Bacteriological Studies of Suspected Infertility in Cattle and Sheep with Particular Reference to Vibrio, Brucella and PPLO

Thesis
Submitted to the Magadh University in Partial Fulfilment of the Requirements for the Award of MASTER OF SCIENCE (Veterinary) Degree in BACTERIOLOGY

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Bacteriological Studies of Suspected Infertility in Cattle and Sheep with Particular Reference to *Vibrio parahaemoliticus* and PPLO

Thesis
Submitted to the University in Partial Fulfilment of the Requirements for the Award of MASTER OF SCIENCE (Veterinary) in Bacteriology

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I certify that this Thesis entitled
"Bacteriological studies of suspected infertility
in cattle and sheep with particular reference to
Vibrio, Brucella and PPLO" has been prepared under
my supervision by Sri Gopal Saran, a candidate for
the M.Sc. (Vet.) with Bacteriology as major subject,
and that it incorporates the results of his independent
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INTRODUCTION
INTRODUCTION

"Few, if any, fields of study entertain more opinions with less corroborative evidence to support them than that dealing with infertility".


True, opinions alone, however ingenious, can not stand long without an appeal to experiment or a wide and detailed observation.

Infertility, a state of barrenness, in regard to reproduction, cuts at the very root of the animal husbandry programmes which aim at the economic welfare of a country through raising a healthy livestock population with good capacity to produce healthy progeny, milk and meat and other by-products. If an etiological approach is desired infertility may fall in one or more of the following four categories - (1) genetic, therefore congenital, which may possibly be hereditary, (2) disturbance of sexual functions due to hormonal imbalance, (3) nutritional deficiencies and (4) infections. It may be pointed out that these broad based categories are far from being sharply demarcated.

The relative contribution of these different and divergent factors to breeding troubles, such as, temporary or permanent sterility, low conception rates, decreased fertility,
abortion and stillbirth, etc. is yet to be ascertained. But presumably infection, as a cause of infertility, excels by far the others. Many a author motivated by a desire for etiological research have investigated into the occurrence of microflora in the reproductive tract of both diseased and apparently normal animals. As a result, a host of microorganisms of varying pathological importance have been isolated.

The role played by some of these organisms, such as Brucella and Vibrio, in relation to abortion and sterility in cattle and sheep is well known; whereas in regard to others, such as pleuropneumonia-like organism (PPLO) the state of affairs is a curious mixture of "established fact, half knowledge, hopeful guessings and frank bewilderment".

Brucellosis has been experienced in almost all the countries of the world. Some of them have adopted measures to keep it under control with varying outcome. The economic loss due to this disease is fourfold: loss due to abortion or birth of dead or weak calves, decreased milk yield, impaired fertility and the interference with desired sequence of calvings. In 1949, a Special Committee in U.S.A. estimated an overall loss of $7 million dollars due to brucellosis in cows.

The study of virosis received little attention until 1940 because of the emphasis being placed on brucellosis (Binns and Fincher, 1954). Since then interest has grown and
extensive research is being carried out to determine the occurrence, its significance in relation to reproductive disturbances and a more accurate and convenient method for diagnosis both in cattle and sheep. In 1954, the U.S. Agricultural Research Service ascribed a loss of 138 million dollars to this disease as a result of decreased reproductive activity and milk production and high replacement cost. In England, vibriosis is no longer considered a national problem though it may exist in individual herds (Lawson, 1963).

Pleuropneumonia-like organisms (PPLO) have been isolated from the genital tract of many species of domesticated animals, some of them having definite pathological significance. In man, PPLO has been recovered from the cases of non-specific (non-gonococcal) urethritis as well as from the vagina of normal human females.

In India, several authors have recorded the incidence of brucellosis and it is still one of the major problems on many farms. Several states, including Bihar, have or had run schemes for investigation into the incidence and control of brucellosis. Not much work appears to have been done to ascertain the present position of vibriosis in this country and only meagre information is available about the pleuropneumonia-like organisms in cattle and sheep.

The present research, therefore, was undertaken with
a view to determine the incidence, if any, of *Brucella*, *Vibrio* and *Pleuropneumonia*-like organisms by cultural and serological means, particularly from animals having a history of "repeat breeding". It is humbly hoped that this investigation will, at least in some measure, lead to valuable information on the important problem of infertility in cattle and sheep.
REVIEW OF LITERATURE
REVIEW OF LITERATURE

(A) VIBRIO

HISTORICAL:

Vibrionic organism was first associated with abortion in sheep by McFadyean and Stockman (1909 - 1913) in England. During the same period they also succeeded in isolating the organism from two out of five cows they examined. But much of the fundamental research on this disease was carried out by Smith and his associates in America between the period 1918 - 1923 and it was Smith and Taylor (1919) who named the organism *Vibrio fetus*.

Cotton (1919) reported the isolation of a spirillum by Buck and Creech in pure culture from the aborted foetuses of four cows (cited by Rhoades and Hardenbrook, 1947).

Since then, many authors including Thomsen (1920), Gmuinder (1922), Poppe (1922), Traum (1923), Witte (1923), Klarin (1926), Lorsche (1927), Barger (1928), Yakimoff (1930), Snyman (1931), McEwen (1940), Plastridge (1941), Plastridge, Williams, Petrie (1947) and Plastridge et al (1951), isolated *Vibrio fetus* from aborted bovine foetuses in varying numbers.

REVIEW:

Hindmarsh (1942) reported the isolation of one strain
of *Y. fetus* from the only case of abortion in a herd within a period of three years.

Based on the examinations carried out between the period of 1924 - 1943, Olsen (1946) recorded the recovery of 2,105 strains of *Y. fetus*.

Rhoades and Hardenbrook (1947) reported the isolation of *Y. fetus* from the stomach contents of three aborted foetuses in the Holstein unit of a large dairy herd. They failed to produce abortion in three pregnant cows experimentally by administering the isolated vibrio by intravenous, oral and intra-uterine routes. The possible explanation put forward was attenuation of the organism on laboratory media.

Canham (1949) isolated nine strains of *Y. fetus* from the animals belonging to one herd in which there was no history of infertility. Wilson and Mc Diarmid (1950) isolated seven strains from 20 herds having similar history.

Sjollem, Stegenga and Terpstra (1949) recovered *Y. fetus* from the vaginal mucus of four heifers experimentally mated with a bull the semen of which was culturally positive for vibrio infection. *Y. fetus* was also isolated from a cow which had aborted and the vaginal mucus of a large number of heifers, all of which had been served by this bull.

Moore (1950) reported the isolation of several strains of *Y. fetus* from herds in which abortion rates averaged 12 per cent and there was high incidence of retained membranes.
Stegenga (1950) isolated *V. fetus* from the vagina of two cows after they were served by an infected bull.

Lawson and MacKinnon (1952) carried out investigations in ninety herds between 1947 to 1951. They examined 1,302 bovine foetuses and isolated *V. fetus* from 112 of them.

Florent (1953) isolated a new saprophytic species of vibrio from the vaginal discharges of cows and semen of bulls. Thouvenot and Florent (1954) isolated this species again and named it *Vibrio bubulus*. This species was a strict anaerobe and shown to be antigenically different from *V. fetus*.

Plastridge (1961) suggested the need of differentiating *V. fetus* from *V. bubulus*.

Ristic and Morse (1953) reported the isolation of *V. fetus* from the gall bladder of experimentally infected guinea-pigs.

Sims and Fincher (1954) isolated *V. fetus* from the semen of one and preputial mucus of two bulls. On retest, they isolated *V. fetus* from the semen of four and preputial mucus of three bulls. Out of 121 cows subjected to first test they isolated *V. fetus* from the vaginal mucus of only one. On retest, *V. fetus* was isolated from the vaginal mucus of three out of 47 cows. No vibrio could be recovered from the uterine mucus of nine and seven cows subjected to test and retest, respectively. They observed that even in a herd known to be infected, *V. fetus* was isolated infrequently from the vaginal...
mucus of cows, but could be found more readily on the bacteriological examination of semen and preputial mucus of bulls.

Kawashima et al. (1954, 1955) isolated V. fetus from 26.5 per cent of 561 bovine foetuses examined in the period 1956-53. They also isolated vibrio organisms from the female genital tract of infertile cattle.

Frank and Bryner (1953) in a study of the bacterial flora of the bovine genital tract noted that V. fetus was the only bacterium found commonly in relation to "repeat breeding" and they recovered vibrio organism from 26 out of 46 herds examined. They, in 1954 and Bryner and Frank (1955) examined 164 strains of vibrios originating from aborted bovine foetuses, reproductive tracts of infertile male and female cattle, vaginal mucus of normal cows, semen and genital tracts of fertile and virgin bulls. Based on certain behaviour of the vibrios, these authors divided them into two groups. The vibrios coming from the normal animals were catalase-negative, produced large quantities of hydrogen sulphide and grew well in deep stab cultures in semi-solid Thiol media. These were regarded as saprophytes. The other vibrios were catalase-positive, did not produce hydrogen sulphide and grew only on or near the surface of deep stab cultures of the above media. These were regarded as strains of true V. fetus. Serologically, the two groups were unrelated.

Mundt (1955) examined various sites of the reproductive
tract of cows and bull to determine the localisation of *Y. fetus* infection. In cow he isolated the organisms from the vaginal vestibule, the sub-urethral diverticulum, the cervix, the right uterine horn and fallopian tubes. From the bull positive isolation was done from the prepuce, the penis and the anterior portion of urethra at about a distance of 12 inches from the orifice.

Hubrig and Mohanka (1956) reported the isolation of *Y. fetus* from the genital tracts of thirty-one bulls and cows and 29 strains from aborted foetuses.

Morgan (1957) frequently isolated *Y. fetus* from the anterior vaginal mucus of recently infected cows and heifers. Biopsy materials from the uterus did not yield so successful results. He observed that catalase-negative *Y. fetus* could be isolated from cows, unmated heifers or heifers inseminated with the semen of known uninfected bulls, from the sheath and semen of bulls, though the rate of recovery was never high. He suggested the differentiation of catalase-negative vibrios from *Y. fetus* on the basis of biochemical tests.

Lindquist (1957) isolated seven strains of catalase-negative and hydrogen sulphide-positive vibrios from the semen and cervical mucus. These vibrios failed to infect maiden heifers.

From slaughtered cows Grogel (1957) collected vaginal mucus for the isolation of vibrio by three different methods. The two of them when combined resulted in detection of 95 per cent
of the positive cases. He identified vibrios microscopically in 96 out of 300 specimens of placentas and foetuses. In addition of vibrios, he also isolated 18 Brucella, three Corynebacterium pyogenes and six fungi out of 38 cases of abortion.

Florent et al (1958) while studying the course of Y.fetus infection in experimental heifers, noted that the vibrios at first remained confined to the vagina. From uterus these organisms were recovered seven days after infection and were present in the whole of the reproductive tract up to seven weeks.

Wandenplasche (1958) obtained regular positive isolations of Y.fetus from the uterus of heifers slaughtered between five to thirtyfive days after service and irregularly from those slaughtered after 50 days to eight months.

Florent (1959) described two types of genital vibriosis in cattle, one due to a vibrio known as Vibrio fetus venerealis and the other as Vibrio fetus intestinalis. The latter was believed to cause only sporadic abortion and no infertility.

Bryans and Smith (1960a) reported the isolation of two strains of a new variety of vibrios from the aborted foetuses of sheep. Later, they (1960b) studied the physiological properties of fiftyfive strains of both pathogenic and non-pathogenic vibrios isolated from cattle, sheep and chickens. These strains were examined with regard to catalase activity, hydrogen sulphide production, reduction of nitrate, tolerance of 4 per cent
sodium chloride, bile salt and sodium selenite. According to their origin they were placed in four groups. Group I comprised of seventeen strains from aborted foetuses (bovine); Group II of fourteen strains from aborted ovine foetuses; Group III of three strains from hepatitis in chickens and Group IV of twenty-one strains from semen, preputial and vaginal mucus samples from cattle and sheep. Strains belonging to the first three groups were considered pathogenic on the basis of origin, production of catalase, failure to grow in 4 per cent sodium chloride and growth on ox bile medium. Only two from Group I, half from Group II and all from Group III produced hydrogen sulphide.

Catalase-positive strains of Group IV and Group I behaved similarly. Catalase-negative strains produced hydrogen sulphide and grew in media containing four per cent sodium chloride and 0.1 per cent sodium selenite. Catalase-negative strains from sheep and cattle behaved alike except that the cattle strains did not grow in ox bile medium but the sheep strains did. No correlation was found between the production of hydrogen sulphide and pathogenicity.

In Congo, Lambelin (1950) were successful in isolating 12 strains of *V. fetus* out of 231 samples of vaginal mucus from European cows. From foetuses three *V. fetus* strains were recovered. However, no vibrio could be isolated from 154 samples of vaginal mucus of indigenous cows, although six mucus samples out of thirty-three gave positive agglutination reaction.
Park (1961) observed morphological differences between *V. fetus* and non-pathogenic and other types of vibrios originating from cows and bulls. He, in 1962 and Florent (1963) noted that type "B" vibrio strains and *V. fetus intestinalis* established and remained for long periods in the genital tract of non-pregnant heifers and cows.

Grigore and Darie (1963) described three variants of vibrio on grounds of morphology and type of colonies on blood agar. One was comma shaped, smooth and stable but difficult to grow; the second, filamentous, fairly stable and easy to grow and the third was polymorphous and unstable and had various stages of dissociation. Morphological variations have been described by several authors (Vizy, 1937; Ogg, 1962; Barner and Oberst, 1950; Barger, 1928; Ryff and Lee, 1945; Prier, 1952 and Lawson, 1959).

In India, Parnaik et al (1957) isolated one vibrio from the placenta of a buffalo which had aborted in the fourth month of pregnancy. They also isolated *V. fetus* from two cows having granular vaginitis. But the authors withheld the final verdict regarding the identity of the strains because the typing was still in progress.

Prasad (1965) isolated, from buffaloes, three strains of *V. fetus* from the vaginal and cervical mucus of slaughtered animals. These strains were claimed to be pathogenic because they produced catalase and not hydrogen sulphide.
Different authors have used media with various composition for the isolation, maintenance, bulk growth and antigen production of vibrio organisms. The results too have been equally variable.

Barger (1928) found relatively good results by enrichment of media with body fluids particularly with whole blood or serum.

For cultivation of *V. fetus* Plastridge and Williams (1943) used a soft agar medium with liver infusion as base in which peptone one per cent and agar 0.3 per cent were added. The pH of the medium was adjusted to 7.4. They suggested that materials suspected for *V. fetus* should first be examined microscopically and subsequently cultured on soft agar and blood agar. They cited McFadyean and Stockman (1913) who cultured *V. fetus* in deep gelatin serum agar and about placing the inoculum in the water of condensation in slants of fresh liver infusion agar but commented that these methods were of limited value. In 1961, Plastridge found better results in isolation of *V. fetus* by incorporating 2 units per ml. of bacitracin and two microgram per ml. of novobiocin in routine blood agar medium. But the number of vibrio-like colonies on this medium was reduced. He observed that bacitracin alone was less effective than novobiocin and recommended the use of bacitracin-novobiocin blood agar medium for cultivating semen.

Huddleston (1949) found thiol (Difco) medium satisfactory for isolation, cultivation and maintenance of *V. fetus*. He
observed that in this medium vibrios would remain alive for 150 days at room temperature. Thiol media was also found satisfactory by Plastridge and Williams (1948), Adler (1952), Hansen, Price and Clements (1952), Hughes and Gilman (1953) and Binns andFincher (1954). Some of these authors used thiol medium in semi-solid form.

Sjollema, Stegenga and Terpstra (1949) used a culture medium for V.fetus which consisted of 1.5 per cent agar to which 10 per cent defibrinated cow’s blood was added. They advised to use a large series of culture plates as a precaution against the possibility of contamination.

Prier (1952) used tryptose agar (Difeo enriched with 0.05 per cent thiamine hydrochloride) routinely and on this medium V.fetus grew in fine dew-drop like colonies on the surface of agar. He observed that particularly heavy growth was present along the agar-glass junction and water of syneresis.

Terpstra (1953 & 1954) devised a medium containing alkyl-aryl-sulphonate, 1:1000 and aesculin,1:200 for isolation of V.fetus from contaminated material. For isolation of V.fetus from similar sources Florent (1956) used nutrient agar with brilliant green, 1:40000 and Kuzdas and Morse (1956a) used Albimi agar containing ox bile, ethyl violet, bacitracin, polymyxin B and actidione.

Schneider and Morse (1955) cultivated a majority of
Y. fetus and unidentified vibrio strains in media containing 10 per cent bile. The stock cultures were best maintained at room temperature (21°C) and transferring them every four weeks to fresh medium. They claimed that incorporation of bile in media led them to develop a selective media for the isolation of Y. fetus from experimental infections in laboratory animals and observed that Y. fetus strains isolated from bovine vaginal mucus or bull semen were more adversely affected than the strains cultured from aborted bovine and ovine foetuses. For storing purposes they found a temperature near freezing point to be detrimental to Y. fetus.

Nowak (1955) observed that Bartlett’s medium was most suitable for the growth of Y. fetus. In this medium organisms were large sized and they appeared twisted in the early stage of growth.

Laing (1958) advised bovine blood-agar and modified Plaetridge medium containing Bacothiol, glutathione and agar to which ten per cent defibrinated guinea-pig blood was added at the time of use for isolation and bovine blood-agar and peptone broth for maintenance of stock cultures.

Gottz (1956) used thiol medium with 0.5 per cent agar for culturing vaginal mucus from cows and preputial washings from bulls. He found that samples obtained by irrigation of vagina with physiological saline to be better for isolation than samples obtained after the slaughter of the animal.
He also noted that an interval of 24 hours between the collection and cultivation of the materials reduced the chances of isolation by half. Similar observation regarding the collection of material from vagina of cattle and sheep was made by Liese (1958) while working on isolation and differentiation of vibrios.

Morgan (1957) used blood agar with 0.1 per cent sodium thioglycollate and pH was adjusted to 7.6 for primary isolation of \( Y.\text{fetus} \) from all sources. For the maintenance of pure cultures he found Brewer's medium (pH 7.2) to be the most satisfactory.

Mundt (1958) devised a special medium for culturing preputial washing of bulls, which contained peptone, sodium phosphate, glutathione, yeast extract, glucose, streptomycin sulphate and modified Ringer's solution (pH 7.4). The organisms were reported to survive up to 4 days and 112 hours at room temperature and 4°C, respectively.

Lawson (1959) reported that the medium for isolation and cultivation of \( Y.\text{fetus} \) at Weybridge was blood agar - consisting of 1.8 per cent Davis nutrient agar and 10 per cent defibrinated ox blood, adjusted to pH 7.8 to 7.6, both plates and slopes being used and several ml. of defibrinated ox blood was added to the slopes after inoculation. The stock cultures were maintained in peptone blood broth.

Witte (1963) allowed the swab to remain for one hour near os uteri to collect mucus and cultured the samples on
blood agar plate containing brilliant green, crystal violet, alkyl-aryl-sulphonate and polymyxin B.

Shepler, Plumer and Faber (1963) found that a combination of antibiotics, such as, bacitracin 15 units/ml., polymyxin 1 unit/ml. and novobiocin 5 microgram/ml in a basal medium consisting of brain-heart infusion agar (produced by Baltimore Biological Laboratories, Baltimore), and added 10 per cent defibrinated bovine blood to be most effective in the isolation of V. fetus from bovine preputial samples.

In addition, they also cultured the samples after filtering through millipore filters in an attempt to assess the relative efficacy of the two techniques, for purposes of isolation. Out of 56 samples, they isolated, separately, V. fetus from 43 samples on antibiotic medium and 41 by filtration through millipore filter. Fortynine samples were positive when the two methods were used jointly.

In India, Prasad (1965) used the modified Elastridge medium and blood agar with 0.4 per cent thiol and brilliant green 1:40,000 for processing samples from slaughtered she-buffaloes.

Several methods have been employed for the diagnosis of V. fetus infection in cattle and sheep. The most reliable of all of them is the isolation of the organism from the suspected animals, aborted foetuses or placental membranes (Prier, 1952). The other methods include test mating of
heifers with suspected bulls (Frank and Bryner, 1953; Rasbech, 1951; Adler, 1952 and 1954), serum agglutination test (Sjollem et al, 1949; Plastridge et al, 1951; Plastridge and Easterbrooks, 1953 and Parnaik et al, 1957), a modified complement fixation test (Trilenko, 1956), an indirect haemagglutination test (Te Runga, 1956), and agglutination test with the vaginal mucus of females and semen and preputial washings of males.

While serum agglutination test was an useful tool in the hands of the above cited workers, others (Stegenga and Terpstra, 1949; Adler et al, 1952 and Boyd, 1955) found it of limited or doubtful value. The suggestion of Kerr (1954) that a barrier exists in the uterus through which the antibodies of such genital infections as Br. abortus, V. fetus and Trichomonas foetus do not pass into blood circulation except under exceptional circumstances, may explain the variation in the results of different workers.

The technique for collection of vaginal mucus and preputial washings and procedure for agglutination test have been described by Terpstra (1949), Hughes (1953), Binns and Fincher (1954), Mac Kinnon (1954) and Laing (1956 and 1962). The test has been found very useful and reliable for routine diagnosis in cattle (Leaver and Hart, 1960).

The interval between infection and appearance of antibodies in mucus may vary and Lawson and Mac Kinnon (1952) and McEntee et al (1954a) found it to be between 2 - 15 weeks
and 30 - 70 days, respectively. Various figures have been given for the persistence of agglutinins in mucus by Lawson and Mackinnon (1953; 2½ to 14 months), McEntee et al (1954a; approximately 7 months) and Blobel et al (1957; 4 - 12 months). It has been suggested that the vaginal mucus should not be collected at oestrus because agglutinins tend to disappear, entirely or partially, which may give false negative results (Laing, 1956 and Lawson, 1959).

Much of the success of this test depends upon a suitable antigen. Different vibrio antigens have been found to give variable results (McEntee et al, 1954a; Binns and Fincher, 1954) and Laing (1956) suggested that generally better results are obtained with antigens prepared from locally isolated strains.

Lawson (1959) emphasized the need of interpreting the result of mucus agglutination test on herd basis and not for diagnosis of infection in individual animals.

Several authors have employed mucus agglutination test for diagnosis of V. fetus infection in cattle (Stegenga and Terpstra, 1949; Hughes, 1953; McEntee et al, 1954 a,b; Binns and Fincher, 1954; Boyd, 1956; Hunter, 1956; Leaver and Hart, 1960; Jacotot and Vallee, 1960).

In India, Mohan (1954) carried out vaginal mucus agglutination test in 89 cows, buffaloes and sexually mature calves from three herds having various degrees of breeding
trouble. Ill-defined and weakly positive agglutination reaction was detected in 20 cows and sexually mature calves. In only one buffalo marked agglutination was found. On the basis of this finding the author suggested the probability of *Y. fatus* infection in India. Parnaik *et al* (1957) subjected vaginal mucus samples from 15 cows to mucus agglutination test and found one to be positive.

(B) **BRUCELLA**

**HISTORICAL:**

The infection by various members of *Brucella* in animals and man is known from ancient times (Stableforth, 1959). As early as the beginning of nineteenth century it was regarded as a contagious disease and by the end of the century this view as strongly supported by Zundel (1871) and Styrl (1875). Transmission experiments with infected materials were successfully carried by several authors (Brauer, 1873, 1880; Lehner, 1878 and Woodhead *et al*, 1889). Bruce (1887) isolated the causative agent of Malta fever in man and subsequently named the organism *Micrococcus melethensis*. In 1897, Bang, in collaboration with Stribolt, isolated a small bacillus from cows suffering from infectious abortion and named it *Bacillus abortus*.

Alice Evans (1918) was the first to recognise the close morphological, biochemical and serological resemblance between
the Micrococcus maletensis of Bruce (1897) and Bacillus abortus of Bang (1897). This view was supported by Bevan (1921-22) and Keefer (1924) and several other workers from many parts of the world (Stableforth, 1959). These closely related organisms have now been placed in Genus Brucella, a name given on the suggestion of Meyer and Shaw (1929) in honour of Sir David Bruce.

**REVIEW:**

Brucellosis has been recorded in a large number of countries including Norway, Sweden, Denmark, England, U.S.A., U.S.S.R., Canada, Japan, New Zealand, Ceylon, France (Stableforth, 1959 and many other workers) and India (Maddow, 1934; Polding, 1943, 1947a, b and others).

Though *B. abortus* is mostly incriminated for bovine abortions (Lawson, 1962), cattle can also be infected, both naturally and experimentally, with *B. maletensis* (Orlov and Karneeva, 1939) and *B. suis* (Schroeder and Cotton, 1925) but abortions following the infection with *B. maletensis* and *B. suis* appear to be less frequent than with *B. abortus* (Stableforth, 1959).

Incidence of ovine brucellosis has been recorded in Algeria, Mongolia, Morocco, Yugoslavia, Turkey, Tunisia, etc. (Lawson, 1962), U.S.A. (Bruce, 1930), Germany (Karsten, 1950), England (Young, 1953) and India (Das et al., 1961) and has been found to be a serious problem in Germany, France Greece,
Italy and some parts of U.S.S.R. (Lawson, 1962). The infection in sheep is mostly due to *Br. melitensis* but *Br. abortus* has also been isolated from ovine foetuses (Stableforth, 1959 and Lawson, 1962). Sheep can be infected artificially with *Br. abortus* (Stableforth, 1959) and sporadic natural infection with this organism have been recorded (Bruce, 1930; Karsten, 1950 and Angelov, 1953).

Fitch *et al.* (1930) recorded that after abortion brucella organisms were excreted in the uterine discharges up to an average of 12 days. In 1931, they found that after parturition *Br. abortus* was not excreted from vagina beyond three or four weeks and, if excreted they were non-viable. In 1938, they examined repeatedly the vaginal swabs from an infected cow and noted that in absence of vaginal discharge and till the uterine seal was intact *Br. abortus* was not found in the vagina.

In an attempt to determine the seat of infection in non-pregnant animals Wackerbarth (1937) examined 300 uteri and failed to isolate brucella either culturally or by guinea-pig inoculation from any, whereas Fitch *et al.* (1939) isolated brucella organisms from 24 out of 64 uteri showing no evidence of parturition and thus demonstrated that *Br. abortus* could be found in non-gravid uteri.

Seelmann *et al.* (1951 and 1961) isolated *Br. abortus*, *Br. melitensis* and *Br. suis* from cows clinically free from
brucellosis. They made the interesting finding that a cow which had yielded \textit{Br. abortus} in 1955 showed \textit{Br. suis} in 1956 and again \textit{Br. abortus} in 1958.

Lerche and Entel (1953) recorded the isolation of brucella from the uterus, milk sample and blood of slaughtered cows which were serologically positive for brucellosis. In a few cases guinea-pig inoculation was found necessary for the isolation of the organisms.

For the recovery of brucella from placentas, Bouvier (1960) treated them with slaked lime or super phosphate in varying concentrations. After centrifugation, the sediment was washed in saline, cultured on \textit{W'} medium and incubated under partial carbon dioxide tension. A high percentage of success was claimed.

From the foetuses of cows and she-buffaloes Kamel and Abdel-Fattah (1961) isolated an unusual type of brucella. It had the biochemical characters of \textit{Br. melitensis} but serologically it behaved like \textit{Br. abortus}. Similar findings were reported by Stableforth (1959) who also pointed out that strains with cultural and biochemical characters of \textit{Br. abortus} and serological character of \textit{melitensis} were recovered in Palestine, U.S.A., England, France, India and elsewhere.

Smith \textit{et al} (1961) observed that at the time of abortion in experimentally infected pregnant cows brucella organisms were almost entirely confined to foetal cotyledons,
fluids and chorion. Pearce et al (1962) isolated erythritol from the bovine foetal fluids and held it responsible for the intracellular and preferential growth (in foetal tissue) of Br. abortus. Williams et al (1962) confirmed the above view and found that erythritol, even in small fractions, stimulated the in vitro growth of Br. abortus.

Henry et al (1932) used chocolate or cooked blood agar for the primary isolation of brucella organisms. They added gentian violet for contaminated materials. Klimer (1939) tried sephirol, malchite green, victoria blue and gentian violet. Victoria blue coloured the colonies slightly and, therefore, had an additional advantage in detecting them. Kerby and Falder (1940) attempted to isolate brucella organisms on a milk medium in which crystal violet was incorporated.

Liver infusion agar, with or without the addition of blood was found suitable by Paterson (1940), Huddleson et al (1941) and Mackie and McCartney (1959). Huddleson (1955) observed that addition of a sterile bacterial extract, aged blood serum or serum albumin enhanced the growth of fastidious carbon dioxide dependent Br. abortus.

Boyd and Caeman (1951) noted complete inhibition of the growth of Br. abortus when tryptose agar was filtered through 15 different brands of commercial absorbent cotton.
The inhibition, which could be prevented by the addition of starch in the medium, was probably caused due to a fatty acid like substance present in the cotton.

For the isolation of brucella from contaminated material Jones and Morgan (1958) found serum dextrose agar to be very effective. Renoux (1959) obtained better results with potato agar, to which 10 per cent horse serum was added, than Albimi brucella agar, tryptose agar and liver agar, whereas Morgan (1960) found Albimi brucella agar plus antibiotics better in comparison to serum glucose plus antibiotics and ethyl violet, thio blood agar plus antibiotics, Morris medium and serum glucose plus dyes.

Stableforth (1959) advocated the use of modified Kuzdas and Morse medium consisting of three per cent agar made with Albimi peptone and per ml. each of 0.1 mg. actidione, 6 units polymyxin B, 25 units bacitracin, 15 units cireculin and 1/800,000 ethyl violet for heavily contaminated materials. He also cited that good results were obtained with the medium of Morris (1958), which is made with either tryptose agar or Hartley’s agar and contains 1/30,000 v/v nitrofurfuryl methyl ether (Furaspor), 25 units/ml. bacitracin, 4 units/ml. polymyxin B and 1/100,000 w/v actidione. But he noted that the dye sensitive types of brucella are inhibited by these media and suggested the use of the medium devised by Jones and Morgan (1959) which consists of horse serum dextrose agar with 0.1 mg/ml. actidione, 6 units/ml. polymyxin B and
25 units/ml. bacitracin.

Gay and Damon (1950) used 3-, 4- and 5-day old embryonating eggs for the primary isolation of brucella from the bloodstream. All the three types of brucella were reported to have been isolated and the technique was claimed to be superior to guinea-pig inoculation and culture in many instances.

Van der Schaaf and Jaartsvel (1958) used a detergent 'Abro', which is composed of a secondary alkyl sulphonate, alkyl-aryl-sulphonate and a non-ionic component, in blood agar. The detergent checked the spreading of proteus organisms. Entel (1960) isolated brucella from the blood of cattle and sheep by using the cation-exchange resin "Duolite". This substance was believed to enhance the growth of brucella.

Wejzlar and Duniewicz (1961) employed a semi-synthetic agar medium with a number of growth factors, vitamins, antibiotics (polymyxin and bacitracin), tryptose phosphate and filtrate of chick embryo extract for the isolation of Br. abortus from the sternal biopsy samples of infected human patients.

Many tests have been employed for the diagnosis of brucella infection in animals. Some of them have been more widely used because of their reliability and convenience of procedure. Their use is also dictated by the purpose for which they are required, e.g., for testing a herd or flock or
individual animals.

Tube serum agglutination test is one of the most widely used and a test of choice both in man and animals, particularly cattle (Stableforth, 1959) and in fact it was in the search of a diagnostic test for this disease that tube agglutination test was discovered (Wright and Smith, 1897). Stableforth (1959) noted that many infected sheep and goats, however, may not show a positive reaction to tube agglutination test, though they may be positive to other tests. He also mentioned that some of the factors influencing the result of agglutination test quantitatively are: number of organisms present in the antigen and their sensitivity, roughness which increases the titre and gives non-specific reaction, presence of dissolved agar leading to increased sensitivity, temperature and the period of incubation and the presence of haemoglobin in the serum which is responsible for non-specific reaction. Ferreira de Abreu and Mario (1960) claimed reliable results by centrifuging the haemolysed blood samples and precipitating the haemoglobin by phenol.

The diagnostic titre of tube agglutination test is now expressed in international units (I.U.). The titre of 80 to 100 I.U./ml. is considered to be positive (Report, 1954) and this has been confirmed by the F.A.O./W.H.O. Expert Committee on Brucellosis at their meeting in 1957 (Report, 1958). Animals
showing half this titre are regarded as doubtful and they should be re-tested within a month (Stableforth, 1959).

Some of the other commonly used tests are: plate or rapid agglutination test (Huddleston, 1920, 1932 and 1943a) for routine test in some countries; Abortus ring test (A.B.R.) introduced by Fleischauer (1937) for herd test in many countries including India and whey test carried out in the same way as the tube agglutination test.

In India, Folding (1943) isolated 46 strains of brucella from various sources. Out of these 22 strains were pure abortus type, 18 intermediate between abortus and melitensis and 3 were melitensis type. The remaining three strains were either not typed by then or lost. He observed that incidence of brucellosis was more in animals having higher proportion of European blood and that the susceptibility of Indian cattle to brucellosis was either decreasing or the spread of this disease was being limited by better management practices. The susceptibility of sheep and goat to the Brucella types present in this country was found to be low.

In 1947(a) he recorded that incidence of brucella infection in village cattle was mainly of indigenous type. In an attempt to analyse the factors responsible for the dissemination of the infection he found a direct correlation
between rainfall and occurrence of brucellosis. Infection was more in farm animals in areas where total rainfall was high. "The rate of village infection was negligible in semi-desert areas but considerable in humid coastal belts". He later (1947b) classified 49 Indian *Brucella* strains as: typical *Br. abortus* 26, *Br. melitensis* 3 and intermediate type 20 strains. The intermediate strains were termed as *Br. abortus/meletensis* (A/M type). He isolated six strains of brucella from village cows and all these were of A/M type. One unclassified strain was isolated from a farm cow at Patna. In 1948, he extensively reviewed his previous work and gave a detailed account of epidemiology, bacteriology, diagnosis, public health significance and control of brucellosis in India and discussed infectious and non-specific abortions.

Dhanda and Rajgopalan (1949) recorded an incidence of up to 20 per cent in the farms of Calcutta and coastal Bombay and up to 50 per cent in the villages of south. The overall percentage of incidence was 1.5.

According to the report of the Indian Council of Agricultural Research (1951-52) the highest incidence of brucellosis was recorded in Orissa. The percentage of incidence for Bombay State and Madhya Pradesh were 20.2 and 11.3, respectively.

Lall and Bakshi (1960) noted that a large number of cows having a history of abortion did not show positive
sero-agglutination reaction with brucella antigen, but microscopical, cultural and serological examinations at the time of abortion indicated that most of the abortions were caused by brucella. Later, they (1961) found negative cultural result with the milk of sixteen cows having serum agglutination titres of 1:20 to 1:320. Guinea-pig inoculation also with the milk of two cows proved infructuous. The guinea-pigs, however, showed a titre of 1:640 and 1:40 respectively, but no thermal or pathological reaction was noticed.

Das, Panda and Biswal (1962) subjected a total of 249 bulls, bull calves and bullocks and 20 buffalo bulls and buffalo bull calves to quick and tube agglutination tests. They found 5 bulls, 6 bull calves, 2 bullocks and none of the buffalo bulls and buffalo bull calves positive for brucellosis.

Pargasonkar and Raj (1962) while carrying a survey in Hyderabad, Secunderabad and a few adjoining villages with ABR test found the percentage of positive samples to be 3.5.

Das, Panda and Dutta (1961) briefly reviewed the works of various authors (such as Haddow, 1934; Milakantan and Pande, 1949; Polding, 1949; Mathur, 1953, 1959 and Sharma, 1958) regarding incidence of brucellosis in sheep and goats. They examined 154 goats and 81 sheep by a combination of quick and tube agglutination tests with positive finding in eight and two, respectively.

Mathur (1962) reviewed the epidemiology, various
serological and bacteriological tests and incidence of brucellosis in man, cattle, sheep, goats and pigs. He discussed the measures of control in man and animals and made certain recommendations. He also compiled the figures of several authors to indicate the number of cows, buffaloes and goats, both of organised farms and villages tested for brucellosis and found positive.

In the "Final Report of the Scheme for the Investigation of Infectious Abortion (Brucellosis) in the Uttar Pradesh for the period 1953-64", the incidence of brucellosis in 16 state livestock farms in the year 1956-57 was found to be 6.2 per cent. The incidence was slightly higher in cattle than buffaloes. Brucella infection had been found in the animals of Goshalas as well. Flocks of sheep and goats maintained at 17 state farms were found negative on tests using \textit{Br. abortus} antigen. But the examination of sera samples of sheep and goats from different slaughter houses and a village flock revealed the incidence of about 0.5 and 11 per cent, respectively. The local strains of \textit{Br. abortus} were subjected to various tests.

A scheme, "Investigation and control of Brucellosis in cattle in Bihar" was run between the period 1953-65. 10,110 samples of sera from cattle and buffaloes of different Government Cattle Farms and Goshalas were tested by tube agglutination method. Out of these, 103 or 1.018 per cent were
found to be positive. The yearwise percentage was: 1.2, 0.9, 0.5, 1.7 and 0.79 for the years 1959-60, 1960-61, 1961-62, 1962-63 and 1963-64, respectively. During 1960-62, 369 sheep were tested and 34 or 9.9 per cent of them were found positive. In 1962-63, all the 41 sheep re-tested and in the year 1963-64 all except one sheep out of 94 sheep and goats, were found negative. 533 goats were tested between 1960-63 and only one was positive. (The results of tests carried out at Government Cattle Farm, Patna, from where some of the materials for the present work were collected, has been given on appendix A).

Several samples from cases of abortion were examined bacteriologically but no brucella could be isolated. Only one strain of *Br. abortus* was, in the period under review, isolated from the semen of a bull.

Purbey (1965) carried out serological test for brucellosis in the animals of Cattle Breeding Farm, Igatpuri and found 16 per cent of the cows and some heifers, bullocks and bull calves to be positive reactors. He also studied two cases of abortion and described the clinical findings. No attempt appears to have been made to isolate the organisms.

Prasad (1965) isolated two strains of *Brucella* from the vaginal mucus of buffaloes. These strains were indistinguishable from *Br. melitensis* on cultural, biochemical and metabolic tests.
(c) PLEUROPNEUMONIA-LIKE ORGANISMS  
(FPLO)

HISTORICAL:

Nocard and Roux (1898) were the first to recognise and cultivate the causal agent of pleuropneumonia of cattle. Bordet (1910) and Borrel et al (1910) described the complex morphology of this organism. On the basis of the infectivity of Berkefeld filtrate, some of the earlier authors believed this organism to be a filterable virus (Wilson and Miles, 1935). The second species of this group was isolated and described by Bridre and Bonatien (1923) from the cases of contagious agalactia in sheep.

Klienesberger (1935) isolated some organisms from the stock cultures of Streptobacillus moniliformis which she initially believed to be FPLO living in close symbiosis with the bacteria. However, the investigations of Dienes (1939) and others showed that they were the dissociated forms of bacteria. These forms, usually designated as 'L-form' or 'L-phase' of bacteria, have been recovered from many organisms, such as, Proteus, Salmonella, Shigella, Neisseria, Haemophilus, Bacillus and Clostridium.

REVIEW:

A number of pleuropneumonia-like organisms, both pathogenic and non-pathogenic, closely similar in morphology and cultural characters have been isolated from the respiratory
and urogenital tracts of rats, mice, dogs, cattle and man (Wilson and Miles, 1955). Saprophytic PPLO were shown to exist in sewage by Laidlaw and Elford (1936) and Seiffert (1937a, b).


The study of PPLO as a pathogen of bovine genital tract is of recent origin. Since Edward, Hancock and Hignett (1947) first reported the isolation of PPLO from the genital tract of cattle, several authors including Edward (1950), Hignett (1949), Nielsen (1949), Szabo (1951), Florent (1953), Terpetra (1953), Bakos et al (1959), Olson et al (1960) and Speck (1962) obtained these organisms from the same source.
Jayaraman (1961) claimed to have isolated for the first time in India, one strain of *Mycoplasma bovis* genitalium from the reproductive tract of a cow, whereas Prasad (1965) failed to isolate any PPLO in his study of 1109 samples from the different portions of the female genital tract of buffaloes.

Edward (1960a) isolated two types of PPLO from the genital tract of cattle. One of these produced larger colonies, grew at room temperature and did not require the addition of serum or ascitic fluid in the culture media for its growth - the characteristics very similar to those possessed by saprophytic strains isolated from soil or sewage, and, therefore, this PPLO was provisionally designated as 'S' strain. The other, required additional enrichment of the culture media, did not grow at room temperature and was isolated from animals having inflammatory condition of the genitals or breeding trouble. Such PPLO was called, provisionally again, as 'P' strain. Isolation of the 'P' strain was confirmed by the findings of Hignett (1949), Weinhofener (1961), Florent (1962) and Terpstra (1963).

Flynn (1952) in his study of the organisms in relation to infertility in cattle found PPLO to be of doubtful importance.

Hartman et al (1964) studied the experimental infection of the uterus of mature heifers with an unclassified PPLO
found responsible for severe mastitis in cows. Various degrees of pathological changes were noted in the uterus, the fallopian tube and the peritoneum. On the basis of this observation the authors concluded that a PPLO pathogenic for the bovine udder was also pathogenic for the bovine genital tract, but it still remained to determine whether infertility was caused by this or other pathogenic PPLO under field conditions.

Klieneberger (1954) and Edward (1964) reviewed the literature on PPLO in great detail and made some valuable suggestions.

The significance of PPLO in the human genital tract has been discussed by Klieneberger (1939), Beveridge (1943), Dines (1940) and Morton et al (1952).

Edward (1947) devised a selective medium consisting of ox heart infusion broth to which were added peptone one per cent, horse serum 20 per cent (v/v), yeast extract (± 250 gm/l) 10 per cent (v/v), thallium acetate (1:2000 for broth and semi-solid media and 1:8000 for agar plates) and penicillin. The final pH was adjusted to 8.0. He advised the use of media in three forms - broth, agar plates and sloppy agar. He also tested the relative efficacy of sodium azide, potassium tellurite, thallium acetate, gentian violet, brilliant green and penicillin in the isolation of PPLO.

Edward and Fitzgerald (1962) noted that 'P' strains
of PPLO from the genital tract needed a specific growth factor for primary isolation and it can be supplied by hog gastric mucin or sodium salt of thymic nucleic acid.

White (1952) used a medium consisting of thallium acetate, sulphanethazine, crystal violet and penicillin in Bennett's broth (1932).

Chicken embryo has also been employed for cultivation of PPLO by some authors (Florent, 1953; Keller and Morten, 1954).
MATERIALS AND METHODS

The materials and methods utilized in this study were designed to facilitate the extraction of data from a variety of sources. The selection of methods was guided by the objectives of the research, ensuring that the methods were appropriate and effective for the study's purpose.

The raw materials and methodological aspects of the study were carefully selected and executed to achieve the desired outcomes. The results obtained from these materials and methods are presented in Tables I and II.

The detailed description of the materials and methods used in this study is provided in Section III.
MATERIALS AND METHODS

The materials for this experiment were collected from the genital tract of cows with a history of "repeat breeding" and sheep. The criterion laid down for cows was that these animals had not conceived after three or more artificial inseminations or natural services. Materials were also collected from a few cows having no such history for comparison. From sheep, the sample was collected on the basis of a prior finding that some of them in the flocks at Sheep Breeding Farm, Tekuna were found positive for brucellosis on tube agglutination test. Most of these sheep were later transferred to the premises of Red Sindh Cattle Breeding Farm, Gauriakarma. Consequently, materials were collected from both of these places.

The cows brought to the Gynaecology Section of Bihar Veterinary College, the Artificial Insemination Centre and those of the Cattle Breeding Farm, Patna, were included in this study.

A description of the materials collected from various sources is given in Tables 1A and 1B.

History of the animals:

A brief history of each cow regarding age, number of calves, both alive and dead; abortions, if any; number of
### TABLE- IA

**Samples collected from cattle**

<table>
<thead>
<tr>
<th>Source of collection</th>
<th>No. of vaginal swabs</th>
<th>No. of vaginal discharges</th>
<th>Aborted foetus</th>
<th>Semen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gynaecology Section, Bihar Veterinary College, Patna.</td>
<td>96</td>
<td>27</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Artificial Insemination Centre, Patna.</td>
<td>23</td>
<td>8</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Cattle Breeding Farm, Patna.</td>
<td>60</td>
<td>20</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Other sources</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td><strong>Total:</strong></td>
<td>179</td>
<td>55</td>
<td>2</td>
<td>9</td>
</tr>
</tbody>
</table>

### TABLE- IB

**Samples collected from ovine female genital tract**

<table>
<thead>
<tr>
<th>Source of collection</th>
<th>No. of swabs</th>
<th>No. of vaginal discharges</th>
<th>Aborted foetus</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep Breeding Farm, Tekuna.</td>
<td>21</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cattle Breeding Farm, Gauriakarma.</td>
<td>45</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Other sources</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>Aborted foetus of a goat.</td>
</tr>
<tr>
<td><strong>Total:</strong></td>
<td>66</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

artificial inseminations or natural services; oestrous; presence of vaginitis, cervicitis or discharges, etc., was recorded. Where possible, the condition of the internal genital organs was also noted.

The history has been summarised in Table II.

Methods of collection of the materials:

Cotton swabs were made on a straight copper wire. It was placed in a test tube (6"x3/4") and plugged with cotton. Later, the shape of the copper wire was modified. One end was made in a small ellipsoidal loop and bent to an angle of about 20°. The swab was so made that a small portion of the loop remained exposed. This was devised so as to dislodge and collect mucus when the swab was moved back and forth and rotated in the genital tract.

For aspirating mucus or discharge from the vagina glass pipette was prepared on the lines of Binns and Fincher (1954) except that the opening was at the end. A glass tubing having an external diameter of 9 mm. was drawn into pipettes. The pipette end was bent to an angle of 20°. The swab was sterilized in autoclave at 15 lbs. for half an hour and the pipette at 180°C for 1/2 hr. in hot air.

At the time of collection of the material, the vulva and perineum were washed with soap and water and dried with a piece of sterile cotton. Rectified spirit was then applied on the vulva through cotton swab and sufficient time was allowed for the spirit to dry. The plug of the tube containing the swab was opened over the flame of spirit lamp as near the vulva.
of the animal as possible. The lips of the vulva were parted
and the swab was inserted into the genital tract. It was gently
pushed as forward as possible and moved back and forth and
rotated several times. It was left in situ for 15 min. It was
then taken out and replaced in the test tube over the flame
of the spirit lamp.

Mucus or discharge was withdrawn in the pipette with a
2 ounce bulb rubber test attached to it and deposited in sterile
screw-capped glass containers or test tubes. Semen when desired,
was collected in sterile test tubes through artificial vagina.
From a few aborted foetuses that were available during this
experiment specimens of stomach content, liver, spleen and
heart were separately collected with sterile precautions.

Attempt was made to inoculate the materials thus
collected on appropriate media as quickly as possible. In case
where delay was inevitable the materials were either placed in
ice or stored in refrigerator, till they were used.

Media used and their preparation:

The medium for the primary isolation of vibrio organi-
isms was prepared on the lines suggested by Morgan (1957). It
had the following composition:-

- Agar - 25.0 gm.
- Sodium chloride - 5.0 gm.
- Peptone - 10.0 gm.
- Lab. leuco - 10.0 gm.
Sodium thioglycollate - 1.0 gm.
Water - 1,000 ml.

The ingredients were dissolved and filtered through a pad of cotton placed between the folds of muslin. The pH was adjusted to 7.8 (which falls to 7.2 when incubated in the presence of 10 per cent carbon dioxide). The medium was autoclaved at 120°C for half an hour. Prior to pouring the plates, brilliant green to a final concentration of 1:40,000 and 10 per cent of fresh defibrinated blood was added and thick plates were poured. For a limited number of samples 0.4 per cent thiol was used in the above media and then sodium thioglycollate was omitted.

Brewer's medium, as suggested by Morgan (1957), was prepared as follows for the maintenance of the organisms:

Peptone - 10.0 gm.
Lemco - 10.0 gm.
Sodium chloride - 5.0 gm.
Dextrose - 5.0 gm.
Agar - 5.0 gm.
Sodium thioglycollate - 1.0 gm.
Methylene blue(1%) - 6.0 ml.
Water - 1,000 ml.

pH 7.2.

The medium was kept in 40 ml. quantities in 100 ml. conical flasks. It was boiled and cooled just before use.
For the primary isolation of brucella organisms, liver infusion agar as described by Mackie and McCartney (1939) was prepared as follows:

Fresh bovine liver was freed from the capsule, fat and larger bile ducts. It was cut into small pieces and pulped in a pulping machine. To 500 gm. of the pulp, 500 ml. of distilled water was added. The mixture was kept in refrigerator for 24 hours and then steamed for one and a half hours. It was filtered through the folds of muslin. No cotton was used for filtration. To 500 ml. of the liver extract the following ingredients were added.

- Washed agar 25.0 gm.
- Peptone 5.0 gm.
- Sodium chloride 5.0 gm.
- Distilled water 500 ml.

The mixture was heated to dissolve the ingredients. The pH was adjusted to 7.2 and the medium autoclaved at 120°C for half an hour. Before plates were poured, gentian violet to a final concentration of 1 in 2,50,000 was added.

Three types of media were used for PPLO. These were broth, agar plates and semi-solid agar and prepared on the lines suggested by Edward (1947,1950).

Bovine heart was freed from fat, pericardium and tendons, cut into small pieces and finely minced in a meat mincer. To 500 gms. of this, 1,000 ml. of tap water was added and the mixture kept in refrigerator for 24 hours. The extract
was strained through muslin on which the coarse particles were retained. These were expressed through the muslin and discarded. The fluid was steamed for half an hour and filtered. The pH was adjusted to 8.4 by addition of caustic soda, incubated at 37°C overnight and filtered again to remove the precipitate. To this was added 1 per cent peptone and 0.5 per cent sodium chloride. The final medium was prepared as follows:

**Agar**

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washed agar</td>
<td>2.5%</td>
</tr>
<tr>
<td>Sterile horse serum (v/v)</td>
<td>20%</td>
</tr>
<tr>
<td>Yeast extract (v/v)</td>
<td>10%</td>
</tr>
<tr>
<td>Thallium acetate</td>
<td>1 in 8,000</td>
</tr>
</tbody>
</table>

To each of the PP10 agar plate, two drops of penicillin containing 1,000 units per ml. was spread with a sterile glass spreader. The plates were allowed to dry before the inoculation of the material.

**Semi-solid agar**

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washed agar</td>
<td>0.3%</td>
</tr>
<tr>
<td>Sterile horse serum (v/v)</td>
<td>20%</td>
</tr>
<tr>
<td>Yeast extract (v/v)</td>
<td>10%</td>
</tr>
<tr>
<td>Thallium acetate</td>
<td>1 in 2,000</td>
</tr>
<tr>
<td>Penicillin</td>
<td>100 units/ml.</td>
</tr>
</tbody>
</table>

**Broth**

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile horse serum (v/v)</td>
<td>20%</td>
</tr>
<tr>
<td>Yeast extract (v/v)</td>
<td>10%</td>
</tr>
</tbody>
</table>
Thallium acetate  -  1 in 2,000
Penicillin      -  100 units/ml.

Penicillin in the above two media was added just before use. The final pH of all the three media was kept at 8.0. Thallium acetate and penicillin were omitted when these were required for purposes of subculturing.

**Yeast extract**

25 gms. of Difco yeast extract was weighed. To this, a little of distilled water was added and was thoroughly beaten so as to make a thick paste. Gradually, more of distilled water was added to a final of 100 ml. This was warmed slightly and then placed in refrigerator till frothing had ceased. It was sterilized by filtration through seltz ER pads and utilized within seven days of preparation.

It was observed that after the addition of yeast extract the pH of the media was rendered acidic. A calculated amount of caustic soda was, therefore, incorporated in the heart infusion broth so that the final pH would be 6.0. After addition of caustic soda in the broth, precipitate used to appear in it. It was, therefore, filtered, sometimes twice or thrice, to remove the precipitate.

**Preparation of inoculum and inoculation of culture media:**

**Vibrio and Brucella**

The swab containing the material from the genital tract was first directly inoculated on half of the plates of vibrio
and brucella culture media. Where possible, a portion of mucus was cut with the help of a sterile scissors and allowed to remain there after smearing it on the culture medium. The swab was next placed in a test tube containing 3 ml. of nutrient broth (pH 7.0) and shaken thoroughly. These were left in the broth tubes for about 2 hours and shaken frequently. After this period, the swab was taken out and discarded. The broth was mixed thoroughly and a loopful was inoculated on the rest half of each plate.

The broth tubes were routinely examined for the presence of bacteria after staining by Gram's method and for motility.

FPIO

A separate swab was used for the examination of FPIO. The swab was first directly cultured on half of the FPIO agar plate and then placed in a tube containing 3 ml. of heart infusion broth (pH 8.0), mixed well and left for about two hours with frequent shaking. Where possible a portion of mucus was left on the FPIO agar plate. After two hours, a loopful of the heart infusion broth was inoculated on the rest half of the plate. The semi-solid agar and broth media were inoculated with 8 to 10 drops of the inoculum through sterile Pasteur pipettes. Sometimes, 2 to 3 samples were inoculated on the same agar plate for purposes of economy.

Vaginal mucus and discharges collected through pipettes...
the stomach content of the aborted foetuses and semen samples were directly cultured on different media. Samples of heart, liver and spleen were triturated with sterile sand and normal saline solution and then cultured as usual.

**Incubation of the culture media:**

**Vibrio and Brucella**

After inoculation of the culture media, a 3-inch square blotting paper, previously sterilized, was placed with sterile precautions on the lid of each Petri dish (diameter 4 inches) in order to remove moisture which has a tendency to deposit on the lid of the plate (Mackie and McCartney, 1959). For larger or smaller Petri dishes the size of the blotting paper was adjusted accordingly. The Petri dishes were then placed in anaerobic jar and 10 per cent carbon dioxide was introduced. The jars were incubated at 37°C for 3 days.

**Carbon dioxide**

It was prepared in Wolfe's bottle from marble chips and hydrochloric acid and collected in a flat-bottomed flask up to a measured volume by displacement of water. From the flask it was transferred in the anaerobic jar by replacing water in the flask.

Later, in this experiment a technique described by Mackie and McCartney (1959) was followed. A test tube containing 8 ml. of 25 per cent hydrochloric acid was placed in the
anaerobic jar along with the inoculated plates. Approximately 0.6 gm. of marble was dropped in the tube, the lid of the jar replaced quickly and the screw tightened. For brucella, the jars were left as they were because the slight increase in pressure due to carbon dioxide is of no consequence (Mackie and McCartney, 1959). For vibrio, the outlet was slightly opened so as to release the extra pressure.

**PELO**

Agar plates were placed in a tin container with lid and incubated at 37°C for 3 days. A piece of cotton soaked in water was also kept in the container so as to prevent loss of moisture from the plates.

The semi-solid agar and broth tubes were either placed in wire baskets or test tube racks and incubated at 37°C, for 3 days.

**Bacteriological examination:**

**Vibrio**

The plates were examined after 3 days' incubation. If there was no evidence of growth, they were re-incubated for a further period of three days before discarding them as negative. Each colony appearing on the plate was examined for colonial characters and staining reaction to Gram's stain, morphology and motility of the organisms. For catalase production 10 per cent hydrogen peroxide in distilled water was dropped on the colony and effervescence noted. For detection of hydrogen sulphide, lead acetate paper was used.
Colonial characters:

The colonies were examined with regard to their size, shape, elevation, structure, surface, edge, colour, consistency, differentiation and haemolysis. Small, glistening, semi-translucent, conical, flattened, pale grey and non-haemolytic colonies were particularly looked for. In case there was no apparent growth, the plate was examined for the "frosting" effect on the surface of the medium, both by naked eye and with the aid of a hand lens. Also, from such plates, two separate subcultures were made, one incubated aerobically and the other under 10 per cent CO₂. These plates were examined in the same way as described above.

Staining reaction and morphology:

Smear was made from all types of colonies appearing on a plate as well as from those on which there was no apparent growth. They were stained by Gram's method and examined under oil immersion lens. Particular attention was paid to Gram-negative, comma, S-shaped, spiral or filamentous organisms having a length and breadth of 1.5 to 5.0 microns and 0.2 to 0.3 micron, respectively.

Motility:

On a clean and grease-free slide a moderately dense suspension of each Gram-negative organism was made in two drops of normal saline solution. The suspension was covered with a clean cover slip with the precaution to exclude air
bubbles. The preparation was first examined under low power (10X) and then under high power (40X) of the microscope. In case where there was doubt regarding the motility, a hanging drop preparation was examined. The characteristics of motility, such as, active, sluggish, darting or swirling, was noted.

**Brucella**

**Colonial characters:**

The plates were incubated at 37°C for three days and the colonial characters studied under the same headings as for vibrio. Particular attention was paid to the colonies with the following characters: - round, 0.5 to 1 mm. in diameter, convex, entire edged, translucent, glistening, smooth-surfaced, slightly greyish white by reflected light and colourless or bluish-green by transmitted light or rough, dull, granular, flatter, more opaque, yellowish or whitish both by reflected and transmitted light.

**Staining reaction and Morphology:**

Preparation of smear and staining was done in a similar way as for vibrio. Gram-negative organisms with the following morphological characters were looked for: - coccoid or cocco-bacillary rods with somewhat sharpened ends, lying singly or in groups and having a length and breadth of 0.6 to 2.0 μ and 0.3 to 0.5 μ, respectively.

**Motility**

Examination for motility was done as described earlier.
Agar plates were incubated at 37°C and examined daily from third day onward till the sixth day for the appearance of colonies. Plates on which there was no apparent growth, were examined with the help of a hand lens and under low power (5X) of the microscope.

The typical colonies, dark centre and light periphery ("nipped" appearance) seen under the microscope, were stained by Biene's technique for the characteristic reaction. A colony was touched with the platinum loop so as to ascertain if it had burrowed in the medium. Smear was made from these colonies on clean slides and stained by Gram's and Giemsa's stain.

For subculturing, a small piece of agar containing the colonies was cut. It was inverted on the surface of a fresh medium and smeared gently. The piece of agar (which usually used to break) was left on the surface of the medium. It was incubated and examined as before.

The semi-solid and the broth media were also incubated for 3 days at 37°C and examined for any visible growth from 3rd to 6th day. Subculture from each tube was done on PPO agar plates on fourth, fifth and sixth day. Three subcultures were done from each tube of semi-solid and broth media before discarding them as negative.

Smears in duplicate were prepared on each day from the third to the sixth day. One of them was stained by Gram's method and the other by Giemsa's and examined.
Giemsaa's stain:

**Composition**

- Giemsaa's powder - 1.2 gm.
- Methyl alcohol (A.R.) - 100 ml.
- Glycerol (A.R.) - 100 ml.

Methyl alcohol and glycerol were mixed well. The Giemsaa's powder was decanted over the surface and allowed to settle. The mixture was placed in the hot air oven at 55°C for 3 days. The bottle was shaken 6 to 7 times each day.

**Buffer solution (pH 7.0):**

- Disodium hydrogen phosphate (anhydrous) - 5.447 gm.
- Potassium dihydrogen phosphate - 4.752 gm.

The two salts were mixed in a mortar and stored. One gram of the mixture was added to 2 litres of distilled water.

**Staining procedure:**

The smear was fixed in methyl alcohol for 3 min. A 1 in 20 dilution of the Giemsaa's stain was made in a Petri dish. Two sticks, about 2" apart and parallel to each other were placed in the Petri dish. The slide, after fixation was kept, smear downward, on the sticks. This was done to prevent deposition of precipitate on the smear. The slides were stained overnight, washed with the buffer solution, dried in air and examined.

**Dienes' stain for the study of colony characteristics:**

- Methylene blue - 2.5 gm.
Azure II - 1.25 gm.
Maltose - 10.00 gm.
Sodium carbonate - 0.25 gm.
Water - 100 ml.

With a cotton swab the stain was applied in a thin film on clean coverslips. These were allowed to dry and stored in a closed container. The coverslips were used within one month.

**Staining procedure:**

Agar blocks about 1 square cm. were cut and placed, colony side up, on a slide. A stained coverslip was laid on it so that the stained surface and the colony were in contact. The margins of the coverslip were sealed with hot paraffin. Staining was complete in 1 to 2 min. Bacterial colonies rapidly decolourised the stain whereas the PPLO colonies retained the stain and they appeared blue under the low (5X) magnification of the microscope.

Suspected colonies of vibrio, brucella and PPLO were subcultured on appropriate media and biochemical and other tests for identification were performed as usual.

**SEROLOGICAL TESTS**

**Vibrio**

Vaginal mucus agglutination test as described by Laing (1956) was done.
Collection of the sample:

Mucus was collected on gauze tampons. The vulva and adjacent parts were washed with soap and water. The lips of the vulva were parted. A metallic tube containing the tampon and a loose wooden plunger was inserted and pushed as forward in the vagina as possible. The tampon was deposited there by means of the plunger. The plunger and the metallic tube were then withdrawn keeping the end of the tube closed by thumb to prevent entrance of air which causes ballooning of vagina. The free end of the string attached to the tampon was placed inside the vulva to prevent soiling by faeces. The tampon was left there for 20 min. to collect adequate amount of mucus for the test. It was then removed with the help of the string and placed in a previously weighed glass container. A mark of identification was given on each container. A separate outfit of tampon was used for each animal.

The weight of the mucus collected on the tampon was determined. For the 1-gm. samples 11.5 ml. of formol-saline (0.3% formalin and 0.8% sodium chloride) was added in the glass container. For larger samples (over 1.5 gm.) formol-saline was added in such volumes so as to give an initial dilution of 1:12.5. For smaller samples, the volume of formol-saline was accordingly adjusted.

Technique of the test:

For each sample five tubes (50 mm. x 8 mm.) were set up in a test tube rack. The first tube was left empty. In each of
the rest four tubes 0.5 ml. of 0.5% phenol-saline was put. In the first two tubes 0.5 ml. of the formol-saline extract was added. The contents of the second tube was mixed well and 0.5 ml. transferred to the third tube. The process was continued till the fifth tube from which 0.5 ml. was discarded. Then 0.5 ml. of the antigen was added in each of the five tubes and mixed well by rotating between the palms. As control, one tube containing 0.5 ml. of formol-saline and 0.5 ml. of antigen was set up.

The tubes were incubated at 37°C for 18 hours and read by ordinary transmitted light against a dark background.

The results were interpreted as follows:-

- Water clear = +++
- About 75% clear = +++
- About 50% clear = ++
- About 25% clear = +

"Any agglutination at all is regarded as denoting suspicion of infection, but a positive report is not given unless there is at least 75 per cent clearing in the first two tubes". - Laing, 1956).

**Antigen**

It was kindly supplied by Dr. G.M. van Waveren, Director of the Central Veterinary Institute, Rotterdam.

The ampoules containing the antigen after receipt were stored in refrigerator prior to use. For test, as suggested by Dr. van Waveren, one ml. of the antigen was diluted to 50 ml. with formol-saline (formol 0.5%).
Brucella:

Tube agglutination test with the sera of cows was performed.

Technique of the test:

Five agglutination tubes were set up as described earlier. In the first tube 0.8 ml. and in the rest four tubes 0.5 ml. of phenol-saline was added. 0.2 ml. of the serum was added in the first tube and mixed well. From this 0.5 ml. was transferred to the second tube and the process was continued up to the fifth tube from which 0.5 ml. was discarded. In all the five tubes 0.5 ml. of Br. abortus antigen was added and mixed by shaking. A control with known positive serum was set up for each batch of the test.

The tubes were incubated at 37°C for 18 hours and examined by ordinary transmitted light.

Result:

The results of the test were expressed in the international unit.
RESULTS

In course of this study it was endeavoured to isolate Vibrio, Brucella and the pleuropneumonia-like organisms from the genital tract of cows and sheep to assess the prevalence of these organisms among the animals particularly with a history of infertility. It was, consequently, proposed not to undertake the study of all the bacterial flora that may be found in the genital tract. The breeding history, as far as available, of each cow was recorded and has been summarised in Table II. The percentage of various disorders has been shown in Table III.

From sheep, samples were collected from Banaur, Chotanagpuri, Marwari and Romni Mars breeds. Out of the 10 Romni Mars included in this experiment, only two had lambed once since their arrival in the Farm. Among the rest of the breeds no such history was evidenced in particular, but, as mentioned earlier, some of the sheep from these flocks had shown positive reaction to tube agglutination test for brucellosis and were segregated. The general conditions of management of the flocks was found satisfactory.

Eight out of the nine bulls, the semen samples of which were examined, had a history of breeding trouble. One of them was found on previous occasion positive for brucellosis on serological test and also Br. abortus was isolated from its semen.
<table>
<thead>
<tr>
<th>Source</th>
<th>37</th>
<th>48</th>
<th>48</th>
<th>12</th>
<th>12</th>
<th>12</th>
<th>12</th>
<th>12</th>
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<th>12</th>
<th>12</th>
<th>12</th>
<th>12</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gynaecology Section, Bihar Veto College, Patna</td>
<td>(96)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Artificial Insemination Centre, Ranchi</td>
<td>(23)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Cattle Breeding Farm, Patna</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
</tbody>
</table>
### Table III

Table showing the percentage of various disorders in cows

<table>
<thead>
<tr>
<th>Number examined</th>
<th>Discharge</th>
<th>Abortion</th>
<th>Irregularity</th>
<th>Cervicitis</th>
<th>Metritis</th>
<th>Persistent corpus luteum</th>
<th>Cystic ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>179</td>
<td>41.3</td>
<td>21.2</td>
<td>3.9</td>
<td>30.7</td>
<td>19.5</td>
<td>12.2</td>
<td>5.02</td>
</tr>
</tbody>
</table>

In all, 328 samples were processed for the isolation of the micro-organisms. They comprised 179 samples of vaginal swabs, 55 discharges of mucus or pus from cows, 4 samples each of two aborted bovine foetuses, 9 samples of bull semen and from sheep, 66 vaginal swabs, 4 discharges of mucus or pus and four samples from an aborted foetus of a goat. Out of these, 242 samples from cows, 9 bull semen and 74 samples from sheep were examined for vibrio and PPLO. 163 samples from cows and all the 74 from sheep were examined for brucella. A total of 40 samples of cattle and 15 of sheep did not show any growth on liver infusion agar with gentian violet and blood agar with brilliant green. 38 and 9 samples of cattle and sheep, respectively, were excessively contaminated. The details of growth on different media are shown in Tables IV and V.

In all, 742 bacterial isolates including Gram positive and Gram variable cocci, Gram negative cocci, Gram positive and Gram negative rods were made on liver infusion and blood agar media.
### TABLE - IV

Table showing the number of samples giving no growth, excessive contamination and yielding bacterial isolates on liver infusion and blood agar media.

<table>
<thead>
<tr>
<th></th>
<th>Liver infusion agar</th>
<th>Blood agar with</th>
<th>Blood agar with brill. green</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cattle</td>
<td>Sheep</td>
<td>Cattle</td>
</tr>
<tr>
<td>Samples showing no growth</td>
<td>15</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>Samples with excessive contamination</td>
<td>18</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>Samples yielding bacterial isolates</td>
<td>130</td>
<td>65</td>
<td>206</td>
</tr>
</tbody>
</table>

### TABLE - V

Table showing the number of samples giving no growth, excessive contamination and yielding bacterial isolates on different PPLO media.

<table>
<thead>
<tr>
<th></th>
<th>Agar plate</th>
<th>Broth</th>
<th>Semi-solid agar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cattle</td>
<td>Sheep</td>
<td>Cattle</td>
</tr>
<tr>
<td>No growth</td>
<td>92</td>
<td>13</td>
<td>96</td>
</tr>
<tr>
<td>PPLO</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Other than PPLO</td>
<td>156</td>
<td>61</td>
<td>155</td>
</tr>
</tbody>
</table>

The details of these and the isolates on the PPLO media are being given in Table VI.
### TABLE VI

Table showing the details of isolates on different media.

<table>
<thead>
<tr>
<th>Types of bacterial growth</th>
<th>Liver infusion agar</th>
<th>Blood agar</th>
<th>PPLO media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cattle</td>
<td>Sheep</td>
<td>Cattle</td>
</tr>
<tr>
<td>Gram Positive cocci</td>
<td>78</td>
<td>19</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>147</td>
<td>58</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>152</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Gram Negative cocci</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gram Positive rods</td>
<td>106</td>
<td>49</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gram Negative rods</td>
<td>71</td>
<td>28</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Out of the 106 Gram negative and motile bacteria isolated on the blood agar with brilliant green 1:40,000 (and on the limited number of thiol medium) none showed the characteristic colonies, motility and morphology, as described earlier, for vibrio. A majority of them produced catalase, all fermented various sugars, some fermenting lactose as well. Based on the biochemical tests also none of them was found to conform to the characteristics of *Vibrio-fetus*.

Of the 24 Gram negative non-motile bacteria isolated on liver infusion agar with gentian violet 1:2,50,000, 13 were found to be *Aerobacter*, 5 *Klebsiella* and none as *Bacillus* on biochemical tests. The remaining six strains were untypable.

From the vaginal swabs of three cows, 3 strains of PPLO were
isolated on agar plate. No PPLO could be isolated from the rest of the cows, sheep or semen samples of nine bulls.

The colonies of PPLO were small, circular, raised and opaque. When touched with platinum loop a part of the colony remained adhering to the surface of the agar medium. Under low magnification they showed dark centre and light periphery. When treated with Diene's stain they looked blue. By Giemsa's method the organisms appeared like cocci and globules but were barely visible by Gram's staining. No filamentous forms were seen. Attempts to subculture these strains on the third day after the primary isolation of the colonies was unsuccessful.

The result of the isolation of vibrio, brucella and PPLO has been shown in Table VII.

**TABLE VII**

Table showing the results of isolation of vibrio, brucella and PPLO.

<table>
<thead>
<tr>
<th></th>
<th>Vibrio</th>
<th>Brucella</th>
<th>PPLO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Sheep</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The remaining strains of Gram negative organisms and Gram positive cocci and rods have been kept for future study.

Since the PPLO strains failed to grow on subculture further biochemical and other tests could not be carried out.
SEROLOGICAL TESTS

Vaginal mucus agglutination test:

Fifty samples of vaginal mucus of the cows of the Government Cattle Farm, Patna and 16 of those brought to the Gynaecology section of Bihar Veterinary College, Patna, were subjected to agglutination test with the Weybridge strain of vibrio antigen obtained from the Central Veterinary Research Institute, Rotterdam. The results are being given in Table-VIII. Only one, cow no. 52/56 showed '1+' in the first tube. This animal had aborted in the third month of pregnancy about a fortnight before the test. The mucus samples from the rest of the animals did not show any agglutination.

Agglutination test for brucellosis:

Tube agglutination test was performed with the sera samples of 50 cows belonging to the Government Cattle Farm, Patna, as listed from 1 to 50 in Table-VIII. Br. abortus antigen supplied by I.V.R.I. was used. Only two cows, nos. 19/6 and 22/4 at serials 24 and 26 in Table-VIII, showed agglutination in the first tubes (dilution 1:10) and the rest samples, no agglutination in any tube. Thus all the 50 cows were found negative for brucellosis. Agglutination test for brucellosis could not be carried out into the animals brought to the Gynaecology section of Bihar Veterinary College, Patna due to some unavoidable reasons.
<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Cow no.</th>
<th>Tube 1</th>
<th>Tube 2</th>
<th>Tube 3</th>
<th>Tube 4</th>
<th>Tube 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49/6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>62/5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8/4</td>
<td></td>
<td></td>
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<tr>
<td>4</td>
<td>87/1</td>
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<tr>
<td>9</td>
<td>82/55</td>
<td>+</td>
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- = no agglutination; + = about 25 per cent clearing.
DISCUSSION AND CONCLUSIONS
DISCUSSION AND CONCLUSIONS

Frank (1962) observed that an average normal breeding herd requires about 1.5 services per conception, whereas the repeat breeders require two or more. It is felt that the Indian average normal breeding herd requires a little higher number of services per conception. Therefore, cows not conceiving after three or more inseminations or services were taken as "repeat breeders" for the purposes of this experiment.

At present very little information is available with regard to the incidence of vibriosis in this country. On the basis of agglutination test carried with the vaginal mucus of cows and buffaloes, Mohan (1954) had suggested the probability of *V. fetus* infection in India. Parwani et al (1957) also found serological evidence of vibriosis in buffaloes of a milk colony in Bombay and isolated vibrios from two cows of a locality where infertility and frequent abortion was prevalent. The exact identity of the organisms isolated was, however, not established. Recently, Prasad (1968) isolated *V. fetus* from the vaginal and cervical mucus of slaughtered she-buffaloes in U.P.

So far no attempt has been made to determine the incidence of vibriosis in Bihar. Seeing to the many cases of
infertility in the cows of Cattle Breeding Farm, Patna and those brought to the Gynaeology Section of Bihar Veterinary College, Patna, it was only desirable to investigate the role of *Y. fetus* in this malady.

In the present investigations no *Y. fetus* could be isolated from 179 cows, both normal as well as those having records of infertility and 66 sheep, some of which had failed to lamb within last 2-3 years. Almost similar report was made in Congo by Lambelin and Rotors (1960) who could not isolate *Y. fetus* from 154 samples of vaginal mucus of indigenous cows although six of them were positive serologically, but they succeeded in isolating the organisms from the vaginal mucus of European cows. It seems likely, therefore, that the different breeds of cattle differ in their susceptibility to infection by *Y. fetus*. The cows examined in the present investigations were those of Mariana and local breeds.

The culture medium employed was, as mentioned earlier, 10 per cent bovine blood agar with brilliant green 1:40,000 (Florent, 1956), which was found very suitable by Morgan (1957) for the isolation of *Y. fetus* from all sources. However, even after the incorporation of brilliant green this medium fails to check the growth of most of the fast growing bacteria. Some of these, such as *Escherichia coli* and *Proteus* have been found inimical to the growth of *Y. fetus* (Morgan, 1957). Antibiotics, such as bacitracin, polymyxin B and novobiocin have been found to inhibit the growth of the contaminating bacteria.
as well as of vibrios other than \textit{V.fetus}. These antibiotics, however, could not be used in the present investigations because they could not be procured in spite of the best efforts.

Also, on serological test with the Weybridge strain of vibrio antigen all the cows, except one, showed no agglutination in any tube. The mucus sample of cow no.82/6 of the Cattle Breeding Farm, Patna, showed slight clearing in the first tube only. According to the criterion laid down by F.A.O. (Laing, 1956), no animal could be declared positive unless it shows at least 75 per cent clearing in the first two tubes. In this context it is difficult to reconcile with the two opposite views put forward by Laing (1956) who suggested that any agglutination at all was regarded as denoting suspicion of infection and Hunter (1956) who found that the value of vaginal mucus agglutination test in diagnosis of pathogenic vibriosis was doubtful unless the number of positive reactors was at least between 30-40 per cent together with a clinical evidence of infection. The issue is further complicated by the suggestion that the interpretation of the results of vaginal mucus agglutination test should be done on herd basis and not as a means of diagnosis of infection in individual animals. Further, there are evidences to indicate that the different vibrio antigens give variable results (McIntee \textit{et al.}, 1954a; Binns and Finch, 1954) in vaginal mucus agglutination test and it is advisable that, as suggested by Laing (1956), the test be carried with antigens prepared from locally isolated strains. Since no local strains were available, antigen was
obtained from the Central Veterinary Research Institute, Rotterdam.

Vibriosis has been found to be a major aetiological factor in the causation of infertility and abortion in some countries, whereas in others, as for example, in England, it is not believed to be a problem though it may exist in some herds (Lawson, 1963). The contention of Prasad (1965) that V.fetus may be responsible for some of the unexplained cases of infertility in cattle and buffaloes will need to be substantiated by further investigations covering a large number of animals in wider areas.

Several authors have attempted to determine the incidence of brucellosis in cattle and sheep in this country and a brief review of their work has been done earlier. The method employed more commonly was the testing of animals serologically with Br.abortus antigen. Varying percentage of incidence has been recorded by different authors. In some localities it has been found to be gravely high.

The yearwise percentage of brucellosis in the cattle of Bihar has been given in the review section of this work. From a perusal of the review it will appear that the incidence has been on a decline and the figure for the year 1963-64 was 0.76 per cent. It implies that the impact of the scheme run for the diagnosis and control of brucellosis in Bihar has been very encouraging. This view is further substantiated by the findings of the present investigation. No brucella could be
isolated from the 179 cows with records of infertility in some of them and 66 sheep subjected to cultural examination in these experiments. Moreover, brucella organisms, and *Br. abortus* in particular, are incriminated largely for abortion in cattle and less frequently infertility which may be caused due to further complications of this infection.

During the course of this study two cases of abortion had occurred in the Cattle Breeding Farm, Patna. One was found to have taken place just after the mass drenching of animals with carbon tetrachloride. Usually animals pregnant over three months are not included in drenching but the possibilities of accident and other causes can not be overlooked. The other cow had aborted in the third month of pregnancy. No brucella could be isolated from the stomach contents or other organs of the aborted foetuses as well as on repeated examinations of the aborting cows.

There were records of abortion in 5 cows that were examined in gynaecology section. In one cow, abortion was reported to have occurred when it had an attack of foot and mouth disease. In the rest four, abortion had taken place 5 to 8 months before they were brought for examination. Attempts to isolate brucella from these animals also failed.

All the 50 cows of the Cattle Breeding Farm, Patna proved non-reactors to tube agglutination test for brucella. It may be pointed out that the animals of the Cattle Breeding Farm, Patna had regularly been tested in past for brucella.
by tube agglutination method. The positive reactors were removed, as early as possible, to the Barwadin Gosadan and the doubtful reactors were segregated immediately and retested thrice before being declared negative. The last batch of brucella reactors was sent to the Gosadan in the month of May, 1964.

The fact that the animals of the Cattle Breeding Farm, Patna are regularly tested and appropriate action taken thereon and the findings of the present investigations suggest the likelihood that brucellosis, at least among the animals tested, is not a problem for the present.

Though some reports are available regarding the occurrence of PPLO in poultry (Pathak and Singh, 1961) and goats (Manjrekar, 1955) in India rather meagre information is available for cattle and sheep. Jayaraman (1961) isolated a suspected PPLO on blood agar from a cow with low fertility. On the other hand, Prasad (1965) failed to recover any PPLO from 326 she-buffaloes he examined in U.P.

In this experiment three strains of PPLO were isolated from three cows. Two of these were suffering from vaginitis and one was having vaginal discharge. The recovery of PPLO from these animals may have some significance because PPLO has been reported to cause inflammatory conditions which sometimes lead to infertility in cattle (Edward, 1950a). However, repeated attempts to subculture these strains of PPLO proved infructuous. This finding was not surprising because several reports of failure of PPLO to grow in subculture are available in the
literature (Edward, 1950a, b and others).

The exact role played by the pleuropneumonia-like organisms in the causation of infertility has not yet been estimated because of the association of some non-pathogenic strains in the genital tract. However, the pathogenic and non-pathogenic types possess some differences that may help in their differentiation. They have been found to differ serologically (Edward, 1950) though this difference in many instances has been overlapping (Beveridge, Campbell and Lind, 1946 and Edward, 1950a). Another difficulty arises from the fact that antibodies appear rather irregularly in the infected animals (Sabin, 1941a). The pathogenic and non-pathogenic types of PPLO have been found to differ in their growth requirements as well, the pathogenic ones needing additional animal protein for their growth (Edward, 1950) whereas the non-pathogenic ones are not so fastidious. Based on his findings (Edward, 1950) concluded that there may exist an inverse relation between the ease with which the organisms grow and the pathogenicity. This finding leads to another problem as to whether the strains maintained in routine subculture are the non-pathogenic variants of pathogenic ones (Edward, 1950). If such is the case, the pathogenicity tests with these strains may lead to erroneous results.

It therefore, warrants further research in the biology, pathogenicity and the occurrence of the PPLO in the genital tract of cattle and sheep before they can be incriminated responsible for breeding troubles in these animals.
CONCLUSIONS

Though no vibrio or brucella could be isolated during the present investigations it seems an untenable proposition to believe that a few strains of vibrio or brucella do not exist in the localities from which the animals were examined. The findings of Prasad (1965), who isolated a few strains of \textit{V. fetus} and \textit{B. melitensis} from the slaughtered she-buffaloes in U.P., stands in support of this view. However, the results of the present experiments indicate the possibility that agents other than vibrio and brucella may be responsible for the causes of infertility in the animals examined. But, it is suggested that further investigations covering larger number of animals should be undertaken to gather more informations on this important problem.

Since all the three strains of PPLO were isolated from animals having vaginitis or discharge it is felt that these organisms may be responsible for at least some of the cases of vaginitis or inflammatory conditions of other parts of the genital tract.

Of the animals examined 19.5 per cent were found to be suffering from vaginitis. It was noted during examinations that most cases of vaginitis were of granular type. It would be worthwhile to investigate the etiology of this condition keeping in mind that agents other than bacteria, such as viruses or fungi might also be associated with this condition.
From the analysis of the history of the animals examined it appeared that a large percentage of cows suffer from irregular oestrous. It is suggested that investigations should be carried out to determine the factors responsible for this condition.
SUMMARY

SUMMARY

A brief review of the salient features of the literature on the immune, isolation and serological status of various animals has been done. Important literature from other sources has been reviewed regarding the calibration, identification and pathogenic significance of the pulmonary-like organisms in the genital tract of animals.

A detailed description of materials collected and analyses employed in the current studies has been given. By history of the animals, as far as available, one recorded previous.

In all, 100 cows and 50 sheep and 4 goats of 60 vaginal discharges, 38 chest infections and 2 samples of bile were used analyzed.

Of the 100 cows, 98 tested negative and 2 tested positive and 4 of the thoracic non-tuberculosis organisms were found to be strains of Mycoplasma, respectively, of bovine, respiratory and bovine vaccines.

Cultures from the culture of pulmonary, lymphoid, and skin lesions were inoculated into various media without yielding any significant or unusual organisms. Further tests with these isolates were not to have revealed that failed to grow in culture.

Vaginal smears and samples in 20 patients were examined in 60 and 50 cases, respectively. All were negative for Mycoplasma but only one had sterile vaginal swab. The patient, however, had a positive culture for Mycoplasma.
SUMMARY

1. A brief review of the salient features of the literature on the incidence, isolation and serological tests for vibrio and brucella has been done. Important literatures have also been reviewed regarding the cultivation, identification and pathological significance of the pleuropneumonia-like organisms in the genital tract of cattle.

2. A detailed description of materials collected and methods employed in the present studies has been given. The history of the animals, as far as available, was recorded and studied.

3. In all, vaginal swabs from 179 cows and 66 sheep and a total of 59 vaginal discharges, 3 aborted fetuses and 9 samples of bull semen were examined.

4. In all, 742 bacterial isolates of Gram positive and Gram negative cocci and rods were obtained. Out of the 106 Gram negative motile and 24 Gram negative non-motile organisms none was found to be vibrio or brucella, respectively on cultural, morphological and biochemical tests.

5. Three strains of pleuropneumonia-like organisms were isolated from three cows suffering from vaginitis or having vaginal discharge. Further tests with these isolates could not be done because they failed to grow in subculture.

6. Vaginal mucus and serum agglutination tests were carried in 66 and 50 cows, respectively. All were negative for brucellosis and only one had shown partial clearing in
the first tube in mucus agglutination test for vibriosis.

7. Possible reasons for failure to isolate vibrio and brucella have been discussed.

8. Suggestions for further studies on the important problem of infertility in cattle and sheep with a view to assess the role of bacterial or other agents have been made.

9. The remaining strains of Gram negative rods and the Gram positive cocci and rods have been kept for future study.
REFERENCES


REFERENCES


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   Ibid. 39, 30.


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