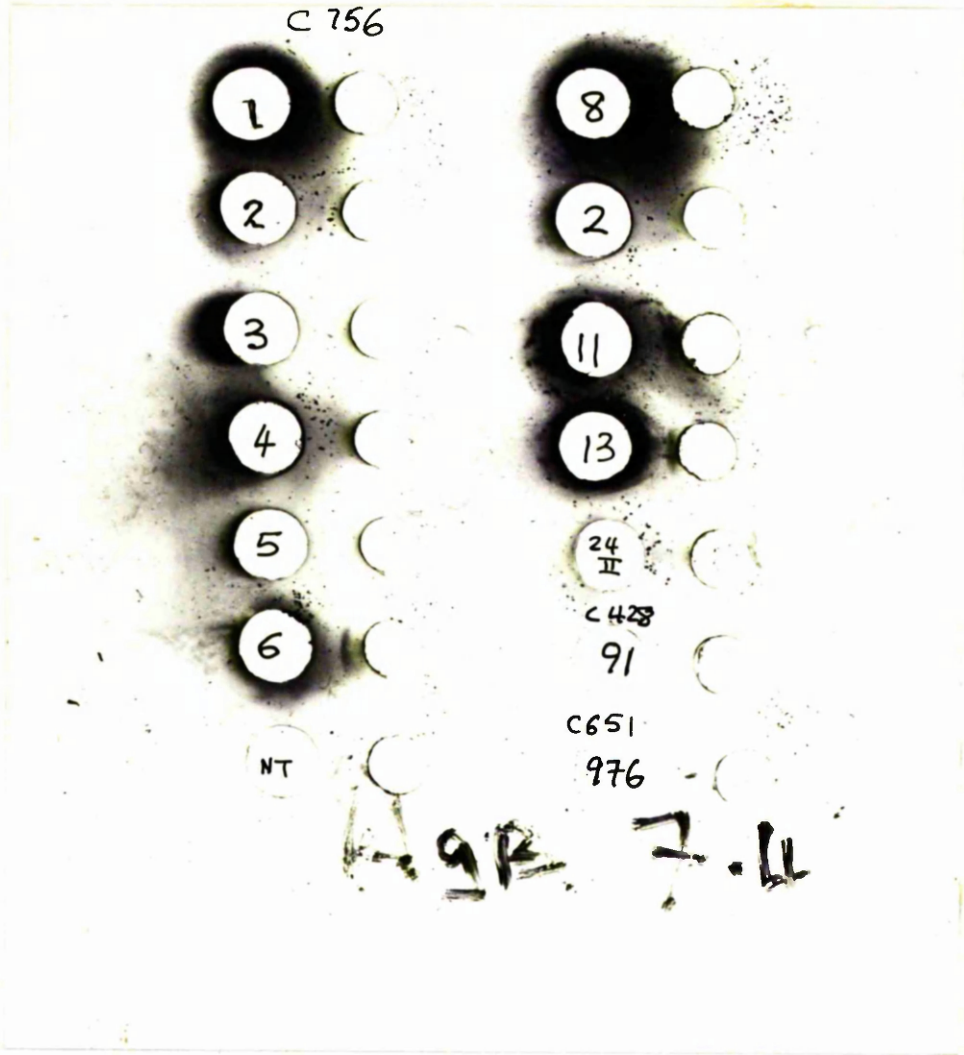


Figure 4b-7c: Agarose B, pH 7.4 barbitone acetate. No. 6 was weakly positive before staining and positive after staining. Nos. 11 and 13 were difficult to interpret before staining and positive after staining.

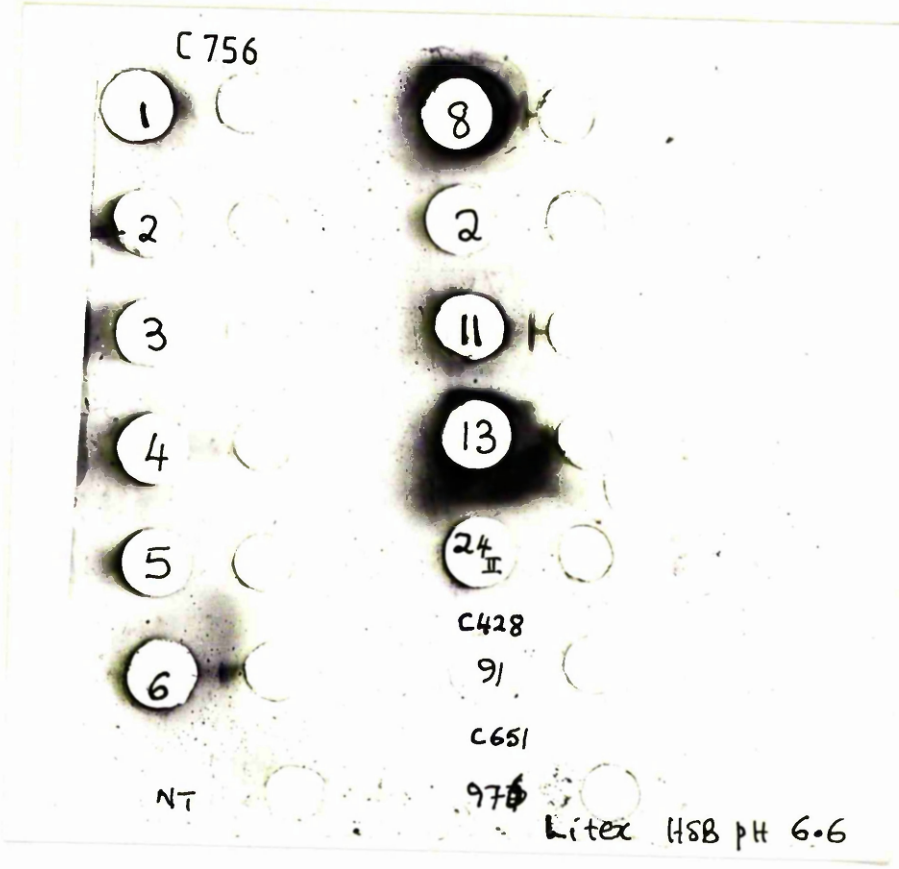


Figure 4b–7d: Litex HSB gel, pH 6.6 barbitone acetate. Notice that now only No. 11 forms a clear precipitate although weak ones can just be seen in No. 8 and No. 6. Again C428 no. 91 is negative (cf Litex HSB 7.6) at this pH.

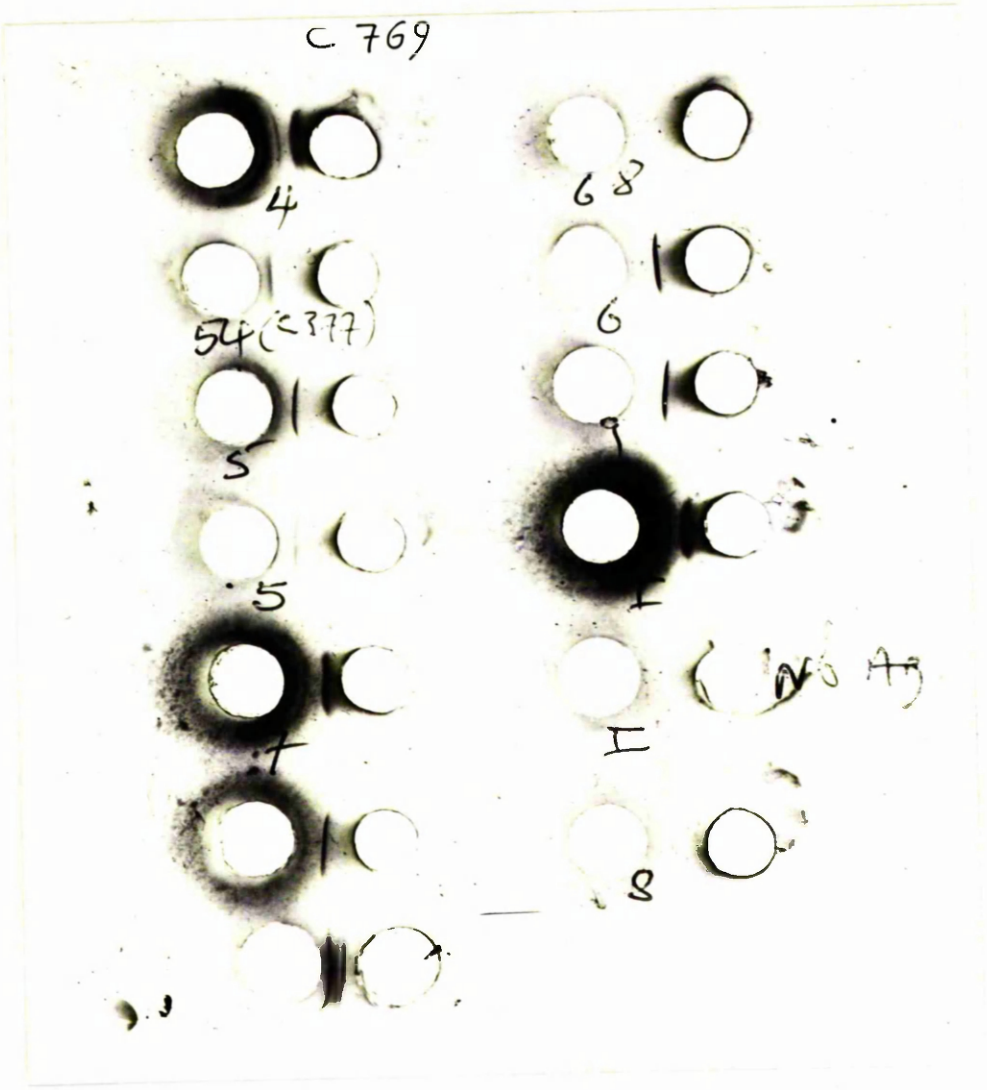


Figure 4b-7e: Litex HSB gel, pH 7.6 barbitone acetate, Sample No. C769 and one sample of C377 - i e. No. 54 (second well on the left). Notice this sample is now detectable by using a sonicated antigen C377-19 in the antigen wells. On the left side too in bottom well Hf 17 was included - the known positive sample from one of the experimental heifers (reported in Chapter 5). Also no. C769-1 does not form any precipitate when no antigen was placed in the right hand side well (Cathodal well) second from bottom but forms line when antigen was added in cathodal well 3rd from bottom right. No. 7 is positive at this pH too. (cf Fig. 4b-7f below).

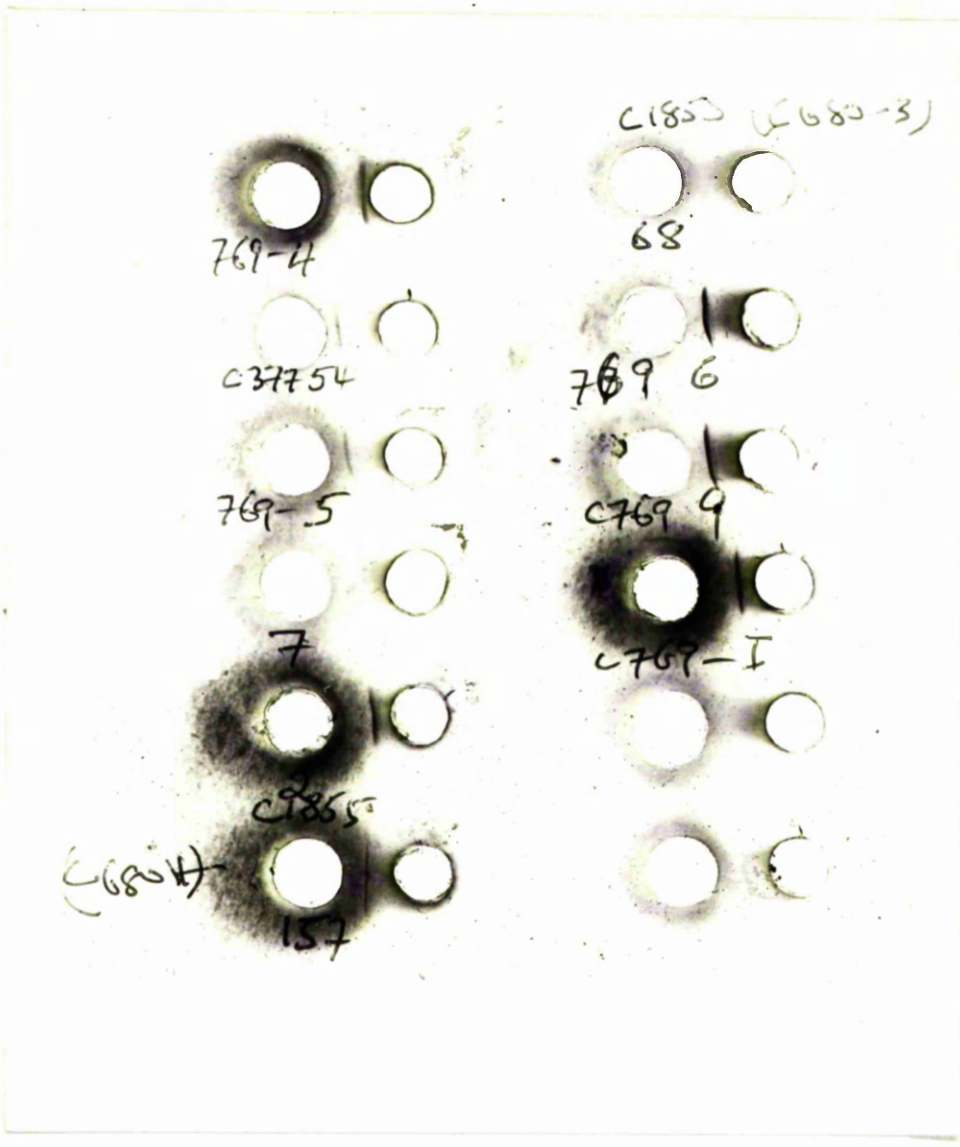


Figure 4b-7f: Litex HSB gel, pH 7.9 barbitone acetate. Notice no. 7 is negative at this pH but C377-54 is still positive even at pH 7.9. No. 2 of C769 was positive here.

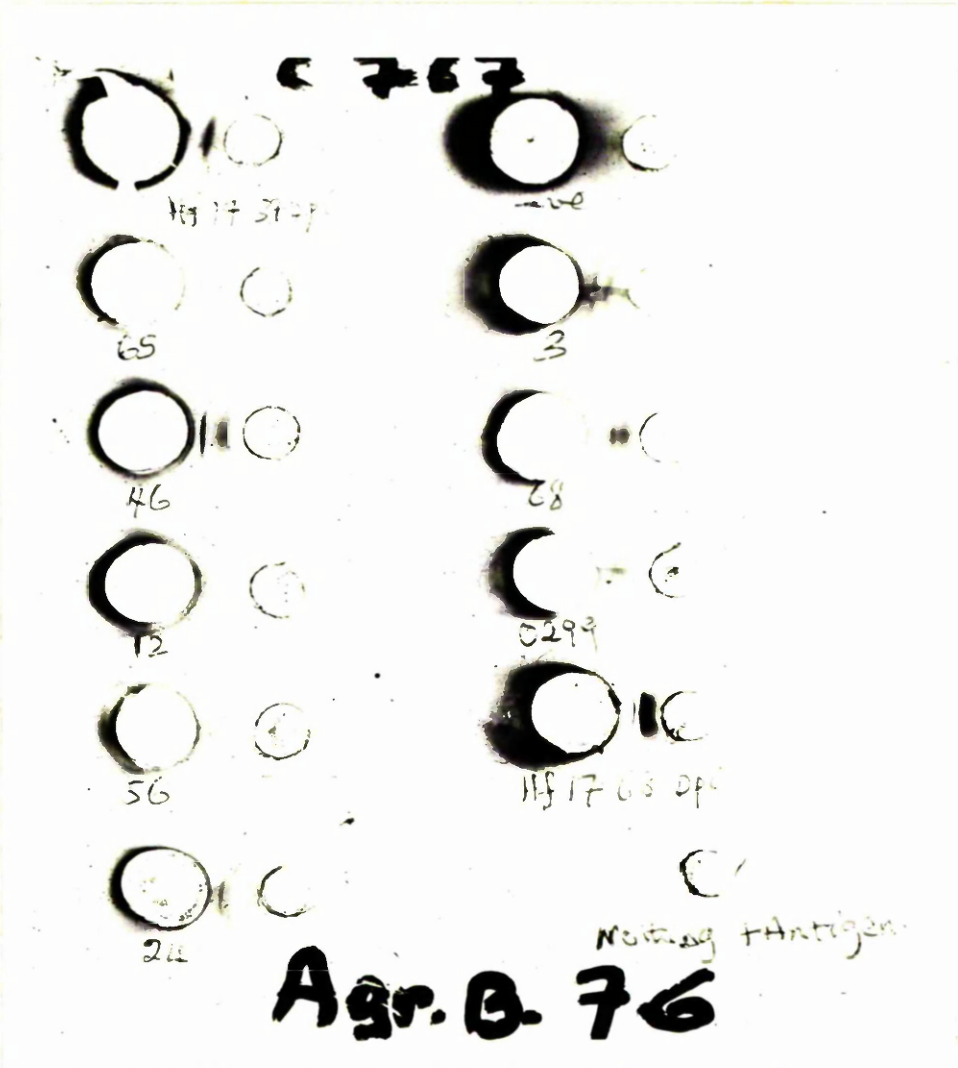


Figure 4b-7g: Sample No. C767 agarose B pH 7.6 barbitone acetate. Note further absence of non-specific precipitation at pH 7.6 in this gel. Also detection of samples No. 46, 24 and 68. Sample Hf 17 (37 Dpi) Hf 17 (68 Dpi) were positive controls. Sample 0299 gives a weak positive result at this pH.

Dpi = Days post infection.

TABLE 4b-1 Litex HSB pH Ranges

Case No. (Farm)	Sample No.	Result at pH Ranges							VMAT Results (Herd)	Cultural Results (Herd)
		5.8	6.0	6.6	7.2	7.4	7.6	7.9		
C841	1	ve+wk AS	ve+wk AS	ve+wk AS	*	ve+wk AS	ve+wk AS	–	+ve	
	11	–	–	–	*	–	–	–	+ve	
	4	Not	tested		*				+ve	C. fetus subsp fetus isolated
	9	–	–	–	*	–	–	–	+ve	
	7	–	–	–	*	–	–	–	+ve	
	10	–	–	–	*	–	–	–	–ve	
	3	+ve BS	+ve BS	+ve BS	*	+ve BS	+ve BS	+ve BS	+ve	
	8	–	–	–	*	–	–	–	–ve	
C351* Not tested										
C275	(124	+ve BS	+ve BS	+ve BS	+ve BS	+ve BS	+ve BS	+ve BS	+ve	C. fetus subsp fetus isolated
C340 or	(118	+ve BS	+ve BS	+ve BS	+ve BS	+ve BS	+ve BS	+ve BS	Suspicious	
C250	(65	+ve BS	+ve BS	+ve BS	*	+ve BS	+ve BS	+ve BS	–ve	
	43	–	–	–	*	–	–	–	+ve	
C377	86	–	–	–	*	–	–	–	–ve	C. fetus subsp venerealis isolated
	99	–	–	–	*	–	–	–	+ve	
	4	–	–	–	*	–	–	–	+ve	

Table 4b-1 Litex HSB pH Ranges (Cont'd)

Case No. (Farm)	Sample No.	Result at pH Ranges							VMAT Results (Herd)	Cultural Results (Herd)
		5.8	6.0	6.6	7.2	7.4	7.6	7.9		
C651	975	–	–	–	–	–	–	+ve AS	+ve	
	982	–	–	–	–	–	–	–	–ve	
	978	+ve AS	ND	ND	ND	+ve AS	+ve AS	ND	Suspicious	C. fetus subsp fetus isolated
	971	–	–	–	–	–	–	–	+ve	
	973	+ve BS	ND	+ve BS	+ve BS	+ve BS	+ve BS	+ve BS	Suspicious	
	976	–	–	–	–	–	–	–	–ve	
	979	–	–	–	–	–	–	–	+ve	
	974	+ve BS	ND	+ve BS	ND	+ve BS	+ve BS	+ve BS	+ve	
	981	ND	ND	ND	ND	ND	–	–	+ve	
C310	61	ND	–	–	–	–	+ve AS	+ve AS	Suspicious	
	31	–	–	–	–	+ve AS	+ve BS 2 lines	+ve AS	–ve	Not cultured but see C849
	36	–	–	–	–	–	–	+ve AS	Suspicious	
	122	–	–	–	–	–	–	ND	–ve	
	151	ND	–	–	–	–	–	–	–ve	
	169	ND	ND	ND	ND	+ve BS 2 lines	+ve BS 2 lines	+ve AS	+ve	

Table 4b-1 Litex HSB pH Ranges (Cont'd)

Case No. (Farm)	Sample No.	Result at pH Ranges							VMAT Results (Herd)	Cultural Results (Herd)
		5.8	6.0	6.6	7.2	7.4	7.6	7.9		
C428	2	ND	–	–	–	–	–	–	+ve	
	195	ND	+ve AS	ND	ND	ND	ND	ND	+ve	Not cultured
	3	–	–	–	–	+ve AS	ND	+ve AS	+ve	
	5	ND	ND	ND	ND	ND	+ve AS	+ve AS	Suspicious	
	4	–	–	–	–	–	–	–	Suspicious	
	91	ND	ND	ND	ND	–	+ve BS	+ve AS	+ve	
Expt. Heifers	Hf 17 (26/9/80)				+ve(wk)	+ve BS	+ve BS	+ve BS	+ve	C. fetus subsp fetus isolated
	Hf 17 (7/10/80)				+ve(wk)	+ve BS	+ve BS	+ve BS	+ve	
C540	874	ND	ND	ND	ND	–	–	+ve BS (wk)	Suspicious	
	873	ND	ND	ND	ND	+ve BS	+ve BS	+ve BS	Positive	Not done see C767 below
	872					–	–	+ve AS	Not recorded	
	871					–	–	–	Suspicious	
C767	56					–	–	–	–ve	
	24					+ve AS	+ve AS	+ve AS	+ve	C. fetus subsp fetus isolated
	65					–	–	–	–ve	

Table 4b-1 Litex HSB pH Ranges (Contd.)

Case No. (Farm)	Sample No.	Result at pH Ranges							VMAT Results (Herd)	Cultural Results (Herd)
		5.8	6.0	6.6	7.2	7.4	7.6	7.9		
C767 Cont'd.	68					–	+ve AS	+ve AS	+ve	C. fetus subsp fetus isolated
	27					–	–	–	Suspicious	
	12					–	–	–	–ve	
	46					+ve AS	+ve AS	+ve AS	+ve	
C849 of 3/11/80	61					–	–	+ve BS	–ve	C. fetus subsp venerealis isolated
	101					–	–	–	–ve	
	2					–	–	–	Suspicious	
	36					–	–	–	ND	
	41					–	–	+ve BS	–ve	
C438	0299					–	–	ND	–ve	ND
C756	1			–	ND	–	+ve BS (wk)	+ve AS	+ve	
	2			–	ND	–	–	–	Suspicious	
	3			–	ND	–	–	–	+ve	FAT on preputial washings from bulls showed many stained C. fetus organisms
	4			–	ND	–	–	–	+ve	
	5			–	ND	–	–	–	–ve	
	6			–	ND	–	+ve BS	+ve BS	–ve	
	8			–	ND	–	+ve BS	+ve BS	+ve	

Table 4b-1 Litex HSB pH Ranges (Cont'd)

Case No. (Farm)	Sample No.	Result at pH Ranges							VMAT Results (Herd)	Cultural Results (Herd)
		5.8	6.0	6.6	7.2	7.4	7.6	7.9		
C756 (cont'd)	11			+ve BS very wk	ND	+ve BS (wk)	+ve BS	+ve BS	Suscpicious	
	13			-	ND	-	+ve AS	+ve BS	+ve	
C769	1						+ve BS	+ve BS	Suspicious	
	2						+ve BS	+ve BS	-ve	
	4						+ve BS (wk)	+ve BS	-ve	
	6						+ve BS	+ve BS	Suspicious	FAT on preputial washings from bulls showed many stained C. fetus organisms
	7						+ve BS	-	Suspicious	
	9						+ve BS	+ve BS	Suspicious	
	5						+ve BS	+ve BS	+ve	
C377	54						+ve AS	+ve AS	ND	C. fetus subsp venerealis isolated
C769	157						-ve	+ve BS	Not done	FAT on preputial washings from bulls showed many stained C. fetus organism

Table 4b-2 Agarose B pH Ranges

Case No. (Farm)	Sample No.	Result at pH Ranges							VMAT Results (Herd)	Cultural Results (Herd)
		5.8	6.0	6.6	7.2	7.4	7.6	7.9		
C841	3	ve +BS	ve +BS	ve +BS	ve +BS	ve +BS	ve +BS	ve +BS	+ve	
	11	–	–	–	–	–	–	+ve AS	–ve	
	9	–	–	–	+ve AS	ND	+ve AS	–	+ve	
Same farm	7	–	–	–	+ve AS	+ ve AS	–	–	+ve	
also as:-	10	–	–	–	–	–	–	+ve AS	–ve	
	4	–	–	–	–	–	–	+ve AS	+ve	Infected
	8	–	–	–	+ve AS	–	–	–	–ve	C. fetus subsp
	1	–	–	ND	+ve AS wk	+ve wk	+ve AS	ND	+ve	fetus isolated
C351	86	–	–	–	–	–	–	–	+ve	
	52	–	–	–	–	–	–	–	–ve	
	11	–	–	–	–	–	–	+ve BS	+ve	
	77	–	–	–	–	–	–	+ve BS	+ve	
	73	–	–	–	–	–	+ve AS	–	+ve	
	107	–	–	–	–	–	–	–	+ve	
	113	–	–	–	–	–	+ve AS	–	Susp.	
	23	–	–	–	–	–	–	–	+ve	
	79	–	–	–	+ve AS	+ve AS	+ve AS	+ve AS	+ve	
	21	–	–	–	–	–	–	–	+ve	

Table 4b-2 (Cont'd)

Case No. (Farm)	Sample No.	Result at pH Ranges							VMAT Results (Herd)	Cultural Results (Herd)
		5.8	6.0	6.6	7.2	7.4	7.6	7.9		
C351 (Contd)	Unmarkd	–	–	–	–	–	–	–	+ve	
C275	+ve (124	+veBS	+ve	+ve BS	+ve BS	+ve BS	+ve BS	+ve BS	+ve	C. fetus subsp fetus isolated
	+ve (118	+veBS	+ve	+ve BS	+ve BS	+ve BS	+ve BS	+ve BS	Susp.	
	con-(65	+veBS	+ve	+ve BS	+ve BS	+ve BS	+ve BS	+ve BS	–ve	
C250	trols(43	+veAS	–	–	–	–	–	–	+ve	As above
C377	86	–	–	–	–	–	–	–	–ve	C. fetus subsp venerealis isolated
	99	–	–	–	–	–	–	–	+ve	
	4	–	–	–	–	–	–	–	+ve	
C651	975			ND	ND	ND	+ve	–	+ve	C. fetus subsp fetus isolated
	982					–	–	–	–ve	
	978			–	+ve AS very wk	+ve BS	+ve BS	–	Susp	
	971			–	–	–	+ve BS	–	+ve	
	973			–	+ve AS very wk	+ve BS	+ve BS	+ve BS (wk)	Susp.	
	976			–	–	–	–	–	–ve	
	977			ND	ND	ND	ND	ND		
	979			–	–	–	–	–	+ve	

Table 4b-2(Cont'd)

Case No. (Farm)	Sample No.	Result at pH Ranges							VMAT Results (Herd)	Cultural Results (Herd)
		5.8	6.0	6.6	7.2	7.4	7.6	7.9		
C651 Contd	974			-	-	+ve BS	+ve BS	+ve AS	+ve	C. fetus subsp fetus isolated
	981			-	-	+ve AS	+ve AS	+ve AS wk	+ve	
Expt. Heifer 17	7/10/80				-	+ve BS	+ve BS	ND		
	48 Dpi				-	+ve BS	+ve BS	+ve BS	Susp.	Experimental Infections
					-	+ve BS	+ve BS	+ve BS		
C310	61				-	-	-	+ve AS	-ve	
	36				+ve AS wk	-	ND	-	Susp.	
C428	3	ND	+ve	ND	+ve AS	+ve AS	+ve AS	+ve AS	+ve	Culture Not Done
	91	ND	ND	-	-	-	+ve AS	+ve AS	+ve	
C767	56					-	-	-	-ve	C. fetus subsp fetus isolated
	24					+ve AS	+ve AS	+ve AS	+ve	
	65					-	-	-	-ve	
	68					+ve AS	+ve AS	+ve AS	+ve	
	27					-	-	-	Susp	
	12					-	-	-	-ve	
	46					-ve	+ve AS	+ve AS	+ve	

Table 4b-2 Cont'd

Case No. (Farm)	Sample No.	Result at pH Ranges							VMAT Results (Herd)	Cultural Results (Herd)
		5.8	6.0	6.6	7.2	7.4	7.6	7.9		
C438	0299					ND	+ve AS wk	ND	+ve	Culture not done
C540	871					-	-	-	Susp	As for C767
C756	1			-	ND	-	+ve BS	+ve AS	+ve	FAT on preputial
	2			-	ND	-	-	-	± Susp	washings from
	3			-	ND	-	-	-	+ve	bulls showed many
	4			-	ND	-	-	-	+ve	stained C. fetus
	5			-	ND	-	-	-	-ve	organisms
	6			+ve very wk	ND	+ve better BS	+ve BS	+ve BS	-ve	
	8			-	ND	-	+ve poor	+ve	+ve Susp.	Culture not done
	11			+ve AS	ND	+ve BS wk	+ve BS	+ve BS	± Prz	
	13			-		Incon-clusive	+ve BS wk	+ve BS	+ve	
C769	1			ND	ND	ND	+ve BS wk	+ve AS	Susp.	As above
	2			ND	ND	ND	+ve BS	+ve BS	-ve	

Table 4b-2 (Cont'd)

Case No. (Farm)	Sample No.	Result at pH Ranges							VMAT Results (Herd)	Cultural Results (Herd)
		5.8	6.0	6.6	7.2	7.4	7.6	7.9		
C769 cont'd	4			ND	ND	ND	+ve 2 lines	+ve 1 live	-ve	Culture not done
	6			ND	ND	ND	+ve BS	+ve BS	Susp.	
	9			ND	ND	ND	+ve BS	+ve BS	Susp.	
	5			ND	ND	ND	+ve BS 2 lines	+ve	+ve	
C377	54			ND	ND	ND	+ve AS	+ve AS	Not done	
C1855	157			ND	ND	ND	ve	+ve 1 line	Not done	
C680-11	7			ND	ND	ND	+ BS	-ve	Susp.	

TABLE 4b-3 SAMPLES TESTED IN AGAROSE OF LITEX HSB AT VARIOUS pH VALUES

pH GRADIENT	5.8	6.0	6.6	7.2	7.4	7.6	7.9
NO. TESTED	27	28	38	18	60	70	68
NO. POSITIVE	8	6	8	5	17	32	37
% POSITIVE	29.6	21.4	21.1	27.8	28.3	45.7	54.4

TABLE 4b-4 SAMPLES TESTED IN AGAROSE B AT VARIOUS pH VALUES

pH GRADIENT	5.8	6.0	6.6	7.2	7.4	7.6	7.9
NO TESTED	26	27	42	40	57	68	66
NO. POSITIVE	5	5	6	13	19	37	34
% POSITIVE	19.2	18.5	14.3	32.5	33.3	54.4	51.5

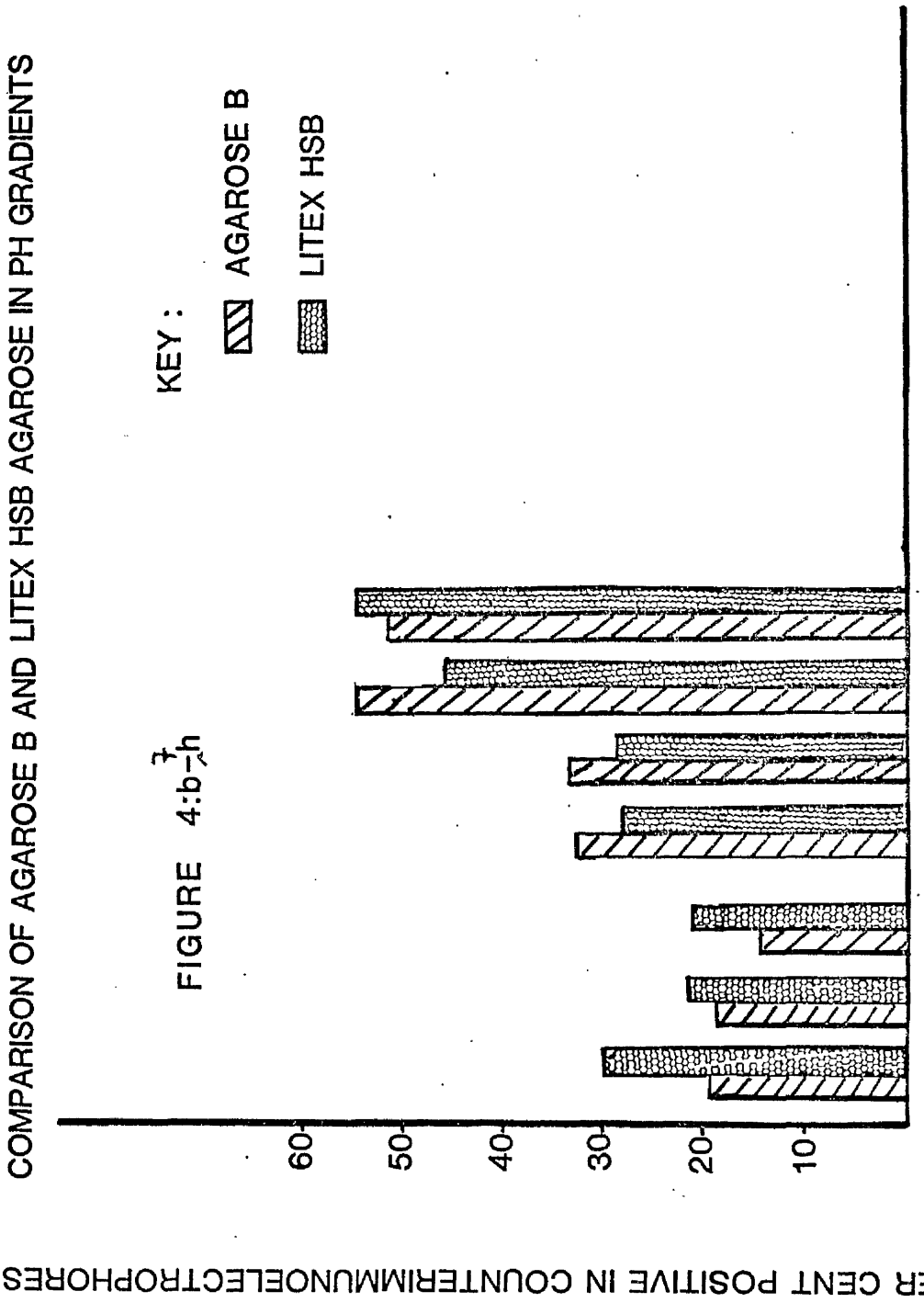

COMPARISON OF AGAROSE B AND LITEX HSB AGAROSE IN PH GRADIENTS
KEY :
AGAROSE B
LITEX HSB
PER CENT POSITIVE IN COUNTERIMMUNOELECTROPHORESIS
60
50
40
30
20
10
5.8
6.0
6.6
7.2
7.4
7.6
7.9
PH GRADIENT

FIGURE 4:b-h

Discussion

A simple rapid and sensitive test for the immunodiagnosis of Campylobacter fetus infection in cattle using the CIE test on vaginal mucus has been developed and applied in field cases. Several technical criteria were essential of which the use of specific agarose types as support media, the buffering of these media at particular pH values, the method of treatment of the vaginal mucus prior to testing and the provision of a wide antigenic spectrum in the crude soluble extract used as the detecting antigen were the most important.

Agarose B and Litex HSB agarose were found suitable. Three other agarose types, Litex HSC, Koch Light Labs Agarose, Gibco Agarose and Difco Noble Agar were tested. Although two of three unsuitable agarose types, Koch Light and Gibco were successfully used in CIE trials with rabbit antisera as reported in Chapter 3, they like Litex HSC agarose were found unsuitable when used with bovine vaginal mucus. Litex HSC agarose was not tested with rabbit antisera. Other workers have found that the quality and purity of agaroses is a major factor in their successful use in diagnostic electrophoretic work (Russell et al 1964 and Hibrawi et al 1977). The latter found that of 22 agaroses tested for the assay of human urinary myoglobin, only six could be used and of these two, Litex HSB and Litex HSC gave best results.

In the system described here, Litex HSC was not suitable, principally because of non-specific precipitation. Vaginal mucus of the cow contains more proteinaceous material than human urine and this could account for the difference. Both Litex HSB and agarose B were found suitable for the test with bovine vaginal mucus as the antibody source. However Litex HSB agarose had the advantage of not allowing excess proteinaceous material in vaginal mucus to move into the reaction space between antigen and antibody well. Agarose B showed non-specific precipitation between

the pH range 5.8 - 7.2. At pH 7.2 and upwards, this non-specific precipitation in agarose B gradually disappeared until at pH 7.6 and 7.9 it was virtually absent. Non-specific precipitation in other agarose types at pH 6.6 to 7.0 in CIE systems has been reported (Holliday, 1980) but it was found that when plates with such non-specific precipitation were washed in alkaline buffers, the non-specific precipitation was removed, implying electrostatic reaction between sample proteins and ionisable impurities in the gels (Hibrawi et al 1977). These unsuitable gels could be purified in polyethylene glycol (Hibrawi et al 1977). Because Litex HSB agarose did not show non-specific precipitation at any pH value studied, it can be recommended. Since agarose B showed no such non-specific precipitation at pH 7.6 and 7.9 at which pH values the majority of positive samples appeared to be detected, it was considered to be as good, provided tests were done only at these two pH values. The one undesirable property of Litex HSB was its brittleness and liability to damage even at 1% w/v concentration. This necessitated the repetition of tests. Other workers (Smyth et al 1976) also reported this disadvantage.

The effect of treatment of vaginal mucus prior to testing in CIE is of practical importance due to the wide variation in the Spinbarkheit of this secretion with the oestrus cycle (Noonan et al 1975). In field samples, from the thin flowing oestral type mucus to the viscid luteal phase material could be collected. In CIE, the mucus has to be solubilized without the breakdown or denaturation of the antibodies. When phenol saline extraction of ground vaginal mucus for antibodies as routinely performed in the U.K. for the VMAT method (MacKinnon 1954) was done, the extracts were non-reactive in CIE even though they contained antibodies which caused agglutination of C. fetus cells. The major reason for this was considered to be due to the effects of dilution in the processing of mucus samples. This was because some 1:4 saline

diluted, homogenized mucus samples gave stainable precipitation lines but when samples were diluted in saline only and then concentrated, not only were the precipitation lines formed but they now became directly visible before staining. Similar effects of concentration of samples prior to CIE testing have been reported (Combridge and Shaw 1971; Jameson 1968; Frey et al 1981). Moreover, staining of immunoplates was found to increase the number of positive reactions detected even after concentration of samples. This was best illustrated by samples of case C767 (24, 46 and 48) all of which were detected by staining although the samples had been concentrated. Thus staining of plates is mandatory for all purposes and agrees with the observations of Combridge and Shaw (1971) and Corkill (1977) that not all apparently negative results as seen at the end of electrophoresis are still negative after staining. Alter et al (1971) however did not find that staining of immunoplates improved the CIE results of sera from patients suffering from Farmer's Lung.

Treatment of samples with sputolysin was found unsuitable. It appeared to lead to breakdown of immunoglobulins as judged by split-line immunoelectrophoregrams obtained when sputolysin treated electrophoresed vaginal mucus antibody was developed with rabbit antibovine (RAB) immunoglobulin sera. However one sample, C993 no. 22, treated for only 15 minutes appeared to have at least the antigen binding parts of the antibody still intact as a broad band precipitation line was formed with this sample which was suspicious in the VMAT when all others treated and negative in the CIE were also negative in the VMAT. That infection actually existed in this farm was confirmed by subsequent visits and isolation of the pathogen. Since the other methods of breaking down the mucus such as use of glass beads and vibrators or sonication also tend to cause shearing forces that may break the antibodies (Creech 1978), the only suitable though tedious method was to homogenize the diluted mucus

in Griffith tubes and concentrate it by carbowax dialysis. Obviously a technical device to break down mucus without interfering with antibodies would greatly help in the application of CIE as it may eliminate the need for concentration.

The pH effects observed in CIE tests is also of great practical value. Most samples that were positive at all pH ranges, 5.8 - 7.9, were also positive at pH 7.6 or 7.9 or both in agarose 8 and Litex HSB irrespective of whether buffer used in the gel was barbitone, barbitone hydrochloride or barbitone acetate. The effect was therefore pH dependent and not buffer dependent. Holliday (1980) had similar experience with CIE for the diagnosis of Legionnaire's disease. Because most samples were detected at pH 7.6 and 7.9 even if not all samples were positive at both, it was decided that not much was to be gained by performing the CIE below pH 7.4. Three samples were positive only below pH 7.4, namely samples C841 no. 8 at pH 7.2, sample C250 No. 43 at pH 5.8 and sample C428 no. 195 also at pH 5.8. All other samples positive at pH 7.4 were also positive at either pH 7.6 or 7.6 and 7.9. It is recommended that samples be examined at both pH 7.6 and 7.9 since some may be positive at one pH and not the other.

This pH effect could involve the antigen specific antibodies or the antigens themselves. At pH values equal to or below the isoelectric point (pI) of a given antigen - specific antibody, the antibody would be either stationary or perhaps move towards the cathode by electro-endosmosis. In either case, little or no antibody will move cathodally to react with its anodally migrating antigen and hence give a negative result. This may affect IgA and IgM in particular as both classes are not only electrophoretically trailing cathodally,, but IgM and secretory IgA (SIgA), the latter likely to be the one found in vaginal mucus, would have larger molecular weights of 900,000 and 360,000-400,000 daltons respectively (Tizard 1977 and Schultz 1978). IgG being more electrophorectically heterogenous could have parts of the molecule spread

out into the narrow reaction space between antigen and antibody wells thus still leading to precipitation into the reaction space which in this case was only 4 mm. It has been shown in humans using diptheria and tetanus toxoid immunisation that the same antigen can evoke an immune response in different individuals with production of IgG molecules with pI varying from 5.0-8.5 but which still have the same antigen specificity (Morrow et al 1981). This phenomenon could apply to C. fetus surface antigens which elicit protective immunity so that in different animals even if the same antibody type was produced, it could have different pI and migrate to the cathode at say pH 7.6 and not 7.9 or vice versa. The isoelectric point of bovine immunoglobulins has been reported to be at pH 7.0 with a variation of 1.5 pH units (Lavon 1972; Brownlie and Hibbitt 1972). This range of pH 6.5 - 8.5 covers the area where results appeared and therefore it is possible that only some portions of the antibodies was available for the reaction as some with pIs in this range may not have participated. Alternatively the pH effect could involve the directional electrophoretic mobility of the antigens. Bohac and Derbyshire (1975) showed that the number of antigens in the virus of transmissible gastroenteritis (TGE) of pigs demonstrable by immuno-electrophoresis varied from one antigen at pH 8.6 to 2 at pH 5.2 and 3 at pH 5.8. It is probable that a certain pH might have been more suitable for the anodal migration of C. fetus antigens. Although the anodal antigens demonstrated (Chapter 3) were detected in gels buffered at pH 8.6 and could be different from those actually active at pH 7.6 and 7.9 where most tests proved positive in the CIE test, this possibility was unlikely to have been of importance unless they had pIs above pH 7.6 and below 8.6 so that they became more or less stationary.

Furthermore the phenomenon of pH effects in the stability of antigen-antibody reactions in a given reaction system could also account for the observations noted during this work. Kleinschmidt and Boyer

(1952) in studying egg-albumin (EA) and antiegg-albuminprecipitin reactions noted that complete precipitation occurred at pH ranges 6.25-8.45. Below and above this, solublisation of the precipitates occurred. Steiner (1955) also observed that the antihuman serum albumin and human serum albumin precipitin reactions were stable between pH 6-9. Singer and Campbell (1955) found precipitation in the same system to be at a maximum at pH 7.5. Gill and Doty (1961) and Gould et al (1964) in studying the precipitin reaction with synthetic polypeptide antigens and their antibody found that the best pH range at which the reaction formed stable precipitation was pH 7.6 -8.7 with maximum precipitation occurring at pH 7.6. In this study the best precipitin reaction between C. fetus soluble antigen and its antibody in vaginal mucus occurred at pH 7.6 and 7.9 and thus this system appears similar to the ones reported by the above authors. Although only a few tests were done at pH 8.6 and none at pH 8.0-8.5, the fact that of seven samples from known infected animals all precipitating at pH 7.9, only 4 precipitated at pH 8.6 indicated that loss of precipitating activity could be noted by doing tests at pH 8.6. Further of those four sampleswhich precipitated at pH 8.6, only two formed precipitation lines adjacent to the antibody well indicating that the alkalinity beyond and between pH 7.9 - 8.6 could most probably only have continued to reduce the number of positives. This aspect may need further investigation.

It was interesting to note that acid pH values which were deleterious to CIE did not affect agglutination (VMAT) as the phenol-saline extracts of vaginal mucus used in these tests were found to have a pH of about 5.8. This could partly explain failure to show precipitation lines in such extracts in the CIE test, but as already stated the additional dilution step in phenol-saline extraction could also be applicable. The participation of presumably the same antibodies in the agglutination test

is however consistent with the knowledge that the electrostatic forces associated with agglutination could still be active even at acid pH values, that the presence of serum or other proteins widen the range of acid agglutination of bacterial cells and that at acid pH values, the amount of specific antibody required to cause agglutination is much lowered (Northrop and de Kruif 1922b, 1922c). Such proteins are present in vaginal mucus of cattle.

Although CIE was found to be applicable to the immunodiagnosis of C. fetus infection in cattle, it is felt that further technical improvements are still desirable. In particular it would be desirable to study further the effect of using lower ionic strength buffer in the support media than in the chamber buffer. This has been reported to improve the sensitivity of CIE by allowing a higher electroendosmotic flow that carries the antibodies to the reaction space (Wallis and Melnick 1971; Combridge and Shaw 1971). This would be more appropriate if Litex HSB agarose was the support medium as it is known to have a low electroendosmotic effect (Smyth et al 1976). Further, if the new variant of this test, the radial-counterimmunoelectrophoresis in cellulose acetate membranes (Rao et al 1979; Gupta and Kolam 1981) were adopted then it might be possible to reduce the time needed since by this variation the authors claim that they could diagnose typhoid fever in man by applying a current for only 4-6 minutes. This would lead to the performance and reporting of many tests on the same day. The VMAT method needs at least 72 hours and could still be inconclusive (Garcia et al 1979) and in its place, culture would be more appropriate to confirm the CIE results. To diversify CIE even further by employing the three well pattern used by Bohac and Derbyshire (1975) so that even cathodally migrating antigens would be reactive in the CIE could be another improvement. One advantage of such an improvement would be the participation of the 'O' antigen in the CIE so that the serotype

of the infecting strain might be known also. However Alter et al (1971) pointed out that this three well pattern may lead to false positive results but he worked with a completely different disease, namely Hepatitis B, in which both antibody and antigen can be found in the same serum. If these modifications were applicable without deleterious effects as to the specificity of the test, then CIE could supercede VMAT as a herd test as it has been shown during this work that positive or negative results were in the main associated with actual infection or absence of infection respectively as revealed by cultural test results.

Perhaps the most significant result in the application of CIE in the immunodiagnosis of C. fetus infection in cattle as shown in this work is the demonstration that antibodies produced in response to the infection are not just 'agglutinins' as they have been popularly known since the work of Stegenga and Terpstra (1949). They can be precipitins as well. Aalund (1968) stressed the fact that identification of one antibody specifically as an agglutinin, precipitin or any other is a remnant of the terminology of the past. That these anti-C. fetus antibodies formed precipitins is however in contrast to the findings of Wilkie and Winter (1971) who failed to demonstrate the precipitin reaction with agglutinating vaginal mucus samples from infected animals except with 'O' antigen extracts from a heterologous organism. This could have been due to loss of antibody by double diffusion. CIE is known to be several times more sensitive then immunodiffusion perhaps by allowing all reactants to meet (Alter et al 1971; Peltier et al 1977; Muhammed et al 1978; Rossiter and Mushi 1980). However it could also have been due to inability of the agglutinating antibody, in their case IgA to be able to diffuse through the pores of the gel used since this was most likely secretory IgA (SIgA) whose molecular weight is about 360,000 - 400,000 daltons (Schültz 1978). IgA is also a poor precipitin

(Tizard 1977) but a very potent agglutinin (Eddie *et al* 1971).

Finally the finding that two bacterial strains (148 and C377-19 or C383-157 and C371-19)when sonicated and the products combined provided the largest and best antigenic spectrum to detect subsequent field infections is in accord with the known antigenic heterogenicity of *C. fetus* (Biberstein 1955; Larson and Ringen 1967). Recently the need to incorporate more than one strain of *C. jejuni/coli* in sonicated antigen to detect complement fixing antibodies in sera of persons suffering campylobacter enteritis was reported (Jones *et al* 1980). Moreover the use of three strains of *C. fetus* to detect antibodies in vaginal mucus of infected cows was advocated (Shekov and Resaschka 1971). It is believed that the heterogenicity of *C. fetus* accounted for the false negative CIE in at least one of the two farms reported in the first part of this chapter. That this was so was proved by including antigen C377-19 along with antigen 148 after which not only were samples from the false negative herd detected by CIE but also samples from one other farm (C310, C849, Farm M). *C. fetus* subsp *venerealis* was isolated from the two farms. Thus biotypic specificity was evident. A part from this phenomenon of antigenic specificity as an explanation for failure to detect infection in some farms, failure could also be due to the stage of infection when the sampling was carried out. Recently infected animals may have 'O' antigen specific antibodies (Corbeil *et al* 1974a, b) and these may not be detected by other antigens such as whole cell surface antigens likely to be in the crude soluble extract used.

CHAPTER FIVE

5:0 EXPERIMENTAL REPRODUCTION OF THE DISEASE AND RESULTS OBTAINED IN DEMONSTRATING THE SPECIFICITY OF THE REACTION

5:0:1 Introduction

Although the preceding chapter has indicated the possible application and the conditions of performance of the CIE test in the immunodiagnosis of *C. fetus* infection in the female bovine, the samples were obtained from the field and the duration of infection was not precisely known. The specific antibodies produced against *C. fetus* vary with the time since initial infection. Furthermore they eventually disappear so that the various immunoglobulin classes and sub-classes may or may not be detected depending both on the method used and the time elapsed since infection (Pedersen *et al* 1971; Wilkie *et al* 1972; Van Aert *et al* 1977).

Because the various immunoglobulin classes tend to be antigen type specific (Corbeil *et al* 1974a, b; Border and Firehammer 1980) the nature of the antigen used is a further variable. Since immunoglobulins produced soon after infection are mainly 'O' antigen specific and the later types both 'O' and whole cell (WC) specific, it would appear that early infections may be missed if a WC specific antigen were used. Although the evidence presented in Chapter 3 shows that the crude soluble antigen used in this work had 'O' antigen in it in agreement with other workers (Winter 1963; Winter *et al* 1978) it is also known that the 'O' antigen has a cathodal and not anodal electrophoretic mobility (McCoy *et al* 1975a). Thus it cannot be reactive in the CIE test. Therefore the test may fail to detect early infections if the antigen were wholly WC specific.

Natural anti-*C. fetus* antibodies of the IgA and IgM class have been demonstrated in sera of animals which have not been exposed to infection

(Winter 1965; Wilkie and Winter 1971). Since exudation of antibodies (at least of the IgG class), from serum into the vaginal mucus of cattle is known to occur (Curtain et al 1971; Wilkie et al 1972; Berg et al 1980) the possibility of such being reactive in the CIE test must be considered. Moreover, cross-reactivity among several members of the species C. fetus may occur and evidence to this effect is presented in Chapter 3 and Chapter 6. Brucella abortus is also known to interfere with the immunodiagnosis of C. fetus infection (Laing 1960) presumably through heterospecific antigens (Nicolleti 1969). These factors therefore touch on the concepts of sensitivity and specificity of the test. The definitions of these terms have been taken in the context of that supplied by Martin (1977). It is also relevant that as in brucellosis, during C. fetus infection immunologically negative, bacteriologically positive individuals may be found. This means that the diagnosis of genital campylobacteriosis must be herd based.

To determine immunoconversion it was decided to infect a group of three non-infected heifers and study the appearance and character of antibodies in the vaginal mucus over a period of one year. Further due to their antigenic specificity, enteric members would not be expected to cross-react with specific anti C. fetus antibodies (Kosunen et al 1980). The group known as C. fetus subsp fetus (intestinalis) may however cross-react with C. fetus subsp venerealis (Clark et al 1975b). This chapter reports studies conducted in ten virgin heifers shown to be free of campylobacter infection, the selection of three of these heifers for infection and the subsequent study of their immune response by the VMAT and CIE techniques. Bacteriological examination over a long period was considered mandatory since VMAT and CIE are immunological tests and this would then obey the 'biological independence' of the means of establishing the 'health status' of the test animals as proposed by Martin (1977).

Progesterone assays were carried out on plasma from the heifers from the day of insemination to determine the oestrus cycle phase and if possible, if repeat breeding occurred, the time of loss of the embryo. It was intended that the heifers should be reinseminated if seen on heat after infection but the need to keep the animals tied up made oestrus detection difficult.

5:1:0 Materials and Methods

5:1:1 Experimantal Animals.

Ten virgin heifers about 15 months old, of various breeds from the University of Glasgow Veterinary Field Station Farm, were examined clinically to establish that they were free from infection with C. fetus and that they were anatomically normal and were cycling. Vaginal mucus was collected as already described. Blood was collected from the jugular or tail vein into heparinized containers. The mucus was cultured and tested for antibodies (VMAT and CIE) as described in Chapters 4 and 6 of this work. The blood was centrifuged at 2000 r.p.m. for 20 minutes usually within one hour of collection. The plasma obtained was stored at $-20^{o}C$ until assayed for progesterone by radio-immuno-assay. All ten animals were found to be suitable for experimental infection and three heifers Hf14, Hf17 and Hf24, were selected for the study.

5:1:1:2 Preparation of Inocula

Isolate C383-157 (Appendix 4) was used for infection. This was classified as C. fetus subsp fetus and belonged to serotype A of Morgan (1959) and Berg et al (1971). It had been recently isolated from an infected cow on farm H2 and had been subcultured only once. Typical colonies were inoculated into universal bottles containing 15 ml of thioglycollate broth (Oxoid) and the bottles were incubated for three days at $37^{o}C$ till turbidity was noted visually. Purity was checked by Gram staining and viability by dark field examination of drops from the

culture. No attempt was made to determine bacterial numbers in the growth because previous observations (Agumbah 1977; Schurig et al 1978) had indicated very wide variations in viable counts per ml of broth culture.

5:2:0 Infection of Experimental Heifers

Oestrus was synchronised in the three selected heifers, now proven to be free from infection, using 500 mcg cloprostenol sodium (BP) (Estrumate, ICI) by intramuscular injection on 18th July, 1980 and 29th July, 1980. On 1st August, 1980 each heifer was inseminated with frozen bull semen in the morning. Six to seven hours later each animal was infected by mid-cervical deposition of 5 ml of 3 day growth of C. fetus subsp fetus strain C383-157 in thioglycollate medium, using a Nielsen catheter.

5:2:1 Examination of Infected Heifers

For the next four weeks and thereafter fortnightly the animals were examined per vaginum using an illuminated vaginoscope. On the day of inoculation and at about 3 day intervals for the next four months, blood was collected from the jugular or tail vein into heparinized tubes and plasma for progesterone assay obtained.

From day 6 post-infection (Dpi) and at intervals varying from 4-10 days in the earlier parts of the experiment (see Table 5:1) and later 3-4 weeks, vaginal mucus was collected, cultured and tested for antibodies (VMAT and CIE). For all CIE tests the combined antigen of strain 148 or C383-157 and C377-19 was used in accordance with the findings in Chapter 4b. The antigens were titrated by the chequerboard method of Papp-Vid and Dulac (1979) described in Chapter 3. The vaginal mucus for CIE tests was concentrated by dialysis against carbowax (Sigma Laboratories, U.S.A.) as before.

To eliminate false negatives from arising from mal-allignment of wells a template of the design below (Figures 5:1 and 5:2) was constructed from perspex. Also gelbond film (Marine Colloids, U.S.A.) was used instead of glass plates. The gelbond film was cut in 10 x 10 cm lengths and with the hydrophilic side uppermost, it was sandwiched between the lower plate (1) and the middle plate (2). The two screws (positions A and B) were then inserted and molten Litex HSB or Agarose B was poured over the gelbond film and allowed to set. Finally the top-most plate (3) was positioned. The 15 well pattern was cut out of the gel as before. It was necessary to let the agarose be quite near its gelling point before pouring so as to avoid under-running.

5:3:0 Specificity of the CIE Test

These tests were conducted on the basis that if the test were specific, then specific antibody only would be involved. This could be demonstrated by -

(1) Preventing the antibody-antigen reaction by absorbing out the specific antibody using rabbit anti-bovine globulins.

(2) Demonstrating the failure of such antibodies to react with strains of C. jejuni and/or C. coli.

(3) Demonstrating that the vaginal mucus antibodies are locally produced and are not serum derived.

(4) The appearance of antibodies sequentially after infection had to be followed by their disappearance as a normal decay phenomenon.

These aspects were tested as outlined below.

5:3:1 Effect of Anti-globulin Absorption of Specific Antibody in the CIE Test

Rabbit anti-bovine immunoglobulin sera of the classes IgA, IgM, IgG_1 and IgG_2 were purchased commercially (Miles Research Products, U.S.A). Starting with 1:4 and later 1:2 dilution of the sera in barbitone buffer

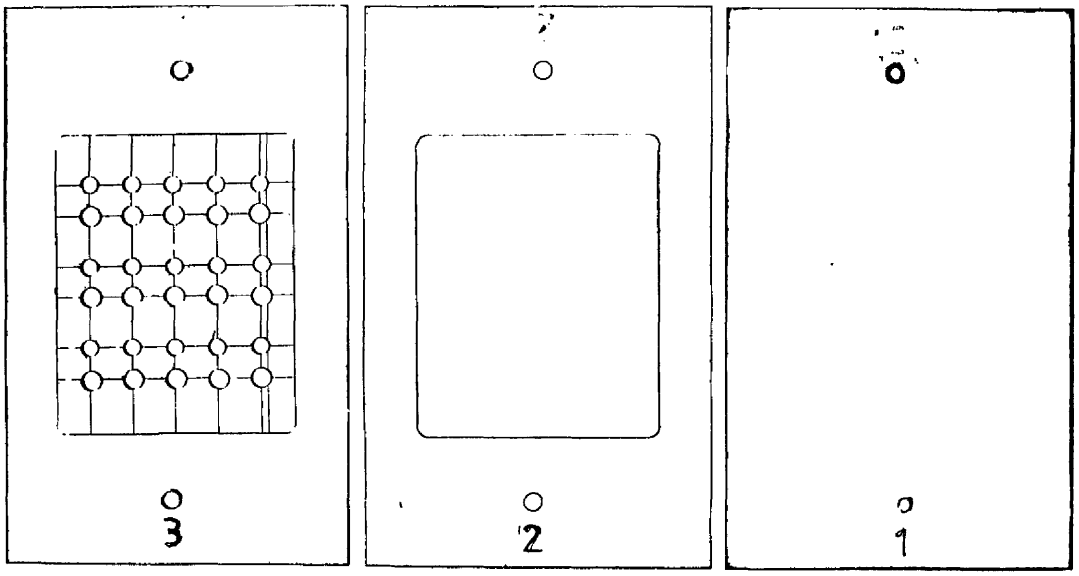


Figure 5-1: Component parts of the CIE template design; the lower plate (1), middle plate (2) and the uppermost plate with the well pattern (3).

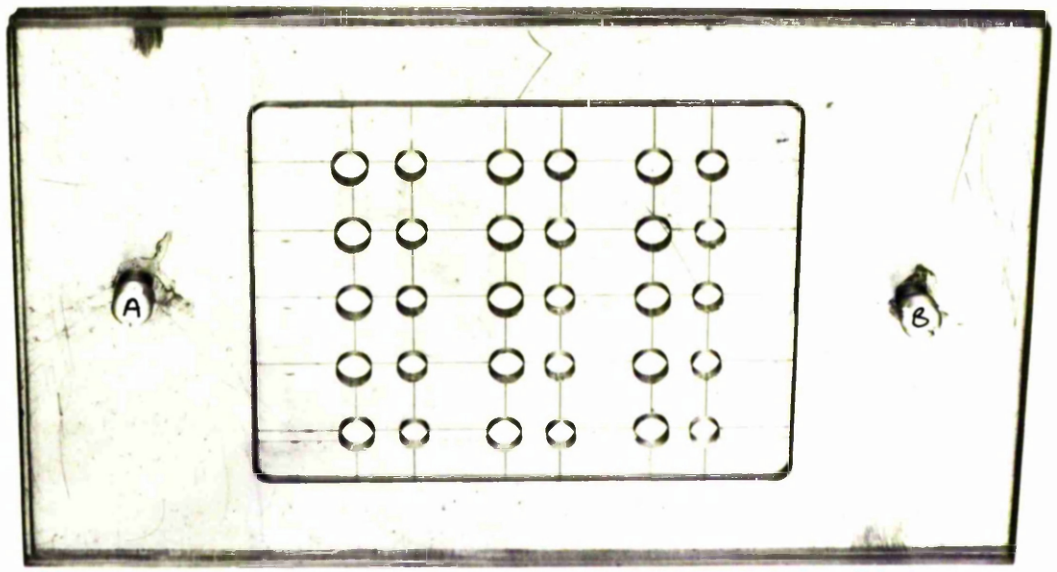


Figure 5-2: Assembled components of the template with the screws A and B not in position.

of the desired pH (i.e. 7.6 or 7.9), the technique of electroimmuno absorption (Daussant and Carfantan 1975) was used. Briefly the wells into which concentrated vaginal mucus was to be placed (anodal) were first filled with diluted rabbit anti-bovine immunoglobulin sera. The plates were placed in a buffer-filled electrophoresis chamber and covered with the lid to prevent the gels from drying out. When the sera had been absorbed into the gels, which took about $2\frac{1}{2}$ hours, the vaginal mucus was placed in the well. The antigen wells were also now filled with titrated antigen, the plates connected to the chamber buffer with Whatman No. 41 filter paper wicks and electrophoresis performed for 3 hours at 7.5 mA/slide. Controls consisted of the same mucus samples put into wells without prior absorption with the rabbit anti-bovine immunoglobulin sera and those into which either saline or corresponding barbitone buffer was allowed to be absorbed into the gel for the same period. If precipitation lines occurred in the controls and not in the anti-globulin treated well pairs, it was interpreted to mean that the particular inhibiting anti-bovine immunoglobulin serum absorbed the corresponding bovine globulin and that the class or sub-class was the major antibody involved in the test.

5:3:2 Reactions of Vaginal Mucus in CIE with Heterologous Campylobacter spp Antigens

Two antigens were used in these tests - namely Belv +c which was classified as C. jejuni/coli group by biochemical tests but did not possess the 'O' antigens A, B and C of Morgan (1959) and Berg et al (1971) (See Appendix 4). This strain had been isolated from a case of human diarrhoea. The other antigen was C. fetus subsp fetus serotype B (NCTC 5850) of Berg et al (1971), but which had some antigens common to isolate 148 (See Chapter 3). Growth and ultra-sonication of antigens have been described in Chapter 3. Appropriate dilutions of both antigens were used as antigen against carbowax concentrated vaginal mucus from infected heifers at various days after infection

and from field clinical cases previously found CIE positive. The test method has been described and either Agarose B or Litex HSB agarose gels at pH 7.6 and pH 7.9 were used in accordance with the findings in Chapter 4b.

5:3:3 Reactivity of Sera from Known Infected Animals in the CIE Test and Comparison with the Corresponding SAT Titres

49 sera were collected from 35 cows known to be infected as were ten from presumably non-infected cows. The infected animals were from proven infected herds and had either given positive culture or were positive to the VMAT test. These sera were tested by CIE against the titrated antigen 148 or C383-157 and C377-19 combined. The sera were tested undiluted since prozone effects due to excess antibody apparently do not affect CIE tests (Cho and Ingram 1973), unlike excess antigen (Crowle 1973). Sera were placed in anodal wells of Agarose gels cast in gelbond film as described above. The conditions of the test were as those found optimal for vaginal mucus in Chapter 4b. Precipitation was recorded after the test and all plates were then washed, dried and stained as before. When precipitation lines were noted before or after staining the serum was considered positive by CIE. Positive controls were of known reactive (CIE) vaginal mucus and negative controls, known CIE negative vaginal mucus. Attempts were made to test Brucella positive sera to determine the possible effect of heterospecific antibodies but only two Brucella positive sera could be obtained. Further, all sera were tested for anti-*C. fetus* antibodies by the serum agglutination test (SAT) (Plastridge *et al* 1947; Van Aert *et al* 1977) using whole cells (formolized) of *C. fetus* subsp *venerealis* strain C377-19 and 'O' antigen of the same organism C377-19 (see Appendix 4 for full classification of this isolate). It was considered that if titres exceeded WC titres (e.g. four fold dilution) then the antibodies were 'O' antigen directed as whole cell 'immunisation' also leads to

'O' antigen antibody formation (see Chapter 6). On the other hand if 'O' titres were lower or absent then it would mean WC specificity. This was considered helpful in interpreting CIE results of the sera since in CIE reactions no 'O' antigen activity would be expected, this antigen being cathodal in its electrophoretic mobility (McCoy et al 1975a).

5:3:4 Pregnancy Diagnosis and Progesterone Assays

Rectal palpation was performed at 37 days post insemination on the 3 heifers, 14, 17 and 24 and again at 48 days to confirm the findings of the first examination.

The stored plasma was assayed for progesterone levels using an LKB Rack Beta Liquid Scintillation Counter and a commercial assay kit (Prog K - International CIS, France).

These assays were performed by Mr C.N. Taylor, F.I.M.L.S., Higher Scientific Officer, Veterinary Laboratory, West of Scotland Agricultural College, Ayr.

Whenever the heifers were seen to return to oestrus they were re-inseminated and the rectal examination and progesterone assay procedure repeated.

5:5 Results

5:5:1 Pre-infection Examination of Heifers

None of the group of 10 virgin heifers showed evidence of infection as revealed by failure to culture Campylobacter spp. from the vaginal mucus and by the absence of any immune response when examined by VMAT and CIE tests.

The 3 chosen heifers were considered to be capable of conceiving since no genital abnormalities were detected and they appeared to be cycling.

Three subsequent cultural and immunological examinations at weekly intervals of vaginal mucus from these 3 heifers failed to reveal any indication of Campylobacter fetus infection.

5:5:2 Post-infection Clinical Observations

The only clinical findings which could be of significance were the observation of an occasional flow of slightly opaque vaginal mucus from heifer 17 and 24 during the first and second week post-infection and the observation in heifer 24 by vaginoscope of an acute cervicitis on day 15 post-infection.

Rectal examination at Dpi 37 and 48 showed that heifer 14 was pregnant to her first insemination. She subsequently delivered a live male calf after 285 days gestation. Heifer 24 and 17 were not pregnant. Heifer 24 continued to return to oestrus on a further 3 occasions but did not conceive following insemination.

The dates of her insemination and the inter-service intervals are shown below:-

Heifer 24

Service number	Date of Insemination	Inter-service interval (days)
1	1. 8. 80	-
2	20. 8. 80	19
3	18. 9. 80	29
4	10. 10. 80	22

The results of the progesterone assays for the 2 non-pregnant heifers are shown in figures 5-3a and 5-3b.

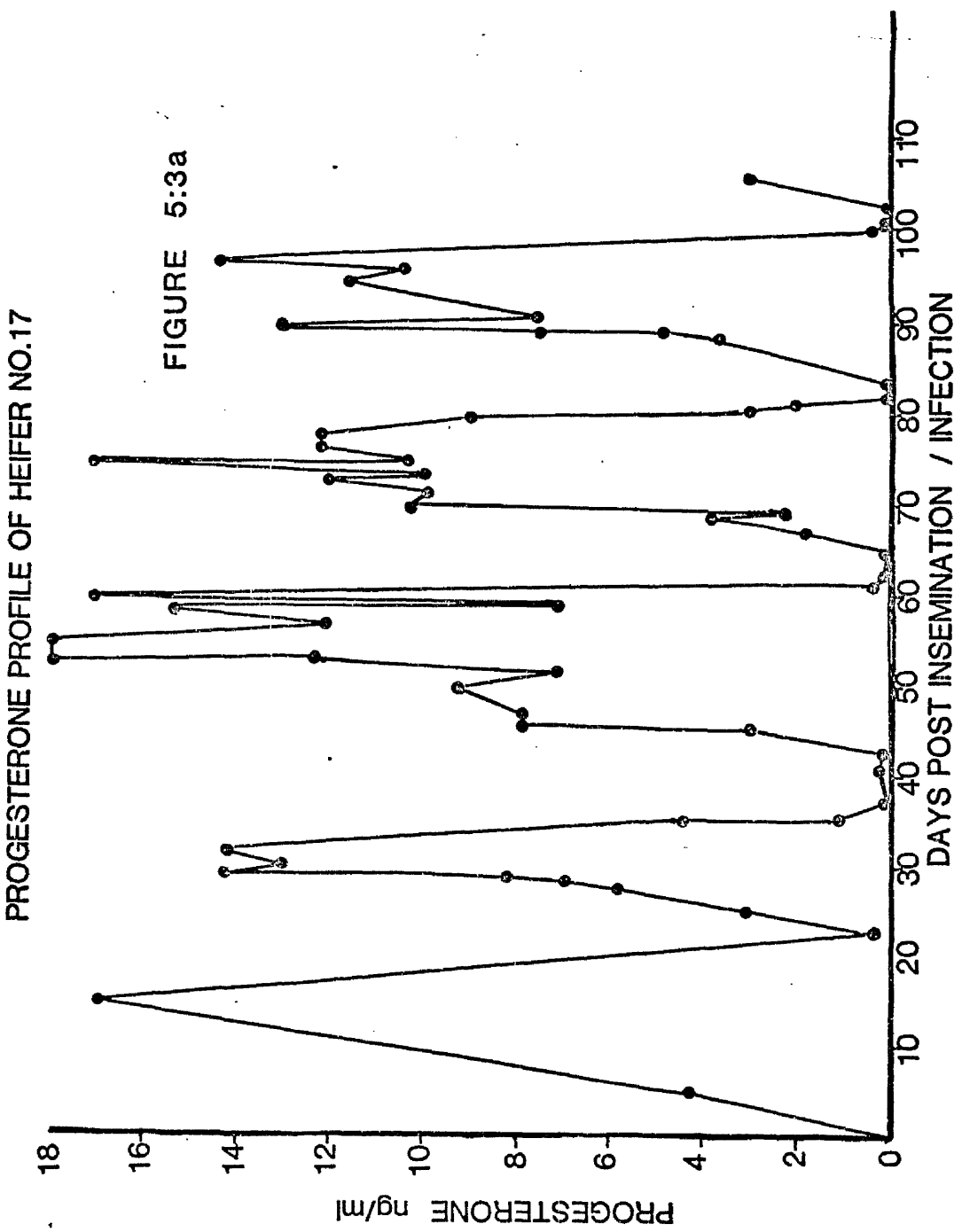
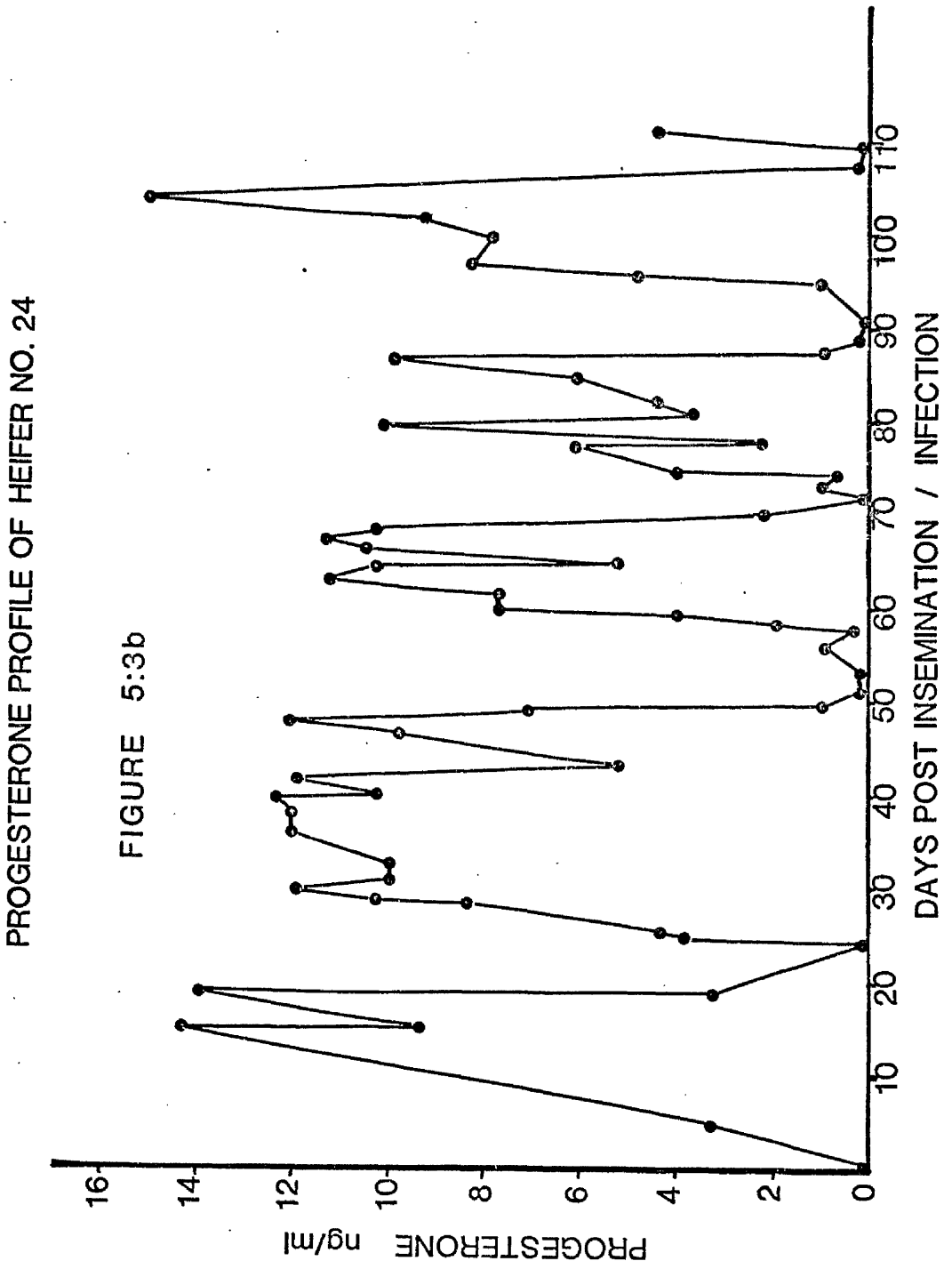

PROGESTERONE PROFILE OF HEIFER NO.17
PROGESTERONE ng/ml
0
2
4
6
8
10
12
14
16
18
10
20
30
40
50
60
70
80
90
100
110
DAYS POST INSEMINATION / INFECTION

FIGURE 5:3a


PROGESTERONE PROFILE OF HEIFER NO. 24
FIGURE 5:3b
PROGESTERONE ng/ml
0
2
4
6
8
10
12
14
16
10
20
30
40
50
60
70
80
90
100
110
DAYS POST INSEMINATION / INFECTION

5:5:3 Results of Bacteriological Examinations of Vaginal Mucus from Experimental Heifers after Infection

The results are shown in detail in Table 5:1. An indication of 'positive culture' (+) in the table, means that an isolate of C. fetus subsp fetus biochemically identical to the infecting strain was recorded. Heifer 14 (Pregnant) C. fetus subsp fetus was first isolated 6 days after infection and excretion of the organism continued throughout pregnancy and until 36 days after parturition, i.e. 321 Dpi. Of 27 samples examined during this period 18 yielded the organism and one culture was overgrown with fungus. From 341 to 383 Dpi 3 consecutive samples were negative. Heifer 17 C. fetus subsp fetus was first isolated 6 days after infection and was isolated until Dpi 127. Of the 13 samples examined in this period 8 yielded positive cultures of C. fetus subsp fetus. Two culture were overgrown with fungus. The organism was not isolated during the remaining 256 days of the experimental period.

Heifer 24 C. fetus subsp fetus was first isolated 6 Dpi and repeatedly until 321 Dpi. Two samples examined towards the end of the experimental period, 341 and 383 Dpi, were negative. Of 25 samples taken up to Dpi 321, 24 yielded the organism. One culture was overgrown with fungus.

5:5:4 Results of Immunological Studies on Vaginal Mucus Following Experimental Infection of Heifers

5:5:4a Vaginal Mucus Agglutination Test

The results are detailed in Table 5-1.

Heifer 14 (Pregnant) The first indication of immunological response was a suspicious reaction 28 days after infection. This was followed by 2 negative test results and the first positive reaction on day 85.

Positive and suspicious results continued until 193 Dpi. From 204 Dpi to the end of the experiment on 383 Dpi, 9 consecutive samples were negative to VMAT. Of 21 samples taken from day 28 until the end of the experiment 5 were positive, 3 were suspicious and 13 were negative.

Heifer 17 The first positive result occurred 23 Dpi. Most samples were positive until Dpi 204 after which only one sample was suspicious at Dpi 302, the rest being negative until the end of the experiment on Dpi 383. During the period 23 to 204 Dpi of 18 samples examined 13 were positive, 2 were suspicious and 3 were negative. Of 8 samples taken between 209 and 383 Dpi, 7 were negative and one was suspicious.

Heifer 24 A suspicious result occurred on 28 Dpi followed by a positive result on 37 Dpi. Subsequently most samples were positive until 302 Dpi. The samples taken on Dpi 321 to the end of the experiment at Dpi 383 were all negative. Of 23 samples examined from Dpi 28 to 383 Dpi, 14 were positive, 4 were suspicious and 5 were negative.

5:5:4b Counter-immunoelectrophoresis

The results are detailed in Table 5-1.

Heifer 14 (pregnant) The first CIE responses were observed on 113 and 120 Dpi when lines of precipitation were detected after staining (AS). These were followed by a sequence of 5 positive reactions before staining (BS) and one reaction after staining from 127 to 180 Dpi. This was followed by one reaction BS at 193 Dpi. From 204 to 302 Dpi all samples were negative. However from 36 days after calving, 321 Dpi, to the end of the experiment at 383 Dpi 3 consecutive samples were positive AS.

Heifer 17 The first response was positive reaction AS on 37 Dpi. This was followed by a sequence of 13 positive reactions, mostly BS, until 193 Dpi. From 204 to 253 Dpi all reactions were negative. However on 291 Dpi a weak, stainable precipitation line was seen at 302,BS and at 341 Dpi a positive reaction, AS, was obtained. The last sample, 383 Dpi, was negative.

Heifer 24 A positive AS reaction was observed on 37 Dpi followed by a sequence of 22 positive reactions, mostly BS, until 383 Dpi when the experiment terminated. However reactions from 302 to 341 Dpi were very faint AS although the sample tested on 383 Dpi showed a readily visible

precipitation line by dark field viewing.

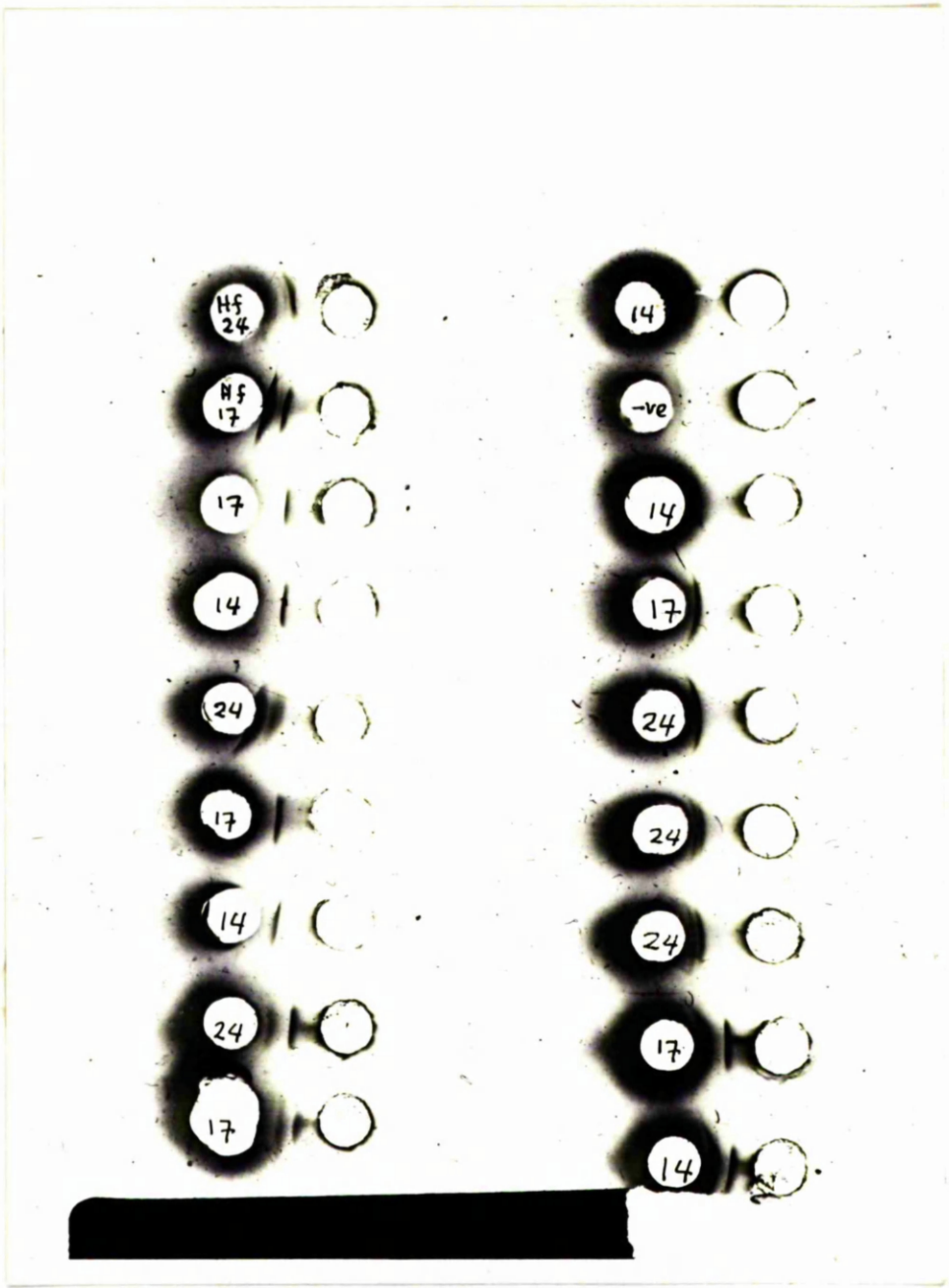


Figure 5-4 shows the CIE reactions for 6 samples collected from each heifer during the period 141-193 Dpi (heifer 14) and 127-193 Dpi (Heifers 17 and 24). During this period all the heifers had been positive to the immunological tests.

5:5:4b Results of Antiglobulin Absorption Tests

These results are summarised in Table 5-2. Figure 5-8a shows that rabbit anti-bovine (RAB) IgG_1 in a 1:4 dilution did not block the reaction. When however a 1:2 dilution was used blocking of the precipitation occurred in all except two samples before staining but after staining partial precipitation lines were detected in some. Figure 5-8b shows a stained immunoelectrophoregram and reveals the presence of precipitation present after staining.

Samples to the right were placed in wells pretreated with RAB IgG_1. This figure shows that the sample in the 2nd well pair on the right was not blocked by RAB IgG_1.

RAB IgA blocked only two samples completely (C756-8 and heifer 24 at 120 Dpi). Six samples were partially blocked and 9 samples revealed no blocking. All these latter nine samples were blocked to some extent by RAB IgG_1. No sample was blocked by RAB IgM or by RAB IgG_2. However in an earlier trial with mucus sample C275-118 used in this work as a known positive (CIE) reactor, both RAB IgA and RAB IgM blocked the reaction but RAB IgG_1 led to formation of a weaker precipitation than was seen in controls.

Table 5:2 excludes the sample C275-118 which had been used to determine the likely usefulness of the method but records subsequent results for RAB IgG_1 and RAB IgA only, since as stated above RAB IgM and RAB IgG_2 did not block any of those samples. Figure 5-9 shows the complete blocking effect of undiluted antiglobulin in samples while saline or buffer (last two well pairs) showed precipitation. Again RAB IgG_1 failed completely to block the sample in the first pair of wells. This was one of the samples completely blocked by RAB IgA.

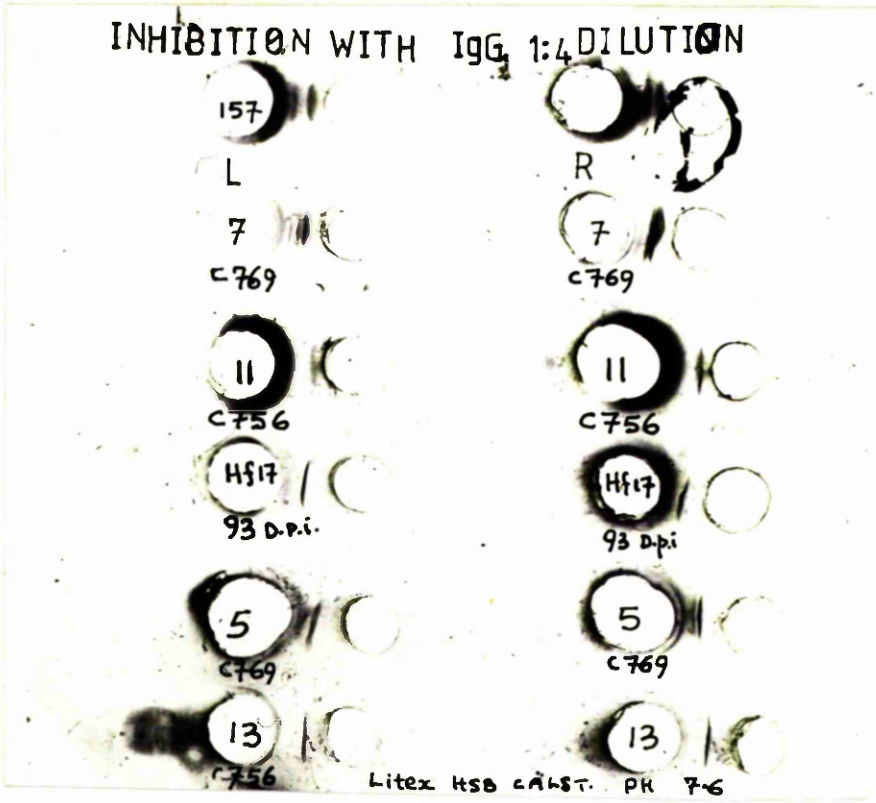


Figure 5-8a: Failure of RAB IgG_1 serum to block CIE positive vaginal mucus samples.

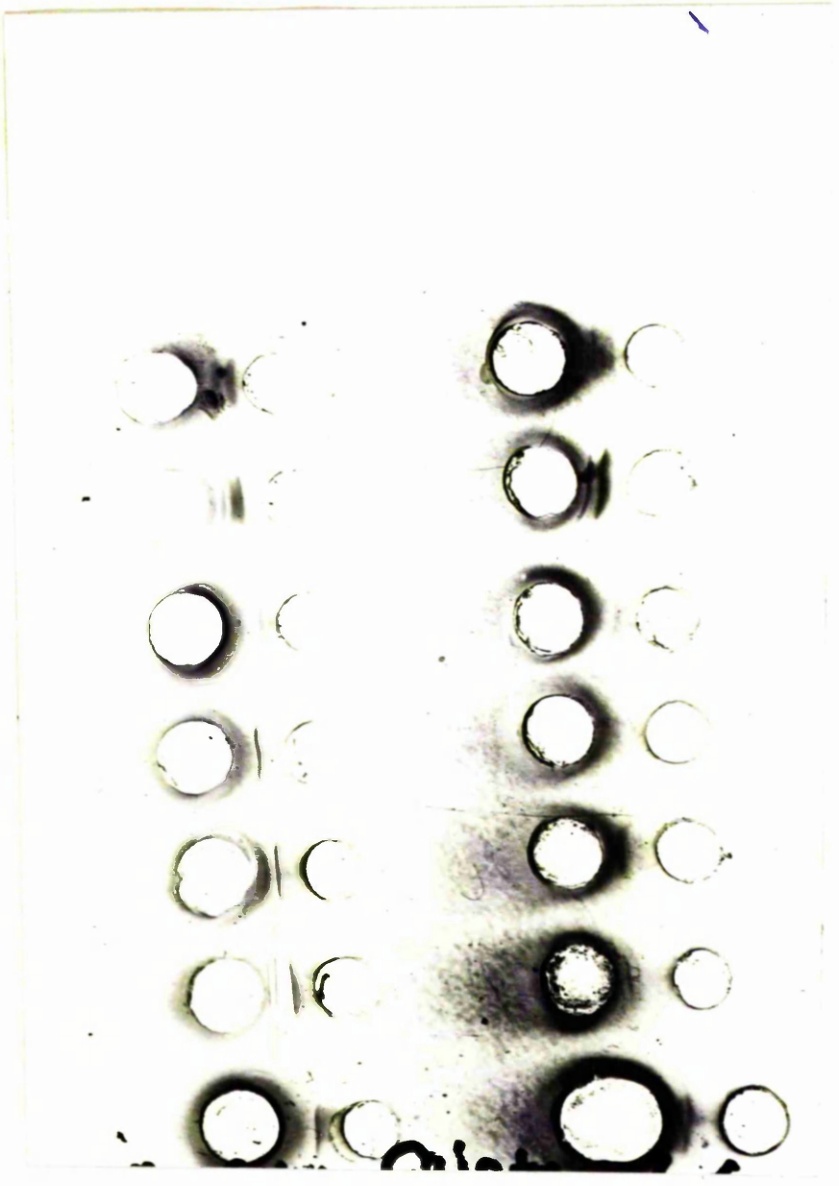


Figure 5-8b: Blocking of some CIE positive samples by 1:2 dilution of RAB IgG_1.

Table 5-2 Samples of Vaginal Mucus with Rabbit Antibovine immunoglobulins. Results for IgG_1 and IgA Inhibition (1:2 dilution)

Sample Identity	Effect of RAB IgG_1 (1:2)	Effect of RAB IgA (1:2)	Presumed predominant precipitating immunoglobulin
C756-8	Not blocked	Completely blocked	IgA
Hf14 113 Dpi	Not blocked	Partially blocked	IgA (others ?)
C769-4	Partially blocked	Not blocked	IgG_1
C769-5	Completely blocked	Not blocked	IgG_1
Hf14-120 Dpi	Completely blocked	Not blocked	IgG_1
C769-9	Completely blocked	Not blocked	IgG_1
C756-13	Completely blocked	Not blocked	IgG_1
Hf24-127 Dpi	Partially blocked	Partially blocked	IgG_1 & IgA
C756-11	Partially blocked	Partially blocked	IgG & IgA
Hf24-120 Dpi	Not blocked	Completely blocked	IgA
Hf17-127 Dpi	Partially blocked	Partially blocked	IgG_1 & IgA
Hf14-127 Dpi	Completely blocked	Not blocked	IgG_1
Hf24-141 Dpi	Partially blocked	Partially blocked	IgG_1 & IgA
Hf17-101 Dpi	Completely blocked	Not blocked	IgG_1
Hf17-120 Dpi	Completely blocked	Not blocked	IgG_1
C756-6	Partially blocked	Partially blocked	IgG_1 & IgA
C769-1	Blocked	Not blocked	IgG_1

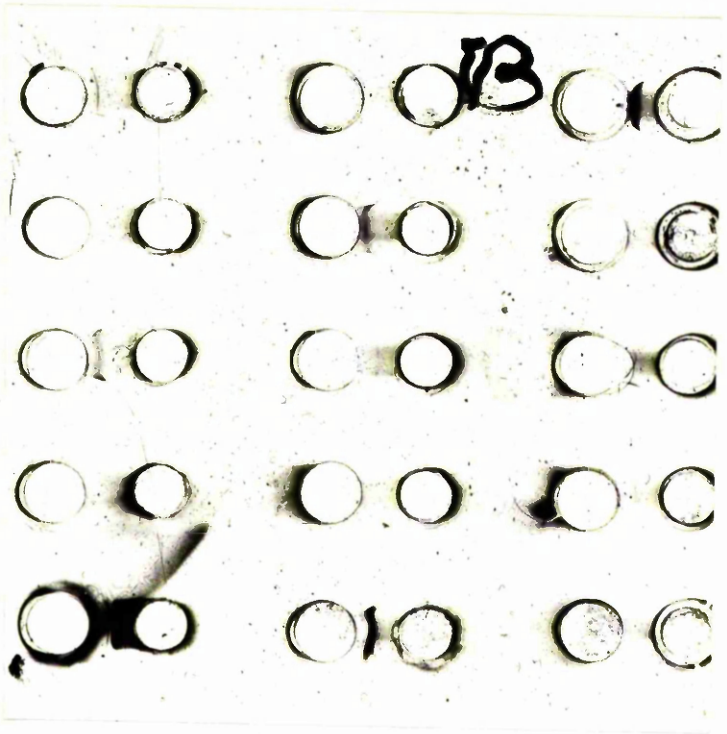


Figure 5-5: Counterimmunoelectrophoregram of known positive vaginal mucus sample, tested against antigen NCTC 5850. Note that a few cross-reactions occurred. (cf Figure 5-6 in which these same samples were tested against the mixed antigen used in this work as control).

Table 5-1 Results of Immunological and Bacteriological Examination of Experimental Infected Heifers

Animal Number	Test Result	Days Post Infection and Insemination																													
		6	14	23	28	37	48	57	68	85	93	101	113	120	127	141	152	162	176	180	193	204	209	239	253	291	302	321	341	358	38
14	Cult.	+	+	+	−	+	+	−	−	+	+	ND	ND	−	−	+	+	OG	+	ND	+	+	+	+	+	+	+	+	−	−	−
	VMAT	−	−	−	±	ND	ND	−	−	+	ND	ND	+	+	+	ND	±	±	−	−	+	−	−	−	−	−	−	−	−	ND	−
	CIE	−	−	−	−	ND	ND	−	ND	−	ND	OG	AS +	AS +	BS +	BS +	BS +	BS +	BS +	AS +	BS +							AS +	AS +	NS	+A
17	Cult.	+	+	−	+	+	+	−	+	+	−	OG	ND	−	+	−	−	OG	−	ND	−	−	−	−	−	−	−	−	−	−	ND	−
	VMAT	−	−	+	+	+	±	+	+	+	+	+	+	+	+	ND	±	−	−	−	+	+	−	−	−	−	±	−	−	ND	−
	CIE	−	−	−	−	+ AS	BS +	ND	BS +	BS +	BS +	+ BS	BS +	BS +	+ ASW	ND	+ BS	+ AS	+ AS	+ve AS	+ BS	−	−	−	−	wk +AS	BS +	−	AS +	ND	−
24	Cult.	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	OG	+	ND	+	+	+	+	+	+	+	+	−	ND	−
	VMAT	−	−	−	±	+	+	+	−	+	+	+	+	±	+	ND	+	+	±	ND	+	+	−	ND	+	±	+	−	−	ND	−
	CIE	−	−	−	−	+ AS	+ BS	+ BS	+ BS	+ BS	+ AS	+ BS	+ AS	+ BS	wk +AS	+ BS	+ BS	+ BS	+ AS	ND	+ BS	wk +BS	wk +BS	ND	+ BS	wk +BS	+ AS	+ AS	BS wk	ND	wk +BS

KEY

ND = Not done, usually sample inadequate or not cultured, too old (>24 hours after collection).

OG = Culture overgrown by fungi.

ASW = After overnight saline wash, direct precipitation seen.

BS = Direct precipitation before staining, always at end of the electrophoresis period.

AS = Precipitation detected by staining immunoplates with Coomasie Brilliant Blue R250.

wk = Weak direct positives, noted by dark field examination of immunoplates.

± = Suspicious

− = Negative

\+ = Positive

5:5:5 Results of Antigenic Specificity Tests with CIE

Thirty mucus samples which showed precipitation lines before staining on CIE(BS) when tested against a mixture of sonicated antigens C842-148 and C377-19, were selected. These mucus samples were tested against a laboratory strain (NCTC 5850) of *C. fetus* subsp *fetus*, serotype B. Eight samples showed precipitation reactions, 5 of these before staining and 3 after staining (Figure 5-5).

Thus 27% of the samples showed cross reactivity. At the same time to ensure that the samples had not been affected by storage 14 of the original 30 samples were tested against soluble combined antigen strains C842-148 and C377-19 and all exhibited precipitation before staining (Figure 5-6).

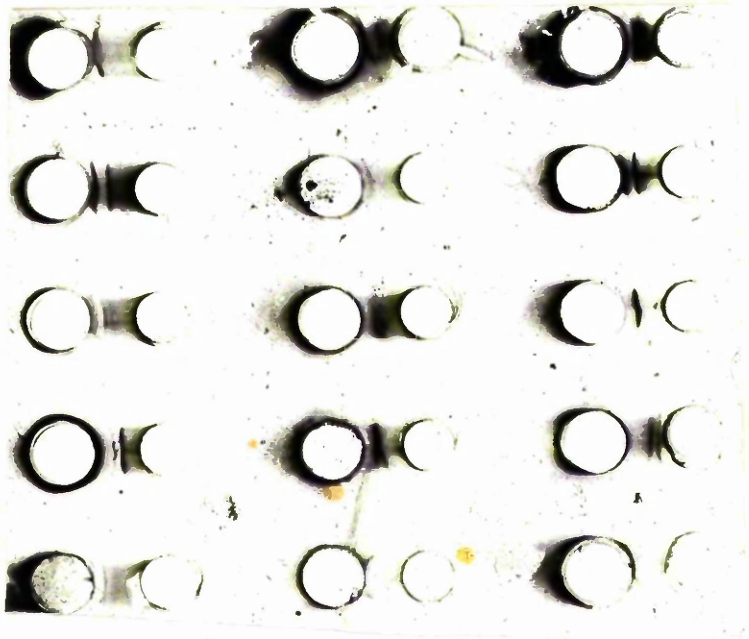


Figure 5-6 : Counterimmunophoregram of positive control samples (as for Figure 5-5) but tested against the mixed antigen 148 and C377-19. All precipitated although the bottom sample in the middle row formed a poor precipitation. This sample precipitated well with antigen NCTC 5850 (see Figure 5-5).

These 14 control mucus samples were also subjected to counterimmuno-electrophoresis using a sonicated extract of C. coli/jejuni (Belv +c) of human origin. With this antigen no sample showed precipitation reactions (Figure 5-7, right hand side). This indicates lack of cross-reactivity whereas with the combined strain 148 and C377-19 precipitation occurred (Figure 5-7, left hand side). For technical reasons the figure shows drawings made from the photographs of the stained precipitation lines.

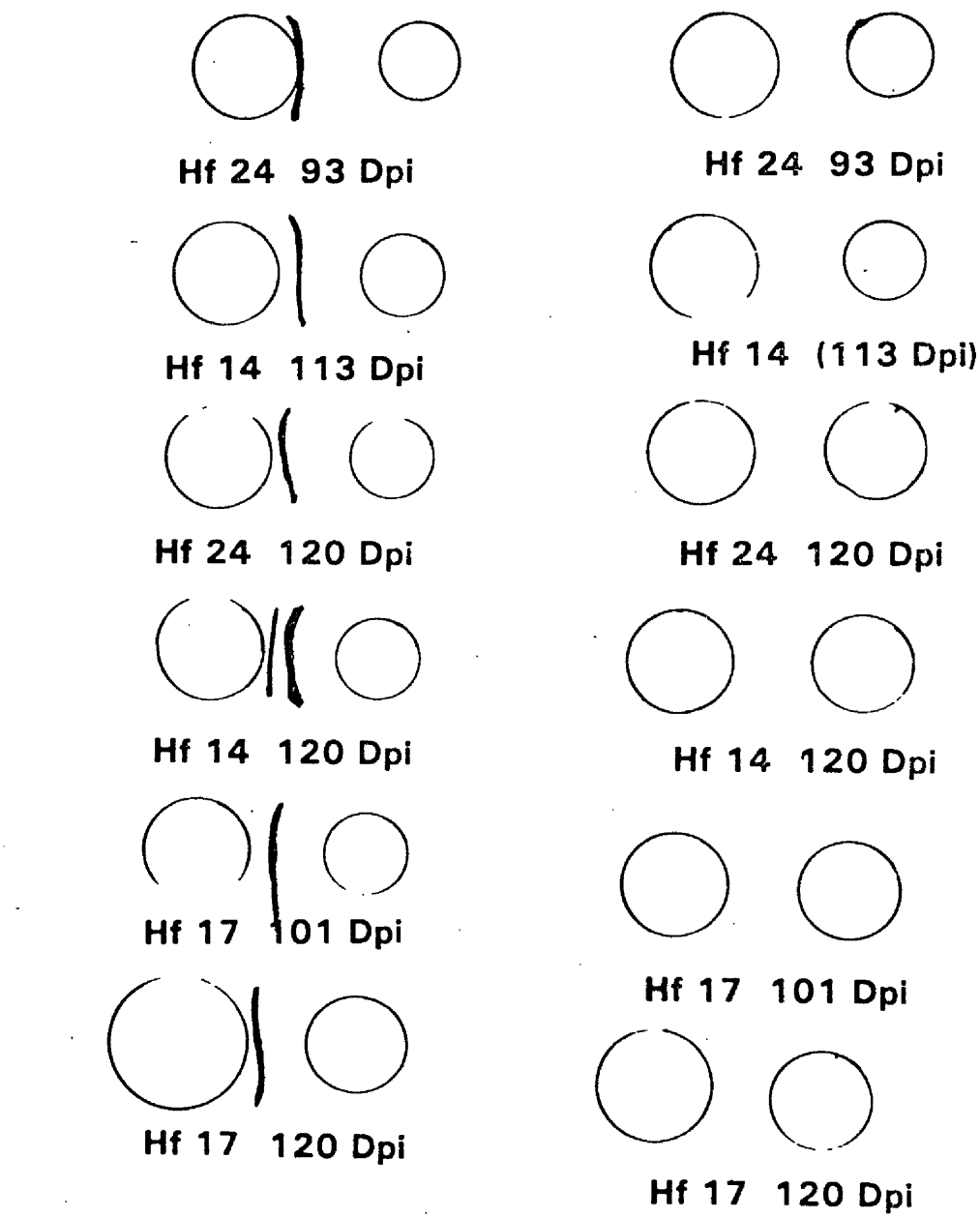


Figure 5-7: Counterimmunoelectrophoregram of drawing/known positives from tested with antigen 148 + C377-19 on the left and with antigen Belv + C on the right. No reaction on the right side indicated absence of cross reactivity of anti-C. fetus antibodies with C. jejuni/coli antigens.

5:5:6 CIE Results with Sera from Known Infected Animals and SAT Results with Whole Cell and 'O' Antigen

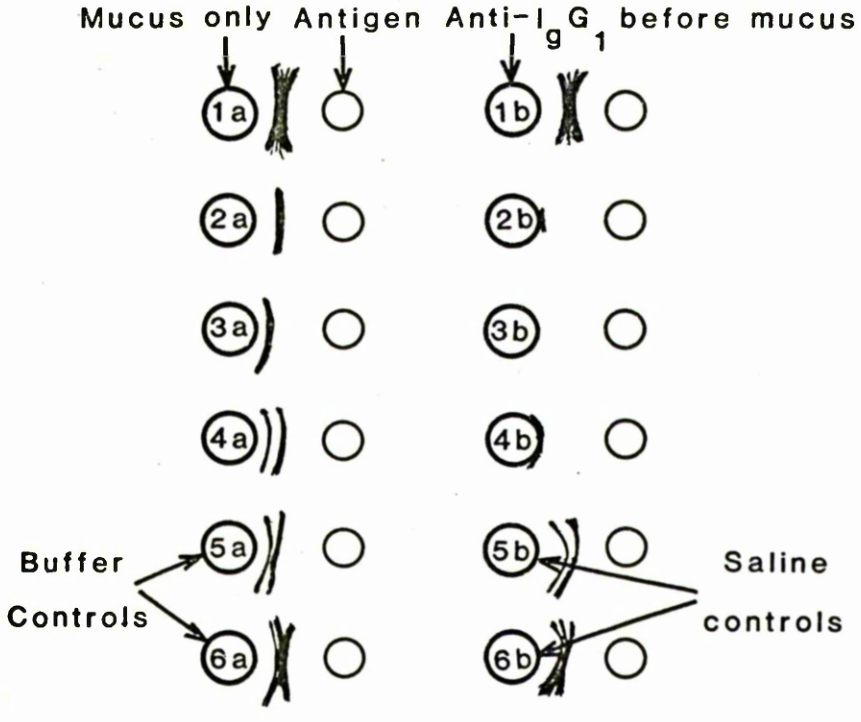
Of the 49 serum samples from the 35 known infected animals (32 naturally infected and 3 experimentally infected) only two gave positive CIE reactions, samples C589-59 and C589-169, both being naturally infected cows. The reciprocal of the whole cell agglutination titre (50 per cent end point) of sample C589-59 was $\geq$ 640 and that of sample C589-169 was 80. The corresponding 'O' antigen titres were 0 and 320 respectively (see Appendix 3). Although the 'O' agglutination titres of the majority of samples ranged from 40-320, these samples did not react in the CIE test, indicating that 'O' antigen specific antibody was not reactive in CIE. Moreover all the animals had this titre range and it would appear that the agglutination was not due to infection, as even the two animals infected with C. sputorum subsp bubulus had similar titres. None of 10 remaining sera from animals of uncertain infection status reacted with antigen in the CIE test. Of these, six were from one farm in which 5 animals were positive to CIE using ground vaginal mucus but which gave inconclusive VMAT results. This is one of the two farms classified as of unknown status in Chapter 4a. The two brucella positive (SAT) samples did not react in the CIE test indicating the fact that anti-brucella antibodies in these samples did not react with the soluble C. fetus antigen used. Figure 5-10 shows the immunoelectrophoregram in which some of the sera were tested. Note that the precipitin formed by the reaction of serum C589-59 and the antigen is centrally placed and is a broad band. That formed by serum C589-169 is at the edge of the antibody well and thin.

5:6 Discussion

The results presented in this chapter showed that vaginal mucus samples taken from 10 virgin heifers had neither agglutinating or precipitating antibodies to C. fetus nor did any of the animals have

INHIBITION of PRECIPITATION BY UNDILUTED ANTI-BOVINE I_gG_1

(Figure 5.9)



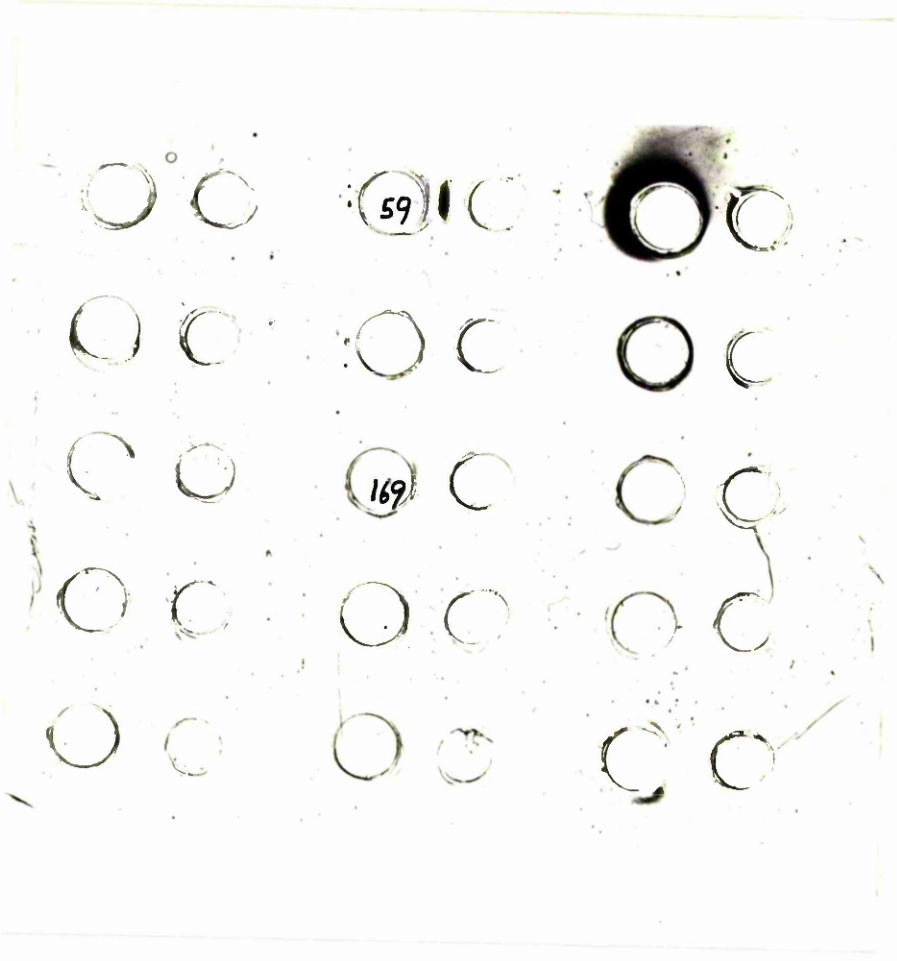


Figure 5-10: CIE positive serum samples C589-59 (first well pair, middle row) and C589-169 just visible (third well pair, middle row). All other sera were negative.

bacteriological evidence of infection. Three of these heifers which were subsequently infected with a newly isolated strain of C. fetus subsp fetus continued to be immunologically negative for 23 days (heifer 17) and 28 days post infection (heifers 14 and 24).

From then onwards up to about 204 days post infection (Dpi), all heifers showed immunoconversion on several occasions. Agglutinating antibodies were first demonstrated at 23 and 28 Dpi and precipitating antibodies were shown for the first time at 37 Dpi. These time intervals agree with previous findings (Newsam 1960; Newsam and Peterson 1964) on the genital immune response to C. fetus infection in the bovine. Variations in immune response by individual animals is known to occur as Newsam and Peterson (1964) showed that some animals had evidence of immune reaction within 6 days of infection.

Positive reactions to the VMAT were the first to disappear while the CIE continued to detect the antibodies for a longer time. In heifer 17, positive CIE reactions were obtained up to 341 Dpi although between 204-253 Dpi the test had been negative. In heifer 14 both tests ceased to detect antibodies after 193 Dpi but from 36 days after calving to the end of the experiment at 383 Dpi, CIE was again positive, albeit weakly. In heifer 24, VMAT ceased to detect the antibodies after 321 Dpi. In this heifer the CIE was positive throughout from 37-383 Dpi although on some occasions the reactions were weak and could only be demonstrated after staining (AS). The failure to detect antibodies by the CIE method until 37 Dpi and the eventual disappearance of the antibodies in two of the experimental heifers were interpreted to indicate that the antibodies were produced in response to genital infection and that with time they decayed or were present in too small amounts to be detected by the method. This phenomenon would indicate the specificity of the test.

The specificity of the CIE test was also illustrated by the fact that pre-treatment of anodal wells with rabbit antibovine immunoglobulin

sera inhibited the precipitation. The inhibition was dose dependent as it did not occur when the sera were diluted 1:4 but occurred at a dilution of 1:2 and with undiluted sera. A similar technique has also been called inhibition counterimmunoelectrophoresis (Milner et al 1972) and was used to determine the occurrence of Dengue Fever precipitating antibodies in human sera (Churdboonchart et al 1981). Although the absorption was not always complete, as revealed by staining immunoplates, it was felt that the degree of inhibition shown was suficient to illustrate that the reaction was that of specific antibody and antigen. Diaz et al (1976) using a similar approach in cases of human brucellosis showed that large volumes of antiimmunoglobulin sera may be required to inhibit the reaction. This might indicate the ability of the test to detect low concentrations of reactants.

On the basis of the inhibition tests discussed above, it would appear that IgG_1 is the major immunoglobulin reactant in CIE and this agrees with the suggestion of Corkill (1977). IgA was also able to block precipitation of some samples either totally or partially and was thus considered to contribute to the reaction. The fact that precipitation lines formed but could not be seen until stained would be compatible with an additive effect whereby in some cases, the observed (direct BS) precipitation was the combined effect of two or more antigen-antibody reactions. The nature of the precipitation line seen at 37 Dpi was diffuse (see Figure 4b-7g). The precipitation lines completely blocked by RAB IgA were also diffuse types. It is likely that the early precipitation could have been mediated by this antibody. It was unfortunate that these early samples were not sufficiently abundant to be used for VMAT, culture and CIE and to leave enough for the inhibition tests which were conducted several months later. The precipitation lines formed at 48 Dpi and later were mainly well defined and thin although one animal Hf17 gave one sharp and one diffuse precipitation line at

68 Dpi (see Figure 4b-7g). These thin sharp lines could be due to the participation of IgG_1 in the reaction or alternatively the reaction between different antibody antigen systems as discussed in Chapter 7. Failure to demonstrate inhibition by IgG_2 in all samples and IgM in all except one sample tested earlier may be considered to indicate absence of the immunoglobulin or their presence in low quantities. Van Aert et al (1977) showed that the appearance of these two immunoglobulins was short lived or variable so it is possible that they were absent during the time of testing.

Antigenic specificity was also demonstrated. The antibodies formed reacted only with antigens from C. fetus organism isolated from field outbreaks of infertility although the organism NCTC 5850 (C. fetus subsp fetus serotype B) cross-reacted with 27 percent of 30 test samples. This was consistent with the known cross-reactivity among C. fetus organisms (Larson and Ringen 1967). This antigenic cross-reactivity among some strains was shown in immunodiffusion studies conducted and reported in Chapter 3. Failure of these antibodies to cross-react with antigens prepared against C. jejuni/coli is consistent with the findings of Kosunen et al (1980) although Véron and Chatelain (1973) reported that isolates from humans that behaved biochemically and serologically as the so called C. fetus subsp intermedius was serologically related to C. fetus subsp venerealis and to some strains of C. fetus subsp fetus . Organisms of C. fetus subsp intermedius were not used in the test and apparently have not been isolated in the United Kingdom (Berg et al 1971). Therefore it is not known if they would cause cross-reactions in CIE tests.

It seems certain the CIE reactive antibodies in vaginal mucus of infected cows are produced in the genital tract because sera of infected cattle were negative to CIE except for two cases. In one of these cases, C589-59, abortion had occurred a few days prior to blood sampling. The vaginal discharge yielded a profuse growth of the organism in pure culture.

It is known that antibodies to C. fetus rise after abortion (Plastridge and Williams 1948) and this was considered a true immunologic response as the whole cell titre of the animals serum was 640 while the 'O' antibody titre was negative (see Appendix 3). This does not explain the CIE reactivity of serum from animal C589-169 whose whole cell titre was only 80. However it can be hypothesised that this animal may have aborted unobserved, and developed an immune response to the infecting organism. At the time of sampling, the immune response may have been wa ning as the animal had attained a negative bacteriological status. Such a situation, however did not occur in the experimental heifer 14 which was culturally positive up to 36 days after calving without serum antibody being demonstrated. The vaginal carrier status of this animal must have led to an anamnestic response as revealed by CIE reactions starting again after she had been negative. Again the local genital nature of antibody synthesis could be illustrated by the CIE reactions in the experimental period 141 -193 Dpi. During this period all 14 of the sera from the experimental animals tested did not react in the CIE test. Vaginal mucus samples collected during this period were abundantly precipitating as revealed by Figure 5-4.

The genital infectivity and tropism of the strains isolated during this work was demonstrated by the ability of one of these strains, C383-157 to colonize the genital tract of virgin heifers for 127 days (heifer 17), 321 days (heifer 24) and 321 days (heifer 14). Heifer 14 carried the organism inspite of being pregnant and calved normally after 285 days of gestation. This has been noted even with C. fetus subsp venerealis organisms (Newsam 1960, 1965b; Frank et al 1964; Clark et al 1968) and may be due to failure of the organism to colonise the uterus, yet become established in the anterior vagina. The organism used here was considered to be C. fetus subsp fetus (intestinalis) and the ability of this strain to proliferate in the genital tract for over 300 days

agrees with the report of Park et al (1962) subsequently confirmed by Florent (1963). That these organisms were also venereally transmitted was revealed by the histories of the cases and is dealt with in the next chapter.

The hormone assays were intended to give information on the ovarian cyclicity during the period of the experiment and possibly to aid in determining whether the time of death of the conceptus could be ascertained as has been suggested by Bulman (1979), Karg et al(1980a). It was unfortunate that with only 3 experimental heifers, one, heifer 14 became pregnant and could not be repeatedly served. The other, heifer 17, did not show overt oestrus or was not detected by the stockman and so was not re-inseminated. During this period rectal palpation for the presence of follicles and corpora lutea indicated that she was cycling. Her progesterone profile Figure 5-3a agrees with the clinical findings. Heifer 24 was served 4 times during the experimental period. From her progesterone profile (Figure 5-3b) she may have conceived at the second service and lost the embryo since her interoestrus interval was also prolonged to 29 days. All her subsequent cycles fell within the normal range of 18-24 days and again her progesterone profile appeared 'normal' during these normal cycle lengths.

However since progesterone profiles reflect the life span of the corpus luteum, they may not necessarily indicate embryonic death or loss. In uterine infections in which inflammatory conditions occur (as would be the case in C. fetus infections) the lifespan of the corpus luteum can either be prolonged or shortened. Prolongation, would give a false impression of the persistence of the luteal tissue being the result of initiation of pregnancy (Hawk et al 1955; Ginther 1968; Shemesh et al 1968). On this basis it is only speculation that the 'abnormal' progesterone profile in the second cycle of Heifer 24 could have been associated with embryonic death since the effect of the inflammation

in prolongation of luteal tissue life could also have been operative in the next 3 months (Dosza et al 1960). During the last 3 cycles, it is possible that the inflammatory condition expected in the uterus may not have been conducive to embryonic attachment so that recognition of the initiation of pregnancy failed and the cycles repeated. It is generally accepted that this is the cause of the repeat breeder syndrome in genital campylobacteriosis as fertilization is known to occur (Adler 1959)

CHAPTER SIX

6:0 DEFINITIVE DIAGNOSIS OF BOVINE VENEREAL CAMPYLOBACTERIOSIS BY CULTURAL METHODS COMPARED WITH IMMUNOLOGICAL METHODS

6:0:1 Introduction

Although immunological methods are of great importance in the screening of samples for presence of antibodies against a given pathogen, the actual isolation and identification of the suspected pathogen is obviously more desirable and unequivocally proves infection (Horlein et al 1964). In bovine venereal campylobacteriosis recent developments indicate that vaccination of bulls and cows or heifers may be the future method of choice for prevention and treatment since vaccination appears to be both protective and curative (Bouters et al 1973). Although vaccination has been more recently found to allow venereal transmission to cows or heifers by a mechanical carrier mechanism (Allan 1972; Clark et al 1975a; Fivaz et al 1978), the vaccinated females apparently did not have reproductive failure and thus for economic purposes it may still be justifiable to perform vaccination. If it were to be the control approach either alone or in combination with other methods vaccination would require the selection of the organism or organisms of a wide biotype and serotype distribution (Clark et al 1976, 1977 and 1979). These can only be known when organisms are isolated. On this basis a multivalenttype of vaccine can be prepared. The identification of biotypes and serotypes has led to the belief that venereal campylobacteriosis is caused by three subspecies of the pathogen classified as —

(a) C. fetus subsp venerealis biotype I of Bryner et al 1962, and same as that of Florent (1959) which is a serotype A organism of Morgan (1959) and Berg et al (1971).

(b) C. fetus subsp venerealis biosubtype I of Bryner et al (1962), and Elazhary (1968) also a serotype A organism of Morgan (1959) and Berg et al (1971).

(c) C. fetus subsp intestinalis serotype B of Berg et al (1971) and described by Park et al (1962) as reported by Berg et al (1971)

On this basis serotype A and serotype C of C. fetus subsp fetus were believed by Berg et al (1971) not to be associated with venereal campylobacteriosis in Great Britain. In Germany, Dedie et al (1977) and in the U.S.A., Whitford et al (1977) also reported that serotype A C. fetus subsp fetus was associated with bovine genital pathology and foetal abortion respectively. The occurrance of bovine genital campylobacteriosis in Scotland has been reported by Deas (1950) and more recently by MacLaren and Wright (1977) and Roberts (1979). In one herd C. fetus subsp venerealis was isolated from 3 aborted foetuses (MacLaren and Wright 1977).

This chapter reports the biotypes and serotypes of campylobacter organisms isolated from herds examined for reproductive failure in South West Scotland. It further points out that serotype A C. fetus subsp fetus is venereally transmitted, has a fetopathogenicity indistinguishable clinically from that caused by serotype A C. fetus subsp venerealis based on breeding records of herds infected with either of the two. Moreover, infection may or may not be detected by the two immunological methods used in this investigation, namely, VMAT and CIE, depending on antigenic and biotype differences. The importance of cultural examination of suspected herds is then discussed with reference to immunological tests and biotyping tests and their use in epidemiology and disease surveillance. During the serological and biotyping tests, faecal campylobacters from cases of human enteritis and from other animal types were included. This was to determine if when using culture techniques in isolating the organism from the field samples of vaginal mucus, there would be any difficulty in

making a decision as to whether an isolate was pathogenic or not without resorting to expensive animal tests.

6:1:0 Materials and Methods

6:1:1 Farms from which Campylobacters were Isolated from Vaginal Mucus: Brief History and Breeding Records of Some Infected Cows

Farm A A herd of 80 milking Ayrshire cattle which had no previous breeding problems except with spring calvers. Three bulls were in use for natural service and one of them, Bull NI, had been in use in another farm where his breeding was apparently satisfactory. Access was denied to this other farm. This bull was introduced to farm A in May 1978 and served about 50 cows within a month, most of which returned to service at irregular intervals. When cows served by bull NI were found not to be in calf, the two other bulls, RF and RT were used. After about 3 more months of poor fertility veterinary advice was requested and the farm was visited on three occasions between November 1978 and March 1979. The vaginal mucus samples designated C749, C842 and C589 in this work were collected from this farm on these visits. Samples of preputial washings were also obtained from the three bulls, NI, RF and RT. As for all farms, both mucus and preputial washings were cultured for campylobacters. The mucus was further examined immunologically using VMAT and CIE as reported in Chapters 4a and 4b.

Farm B A cow from this farm (No. 41) was sent to Farm A and was served by bull NI on 2nd September, 1978. She returned to oestrus on 24th September 1978 and was then served by the farmer's own stock bull (SB). She returned to oestrus a third time on 25th October 1978 and was then served by artificial insemination (AI). Subsequent breeding dates were not available.

On 26th September, 1978 the farmer used his bull (SB) to serve one of his cows No. 26. This cow returned to oestrus on 22nd October 1978. The bull (SB) served another cow (no. 69) on 3rd October, 1978 and she returned on 27th October 1978. At this time the farmer heard that there had been an infectious infertility problem at farm A, and

requested veterinary assistance. The farm was visited on 7th November 1978 and eight vaginal mucus samples taken, three from the cows no. 41, 26 and 69 and five from other cows which had not been served by the stock bull (SB) since the problem became apparent. Preputial washings from the stock bull (SB) were also taken. Samples from this farm are designated C759 in this work.

Farm C A herd of 100 Ayrshire milking cows was mainly served by bulls, 3 Ayrshire and 1 Hereford. Occasionally AI was used. In the autumn 1978 several cows returned 2-3 months after they were thought to be in calf. Occasional abortions occurred in the winter of 1978. No breeding records were kept by the farmer. One of the bulls being used for service had been on loan to another farm for 2 years but was now back. The Hereford bull had been bought from a dealer and had no known breeding history. Because of continued fertility problems, veterinary help was requested. On 20th August 1979 the farm was visited and several vaginal mucus samples collected. These samples are designated as C263 in this work.

Farm D was a commercial herd of 100 Ayrshire and Friesian cows. High yielders were served by AI and low yielders by one of two bulls - a young $1\frac{1}{2}$ year old Friesian bull purchased as a calf and a 6 year old bull which had been disposed of for slaughter 10 days before our farm visit on 24th July 1979. A third bull, an Aberdeen Angus (AA) believed to be fertile was borrowed from another farm and placed with 25 heifers on 20th December 1978. The heifers were noted to be returning to oestrus at which time in early February 1979 the old Friesian bull was now also placed with the same heifers. Pregnancy diagnosis by rectal palpation at the end of March 1979 by the farmer's own veterinary surgeon revealed that less than half of the heifers were in calf. According to the farmer no previous fertility problem had existed in the farm prior to the use of the bull (AA) and in 1977 and

1978 calving indices were 368 and 358 days respectively. It therefore appeared that the problem was of recent introduction. The farmer's veterinary surgeon submitted two mucus samples (A and B C569) for examination for agglutinins of C. fetus by VMAT. As it appeared that the farm was infected on the basis of these results, two other mucus sample collections were made and these are designated C34 and C993.

Farm E was a dairy herd of 180 milking Ayrshire cows which had experienced clinical respiratory disease due to infectious Bovine Rhinotracheitis (IBR) in early December of 1978 to early January 1979. The bulls were apparently not affected by the IBR problem but one heifer had aborted. IBR virus had been isolated from the heifer. Two months later (March to April 1979) a problem of return to service developed and the veterinary surgeon thought that this was due to the IBR infection. However, the infertility problem continued throughout spring and summer until August 1979 when the veterinary surgeon thought that other causes of infertility were worth investigating. On visiting the farm on 28th August 1979 it was found that six of the bulls used in this herd had never been off the farm except for use on another farm in the same ownership in which no problem was evident. Another bull, a 2 year old Ayrshire bull (VR) had, however, been purchased at a bull sale on 24th November 1978. This bull was found to have been reared on farm F, which had direct contact with farm A. The bull (VR) was first used at the end of January 1979 and of 14 cows served, 10 returned to oestrus. Other bulls were used to serve some of these cows. The continuation of the problem then led to the investigation undertaken in August 1979. Mucus samples were collected from 18 cows and preputial washings from two of the bulls (RD and VR) then present in the farm. The samples from farm E are designated C340 and C250. These samples were also examined at the Moredun Research Institute for virus and mycoplasma but no isolations were made.

Farm F was a farm where about 100 heifers and cows under the same ownership as farm A were kept. Bull (NI) from farm A had run with cows and heifers on this farm. Many of these returned to service within 6 weeks and a request to investigate the nature of the problem was received. The farm was visited on 21st August, 1979 and 14 mucus samples were collected. Preputial washings were taken from another bull (C276) present on this farm which had been used when NI had not put many of the animals in calf. Samples from this farm are designated C275.

Farm G A herd of 130 Ayrshire cows. Infertility was noted to follow the use of a bull (CT) borrowed on 15th April 1979, and used subsequently. This bull was from farm M. He served 20 cows of which only two appeared to be in calf, the rest returning to oestrus. Prior to his arrival, three other bulls (CJ, CG and GS) were being used on the farm. Bull CG had previously been used in another farm owned by this farmer's father, in which bull CG had been used for 2 years. When CT failed to put the cows in calf, the bull CG was then used to serve them but they kept on returning. Bull CG also now had poor fertility. Bull CJ was now used to serve cows failing to be pregnant to both CT and CG. Bull GS was also used to serve 3 cows which failed to be pregnant to CT and one cow which failed to be in calf to CJ. No improvement in fertility occurred and the farm was visited on 25th February, 1980 to investigate the fertility problem. Eighteen mucus samples were taken from the cows and preputial washings from the two bulls GS and CG which were now present. CT had been moved to another farm which was sharing him. This farm later turned out to be Farm M. The samples from farm G are designated C377 in this work.

Farm H1 and H2 This was a herd of about 150 Ayrshire cows in which AI and natural service were used. At the time of the first visit the farm had eight bulls. Infertility started after 5th November 1979 when an Old Ayrshire bull (OB) bought into the farm on 2nd November

1979 was used to serve two cows. Both returned. The bull was also used to serve 3 heifers and none of them became pregnant. On the second farm (H2) close to H1 and co-owned, this bull served 3 other cows two of which returned. Following this, other farm bulls were now used to serve these returning cows. Two young bulls (BN and TL) now served the three heifers which had been served by the old bought-in bull (OB) but the heifers still did not become pregnant. The poor fertility then spread into the cow herd and the veterinary surgeon noted that many cows were returning irregularly. He submitted 12 mucus samples for examination for campylobacteriosis. These samples are designated C841. Visits were made to both farms and further vaginal mucus samples and preputial washings from the two young bulls (BN and TL) were taken. The samples are designated C351 and C383. The older bull (OB) and several others were not examined as they were being treated following the report from sample C841.

Farm I A young bull purchased from farm F was run with 30 cows for approximately 4 months. When examined for pregnancy only one of these cows was found to be in calf. The bull was examined clinically and found to have orchitis. The semen was dilute and the spermatozoa had several primary morphological abnormalities. Because of its origin from farm F, a representative number of cows was examined for possible C. fetus infection. Both this bull (SJ) and another bull co-resident in the farm (TO) were sampled by preputial lavage and the samples were cultured as detailed in this chapter. Vaginal mucus from 10 cows was also collected for culture, VMAT and CIE. These samples are designated C437 in this work.

Farm L was a suckler herd using natural service from commericial bulls obtained from dealers. In October 1980 the fertility of one bull used on a group of 20 cows was called in question by the farmer. On clinical examination, the bull was found to have ulcerative balanoposthitis.

Swabs taken from the lesions yielded Mycoplasma bovi genitalum and Ureaplasma spp. Semen was not taken on account of the bulls genital tract pathology. Ten days after the visit, the farmer's veterinary surgeon examined 20 cows expected to calve in the spring of 1981 and found only seven to be in calf. He requested that the herd be examined for campylobacteriosis as the origin of the bulls was unknown. Vaginal mucus samples were collected for culture and immunological studies (VMAT and CIE). These samples are designated C651 in this work.

Farm K This was a large dairy herd in which successful natural service had been practised with a mature Ayrshire bull (SW) until May 1979 when this bull was exchanged for another bull from a farm in Cheshire. Fertility in the herd continued to be satisfactory until May 1980. Bull SW was re-introduced to the herd and allowed to serve 8 cows all of which returned to oestrus at irregular intervals according to the farmer. In late September 1980 vaginal mucus samples from 4 of these 8 cows were delivered on the day following collection and of these one was positive and two other suspicious to the VMAT. Two samples, the VMAT positive sample and one suspicious sample, were positive to CIE. The farm was visited in early October 1980 and 12 mucus samples, from the 8 repeat breeder cows and 4 others were collected and subjected to culture, VMAT and CIE. These samples are designated C767.

Farm J This was a commercial suckler herd with between 100 and 150 cows. The farmer had bought two bulls with which he hoped to cross-breed. Following their use in the herd, several cows were noted to be returning to service at times when they were presumed pregnant. The farmer then requested veterinary advice and his veterinary surgeon sampled 8 cows and requested examination for C. fetus infection. These samples are designated C928. Another farm visit was undertaken and further samples collected. These are designated C111 in this work. Brucellosis was then diagnosed in this herd which was then slaughtered.

Farm M This farm had about 120 Ayrshire cows. On 4th September, 1980 six vaginal mucus samples (C310) were received from another laboratory which requested that they be examined for C. fetus antibodies. Animals in this farm which had been running with a bull (later identified as bull CT also used in farm G) were returning to service. By July 1980 it was thought that as many as 50 cows had returned to service. The samples were immunologically tested by the VMAT and CIE tests. One was positive by VMAT and 4 by CIE. On this basis the farm was reported as being infected and freshly collected mucus samples were delivered to the laboratory on 3rd November 1980, within 2 hours of collection, for cultural confirmation. When questioned the farmer revealed that the cows served by bull CT had the worst breeding record (see samples C310 and C849, Cow Nos. 159, 61, 151 and 41).

6:1:2 Collection of Samples

6:1:2a Cows and Heifers

The selection of cows and heifers was made on the basis of the probable time of introduction of the disease suggested by the history. On each farm as many as possible repeat breeding cows and heifers which had been served within the previous six months were sampled. Mucus was collected by aspiration using the method of Pierce (1946). The free ends of the pipettes were then covered with tin foil. Two recently aborted cows from herd A Nos 4 and 59 were similarly sampled 7-12 days after abortion but because of the large quantities of lochia aspirated these were placed in sterile plastic containers and transported to the laboratory and processed within 8 hours of collection.

6:1:2b Bulls

All bulls were restrained and about 100 mls of phosphate buffered saline pH 7.2 was instilled into the preputial cavity under gravity by using a flutter valve apparatus. The composition of the PBS was that of Schutte (1969). The preputial end was then held tightly by

the operator and the contents of the cavity were thoroughly massaged for 2-4 minutes before they were transferred into plastic containers and from these into sterile plastic universal bottles. On some occasions 2.0 ml of this washing was injected into the transport medium of Clark et al (1974) or that supplied by Corbeil (1979) from the Central Veterinary Laboratory, Weybridge, Surrey, England. All the samples were transported to the laboratory and processed for culture within 8-10 hours of collection. Prior to culture they were treated as described below.

6:2 Treatment of Samples from Bulls

All preputial samples were centrifuged at 1500 r.p.m. for 10 minutes to deposit gross debris and then the supernatant was subsequently centrifuged at 3900 r.p.m. for 45 minutes (a modification of the method of Schutte 1969). Both supernatant and deposit from the second centrifugation were used for culture. The samples in transport media were incubated at 37°C for 3 days in the case of that in the medium of Clark et al (1974), or left at room temperature for 3 days as detailed by Corbeil (1979).

6:3:0 Media for Isolation

Solid medium was made by incorporating 10% ovine blood into Columbia agar(Gibco Biocult) and adding antibiotics as specified by Shepler et al (1963). In addition mycostatin was incorporated at 300 units/ml of medium as suggested by Dunn et al (1965). This medium is referred to as BAA throughout this work and had been found to be the best selective medium for isolation of C. fetus from contaminated preputial washings in previous work (Agumbah 1977). Blood agar without antibiotics (BA) was also used to culture the organism from mucus.

6:3:1 Cultural Methods

6:3:1:1 Vaginal Mucus

Vaginal mucus from the tubes was obtained by inserting sterile

swabs through the end of the tube. The mucus was then heavily smeared on the surface of the medium so that mucin tracks would be seen visually and leaving the mucus on the surface. This was essential and is recommended for optimal isolation of this pathogen (Anon 1978). For each mucus sample at least two BAA plates and one BA plate were used.

6:3:1:2 Preputial Washings

The technique used for culturing these samples was that described earlier (Agumbah 1977). 0.1-0.25 ml of the supernatant fluid was pipetted onto the surface of pre-dried BAA and BA plates (30 minutes at 37°C) and by using sterile bent pasteur pipettes as spreaders the fluid was evenly distributed on the medium. Pre-drying aided absorption of the fluid into the agar and prevented spread of contaminants known to be mainly Proteus and Pseudomonas spp (Seger et al 1966). The deposit was spread on the surface of the dry BAA plates only.

6:3:2 Gaseous Environment and Incubation Conditions

All plates inoculated as above were placed into anaerobic jars without catalysts. The jars were evacuated of air and filled with gaseous mixture of 85% N_2, 10% CO_2 and 5% O_2 as recommended by Kiggins and Plastridge (1956), and incubated at 37°C. The earlier samples from clinical cases C842 and C589 were however incubated at 37°C in an atmosphere of 18-20% CO_2 purged into Leec incubators. Later, in the absence of the special gas mixture above, plates were placed in anaerobic jars and the jars were gassed by placing Oxoid anaerobic gas teblets into the jar and adding 10 ml of tap water then tightly closing the jar. All were then incubated for 3-10 days, i.e. plates were examined on day 3 and those without campylobacter-like colonies regassed and reincubated to be re-examined seven days later. Those from which campylobacter colonies were seen and confirmed by Gram staining (observing the characteristic morphology) and dark field microscopy (for motility) were then subcultured onto fresh BAA plates or blood agar (BA) for purification. It was from these latter plates that colonies

for biotyping were obtained.

6:4:0 Biotyping of Isolates

Biotyping Media: Either semisolid brucella broth (ABB) made by incorporating 0.16% Difco agar or solid media of Columbia blood agar was used. The formulations were as follows:-

1. Glycine Tolerance Tests:

a) Gly^1: (1% w/v glycine)

5 gm glycine (BDH Chemicals Ltd., England) was dissolved in 50 ml sterile distilled water and sterilized by Seitz filtration (EK Pads) and stored at $4^{o}C$. 14.0 g brucella broth base (Albimi) and 0.8 g Difco agar was dissolved in 450 ml distilled water and heated to boiling point. This was then autoclaved at 15 lb/sq in for 15 minutes. The medium (ABB) was cooled to $50^{o}C$ in a water bath and then the 50 ml of sterilized glycine solution was added to make 500 ml. The final concentration of glycine in the medium was 1%. The medium was dispensed in 15 ml portions in sterile universal bottles using an automatic dispenser. Campylobacter colonies were picked from BAA or BA plates and inoculated into the glycine medium and incubated at $37^{o}C$ for 3 to 7 days. Growth (+) in this medium constituted tolerance and absence of growth (-) intolerance.

For solid medium: 5.0 g glycine (BDH Chemicals Ltd., England) was dissolved in 50 ml sterile distilled water and Seitz filtered (EK pads). 22 g Columbia agar was dissolved in 400 ml sterile distilled water, heated to boiling point and sterilized by autoclaving as above. This was cooled as before and 50 ml of the glycine solution added.

50 ml of sterile defibrinated ovine blood was then added and the medium was thoroughly mixed. This was dispensed on to Petri dishes at 35-40 ml per plate. Campylobacters from BAA or BA plates were inoculated on to these plates after drying and the plates incubated

at 37°C for 3-7 days in an atmosphere of 85% N2, 10% CO_2 and 5% O_2 (Kiggins and Plastridge 1956). The interpretation was as for ABB above.

(b) Gly^2: (1.5% w/v) glycine

7.5 g glycine (BDH Chemicals Ltd., England) was dissolved in 50 ml distilled water and Seitz filtered (EK pads). This was added to 450 ml sterilized and cooled semisolid ABB. A similar treated solution of 50 ml glycine (7.5g) was also added to 400 ml sterile cooled Columbia agar (22.0g) base and 50 ml sterile ovine blood and poured as described above. Inoculation and incubation conditions were as for ABB 1% (w/v) glycine and Columbia agar 1%(w/v) glycine above. Interpretation was as above.

(3) Hydrogen Sulphide Production Tests

This was tested at three levels.

a) H_2S^1 : (Triple Sugar Iron Medium)

32.5g Triple Sugar Iron agar (Oxoid Code CM277) was dissolved in 500 ml distilled water and heated to the boil, autoclaved at 15 lb/sq in for 15 minutes, cooled and dispensed in 15 ml portions into sterile universal bottles which were then placed so that the medium set as a slope. Campylobacter colonies from ABB or BA were inoculated on the sloped surface and the universal bottles incubated in air (after tightly closing them) for 3-7 days at 37°C. Blackening of the butt from 24 hours to 7 days was observed and if it occurred the isolate was considered H_2S positive (+) at this level. Absence of blackening constituted a negative (-) reaction at this level.

H_2S^2: (Insensitive Semisolid Albimi Brucella Broth)

0.8 g Difco agar and 14.0 g Albimi Brucella Broth base were dissolved in 500 ml distilled water by heating to the boil. This was autoclaved at 15 lb/sq in for 15 minutes and dispensed into 15 ml portions as for the glycine tolerance tests. Campylobacters were inoculated into the medium and incubated in air at 37°C for 3-7 days. H_2S

production was tested by suspending sterile dry filter paper strips which had been soaked in a saturated lead acetate solution over the media. Blackening of the tips of the filter paper indicated H_2S production (+) and absence of blackening no production (-).

H_2S^3: (Sensitive ABB Medium)

(a) 100 mg L-cystine (BDH, England) was dissolved in 50 ml sterile distilled water by magnetic stirring and Seitz filtered (EK pads).

(b) 14.0 g brucella broth base, 0.8 g Difco agar was dissolved in 450 ml distilled water. This was heated to boiling point and then sterilized at 15 lb/sq in for 15 minutes, then cooled to 50°C then portion (a) and (b) above were aseptically mixed and dispensed into universal bottles in 15.0 ml portions. Campylobacters from BAA or BA plates were inoculated in to this and incubated at 37°C in air for 3-7 days. H_2S production was tested with sterile lead acetate paper strips as for H_2S^2 above and the interpretation was the same.

(4) Catalase Production Tests

Semisolid Albimi Brucella Broth (ABB) prepared as for H_2S^2 above was used to inoculate campylobacters from BAA or BA plates in duplicate. Incubation conditions were as for H_2S system. When growth had occurred one tube had 1 ml of 10 vols. H_2O_2 solution (BPC) added to it and the other had 1 ml of 3% H_2O_2 solution added. Two uninoculated tubes were similarly treated. Catalase production was recorded to be present (+) when an immediate bubbling of gas occurred. When no bubbling was noted the isolate was considered catalase negative (-). This was compared with reaction in the two control tubes.

(5) Nalidixic Acid Tolerance Tests ($NALD^1$ = 40 µg/ml, $NALD^2$ = 45 µg/ml)

(a) (i) 20 mg nalidixic acid (Aldrich Chemicals Co., England) was dissolved in 50 ml sterile distilled water and Seitz filtered (EK pad).

(a) (ii) 22 g Columbia blood agar base (Gibco, Scotland) was dissolved in 400 ml distilled water. This was heated to boiling

point and autoclaved at 15 lb/sq in for 15 minutes.

(a)(iii) 50 ml sterile ox blood. After (a)(ii) above was cooled to 50°C (a)(i) and (a)(iii) were added to (a)(ii) and thoroughly mixed. The final concentration of nalidixic acid was 40 µg/ml ($NALD^1$). The medium (35-40ml) was dispensed on sterile petri dishes. Campylobacters were inoculated into the medium which was incubated for 3-7 days at 37°C in an atmosphere of 85% N_2, 10% CO_2 and 5% O_2 (Kiggins and Plastridge 1956).

(b)(i) 22.5 mg nalidixic acid (Aldrich Chemicals, England) was dissolved in 50.0 ml sterile distilled water and Seitz filtered (EK pads).

(b)(ii) 22.0 g Columbia blood agar base was dissolved in 400 ml distilled water, boiled and autoclaved at 15 lbs/sq in. Again as above the Seitz sterilized nalidixic acid and 50 ml of sterile ovine blood were added after cooling to 50°C. The final concentration of nalidixic acid was 45 µg/ml ($NALD^2$). Campylobacters were inoculated in the medium under the same conditions as above. If growth occurred in either $NALD^1$ or $NALD^2$ this was interpreted as tolerance (+) at that level. Failure to grow was interpreted as intolerance (-).

(6) Tetrazolium Chloride Tolerance Tests (TTC^1 = 1mg/ml, TTC^2 = 1.5 mg/ml)

These tests were also performed in solid media of Columbia blood agar plates. Preparation of Columbia blood agar medium is described above. To make TTC^1 (1 mg/ml TTC):- 0.5g 2,3,5, triphenyl tetrazolium chloride (BDH Chemicals, England) was dissolved in 50 ml of sterile distilled water and Seitz filtered (EK pad). 400 ml Columbia agar base autoclaved at 15 lb/sq in precooled to 50°C, together with 50 ml sterile ox blood was added, thoroughly mixed and dispensed into petri dishes (35-40 ml per dish). This produced a final concentration of 1 mg/ml TTC (TTC^1). For TTC^2 0.75g, 2,3,5, triphenyl tetrazolium chloride

was dissolved in 50 ml sterile distilled water and the medium prepared as above. The concentration was 1.5 mg/ml TTC (TTC^2). Campylobacters from BAA or BA plates were inoculated onto both TTC^1 and TTC^2 plates and incubated at 37°C for 3-7 days in an atmosphere of 85% N_2, 10% CO_2 and 5% O_2 (Kiggins and Plastridge 1956). Tolerance (+) at these levels was indicated by growth; intolerance (-) by absence of growth.

(7) Sodium Chloride Tolerance (3.5% Nacl w/v)

17.5g sodium chloride (Analar)(BDH Chemicals, England), 14.0 g of Albimi brucella base and 0.80 g Difco agar were dissolved in 500 ml distilled water. This was heated to boiling point and dispensed into sterile universal bottles in 15.0 ml portions. The latter were autoclaved at 15 lb/sq in for 15 minutes. Campylobacters were inoculated into the medium from BAA or BA plates and the lids tightly closed. They were incubated at 37°C for 3-7 days. If growth (+) of the test strain occurred it was considered salt tolerant. If no growth occurred, it was considered intolerant (-).

(8) Selenite Reduction Test (0.1% Sodium biselenite)

(i) 0.5g sodium biselenite (London Analytical and Bacteriology Media Ltd., England) was dissolved in 50 ml sterile distilled water and steam sterilised for 10 minutes.

(ii) 22.0 g Columbia agar base was dissolved in 400 ml of distilled water, heated to boiling point and autoclaved at 15 lb/sq in for 15 minutes. This was cooled to 50°C and finally 50 ml of sterile ovine blood together with the selenite solution was added to it, mixed and dispensed into sterile petri dishes (35-40 ml per dish). Campylobacters were inoculated into these media and incubated for 3-7 days in an atmosphere of 85% N_2, 10% CO_2 and 5% O_2 (Kiggins and Plastridge 1956). Growth on the medium was accompanied by formation of pink colonies indicating selenite reduction. When no colour change occurred this indicated non-reduction.

(9) 8% Glucose Tolerance Tests

(i) 40 g glucose (Analar)(BDH) was added to 100.0 ml sterile distilled water, dissolved and Seitz filtered as above.

(ii) 14.0 g Albimi brucella broth base and 0.8 g Difco agar were dissolved in 400 ml distilled water, heated to boiling point and autoclaved at 15 lb/sq in for 15 minutes. This was cooled to 50°C in a water bath and (i) and (ii) mixed before dispensing in 15 ml portions in sterile universal bottles. Campylobacters were inoculated into the medium, the caps screwed tightly and incubated at 37°C for 3-7 days. If growth occurred (+) the strain was considered tolerant and if no growth occurred (-) it was considered intolerant.

(10) Brilliant Green Tolerance (BG_1 = 1:33,000, BG_2 = 1:100,000)

This was tested at two levels namely BG_1 in which Seitz sterilised brilliant green was added to sterilised and cooled Columbia blood agar to give a 1:33,000 concentration and BG_2 in which the same dye was added to give a final concentration of 1:100,000. Growth (+) in BG_1 and BG_2 constituted tolerance while failure to grow(-) intolerance. The organisms were inoculated on to these media and the media incubated in the gas mixture of 85% N_2, 10% CO_2 and 5% O_2 (Kiggins and Pastridge 1956) as for all other solid media.

(11) Temperature Tolerance Tests - were performed in semisolid Albimi Brucella Broth (ABB). 0.8 g Difco agar and 14.0 g brucella broth base were dissolved in 500 ml distilled water, heated to boiling point and autoclaved at 15 lb/sq in for 15 minutes, cooled and dispensed in 15 ml portions in sterile universal bottles. These were inoculated with campylobacters, the lids screwed down tightly and incubated in air at 25°C, 37°C and 42°C. If growth occurred at any of these temperatures (+) the organism was considered tolerant at the appropriate temperature. Absence of growth was scored as intolerance (-).

During these tests it was noted that isolates from the cases C377(Farm G)

and C849 (Farm M) did not grow well in semisolid ABB alone or in ABB to which the chemicals had been added. These strains were therefore re-tested in thioglycolate broth (Oxoid) to which 0.16% Difco agar had been added. They then grew well and could be biotyped. As noted in the results below, these isolates were C. fetus subsp venerealis of Florent (1959) and serotype A of Morgan (1959) and Berg et al (1971). The similar types e.g. NCTC 10354 and MSU 18567 from Montana, Bozeman U.S.A. all grew in semisolid ABB.

In all cases when growth occurred in the test media smears were stained by Gram's method to exclude the possibility of mistyping. This identified the typical campylobacter morphology in pure forms. Whenever contamination occurred the tests were always repeated.

6:4:1 Serotyping of Isolates

6:4:1a Immunising Antigens and Immunising Procedure

The strains of C. fetus designated Langford and 64/3 (serotype B of Morgan 1959) and Grant, serotype A of Morgan (1959) were grown on blood agar plates with antibiotics (BAA) for 3 days, washed off with 0.25% formol saline and allowed to stand overnight at $4^{o}C$. These were then centrifuged, washed twice in sterile saline (PSS) and resuspended in 0.25% formal saline and kept at $4^{o}C$ for one week. They were then centrifuged once more and resuspended in sterile saline to an optical density of 0.55 ± 0.01 ($E^{1\ cm}_{525}$) just prior to use for the inoculation of white male New Zealand rabbits. The rabbits were given ten intravenous injections at the rate of 0.5, 1.0, 2.0, 3.0 and 4.0 ml (the latter six times) at 3 day intervals. This procedure was a modification of that of Walsh and White (1968). This regime was found to give very high titres of antibody. Seven days after the last injection the rabbits were exsanguinated by cardiac puncture and the blood collected, allowed to clot, centrifuged and the serum removed. The antisera were tested using homologous and heterologous strains (see table 6:3a, 6:3b below)

before and after absorption.

6:4:1b Specific 'O' Sera Absorption Technique

The whole cell antisera produced as above were absorbed (Berg et al 1971). Absorption for specific 'O' typing antisera was obtained by producing 'O' antigens by boiling whole cell antigens for 2 hours in sterile saline, washing the cells at least twice in saline and resuspending in 0.25% formol saline to an OD ($E^{1\ cm}_{525}$) of about 0.4 for agglutinating antigens. Simultaneously, a thick suspension of the boiled antigens OD ($E^{1\ cm}_{525}$) of about 1.85 was made as stock for absorption purposes. One ml of the antiserum and 4.0 ml of the thick suspension were mixed together in 10 ml plastic tubes shaken together and the tubes were capped and incubated at 37°C overnight. The following day, the tubes were centrifuged at 4,500 r.p.m. at 4°C (Chilspin MSE) for 30 minutes. The supernatant was tested for agglutinating activity against the strains used in preparing it and related ones (i.e. serotype A against their antisera) and against unrelated ones (i.e. serotype B antisera against serotype A antigens and vice versa). This trial absorption was found to be sufficient but to remove any possible doubt the antisera used were then absorbed at the rate of 1 ml of the antiserum and 9 ml of the stock absorbing antigen thereby effecting a dilution of 1:10 to the antiserum. The supernatants obtained after the absorption were further diluted, retested and when there was no cross-reactions, they were stored and used for serotyping the isolates from the farms (see table 6:4). The strains 64/3, Langford and Bridge were used to prepare reference serotype B agglutination antigens and the strains Grant and 156AH 177 for reference serotype A antigens (Morgan 1959; Berg et al 1971).

6:4:1c Serotyping Procedure

A set of racks was set up such that each row had seven agglutination tubes 1-7 to which 0.5 ml of 0.5% phenol saline was added. A further

two tubes were also placed for antigen stability. In the first tube 0.5 ml of 1:20 dilution of the absorbed serotype A serum was placed and from this doubling serial dilution made to the 7th tube. The first tube had therefore a 1:40 dilution before antigen addition. A second rack was similarly prepared except that serotype B antiserum was used. 0.5 ml antigens of the various isolates prepared as described earlier and diluted to approximately OD ($E^{1\ cm}_{525}$) 0.4 was added to all tubes. Thus all first antiserum tubes had a 1:80 dilution. All antigens were tested against both serotype A and serotype B antiserum made by the author. The tubes were then, after mixing thoroughly, incubated at 37°C for 18-20 hours. The tests were then read in the Weybridge agglutination box and the titre was taken as the last tube giving a 50% clearing.

6:4:1d Serotyping Confirmation by Using Antisera Provided by Dr. M. Border and Professor Firehammer, Montana State University, U.S.A.

Because (as reported in the results below) the isolates obtained in many field outbreaks of campylobacteriosis in South West Scotland were typed as C. fetus subsp fetus and since as reported by Berg et al (1971) only serotype B strains of the C. fetus subsp fetus biotype were venereally transmitted, it became necessary to determine the serotypes of these isolates. Furthermore since our findings reported below indicated that one of the strains NCTC 10348 isolated by Park et al (1962) was also serotype A (by our serotyping procedure) yet Berg et al (1971) classified organisms of this type as serotype B, it became necessary to confirm these serotyping results using the Montana antisera. Antisera were obtained specific for serotype A, serotype B and serotype C and for heat labile antigens. At the same time we obtained isolates (see table below) from the Montana State University Veterinary Research Laboratory, U.S.A. (courtesy of Dr. Border and Professor Firehammer). Antigens were prepared from these isolates as well and tested both

against our own antisera specific for serotype A and serotype B and against Montana antisera. Six antigens were also prepared from isolates from human diarrhoea, one from equine faeces, one from dog faeces and one from an aborted ovine foetus. The purpose was to determine if serotyping of isolates would be of clinical epidemiological significance in bovine cases as it is claimed by several authors (Florent 1959; Neill et al 1978) that campylobacters of faecal or intestinal origin of the biotype C. fetus subsp fetus may interfere with clinical decision-making based on biotyping of isolates from the genital tract as organisms typeable as C. fetus subsp fetus could also be of faecal origin. Others (Smibert 1978) also claim not to have isolated C. fetus subsp fetus organisms from faeces of cattle and Hawari (1979) found only one organism out of 18 to be like C. fetus subsp fetus (intermediate) from faecal isolates. The procedure used in serotype confirmation was the same as the one reported above.

6:5 Results of Biotyping and Serotyping of Field Isolates and Other Campylobacters Acquired for Comparative Purposes

The criteria used in the biotyping of isolates during this work are tabulated in Table 6:1 in which the biochemical reactions of the various species of campylobacter found by the different authors is cited. Table 6:2 shows the total number of isolates which belonged to a particular biotype and their reactions in the various media. The details of reactions of each individual isolate are presented in Appendix 4 of this work. A total of 57 campylobacters were isolated from the genital tract of cows and bulls examined. Of these 39 from 8 herds were classified as C. fetus subsp fetus. A further two behaved as C. fetus subsp fetus but later grew in 1.5% glycine making them classifiable as 'true' C. fetus subsp fetus (intestinalis). Only

one isolate C377-4SN +ve was classified as 'true' C. fetus subsp fetus (intestinalis) outright. The epidemiology and history of three farms from which the isolates were obtained indicated direct or indirect venereal transmission from farm A. The epidemiology indicated that bull NI was involved in disease transmission in all 3 herds. Seven isolates from two farms were typed as C. fetus subsp venerealis and again both farms (G and M) were connected through the use of bull CT. Figures 6-1 and 6-2 illustrate the epidemiological facts connected with these bulls and farms and strongly suggest that both C. fetus subsp fetus and C. fetus subsp venerealis were capable of venereal spread. Three other organisms (C263-92, C263-21 (Farm C) and SR-JPR (Farm I)) were also classified as C. sputorum subsp bubulus, the latter being isolated from a bull used for service in Farm I. The organism C250-5 (Farm E) has been classified as C. fecalis on the strength of catalase production, H_2S production at all levels and growth at 42°C. However repeated tests showed that it was intolerant to sodium chloride at 3.5% - a criterion considered typical of C. fetus (Jackovljevic 1973) and C. jejuni/coli (Véron and Chatelain 1973). That it was not related to the other organisms isolated in the same farm was confirmed by the serotyping results as shown in Table 6-4. Two isolates, one selenite positive (C377-SN+) and identified as C. fetus subsp fetus, and the other, selenite negative (C377-4) and classified as C. fetus subsp venerealis were isolated from the same vaginal mucus sample.

Table 6-3a summarises the reaction of homologous and heterologous antisera and antigens used in this work before absorption. Table 6-3b shows the effect of absorption by heterologous serotype antigen. Thus O-antigen specific antisera were prepared against types A and B (Morgan 1959 and Berg et al (1971). All isolates classified as C. fetus subsp fetus and C. fetus subsp venerealis belonged to serotype A.

Table 6-4 gives the titres of 'O' antigens prepared from these isolates and those acquired for comparison in the absorbed sera prepared by the author and those supplied by Border and Firehammer and classified by the method of Berg *et al* (1971). The findings were the same as those of Morgan (1959) and Berg *et al* (1971) in types A and B sera. None belonged to serotype C. The strain NCTC 10348 used here is one of the organisms isolated by Park *et al* (1962) and was found to be serotype A and not serotype B.

Table 6-5 summarises the breeding records of cows which had a pathogenic campylobacter (that is *C. fetus* subsp *fetus* or *C. fetus* subsp *venerealis*), isolated from them or were VMAT positive and Table 6-6 summarises the interservice intervals from those cows. It should be noted that most of these had had three or more services at the times of examination. Although in a few cases the interservice interval was prolonged, in the majority of cases it ranged between 22-24 days - this being considered as being within the normal oestrus cycle length of the bovine (Salisbury *et al* 1980).

Finally Table 6-7 lists the bulls from which preputial samples were cultured, the farms of origin and the types of *C.* spp. isolated from them, if any. As can be seen, a pathogenic campylobacter was isolated from only two bulls (NI and SW) from Farms A and K. The reason for this poor bacteriological result was not related to inadequacy of isolation methods but to the fact that the bulls, as revealed later, had been treated prior to sampling. Only in farm A was a pretreatment sample taken. In Farm K, the preputial sample was sent to the laboratory by the veterinary surgeon together with several vaginal mucus samples (See C767) following suspicion of *C. fetus* infection.

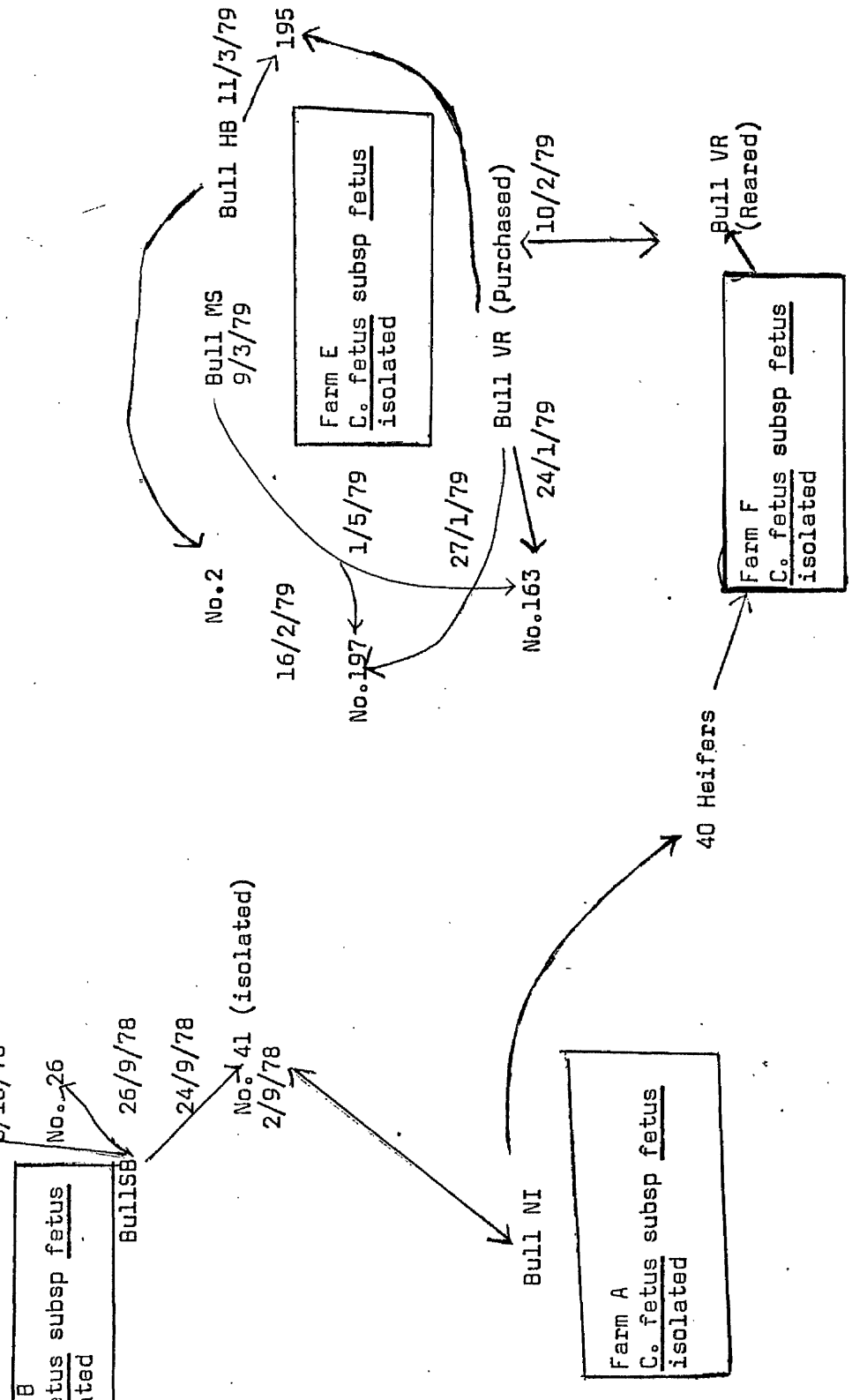


Figure 6:1 Evidence of venereal transmission of C. fetus subsp fetus involving Farms A and B, Farms A, E and F through bulls NI, SB and bull VR (indirect). One cow in Farm E (No. 163) served by bull VR at first (and later by MS) and two other cows (Nos. 195 and 2) served by yet another bull (HB) after VR, are also included. The same type of organism was isolated from both No. 163 and No. 2. Thus it was likely that No. 2 was infected by bull HB (after serving cow No. 195 served by bull VR) and bull MS (later found infertile in this farm) was probably infected by cow No. 197 following the service by bull VR. Moreover bull VR appears to have served in farm F where he was reared.

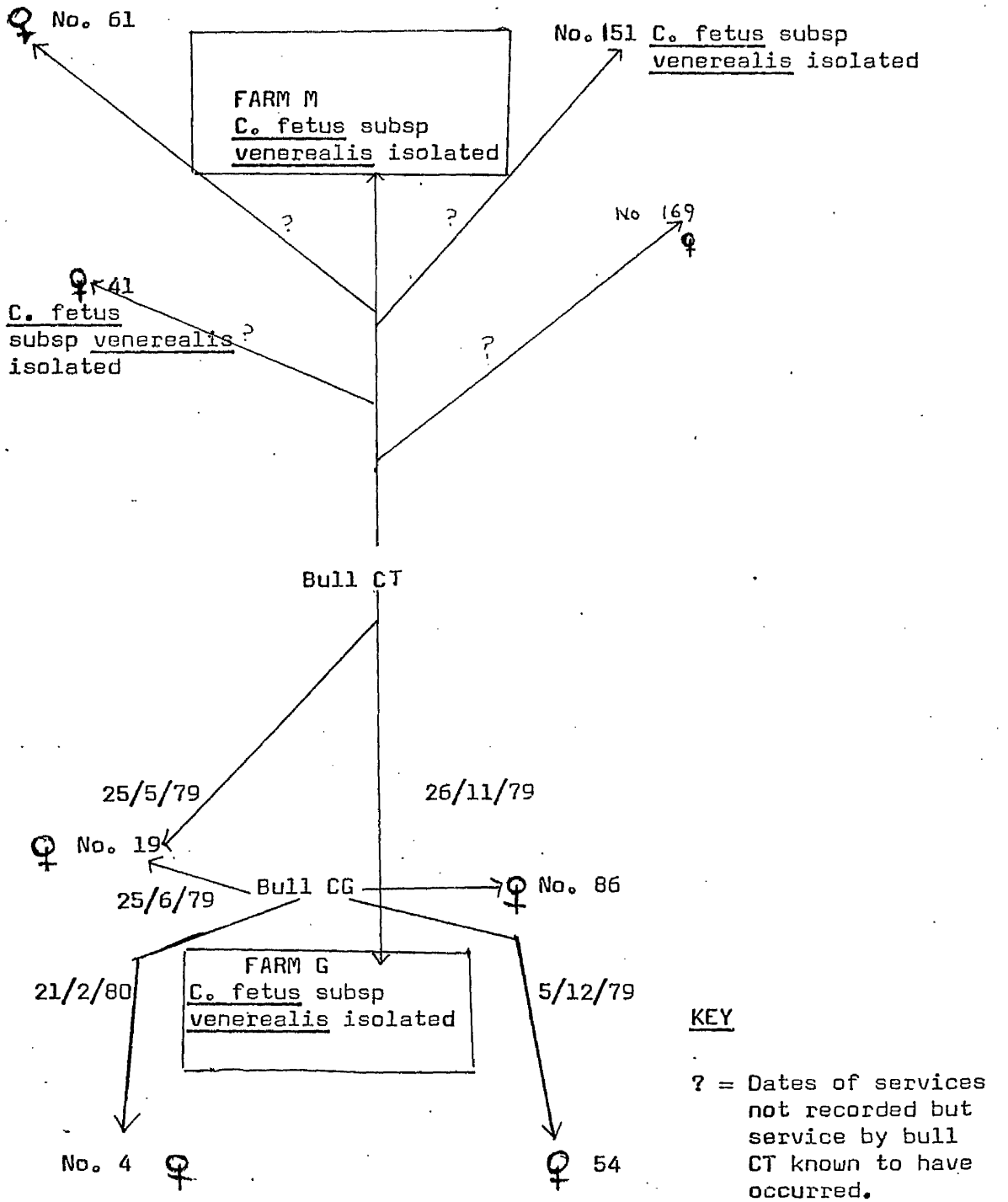


Figure 6:2 Venereal transmission of C. fetus subsp venerealis between Farms G and M through bull CT. Bull CG in Farm G must have been infected by the service of cow No. 19 served earlier by bull CT. Cows No. 4, 54 and 86 served by bull CG later all became infected and the same pathogen was isolated from all three and cow No. 19. Cows served by bull CT in Farm M had immunological evidence of infection (VMAT and CIE) and from two (Nos. 41 and 151) the pathogen was isolated.

TABLE 6-1 Currently Acceptable Biochemical and Tolerance Characteristics of the Species of the Genus Campylobacter.

(Ref. Véron and Chatelain 1973; Neill et al 1978; Firehammer 1965; Loesche et al 1965; Florent 1953; Bryner et al 1962; Anon 1979)

Species	BIOCHEMICAL AND TOLERANCE CHARACTERISTIC												TEMPERATURE TOLERANCE		
	Catalase Production	H_2S^2	H_2S^3	$Glyc^1$	$Glyc^2$	40µg/ml NALD	45µg/ml NALD	1mg/ml TTC	3.5% NaCl	0.1% Selenite Reduction	1:33,000 brilliant green	1:100,000 brilliant green	25°C	37°C	42°C
C. (V) fetus subsp fetus (Smith & Taylor 1919)	+	-	+	+	-(1)* NR +(2)*	+	+	-	-	+	+	+	+	+	-
C. (V) fetus subsp venerealis biotype I (Florent 1959)	+	-	-	-	(NR) -	+	+	-	-	-	+	+	+	+	-
C. (V) fetus subsp venerealis X biosubtype I or biotype intermedius (Bryner et al 1962, Elazhary 1968)	+	-	+	-	(NR) -	+	+	-	-	(+) -	+	+	+	+	-
C. (V) coli (Doyle 1948)	+	d	+	+	(NR) +	-	-	+	-	+	-	+	-	+	+
C. (V) jejuni (Jones et al 1931)	+	W +	+	+	(NR) +	-	-	W +	-	W +	-	-	-	+	+
C. (V) sputorum subsp sputorum (Prevot 1940; Loesche et al (1965)	-	+	+	W +	(NR)	(NR)	(NR)	+ -	-	?	?	+	+	+	-
C. (V) Sputorum subsp bubulus (Florent 1953)	-	++	++++	+	+	+	+	-	+	+	+	+	±	+	-
C. (V) fecalis X (Firehammer 1965)	+	+	+	+	+	+	+	(NR)	±	+ (NR)	+ (NR)	+ (NR)	-	+	+
C. sputorum subsp mucosalis X (Lawson and Rowland 1974)	-	(NR) +	+	-	-	(NR)	(NR)	-	-	(NR)	(NR)	(NR)	N	+	?

KEY -(1)* = The 1.5% glycine (w/v) negative strain known as 'intermediate' in the UK, (Bracewell quoting J.A. Morris Ph.d Thesis, Reading, (1971), Anon (1979). They behave as strains isolated by Park et al (1962), see NCTC 10348 which has been described as intestinalis. They are described as C. fetus subsp fetus

-(2)* = Type II C. fetus subsp fetus or true intestinalis of Florent (1959).

d = Various results as reported by Véron & Chatelain (1973). When no other symbol present then information not available.

NR = Not recorded as reported by Véron & Chatelain (1973) etc.

X = These organisms are not included in the official nomenclature of the genus Campylobacter, Skerman et al (1980).

? = Information not available or unknown.

W = Weak positive reaction.

TABLE 6:2 CONDENSED RESULTS OF BIOCHEMICAL TYPES OF ORGANISMS ISOLATED FROM INFERTILE HERDS OF SOUTH WEST SCOTLAND

Biochemical And Tolerance Test Results																Classification Into The Biotypes of Campylobacter								
																Number Reacting As:-								
Item	Catalase	H_2S^1	H_2S^2	H_2S^3	Gly^1	Gly^2	$NALD^1$	$NALD^2$	TTC^1	TTC^2	3.5% Nacl	0.1% Selenite	1% Glucose	BG_1	BG_2	Fetus		Venerealis						
																Fetus	Intermediate	Biotype I	Biosub. I	bubulus	coli	jejuni	faecalis	
A	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	0	11	0	0	0	0	0	0	
B	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	0	02	0	0	0	0	0	0	
C	−	+	++	++++	+	+	+	+	−	−	+	+	+	+	+	0	0	0	0	2	0	0	0	
D	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	0	03	0	0	0	0	0	0	
E	+(1) +(3)	+ −	+ −	++ +	+ +	− −	+ +	+ +	− −	− −	− −	+ +	+ +	+ +	+ +	0	(+1) 03*	0	0	0	0	0	1 (See Appendix 4)	
F	+	−	−	+	+	(2)+a	+	+	−	−	−	+	+	+	+	(2)	06	0	0	0	0	0	0	
G	+(1) +(5)	− −	− −	+ −	+ −	+ −	+ +	+ +	− −	− −	− −	+ −	+ +	+ +	+ +	1	0	05	0	0	0	0	0	
H1+ H2	(1) +(6)	+ −	++ −	++++ +	+ +	+ −	+ +	+ +	− −	− −	+ −	+ +	+ +	+ +	+ +	0	06	0	0	1	0	0	0	
I	−	+	++	++++	+	+	+	+	−	−	+	+	+	+	+	0	0	0	0	03 (2 lost)	0	0	0	
J	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?			1?	? (0)	0	0	0	0	
K	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	0	04	0	0	0	0	0	0	
L	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	0	03	0	0	0	0	0	0	
M		+	−	−	−	−	+	+	−	−	−	−	+	+	+	0	0	02	0	0	0	0	0	
TOTAL																03?.	39	8(+1)	0	6	0	0	1	

TABLE 6:3a – REACTIVITY OF ANTISERA PRODUCED IN RABBITS WITH VARIOUS ANTIGENS PRIOR TO ABSORPTION WITH BOILED ('O') ANTIGENS TO PRODUCE 'O' ANTIGEN SPECIFIC SERA.

Whole Cell Antiserum Against	Agglutinating Test Antigens	Dilutions of Antiserum to Determine Titres (Homologous & Heterologous) (i.e.) Cross-reactions)										
		40	80	160	320	640	1280	2560	5120	10240	20480	40960
C377-19	C377-19	++++	++++	++++	++++	++++	++++	++++	+++	++	++	±
	64/3	++++	++++	++++	+++–	–	–	–	–	–	–	–
	Langford	++++	++++	++++	++++	++++	+++–	–	–	–	–	–
	Grant	++++	++++	++++	++++	++++	++++	+++	–	–	–	–
64/3	64/3	++++	++++	++++	++++	++++	++++	++++	++++	++++	++	–
	C337-19	++++	+++	+++	++	++	–	–	–	–	–	–
	Langford	++++	++++	++++	+++	+++	++	+	–	–	–	–
	Grant	++++	++++	++++	++	+	–	–	–	–	–	–
Langford	Langford	++++	++++	++++	++++	++++	++++	++++	++++	++++	++	±
	C337-19	++++	++++	++++	++++	++++	++++	+++	+	–	–	–
	64/3	++++	++++	++++	++++	++++	++++	+++	–	–	–	–
	Grant	++++	++++	++++	++++	++++	++	+	–	–	–	–
Grant	Grant	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	–
	C377-19	++++	++++	++++	++++	+++	–	–	–	–	–	–
	64/3	++	++	++	++	++	–	–	–	–	–	–
	Langford	++++	++++	+++	++	++	+	–	–	–	–	–

TABLE 6:3b - REACTIVITY OF THE ANTISERA PRODUCED IN RABBITS AFTER ABSORPTION ('O' TYPE SPECIFIC SERA)

Antiserum Against	Testing	Dilutions of the Absorbed Sera											Absorbing Antigen
		40	80	160	320	640	1280	2580	5120	10240	20480	40960	
Grant	Grant 'O'	++++	++++	++++	+++	+++	-	-	-	-	-	-	64/3 'O' (serotype A specific antiserum this was used in testing isolates
	64/3 'O'	-	-	-	-	-	-	-	-	-	-	-	
	Langford 'O'	-	-	-	-	-	-	-	-	-	-	-	
	156AH 177'O'	++++	++++	++++	++++	+++	++	-	-	-	-	-	
Langford	Langford 'O'	++++	++++	++++	+++	-	-	-	-	-	-	-	Grant 'O' (serotype B specific)
	64/3 'O'	++++	++++	++++	+++	-	-	-	-	-	-	-	
	Grant	-	-	-	-	-	-	-	-	-	-	-	
	156AH 177'O'	+++	++	-	-	-	-	-	-	-	-	-	
64/3	64/3 'O'	Pr.	Pr.	++++	++++	+++	++	-	-	-	-	-	Grant 'O' (serotype B specific)
	Langford 'O'	++++	++++	++++	++++	++	-	-	-	-	-	-	
	Grant 'O'	-	-	-	-	-	-	-	-	-	-	-	
	156AH 177'O'	-	-	-	-	-	-	-	-	-	-	-	
C377-19	Grant 'O'	++++	+++	+++	+++	++	+	-	-	-	-	-	Langford 'O' (serotype A specific)
	156AH 177'O'	++++	++++	++++	+++	++	-	-	-	-	-	-	
	64/3 'O'	-	-	-	-	-	-	-	-	-	-	-	
	Langford 'O'	-	-	-	-	-	-	-	-	-	-	-	

TABLE 6:4 - RESULTS OF SEROTYPING OF REPRESENTATIVE ISOLATES OBTAINED DURING THE STUDY AND THOSE ACQUIRED FOR COMPARISON

Source of Isolates	O-Antigen	Titres Against Specific Antiserum Types					Serologically unclassified by the system
		Serotype 'A'		Serotype 'B'		Serotype 'C'	
		Serotype 'A' (Author)	Serotype 'A' (Border & Firehammer)	Serotype 'B' (Author)	Serotype 'B' (Border & Firehammer)	Border & Firehammer	
C842 or C589 or C749 (Farm A)	No. 16	2560	1280	0	0	0	
	No. 59	1280	640	0	0	0	
	No. 148	1280	640	0	0	0	
	No. 4	1280	640	0	0	0	
	No. 34	1280	ND	0	0	0	
C275 (Farm F)	7558	5120	640	0	0	0	
	106	5120	640	0	0	0	
	83	2560	1280	0	0	0	
	74	2560	320	0	0	0	
	32494	2560	ND	0	0	0	
	204	2560	1280	0	0	0	
C34 (Farm D)	84	2560	1280	0	0	0	
	10	640	320	0	0	0	
	UNK	2560	1280	0	0	0	
C351 & C383 Also as C841 (Farm H_1 and H_2)	11	5120	1280	0	0	0	
	77	2560	640	0	0	0	
	22231/D	2560	640	0	0	0	

TABLE 6:4 (CONT'D)

Source of Isolates	O-Antigen	Titres Against Specific Antiserum Types					Serologically unclassified by the system
		Serotype 'A'		Serotype 'B'		Serotype 'C'	
		Serotype 'A' (Author)	Serotype 'A' (Border & Firehammer)	Serotype 'B' (Author)	Serotype 'B' (Border & Firehammer)	Border & Firehammer	
C351 & C383 Also as C841 (Farm H) cont'd.	157	1280	640	0	0	0	
	UNKM	1280	640	0	0	0	
C250 and/or C340 (Farm E)	65b	2560	320	0	0	0	Unclassified
	5	0	0	0	0	0	
	163	2560	1280	0	0	0	
	65	2560	640	0	0	0	
C377 (Farm G)	4	2560	1280	0	0	0	
	54	2560	320	0	0	0	
	86	1280	320	0	0	0	
	92	2560	ND	0	0	0	
	4SN+	2560	ND	0	0	0	
	19	640	640	0	0	0	
C767 (Farm K)	46	1280	320	0	0	0	
	52	2560	640	0	0	0	
	SW	2560	640	0	0	0	
C651 (Farm L)	972	1280	160	0	0	0	

TABLE 6:4 (CONT'D)

Source of Isolates	O-Antigen	Titres Against Specific Antiserum Types					Serologically unclassified by the system
		Serotype 'A'		Serotype 'B'		Serotype 'C'	
		Serotype 'A' (Author)	Serotype 'A' (Border & Firehammer)	Serotype 'B' (Author)	Serotype 'B' (Border & Firehammer)	Border & Firehammer	
C849 (Farm M)	41	1280	640	0	0	0	
C263 (Farm C)	92	0	0	0	0	0	Unclassified
	21	0	0	0	0	0	
Ovine abortion	OBAN-C	0	0	1280	640	0	
Human enteritis	BELV+C	0	80	0	0	0	
	A.Smith	0	0	0	0	0	
	58395	0	0	0	0	0	Unclassified
	54640	0	0	0	0	0	
	6338	0	0	0	0	0	
	54864	0	0	0	0	0	
Reference strains acquired for comparison	64/3	0	0	640	1280	0	
	Langford	0	0	320	1280	0	
	NCTC5850	0	0	320	640	0	
	NCTC10354	2560	1280	0	0	0	
	NCTC10348	1280	320	0	0	0	
	MSU18756	0	80	0	0	320	

TABLE 6:4 (CONT'D)

Source of Isolates	O-Antigen	Titres Against Specific Antiserum Types					Serologically unclassified by the system
		Serotype 'A'		Serotype 'B'		Serotype 'C'	
		Serotype 'A' (Author)	Serotype 'A' (Border & Firehammer)	Serotype 'B' (Author)	Serotype 'B' (Border & Firehammer)	Border & Firehammer	
Reference strains acquired for comparison	MSU14840	2560	640	0	0	0	
	BRIDGE	0	0	640	1280	0	
	MSU17535	0	0	0	0	640	
	Grant	640	1280	0	0	0	
	156AH177	1280	1280	0	0	0	
	MSU18567	1280	1280	0	0	0	
	MSU14840	1280	640	0	0	0	
Animal Faecal or enteric types	Canine	0	0	0	0	0	Unclassified
	Equine M874	0	0	0	0	0	
	Bovine 24/1980	0	0	0	0	0	

1. The strain NCTC 5850 and NCTC 10354 were obtained from the National Collection of Type Cultures.
2. Strains Langford, Bridge, Grant, 64/3 and 156AH177 were obtained from Central Veterinary Laboratory, Weybridge, Surrey, England.
3. Strains MSU18756, 17535, 18567 and 14840 were obtained from Montana State University, Bozeman Veterinary Laboratories, Department of Veterinary Science, courtesy of Dr. M. Border and Professor B.D. Firehammer.
4. The human strains 58895, BelV+C, 6338 were obtained from Irvine General Hospital, Ayrshire or Belvedere Hospital, Glasgow, courtesy of Dr. C.L.

TABLE 6:5 - Breeding records of some cows from which pathogenic Campylobacter Fetus was isolated or that were serologically positive : Service Dates and Methods

Sample and Cow No.	Farm of Origin	Service dates and method up to isolation or serological testing time						
		1st	2nd	3rd	4th	5th	6th	7th
C340-65	E	1/4/79 (N)	24/4/79 (N)	9/6/79 (AI)	29/6/79 (AI)	21/7/79 (AI)	-	-
C340-163	E	28/3/79 (N)	1/5/79 (N)	1/7/79 (AI)	22/7/79 (AI)			
C340-5	E	20/1/79 (N)	11/2/79 (N)	4/3/79 (N)	25/3/79 (N)	14/4/79 (N)		
C340-2	E	9/3/79 (N)	2/4/79 (N)	23/4/79 (N)	13/5/79 (AI)	3/6/79 (AI)	24/6/79 (AI)	
C34-10	D	11/4/79 (N)	6/5/79 (N)	9/6/79 (N)	8/7/79 (N)			
C34-84	D	7/5/79 (AI)	28/5/79 (N)	26/6/79 (N)				
C34-103	D	24/2/79 (N)	18/3/79 (N)	29/4/79 (N)	22/5/79 (N)	17/6/79 (N)		
C351-11	H_1	4/1/80 (N)	4/2/80 (N)	27/2/80 (N)	21/3/80 (N)	7/4/80 (AI)		
C351-77	H_1	20/11/79 (N)	7/1/80 (N)	2/2/80 (N)	21/3/80 (N)	4/5/80 Not Served		
C351-79	H_1	5/12/79 (N)	21/21/79 (AI)	14/3/80 (N)	10/4/80 (AI)			
C383-157	H_2	10/12/79 (N)	3/1/80 (N)					

(N) = Natural Service (Bull) (AI) = Artificial Insemination

TABLE 6:5 CONT'D. - Breeding Records of some cows from which Pathogenic Campylobacter Fetus was isolated or that were serologically positive

Sample and Cow No.	Farm of Origin	Service dates and method up to isolation or serological testing time						
		1st	2nd	3rd	4th	5th	6th	7th
C383-22231/D	H_2	Not recorded with bull several months	Not Pregnant					
C759-69	B	3/10/78 (N)	27/10/78 ?		-	-	-	-
C759-41	B	2/9/78 (N)	24/9/78 (N)	15/10/78 (AI)	-	-	-	-
C377-4*	G	19/11/79 (N)	22/1/80 (N)	21/2/80 (N)	-	-	-	-
C377-54	G	31/12/79			-	-	-	-
C377-86	G	26/11/79 (N)	19/12/79 (N)	11/1/80 (N)	-	-	-	-
C377-19	G	30/4/79 (N)	25/5/79 (N)	25/6/79 (N)	18/7/79 (N)	-	-	-
C377-92+	G+	17/12/79	?	?	?	?	?	?

* From this animal was also isolated a Campylobacter subsp biotyped as true intestinalis and belonging to serotype A of Morgan (1959) and Berg et al (1971)

+ = Only one service record available. Likely to have been confused as there were two animals with the same number in the Farm.

(N) = Natural Service (Bull) (AI) = Artificial Insemination ? = Heifer left with bull for several months, found not pregnant. This bull suspected of introducing disease into farm.

TABLE 6:5 CONT'D. - Breeding records of some cows from which pathogenic Campylobacter Fetus was isolated or that were serologically positive

Sample and Cow No.	Farm of Origin	Service dates and method up to isolation or serological testing time						
		1st	2nd	3rd	4th	5th	6th	7th
C377-99	G	16/10/79 (N)	8/11/79 (N)	30/11/79 (N)	2/1/80 (N)	-	-	-
C377-102	G	6/11/79 (N)	27/11/79 (N)	16/1/80 (N)	-	-	-	-
C377-62	G	6/11/79 (N)	30/11/79 (N)	23/12/79 (N)	17/1/80 (N)	-	-	-
C377-11	G	17/7/79 (N)	4/10/79 (N)	14/11/79 (N)	-	-	-	-
C849-169 a	M	With	Bulls	Throughout	The	Period		
C849-61a	M	"	"	"	"	"		
C849-151a	M	"	"	"	"	"		
C849-41a	M	"	"	"	"	"		
C849-36	M	"	"	"	"	"		

(N) = Natural Service

a = These cows from C849 were served by the same bull. Note all were positive serologically and two (41 and 151) culturally.

TABLE 6:6 - Breeding records of some cows from which pathogenic Campylobacter fetus was isolated or that were serologically positive : Interservice Intervals

Sample and Cow No.	Service Interval in Days						Type of Campylobacter isolated
	1-2	2-3	3-4	4-5	5-6	6-7	
C340-65	23	46	20	22			C. fetus subsp. fetus
C340-163	34	61	21				C. fetus subsp. fetus
C340-5	21	52	21	20			This organism was classified tentatively as C. f ecalis
C340-2	24	21	20	25	21		Untyped. Likely to be C.fetus subsp. fetus
C34-10	25	34	29				C.fetus subsp. fetus
C34-84	21	29					" " "
C34-103	22	42	23	27	-	-	Serologically positive
C351-11	30	23	22	17			C. fetus subsp fetus
C351-77	48	27	49	44			" "
C351-79	26	74	27				" "
C383-157	24						" "
C383-22231/D							C.fetus subsp. fetus
C759-69	22	24					" " "
C759-41	22	21	-	-	-	-	C. fetus subsp fetus isolated
C377-4	65	30					C.fetus subsp. venerealis

TABLE 6:6 - Breeding records of some cows from which pathogenic Campylobacter fetus was isolated or that were serologically positive & Interservice Intervals
CONT'D.

Sample and Cow No.	Service Interval in Days						Type of Campylobacter isolated
	1-2	2-3	3-4	4-5	5-6	6-7	
C337-54	?						C. fetus subsp. venurealis
C377-86	24	23					" "
C377-19	25	31	23				" "
C377-92							C. fetus subsp. venorealis
C377-99	22	22					Serologically positive
C377-102	21	20					" "
C377-62	24	23	24				" "
C377-11	79	72					" "
C849-169a	33	39	48	-	-	-	" "
C849-61a	59	22	-	-	-	-	" "
C849-151a	28	Not served missed	49				C.fetus subsp. venerealis
C849-41	68	23	-	-	-		" "
C849-36	Not observed						Serologically positive

? = Heifer let with bull for several months found not pregnant. This bull suspected of introducing disease into farm.

a = These cows from C849 were served by the same bull. Note all were positive serologically and two (41 & 151) culturally.

TABLE 6.7 RESULTS OF CULTURAL EXAMINATION OF BULLS

Identification of Farm	No. of Bulls Examined	Idividual Identification of bulls	Isolation/No. Isolation of a Campylobacter	Biotype of campylobacter isolated
A	3	NI RF RT	Yes (Yes?) No	C. fetus subsp fetus Lost before characterization -
B	1	SB	No	-
D		C34	No	-
E	2	VR RD	No No	- -
F	1	C276	No	-
G	2	CG GS	No No	- -
H_1	2	BN TX	Yes No	C. sputorum subsp bubulus -
I	2	SJ TO	Yes No	S. sputorum subsp bubulus -
K	1	SW	Yes	C. fetus subsp fetus

Discussion

Bacteriological examination of samples from the genital tract of cattle in herds with fertility problems has led to the isolation of 57 strains of Campylobacter spp. On the basis of criteria shown in tables 6-1 and 6-2, 39 of these would be classified as C. fetus subsp fetus of the 'intermediate' type in the nomenclature in the United Kingdom (Bracewell 1974; citing Morris 1971; Anon 1978), 3 as the recognised C. fetus subsp fetus (Skerman et al 1980), 8 as C. fetus subsp venerealis, 6 as C. sputorum subsp bubulus and 1 as C. fecalis. Two of the three isolates identified as C. fetus subsp fetus (Skerman et al 1980) had initially failed to grow in 1.5% glycine but later did grow thus allowing them to be clasified as C. fetus subsp fetus. The other isolate classified as C. fetus subsp fetus was isolated from the same sample as another organism identified as C. fetus subsp venerealis and these have been designated C377-4SN+ and C377-4 respectively (see Appendix 4). Although the term 'intermediate' is used in Table 6-2 it is to be noted that these organisms so identified were not similar to the ones called C. fetus subsp intermedius (Bryner et al 1962; Elazhary 1968). This group is glycine intolerant unlike the isolates reported here.

The use of this term 'intermediate' as proposed by Morris (1971) cited by Bracewell (1974) was found useful in distinguishing between two types of catalase positive, glycine tolerant and hydrogen sulphide positive C. fetus strains - that is those which could also tolerate 1.5% glycine and those which only tolerated 1% glycine. The use of glycine tolerance tests in differentiation of genital isolates was thus not found to be distinctive when correlated with reproductive pathogenicity. This is in contrast to the classification of Florent (1959). Organisms which tolerated 1% glycine such as were found in this study resembled those of Park et al (1962). They have been recently shown to be

potentially pathogenic (Dedie et al 1977; Whitford et al 1977; Agumbah and Ogaa 1979).

Biochemical differentiation of Campylobacter spp and correlation of results with reproductive pathogenicity has therefore not been entirely successful except in the case of two of the four possible pathogenic campylobacters likely to be met in clinical bacteriological examination of genital campylobacters namely C. fetus subsp venerealis biotype I and biosubtype I which are 1% glycine intolerant. Tolerance to glycine appears not to be a fixed characteristic and could be both transducable and acquirable by mere selection (Chang and Ogg 1971). It could have led to the isolates reported here being considered possibly of no significance with regard to fertility. It would appear that tolerance to 0.1% sodium selenite was a more stable criterion of organisms classified as C. fetus subsp venerealis as none tested grew in media so supplemented. The glycine tolerant strains all grew and reduced selenite as judged by pink colony formation. Similar findings have recently been reported (Kotsche 1980). These criteria could therefore be of use in differentiating C. fetus subsp venerealis from C. fetus subsp fetus but there would still be no correlation with reproductive pathogenicity. Although a third 1% glycine tolerant catalase positive organism designated C250-5 was also isolated from the vaginal mucus of a repeat breeding cow it was classified as C. fecalis on the basis of its growth at 42°C, production of hydrogen sulphide at all levels tested and as noted in the serological results, its non-reactivity with 'O' type A and type B antisera. Firehammer and Berg (1965) recorded C. fetus subsp venerealis organisms capable of growth at 37°C and 42°C but they were serotype 01 (or A). No difficulty was encountered with the classification of catalase negative strains (C. sputorum subsp bubulus) although in two farms with infertility problems (Farm C and Farm I) they were the only

campylobacters isolated. Although they are considered nonpathogenic, these organisms have been isolated from aborted foetuses (Amund 1968) and shown experimentally to cause mild endometritis when instilled into the uteri of cattle (Dozsa 1965).

Although it has been claimed recently (Neill et al 1978) that enteric campylobacters could conceivably be isolated from vaginal mucus and by inference also from preputial samples as a result of faecal contamination, it would appear that such a situation would not be of clinical importance since enteric organisms were biochemically distinct from the genital pathogens studied here. The enteric organisms were mainly nalidixic acid (NALD) intolerant and could grow in some cases in media with 1mg/ml of 2,3,5 triphenyl-tetrazolium chloride (TTC) added. The genital pathogens all grew in $NALD^1$ and $NALD^2$ but none grew at any of the TTC concentrations tested. However if catalase production, hydrogen sulphide production, 1% glycine tolerance and salt tolerance tests alone were used, it would have been impossible to distinguish these enteric types from the one genital pathogen isolated here namely C. fetus subsp fetus. Indeed Florent (1957) reported that some of the organisms called Vibrio (campylobacter)coli did not differ biochemically, serologically or in their pathogenicity from the group he called Vibrio (C.)fetus var intestinalis. Mitscherlich and Liess (1958b) considered that enteric calf types could cause abortion. Kashiwazaki et al (1970) showed that porcine enteric types (Vibrio coli) were not biochemically homogeneous and belonged to three biochemical groups on the basis of 1% glycine tolerance, catalase production and hydrogen sulphide production. Florent (1959) and Savov (1964) showed that these so called faecal types could be pathogenic to pregnant cattle. These confusing findings and the aetiological classification of campylobacters has persisted. It is clear from the above that several biotypes must have been classified as Vibrio fetus var intestinalis

(C. fetus subsp fetus) prior to the introduction of the nalidixic acid and TTC tolerance tests (Véron and Chatelain 1973). In recent years when the large array of tests used in this work has been applied most authors have not isolated C. fetus subsp fetus but have recorded C.jejuni. Thus Hawari (1979) studied 18 faecal isolates and found only one behaving as what he classified as 'intermediate' and Smibert (1978) and Firehammer and Myers (1981) also reported that they have not isolated C. fetus subsp fetus from faeces. If this were so then faecal contamination of vaginal mucus or preputial cavity of bulls is unlikely to interfere with biochemical classification of genital isolates provided the tests included in this work are performed.

Because of the earlier inconclusive differentiation of genital isolates noted above, serological typing of isolates was combined with biotyping and it appeared that of the glycine tolerant strains (Biotype II), a distinct serotype, serotype B was the only one which could be venereally transmitted (Berg et al 1971). Although serotype A biotype II was considered abortifacient, it apparently was not venereally transmitted. During this work organisms belonging to serotype A biotype II were found to be associated with infertility as did Biotype I serotype A. Both were venereally transmitted as evidenced by bull movement. The bulls NI and SW yielded C. fetus subsp fetus biotype II serotype A from their preputial washings. Cows in herds served by these bulls were infertile and in one herd, herd A, two cows aborted (Cows no. 4 and 59) and from the vaginal mucus and lochia of these and other cows large numbers of C. fetus subsp fetus (A-2) organisms were isolated. The breeding records of such herds presented in table 6-5 and 6-6 indicated infertility in herds infected by both A-2 and A-1 organisms. Moreover the isolate NCTC 10348 typed in this work was one of those isolated by Park et al 1962 and was found to be A-2. Thus these organisms may also be genitally pathogenic as has been reported recently from

Germany (Dedie et al 1977) and the U.S.A. (Whitford et al 1977). Although Florent (1963) confirmed the genital tropism of such organisms, he did not accept that they were fully tolerant to 1% glycine. In this work they have been shown not only to have been 1% glycine tolerant but that two of the isolates became tolerant to 1.5% glycine. These findings support the report of Park et al (1962). However, it is at variance with the assertion of Berg et al (1971) that only serotype B biotype II organisms are venereally transmitted. The existence and abortifacient potential of serotype B organisms of Biotype II are also acknowledged (Whitford et al 1977). Again as in biochemical typing, serotyping was found to be useful in differentiating not only other genital isolates but also campylobacters of faecal origin. None of the catalase negative strains (C. sputorum subsp bubulus) or the one catalase positive C. fecalis isolated from the genital tract belonged to the serotypes A, B or C in which C. fetus isolates can be grouped, while all pathogenic types isolated during this work and those acquired for comparison belonged to either serotype A or B. C. jejuni isolates studied here from human enteritis did not belong to serotype C indicating the possible occurrence of different serogroups in this species. However some human isolates have been reported as belonging to serotype A and behave more like C. fetus subsp venerealis biosubtype I (Véron and Chatelain 1973). The campylobacter organisms recently isolated from aborted bovine and pig foetuses(Ellis et al 1977; Neill et al 1978, 1979)were biochemically similar to enteric organisms by virtue of their nalidixic acid intolerance, and were shown by these authors to be serologically distinct from C. fetus. Although nalidixic acid intolerance may also be lost or acquired(Butzler and Skirrow 1979) the use of serotyping of isolates could still differentiate them from C. fetus organisms known to have genital pathogenicity. On this basis it is considered justified to recommend the routine inclusion of serotyping

of genital isolates so that when a given serotype can be associated with infertility and animal movement, its possible venereal spread should then become apparent and precautions taken to prevent the spread.

The above discussion has assumed that C. fetus will be readily isolated from the genital tract of suspect or infected animals so that biotyping and serotyping can be performed in order to determine its possible pathogenicity. This assumption is partially justified at present because of technical improvements in media (Shepler et al 1963; Hoffman 1978; George 1978) also a gaseous mixture containing not more than 6% oxygen should be used (Kiggins and Plastridge 1956). However it is most interesting to note that even in modern microbiological text books (Buxton and Fraser 1977) the use of 10% CO_2 in air is still the recommended growth environment for C. fetus. The unsuitability of this environment has been reported by Roberts (1979). However during this work it was noted that when using the Oxoid anaerobic tablets fresh isolates of C. fetus subsp venerealis occassionally grew so poorly that unless the laboratory worker was familiar with the growth characteristics of this organism, plates could well have been discarded as being negative when minute colonies of the organism were in fact present. Since isolation and characterisation of the organisms from only one sample constitutes a definitive diagnosis, it is essential to identify positive plates. Two methods were found rewarding in such situations, namely the re-incubation of all plates for 10 days and the making of smears from sweep-swabs of the mucin tracts in the plates, staining these and examining for campylobacter morphology. However when plates were re-incubated for periods of longer than 10 days, some were found to be overgrown with fungi.

Using these methods 14 preputial washings obtained from bulls in nine herds were also examined. Positive isolation was made from two bulls, NI and SW although a third bull, RF may have been infected.

In this case the campylobacter shaped organism died before it could be typed. This constituted a particularly poor bacteriological result since by using the antibiotic supplemented media (BAA) and the gas environment of Kiggins and Plastridge (1956), Agumbah (1977) isolated the organism from 83% of samples taken from 37 known infected bulls which were examined 3-4 times. Moreover effective transport media (Clark et al 1974; Corbeil 1979) were used during the work. However, subsequent inquiry revealed in many instances that the bulls had been treated before sampling. In the two cases where isolations were made pre-treatment samples were available. Epidemiologically bull treatment should only be recommended when the bulls are not to be used again within the infected herd. Bouters et al (1973) confirmed this contention when they reported massive re-infection of bulls in Belgium in an area which had been supposedly cleared of bull infection four years previously by Schutte (1969).

Other means of control especially vaccination (Bouters et al 1973) should be considered. It is interesting to note that in these infected herds studied the infection was introduced by purchased, borrowed or shared bulls. Deas (1950) reported a similar situation and this illustrates the difficulty of influencing established customs among farmers. It was not therefore surprising to find that farms in the same ownership but several miles apart were simultaneously infected. It has been shown that this was due to free movement of breeding stock between two pairs of farms. This situation was also found to be operative in Kenya (Agumbah 1977). This leaves the veterinary profession with one unsolved question: Should C. fetus infection be a reportable disease?. Although farmers may be unwilling to divulge the possible existence of the disease it has been found that in those countries where the disease was made notifiable the incidence of Campylobacter fetus infertility declined (Safford 1969).

CHAPTER SEVEN

GENERAL DISCUSSION

The primary aim of this study was to determine if other more sensitive immunological methods could be devised to replace the vaginal mucus agglutination test (VMAT) for the diagnosis of *C. fetus* infection in cattle. If such a test was found, its specificity had to equal or surpass that of VMAT and it had to correlate well with actual disease status in infertile herds. To confirm the disease, the pathogen, *C. fetus* had to be isolated and shown to belong to the groups known to cause reproductive failure in cattle. Criteria for such identity were based on biochemical tolerance and serological characteristics of the organism (Berg *et al* 1971; Véron and Chatelain 1973; Neill *et al* 1978).

Because of the simplicity, rapidity and specificity of the CIE test, it was singled out for trial. The primary requirement of the use of this test is the preparation of soluble antigen(s) of the pathogen with anodal electrophoretic mobility at pH ranges in which antibodies to the pathogen would be moving in a support medium towards the cathode (Corkill 1977; Poli *et al* 1980). The occurrence of antibodies to *C. fetus* in the vaginal mucus of infected cattle has been known since the work of Stegenga and Terpstra (1949) and their titres, classes and subclasses have been documented in recent years (Pedersen *et al* 1971; Corbeil *et al* 1974a, b; Van Aert *et al* 1977).

The work reported in Chapter 3 of this thesis has conclusively demonstrated the existence of anodal antigens of *C. fetus* at pH 8.6. It has further been shown that these anodal antigens were serologically reactive with antisera to whole *C. fetus* organisms prepared in rabbits. These anodal antigens have been reported by previous workers (McCoy *et al* 1975a, b, 1976a, b) but their reaction in CIE test system has not been reported. These workers reported two anodal antigens which they named

antigens (a) and (b). A third antigen, named (e) was also shown to have a heterogenous electrophoretic mobility and this could also participate in CIE reactions.

From the strains of C. fetus organisms studied, one, C842-148 had 4 or 5 anodal antigens, another NCTC 5850 had 4 antigens although only 3 could be easily photographed. The remaining strains each had at least two anodal antigens. The characterization of these antigens into the nomenclature of McCoy et al (1975a, b) was not possible, but, because there were two major anodal antigens in all the strains studied and at least two minor ones, it is possible that the major antigens could correspond to those named (a) and (b) by the above authors. If this were so then for antigen C842-148, the components labelled C and D could be (a) and (b),respectively. Similarly for NCTC 10354, the antigen labelled 3, being quantitatively abundant, may have corresponded to antigen (a). For NCTC 5850, the quantitatively abundant antigen, labelled antigen 4, may correspond to (a). It will be seen that in this strain, antigen 4 behaved as if it had two adjoined molecular moieties each part having a different electrophoretic mobility resulting in the formation of two adjoined peaks. The minor antigen of strain NCTC 10354 labelled 1 had a similar shape. It is possible that these two were the same antigen but present in different quantities. It has recently been speculated that different strains of C. fetus might have immunological and therefore chemical microvariants of antigen (a) (Winter et al 1978).

Whatever the nature of these antigens, they reacted with anti-C. fetus whole cell rabbit antisera to form immunoprecipitates in Agarose gels (Figure 3-7) in which one sharp electrophoretically fast band and one diffuse electrophoretically slow band was seen in some cases (Figure 7-1). This simple reaction opened the way for the application of the CIE test in the immunodiagnosis of C. fetus in cattle. Although initial trials

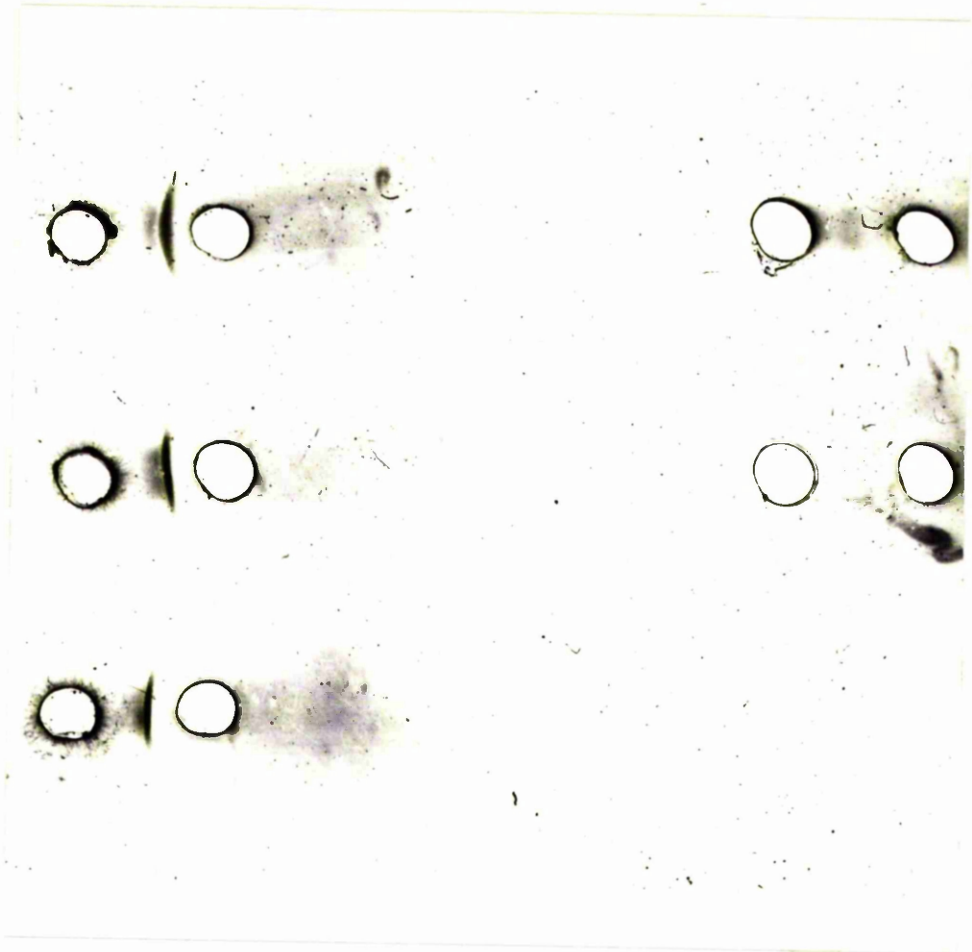


Figure 7-1: Immunoprecipitates between rabbit anti-C. fetus antiserum to C842-72 and its homologous antigen showing the sharp fast precipitation line and the broad slow precipitation line behaving similarly to antigens (b) and (a) respectively.

with phenol-saline extracts were negative, it was to be found in subsequent tests that several factors were essential for the use of this method with cattle vaginal mucus. One of these factors, pH, was encountered early in this study. Rabbit antisera were able to detect certain antigens at pH 7.4 and 7.6 but pH 8.6 was less suitable for the test.

As shown in subsequent trials, the support medium used in CIE test was critical. Thus the two Agarose types, Koch Light and Gibco Agarose, though suitable with rabbit antisera, did not give optimal conditions for use of the CIE with cattle vaginal mucus. The role of electroendosmosis in this phenomenon became apparent when Agarose B now detected the presence of antibodies in saline homogenized samples of known positive vaginal mucus. This phenomenon of electroendosmosis appears to be related to the purity of Agarose as demonstrated by Russell et al (1964), as Difco agar was not satisfactory, there apparently being too fast a movement of antibody proteins towards the antigen well. This may have been due to creation of a local antigen excess which is known to adversely affect CIE reactions (Crowle 1973). The low electroendosmosis agarose, Litex HSB, was considered very satisfactory because it detected about as many positive samples as Agarose B and revealed no false precipitation reactions. This criterion was chosen by Hibrawi et al (1977) as the major requirement for an agarose type to be useful in CIE tests. It would have been interesting to study an equal mixture of Agarose B and Litex HSB agarose because the latter had undesirable quality of brittleness which lead to breakage of the gel and the need for subsequent re-testing. Such a test system might provide the solution to this problem and ought to be tried in the future.

The pH effects in the gel on the test which culminated in the choice of pH 7.6 and 7.9 as the optimal must be considered to be a function of both the nature of bovine anti-C. fetus antibodies and the antigens

used in this test. It appears that CIE reactions are best observed at pH values at or about the isoelectric points of the antibodies concerned since, in a recent application of this test to the detection of Pasteurella multocida antigens, it was found that the most suitable pH at which Pasteurella multocida Avian antisera detected these antigens was around pH 5.2-5.6 which was near the isoelectric point of the antibody (McKinney and Rimler 1981). Similarly the two pH values found suitable for bovine vaginal mucus samples lay near or at the isoelectric points of the antibodies (Lavon 1972; Brownlie and Hibbitt 1972). Since it appeared that the major precipitating antibody in this test was IgG_1 and since IgA also participated in this reaction, a fuller understanding of this phenomenon can be developed by future studies involving isoelectric focussing of anti-C. fetus antibodies and detecting these by their specific antigen binding capacity. Also, as seen in Chapter 3 one serum sample failed to detect cross-reactive antigens at pH 7.4, which suggests that antigens were also affected by pH. As the pH was around neutrality, it can only be speculated that at this pH, the net charge of these antigens was affected and they thus became undetectable. Similar pH effects on antigens have been reported for the antigens of transmissible gastroeneteritis (TGE) of pigs by Bohac and Derbyshire (1975). Again this would require the isoelectric points of the anodal antigens to be determined. When the isoelectric points of the class types of anti-C. fetus antibodies and the isoelectric points of the antigens are both known, it should be feasible to formulate a suitable buffer to permit the migration of all or most of the antibodies to the cathode and all or most of the anodal antigens to the anode. Thus conditions of maximum precipitation should be achieved.

The requirement that more than one strain of C. fetus had to be used in the diagnostic sonicated antigen was not surprising in view of the established antigenic heterogenicity of the organism (Larson and Ringen 1967).

Although the relationship between several strains has been demonstrated by precipitation reactions with sonicated antigens, variations with strains used in the antiserum preparations were also noted (Winter 1963). The same has been illustrated in this work. Antigens C377-19 and C849-41 showed no cross-reacting antigens in immunodiffusion with antiserum C842-148 but showed reactions with antiserum NCTC 5850, but C377-19 must have had a low concentration of anodal antigen(s) detectable by CIE as antiserum C842-148 detected this antigen(s). It was therefore not possible to explain completely why vaginal mucus from cows infected with strain C377-19 did not react in CIE test with antigen 148 above.

The next major item in this study was to ascertain that the CIE test developed was indeed specific. Criteria for this were set out in Chapter 5, and it will be seen that all these were satisfied. Thus known non-infected animals originally negative to CIE and VMAT on 4 occasions prior to experimental infection, continued to be so until about 3-5 weeks after infection. This was considered to be a true immunological response in that the reactive substance in the vaginal mucus of these animals was specific antibody. The inhibition of such a reaction by rabbit antibovine immunoglobulin sera confirmed that these substances were indeed antibodies. Moreover, in CIE reactions, the substance in vaginal mucus moved to the cathode, this being one property of immunoglobulins with which antibody activity is associated (Tiselius and Kabat 1939). The antigenic specificities reported in Chapter 5 went a long way to show that the substance in vaginal mucus was specific antibody and the production of these antibodies at the local genital level and not in the serum was in accord with other reports on the dissase (Van Aert et al 1977).

The two animals which showed positive serum CIE reactions (Chapter 5) were from a known infected herd, herd A and indeed one of these (C589-59) had aborted only a few days before sampling. Although non-specific

precipitation is known to be an occasional problem in CIE tests (Hibrawi et al 1977; Holliday 1980; McKinney and Rimler 1981; Chan and Folds 1981), it was only found to be associated with low pH values in CIE performed in Agarose B gels and did not affect the final results read at pH 7.6 and 7.9. As in all immunodiffusion tests, criteria for judging certain precipitation lines as non-specific are few and the danger has been pointed out by Crowle (1973). The criterion of running the test samples without antigen as proposed by Chan and Folds (1981) has been used many times in this work. The example of sample C769-1 where the sample formed a precipitation when tested with antigen but not without antigen (see Figure 4b-7e), testifies again that the precipitations seen in this work were actually due to antigen-antibody reactions and were not non-specific precipitations.

The isolation and characterization of C. fetus organisms for a definite diagnosis of C. fetus infection in cattle is always advocated (Bearden and Fuquay 1980). Current technical innovations basically concerning media and gaseous requirement for isolations, mean that it can no longer be considered difficult to isolate the organism. The characterization of the isolate is however to some extent problematic. The criteria are those of biochemical, tolerance and serotype characteristics ascribed to genital pathogenic strains of C. fetus. In this work the use of these criteria led to the isolation of one variant strain of C. fetus subsp fetus (Skerman et al 1980) which was not only venereally transmitted but caused extensive infertility in South West Scotland. Most outbreaks of infertility attributed to this strain could be traced back to Herd A although in a few cases the source could not be determined. This organism was used in infection experiments and survived genital habitation for more than 300 days in two of the experimental heifers. Experimental infection led to the production of antibodies in the vaginal mucus of all three animals. Both observations corroborate the report

of Park et al (1962). In recent years, such glycine tolerant and possibly venereally transmitted organisms have been reported from Germany, (Dedie et al 1977), U.S.A. (Whitford et al 1977) and Namibia (Beutler-Schroder 1981). These were all serotype A and therefore differed from similar glycine tolerant venereally transmitted organisms reported by Berg et al (1971). It would appear that this investigation now links them positively with infertility, abortion and venereal transmission. The effect on fertility of these isolates was clinically indistinguishable from that of C. fetus subsp venerealis which was also isolated during this work in South West Scotland. On this basis the use of biochemical criteria, particularly glycine tolerance tests alone or in combination with hydrogen sulphide production tests, should be discouraged as the sole means of typing campylobacters of genital origin. In addition, the nalidixic acid, selenite reduction, 3.5% NaCl tolerance tests and serotyping should be used in conjunction with clinical and epidemiological observations to determine if an isolate classified as C. fetus subsp fetus is likely to be fetopathogenic.

The other catalase positive campylobacters likely to cause problems of characterization if they contaminate the genital tract of cattle (Neill et al 1978) were shown to differ from fetopathogenic types if tolerance to nalidixic acid, 2, 3, 5, triphenyl tetrazolium chloride and serotyping tests were performed. The catalse negative strains are believed to be nonpathogenic and are generally not difficult to identify. Although they were isolated from two farms with fertility problems (Herd 7, Chapter 4a and Farm I Chapter 6) the one VMAT positive mucus from one of these farms (Herd 7) was most likely a non-specific reaction. CIE did not detect antibodies in either farm. The only two occasions when doubt was cast on the validity of the CIE test because it could not be substantiated by culture or by VMAT, were in 2 herds, herds 13 and 19 (Table 4a-3 of Chapter 4a) in which the cause of the infertility was not

understood. With this reservation, this work shows that the CIE test performed on vaginal mucus of repeat breeding cattle is a reliable test enabling a rapid diagnosis of infection with *C. fetus* to be made.

APPENDIX I

DETERMINATION OF PROTEIN CONTENT IN SONICATED ANTIGENS PRIOR TO CONCENTRATION USING THE METHOD OF LOWRY ET AL (1951) AS MODIFIED BY GARVEY ET AL (1977).

Reagents

1. Sodium tartrate dihydrate ($Na_2C_4H_4O_6.2H_2O$) - 2.0 grams

   Distilled water (H_2O) - 100 ml

   Dissolve in a volumetric flask.

   This is reagent A-1.

2. Copper Sulphate Solution

   $CuSO_4.5 \cdot H_2O$ - 1.0 gram

   Distilled water (H_2O) - 100 ml

   Dissolve in a volumetric flask.

   This is reagent A-2.

3. Alkaline Sodium Carbonate Solution

   Na_2CO_3 - 20.0 grams

   0.1M NaoH - 1000 ml

   Dissove the Na_2CO_3 in 500 ml 0.1M NaoH in a volumetric flask.

   Make up to 1 litre with 0.1M NaoH.

   This is reagent A-3.

Working mixture of reagents A-1, A-2, A-3.

1.0 ml of Reagent A-1

1.0 ml of Reagent A-2

Mix thoroughly and

Add 100 ml of reagent A-3. Mix thoroughly.

This is suitable for 50 determinations. Any portion remaining after each days work is discarded.

APPENDIX I (CONT'D)

4. Titration of Folin-Ciocalteu Phenol Reagent (BDH Chemicals, England)

Twenty five ml of the reagent is added to each of six 250 ml conical flasks. Three drops of phenolphthalein solution is then added to each flask and titrated against 0.1M NaoH solution (freshly prepared) and the volume of NaoH solution required to neutralise the reagent noted in each case and the mean volume calculated. (In the determinations performed the mean volume required was 100 ml). This necessitated the two fold dilution of the Folin-ciocalteu phenol reagent to 50.0 ml. The diluted reagent is reagent 4.

5. Preparation of Standard Protein Solution Using Bovine Serum Albumin Factor V (Armour Laboratories, U.S.A.).

Stock Solution :

Bovine serum albumin factor V	-	1.0g
Distilled water	-	200 ml.

Thus the standard solution had 5mg/ml of protein.

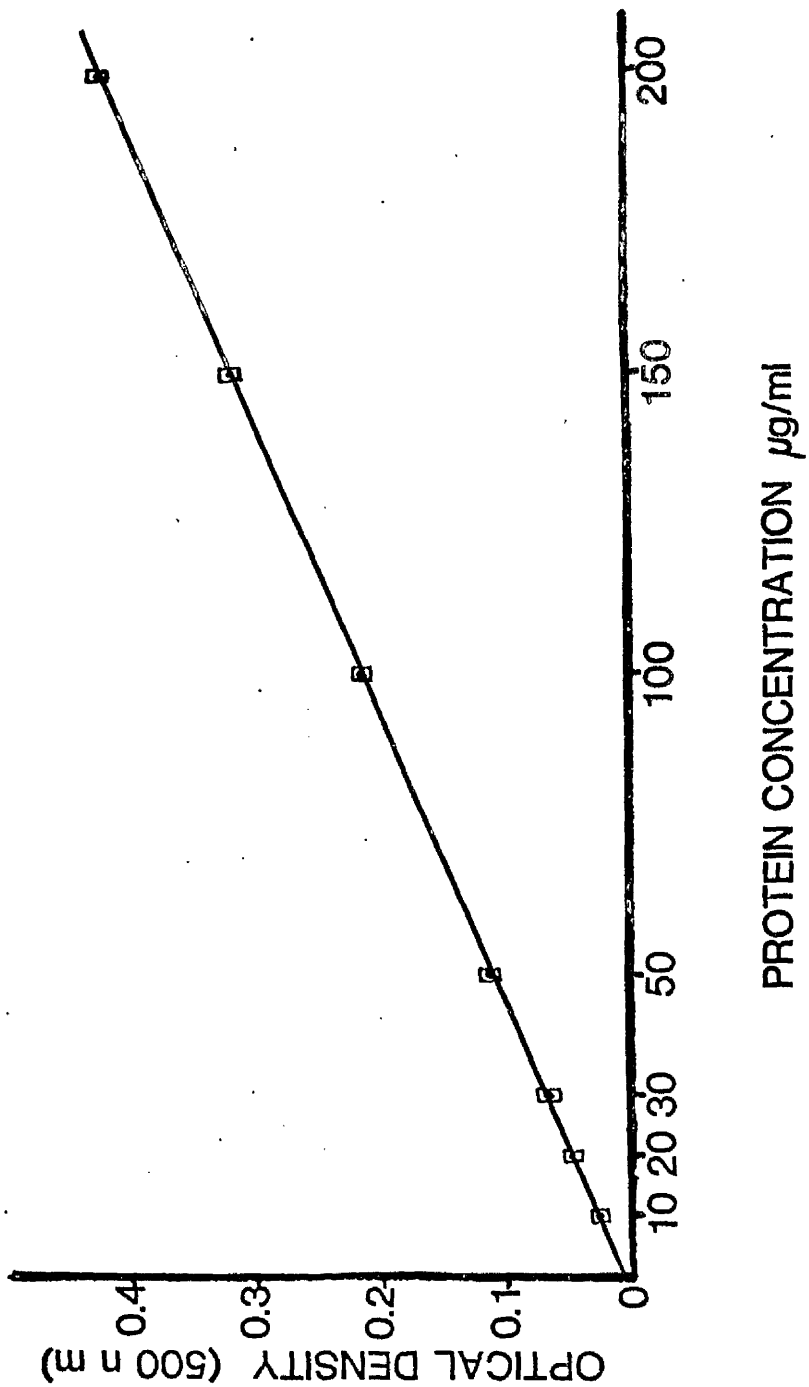
From this solution, standard dilutions are prepared containing 10 µg, 20 µg, 30 µg, 50 µg, 150 µg and 200 µg of protein and the assays are performed according to the method of Garvey et al (1977).

A standard graph is prepared from several readings at each concentration and the assays of dilutions of the sonicated antigens are ploted on the graph and the concentrations calculated by interpolation.

Figure 8 shows a typical standard curve .

STANDARD GRAPH FOR PROTEIN DETERMINATION

FIGURE 8



The table (Appendix 1.1) below shows the protein concentration of the prepared sonicated antigens before and after concentration.

Table Appendix 1:1

Protein Content of Sonicated Antigens Prior to Sonication and After Concentration.

Antigen	Protein Concentration Before Concentration	Protein Content After Concentration
C842-148	370 ug/ml	9.30 mg/ml
C383-157	1.25 mg/ml	10.0 mg/ml
C849-41	985 ug/ml	11.8 mg/ml
NCTC 10354	1.66 mg/ml	13.3 mg/ml
NCTC 5850	1.25 mg/ml	10.0 mg/ml

APPENDIX 2

THE CAMPYLOBACTER FETUS MUCUS AGGLUTINATION TEST (WEYBRIDGE METHOD)

Collection of Mucus Samples

Vaginal mucus is collected by means of a mucus pipette which consists of a glass tube about 50cm in length and 1 cm in diamter. The pipette has slight bend about 10 cm from one end and this end is lightly plugged with cotton wool. The other, straight, end is tightly plugged with cotton wool to act as a stopper. Before use the pipettes are wrapped in greaseproof paper and sterilized by autoclaving.

To collect the mucus a piece of pressure tubing about 50 cm long is attached to the straight end of the pipette, the plug at the bent end is removed and after the vulva has been washed clean the pipette is passed into the vagina as far as the cervix. Then by sucking on the free end of the rubber tubing and moving the pipette backwards and forwards in the vagina, a portion of the mucus is loosened and is drawn into the pipette. The suction should be maintained as the pipette is being withdrawn. The cotton wool plug is reinserted in the pipette and a label with the identity of the animal is attached.

For transportation purposes it is recommended that the mucus should be transferred from the pipettes into containers such as McCartney bottles. By applying pressure on the cotton wool plug at the straight end of the tube with a length of flexible wire the contents of the tube are forced into the container.

Laboratory Equipment Required

For the performance of the test the following equipment is required.

Test tubes (standard 6" x 5/8") Griffiths tubes. M.R.C. 1 oz screwtopped jars. 0.5% phenol saline. 3% agar (Davis' New Zealand Powdered Agar, plus 0.5% sodium chloride). 10 ml pipettes (with the bore at the delivery end widened). Water bath running at 57°C. Incubator running at 37°C. Agglutination tubes approximately 2" x $\frac{3}{8}$". 0.25%

formol saline (0.25 ml formaldehyde solution 40% in 100 ml physiological saline). Positive C. fetus serum, 385M. Measuring cylinder. C. fetus antigen (Strain 1980 or Norman).

Technique of the Test

The mucus is diluted with normal saline in the proportion of one part mucus to four parts saline. The mixture is ground to a homogeneous suspension in a Griffiths tube. (Care should be taken in the grinding process as the mucus may form a seal when the plunger is being withdrawn and thus create such a vacuum that the mucus may be drive through the bottom of the Griffiths Tube). Two ml of this mucus-saline suspension are transferred to a test tube which is placed in a water bath running at 57°C.

The 'Davis' agar is kept in stock in 25 ml quantities in universal bottles. When required, this agar is melted by bringing to 100°C in boiling water. The bottles of molten agar are transferred to a water bath running at 57°C and left there for about five minutes. Two ml molten agar are then added to the 2 ml of the mucus-saline mixture. The resultant mixture of agar-mucus-saline is then shaken vigorously and quickly poured into an M.R.C. 1 oz screw-topped jar. The jar is allowed to stand for about 30 minutes until the mixture is firmly set, when 2 ml of 0.5% phenol saline are layered on. The lid, with a paper washer to avoid excess evaporation, is screwed on tightly and the jar is incubated for 18 hours at 37°C to allow the process of the extraction of the antibodies to take place. The water-clear supernatant fluid is used, as a serum, in a three tube serial dilution agglutination test.

Three tubes are set up in a rack. In the first stage, the first tube is left empty but 0.5 ml of 0.5% phenol saline is measured into each of the other two. In the second stage, 0.5 ml of the supernatant fluid is put into each of the first and second tubes. The contents of the second tube are mixed and 0.5 ml carried over into the third tube where after mixing, 0.5 ml is discarded. In the last and final stage 0.5 ml

of antigen suspension is added to each of the three tubes and mixed.

Controls: An ampoule containing 1 ml dried serum 385 M or 12/9/73 is opened and 1 ml distilled water is added. The ampoule is rinsed out thoroughly. The serum thus reconstituted is made up to 40 ml in a measuring cylinder with 0.5% phenol saline. A strip with seven agglutination test tubes is prepared and into each tube is put 0.5 ml of 0.5% phenol saline. 0.5 ml of the diluted 385 M or 12/9/73 serum is added to the first tube, the contents are mixed and 0.5 ml is carried over to the second tube. The process is repeated to the seventh tube. 0.5 ml antigen is then added to each tube and mixed.

The stability of the antigen in saline is controlled by preparing two agglutination test tubes each with 0.5 ml phenol saline and 0.5 ml antigen suspension.

The tests and controls are incubated at $37^{o}C$ for 18 hours and read by ordinary transmitted light against a dark background. Readings are recorded as follows:-

++++	=	water clear
+++	=	75% clearing
++	=	50% clearing
+	=	25% clearing

The control tubes with positive serum should read:-

++++, ++++, ++++, +++, +++, $^{+}_{+}$ or $^{+}_{+}$, -.

The saline controls should give negative readings.

Interpretation of the Test

It has been the practice of Weybridge to designate the readings of the test as 'Positive', 'Suspicious' or 'Negative', according to the degree of agglutination present. When agglutination has been found to occur in the third tube a 'Positive' result has been returned. When agglutination has been found to occur in any lesser degree a 'Suspicious' result has been recorded.

APPENDIX 3 AGGLUTINATION TITRES OF SERA TESTED FOR PRECIPITATING ANTI C. FETUS ANTIBODIES BY THE CIE TEST

Clinical Case No.	Animal No. Sample No.	Whole Cell (WC) titre	'O' Antigen Titre	Natural/ Experimentally Infected or Specified	Serum CIE Results
C589,	169	80	320	Natural	+ve(AS)
C749	71	80	160	Natural	-ve
& C842	190	40	160	Natural	-ve
	101	40	40	Natural	-ve
	25	40	160	Natural	-ve
	14	40	40	Natural	-ve
	148*	80	80	Natural	-ve
	70	40	80	Natural	-ve
	79	40	160	Natural	-ve
	59*	$\geq$ 640	0	Natural	+ve(AS)
	2*	40	80	Natural	-ve
	34*	40	160	Natural	-ve
	4*	40	160	Natural	-ve
	16*	40	160	Natural	-ve
C275	32494*	40	320	Natural	-ve
	75773*	40	160	Natural	-ve
	204*	$<$ 40	160	Natural	-ve
	124	80	160	Natural	-ve
	96873	40	320	Natural	-ve
	123	80	160	Natural	-ve
	118	$<$ 40	320	Natural	-ve
	83*	$<$ 40	320	Natural	-ve
	106*	40	320	Natural	-ve
	997724*	40	320	Natural	-ve
	74*	40	320	Natural	-ve
C263	21+	40	40	*C. sputorum* subsp *bubulus* isolated	-ve
	92+	40	160	"	-ve
	63	$<$ 40	160	(False +ve? VMAT Chapt. 4)	-ve

APPENDIX 3 (cont'd)

Clinical Case No.	Animal No. Sample No.	Whole Cell (WC) titre	'O' Antigen titre	Natural/ Experimentally infected or Specified	Serum CIE Results
Cairn Hill	37	< 40	160	Unknown	-ve
	48	40	80	Unknown	-ve
	20	< 40	40	Unknown	-ve
	35	0	0	Unknown	-ve
	38	40	160	Unknown	-ve
	44	40	0	Unknown	-ve
C340	65*	< 40	80	Natural	-ve
	192	40	160	Natural	-ve
C34	10*	< 40	160	Natural	-ve
	84*	< 40	160	Natural	-ve
	3096	< 40	160	Brucella +ve	-ve
	3097	< 40	160	(SAT)RBPT	-ve
C377	04*	< 40	80	Natural	-ve
	110	40	80	Natural	-ve
	92*	160	160	Natural	-ve
Hf24*	28 Dpi	40	160	Experimental	-ve
	141 Dpi	40	160	Experimental	-ve
	152 Dpi	< 40	80	Experimental	-ve
	162 Dpi	40	160	Experimental	-ve
	239 Dpi	40	320	Experimental	-ve
	291 Dpi	40	160	Experimental	-ve
Hf17*	152 Dpi	< 40	160	Experimental	-ve
	162 Dpi	< 40	160		-ve
	180 Dpi	< 40	320		-ve
	291 Dpi	40	320		-ve
	302 Dpi	40	160		-ve
Hf14*	14 Dpi	40	160	Experimental	-ve
	28 Dpi	< 40	160		-ve
	141 Dpi	40	80		-ve
	152 Dpi	40	160		-ve
	162 Dpi	40	160		-ve
	239 Dpi	40	320		-ve

* C. fetus (venerealis or fetus) isolated
\+ C. sputorum subsp bubulus

SAT = Serum Agglutination Test
RBPT = Rose Bengal Plate Test
Dpi = Days post-infection

APPENDIX 4 BIOCHEMICAL AND SEROLOGICAL CLASSIFICATION OF INDIVIDUAL C. FETUS ISOLATES STUDIED

Identification of Organism	Biochemical and Tolerance Tests															Temperature Tolerance			Serotype				Clinical Syndrome	Source of Organism	Final Classification
	Catalase	H_2S^1	H_2S^2	H_2S^3	Gly^1	Gly^2	$NALD^1$	$NALD^2$	TTC^1	TTC^2	3.5% NaCL	0.1% Na-Selenite	8% Glucose	BG1 (33,000)	BG2 (100,000)	25°C	37°C	42°C	A	B	C	Unknown			
C842-72	+	-	-	+	+	-	+	+	-	-	-	+	+	+	+	+	+	-	A	0	0	0	Infertility	Vaginal Mucus	C. fetus subsp fetus
C842-16	+	ND	-	+	+	-	+	+	-	-	-	ND	ND	ND	ND	ND	+	ND	A	0	0	0	"	"	"
C842-22	+	ND	-	+	+	-	+	+	-	-	-	ND	ND	ND	ND	ND	+	ND	A	0	0	0	"	"	"
C842-2	+	L	O	S	T	B	E	F	O	R	E	T	E	S	T	S				+	ND	ND	ND	ND	"	"	"
C589-4	+	ND	-	+	+	-	+	+	-	-	-	+	+	+	+	ND	+	-	A	0	0	0	Infertility & abortion	"	"
C589-44	+	ND	-	+	+	-	+	+	-	-	-	ND	ND	ND	ND	ND	+	-	A	0	0	0	Infertility	"	"
C589-34	+	-	-	+	+	-	+	+	-	-	-	+	+	+	+	+	+	-	A	0	0	0	"	"	"
C589-59	+	-	-	+	+	-	+	+	-	-	-	ND	ND	ND	ND	ND	+	ND	A	0	0	0	Infertility & Abortion	"	"
C759-69	+	-	-	+	+	-	+	+	-	-	-	ND	ND	ND	ND	ND	+	ND	A	0	0	0	Infertility	"	"
C759-41	+	-	-	+	+	-	+	+	-	-	-	ND	ND	ND	ND	ND	+	ND	A	0	0	0	"	"	"
C589-148	+	-	-	+	+	-	+	+	-	-	-	+	+	+	+	+	+	-	A	0	0	0	"	"	"
NI	+	-	-	+	+	-	+	+	-	-	-	+	+	+	+	+	+	-	A	0	0	0	Infertility	Bull preputial washing	"
C34-10	+	-	-	+	+	-	+	+	-	-	-	+	+	ND	ND	+	+	-	A	0	0	0	"	Vaginal Mucus	"

APPENDIX 4 (Cont'd)

Identification of Organism	Biochemical and Tolerance Tests															Temperature Tolerance			Serotype				Clinical Syndrome	Source of Organism	Final Classification
	Catalase	H_2S^1	H_2S^2	H_2S^3	Gly^1	Gly^2	$NALD^1$	$NALD^2$	TTC^1	TTC^2	3.5% NaCL	0.1% Na-Selenite	8% Glucose	BG1 (33,000)	BG2 (100,000)	25°C	37°C	42°C	A	B	C	Unknown			
MSU 13140	+	-	-	+	+	+	+	+	-	-	-	+	+	+	+	ND	+	ND	A	0	0	0	?	Montana acquisition	C. fetus subsp fetus
MSU 17535	+	-	-	+	+	+	-	-	N	-	-	+	-	ND	ND	-	+	+	0	0	0	0	?	"	C. jejuni/coli
MSU 18756	+	-	-	+	+	+	-	-	d	-	-	+	-	-	-	ND	+	+	0	0	C	0	Not known	"	"
C383-22236	+	-	-	+	+	-	+	+	-	-	-	+	+	+	+	+	+	-	A	0	0	0	Infertility	Vaginal mucus	C. fetus subsp fetus

APPENDIX 4 (Cont'd)

Identification of Organism	Biochemical and Tolerance Tests																Temperature Tolerance			Serotype				Clinical Syndrome	Source of Organism	Final Classification
	Catalase	H_2S^1	H_2S^2	H_2S^3	Gly^1	Gly^2	$NALD^1$	$NALD^2$	TTC^1	TTC^2	3.5% NacL	0.1% Na-Selenite	8% Glucose	BG1 (33,000)	BG2 (100,000)		25°C	37°C	42°C	A	B	C	Unknown			
C34-84	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+		+	+	−	A	0	0	0	Infertility	Vaginal Mucus	C. fetus subsp fetus
C34-Uknwn	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+		+	+	−	A	0	0	0	"	"	"
C111-72	+			FAILED				TO				GROW								ND	ND	ND	0	"	"	Died during subcultivation
C275-75558	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+		+	+	−	A	0	0	0	"	"	C. fetus subsp fetus
C275-75573	+	−	−	+	+	(+)	+	+	−	−	−	+	+	+	+		+	+	−	A	0	0	0	"	"	"
C275-74	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+		+	+	−	A	0	0	0	"	"	"
C275-997724	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+		+	+	−	A	0	0	0	"	"	"
C275-106	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+		+	+	−	A	0	0	0	"	"	"
C275-32494	+	−	−	+	+	(+)	Low +	Low +	−	−	−	+	ND	+	+		+	+	ND	A	0	0	0	"	"	"
C275-83	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+		+	+	−	A	0	0	0	"	"	"
C263-92	−	+	++	++++	+	+	+	+	−	−	+	+	ND	+	+		ND	+	ND	0	0	0	0	"	"	C. sputorum subsp bubulus
C263-21	−	+	++	++++	+	+	+	+	−	−	+	+	ND	+	+		ND	+	ND	0	0	0	0	"	"	"
C340-2	+	−	−	L O S T																				"	"	
C340-65	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+		+	+	−	A	0	0	0	"	"	C. fetus subsp fetus

APPENDIX 4 (Cont'd)

Identification of Organism	Biochemical and Tolerance Tests															Temperature Tolerance			Serotype				Clinical Syndrome	Source of Organism	Final Classification
	Catalase	H_2S^1	H_2S^2	H_2S^3	Gly^1	Gly^2	$NALD^1$	$NALD^2$	TTC^1	TTC^2	3.5% NaCL	0.1% Na-Selenite	8% Glucose	BG1 (33,000)	BG2 (100,000)	25°C	37°C	42°C	A	B	C	Unknown			
C250-5	+	±	+	++	+	+	+	+	-	-	-	+	+	+	+	-	+	+	0	0	0	0	Infertility	Vaginal Mucus	C. f ecalis (tentative)
C377-4	+	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	-	A	0	0	0	"	"	C. fetus subsp venerealis
C377-92	+	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	-	A	0	0	0	"	"	"
C377-86	+	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	-	A	0	0	0	"	"	"
C377-19	+	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	-	A	0	0	0	"	"	"
SR JPR	-	+	++	++++	+	+	+	+	-	-	+	+	ND	+	+	ND	+	ND	0	0	0	0	"	Bull preputal washing	C. sputorum subsp bubulus
OBAN-C																									
C377-54	+	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	-	A	0	0	0	"	Vaginal mucus	C. fetus subsp venerealis
Quality Control	+	-	-	+	+	+	+	+	-	-	-	+	+	ND	ND	ND	+	ND	ND	ND	ND	ND	Ovine abortion	Unknown (simulated)	C. fetus subsp fetus
64/3	+	-	-	+	+	+	+	+	-	-	-	+	+	+	+	ND	+	ND	0	B	0	0	"	Unknown	"
156 AH 177	+	-	-	-	-	-	+	+	-	-	-	(±)	+	+	+	+	+	-	A	0	0	0	Infertility	"	C. fetus subsp venerealis
Bridge	+	-	-	+	+	+	+	+	-	-	-	+	+	+	+	+	+	-	0	B	0	0	Ovine abortion	"	C. fetus subsp fetus

APPENDIX 4 (Cont'd)

Identification of Organism	Biochemical and Tolerance Tests															Temperature Tolerance			Serotype				Clinical Syndrome	Source of Organism	Final Classification
	Catalase	H_2S^1	H_2S^2	H_2S^3	Gly^1	Gly^2	$NALD^1$	$NALD^2$	TTC^1	TTC^2	3.5% NaCL	0.1% Na-Selenite	8% Glucose	BG1 (33,000)	BG2 (100,000)	25°C	37°C	42°C	A	B	C	Unknown			
Langford	+	−	−	+	+	+	+	+	−	−	−	+	+	+	+	+	+	−	O	B	O	O	Ovine abortion	Unknown	C. fetus subsp fetus
Grant	+	−	−	+	+	+	+	+	−	−	−	+	+	+	+	ND	+	−	A	O	O	O	"	"	"
I. Knew	+	−	±	+	+	+	−	−	+	−	−	±	ND	+	+	ND	+	+	O	O	O	O	Human enteritis	Intestinal/faecal	C. jejuni/coli group
C377-4SN+	+	−	−	+	+	+	+	+	−	−	−	+	ND	+	+	ND	+	−	A	O	O	O	Infertility	Vaginal mucus	C. fetus subsp fetus
C351-11	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	A	O	O	O	"	"	"
C351-77	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	A	O	O	O	"	"	"
C351-79	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	A	O	O	O	"	"	"
C351-UNK	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	A	O	O	O	"	"	"
GBAN-C[2]	+	−	−	+	+	+	+	+	+	−	−	+	ND	+	+	ND	+	ND	Not done				Ovine abotion	Ovine foetus	C.jejuni/coli
C383-157	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	A	O	O	O	Infertility	Vaginal mucus	C. fetus subsp fetus
GR-CAMP	+	±	+	++	+	ND	−	−	±	−	−	+	−	−	+	ND	+	+	O	O	O	O	Enteritis	Canine faeces	C.jejuni/coli
M874	+	±	+	++	+	ND	−	−	+	−	−	+	−	−	+	ND	+	+	O	O	O	O	"	Equine faeces	"
C651-975	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	A	O	O	O	Infertility	Vaginal mucus	C. fetus subsp fetus

APPENDIX 4 (Cont'd)

Identification of Organism	Biochemical and Tolerance Tests															Temperature Tolerance			Serotype				Clinical Syndrome	Source of Organism	Final Classification
	Catalase	H_2S^1	H_2S^2	H_2S^3	Gly^1	Gly^2	$NALD^1$	$NALD^2$	TTC^1	TTC^2	3.5% NacL	0.1% Na-Selenite	8% Glucose	BG1 (33,000)	BG2 (100,000)	25°C	37°C	42°C	A	B	C	Unknown			
C651-972	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	A	0	0	0	Infertility	Vaginal mucus	C. fetus subsp fetus
C767-68	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	ND	+	−	A	0	0	0	"	"	"
C767-52	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	A	0	0	0	"	"	"
C767-46	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	A	0	0	0	"	"	"
C767-SW	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	A	0	0	0	"	Bull preputial washing	"
C849-41	+	−	−	−	−	−	+	+	−	−	−	−	+	+	+	+	+	−	A	0	0	0	"	Vaginal mucus	C. fetus subsp venerealis
Hf 24 (14)	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	A	0	0	0	"	"	C. fetus subsp fetus
Hf 24 (141)	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	A	0	0	0	"	"	"
Hf 17 (6)	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	A	0	0	0	"	"	"
Hf 24 (321)	+	−	−	+	+	−	+	+	+	+	−	+	+	+	+	+	+	−	A	0	0	0	"	"	"
MSU 14840	+	−	−	+	−	−	+	+	−	−	−	ND	ND	ND	ND	ND	+	ND	A	0	0	0	?	Montana Acquisition	C. fetus subsp bubulus (biosubtype I)
MSU 18567	+	−	−	−	−	−	+	+	−	−	−	−	+	+	+	ND	+	−	A	0	0		?	"	C. fetus subsp venerealis
MSU 1309	+	−	−	+		ND	ND	ND	ND	ND	−	+	ND	ND	ND	ND	+	ND	A	0	0	0	?	"	C. fetus subsp fetus

APPENDIX 4 (Cont'd)

Identification of Organism	Biochemical and Tolerance Tests															Temperature Tolerance			Serotype				Clinical Syndrome	Source of Organism	Final Classification
	Catalase	H_2S^1	H_2S^2	H_2S^3	Gly^1	Gly^2	$NALD^1$	$NALD^2$	TTC^1	TTC^2	3.5% NaCL	0.1% Na-Selenite	8% Glucose	BG1 (33,000)	BG2 (100,000)	25°C	37°C	42°C	A	B	C	Unknown			
NCTC 10348	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	A	0	0	0	Infertility	Vaginal Mucus	C. fetus subsp fetus
NCTC 10355	−	+	++	++++	+	+	+	+	−	−	+	ND	ND	ND	ND	ND	+	ND	0	0	0	0	Not known	Bull Semen	C. sputorum subsp bubulus
NCTC 5850	+	−	−	+	+	+	+	+	−	−	−	+	+	+	+	ND	+	ND	0	B	0	0	Ovine abortion	Aborted ovine foetus	C. fetus subsp fetus
NCTC 10354	+	−	−	−	−	−	+	+	−	−	−	−	+	+	+	+	+	−	A	0	0	0	Infertility	Vaginal Mucus	C. fetus subsp venerealis
S. Wood	+	−	+	+	+	+	−	−	+	−	−	+	ND	ND	ND	ND	+	+	0	0	0	0	Human enteritis	Intestinal faecal	C. jejuni/coli
A. Smith	+	−	+	+	+	+	−	−	+	−	−	+	ND	+	+	ND	+	+	0	0	0	0	"	"	"
Belv +C	+	−	+	+	+	+	−	−	+	+	−	+	−	W	+	−	+	+	0	0	0	0	"	"	"
54640	+	−	+	+	+	+	+	+	+	+	−	+	+	+	−	ND	+	+	0	0	0	0	"	"	"
54864	+	−	+	+	+	+	−	−	−	−	−	+	ND	+	−	ND	+	+	0	0	0	0	"	"	"
63348	+	−	+	+	+	+	+	−	−	−	−	+	−	+	+	ND	+	+	0	0	0	0	"	"	"
58395	+	−	+	+	+	+	−	−	−	−	−	+	+	+	−	ND	+	+	0	0	0	0	"	"	"
C250-163	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	A	0	0	0	Infertility	Vaginal Mucus	C. fetus subsp fetus
C250-65b	+	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	A	0	0	0	"	"	"

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