EPIDEMIOLOGY OF CERTAIN BACTERIAL AND VIRAL DISEASES CAUSING NEONATAL MORTALITY IN PUPS

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Thesis submitted in partial fulfilment of the requirement for the degree of

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DECLARATION

I hereby declare that this thesis entitled **"EPIDEMIOLOGY OF CERTAIN BACTERIAL AND VIRAL DISEASES CAUSING NEONATAL MORTALITY IN PUPS"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

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Introduction

1. INTRODUCTION

Man has always cherished the dog as a companion animal and the healthy newborn puppy is a delight to behold. Though mostly in urban areas, these loyal friends have become an integral part of most of the households. This has led to increased popularity in the breeding of dogs. Apart from the social losses, the death of puppies at neonatal age clearly imparts heavy economic losses to the pet owner or breeder.

The scientific community has well established that there are significant physiological differences between neonatal and older pups or adults. Blood sugar, ability to regulate the body temperature and resistance to infection are among the differences to be appreciated.

An understanding of these differences between neonatal pups and puppies three to four weeks old helps to explain why certain management practices lead to higher death rates while others greatly reduce mortality.

An average incidence of 25-30 per cent puppy deaths at the neonatal age has been reported.

Broadly, the causes of puppy death come under two categories, the infectious and the non- infectious. An important category among non-infectious ones is chilling which ranks first place in the causes for death in neonates because they are poikilothermic.

Nutritional, environmental, genetic factors and maternal behaviour may cause death in the newborn dogs even without infectious disease. Agalactia, behavioural inadequacies due to maternal inexperience, placental competition for uterine space and physical inability to compete for feeding space around the nipples in very large litters, and inbreeding are the other important incriminated causes.

The role of infectious causes in puppy mortality has been recognised as early as 1937. However, inspite of remarkable progress during the past two decades in the areas of knowledge about infectious diseases of dogs and in strategies to control them, there has been relatively little improvement in our understanding of the various causes of neonatal deaths and the widespread occurrence of the "fading puppy" remains as a major problem for dog breeders.

Many workers have studied on the role of bacterial pathogens in the neonatal mortality of pups and have isolated beta haemolytic streptococci, *Staphylococcus aureus*, *Staphylococcus intermedius, Escherichia coli*, *Proteus* and *Klebsiella* spp., from the colostrum of bitches and *Esherichia coli*, beta haemolytic streptococci and *staphylococcus* species from puppies. But before an infectious organism can be identified as being concerned in the etiology of the fading puppy complex, it is necessary to fulfill the Koch's postulates, which yet has to be done.

Others have worked on the viral causes of puppy mortality namely canine herpes virus, canine parvovirus (1 and 2) and infectious canine hepatitis virus. Parasites such as *Toxoplasma gondii* and *Neospora caninum* are also considered important etiological agents.

Prevention is much better than any attempts of treatment to stop losses or to cure a disease once it is established. This is particularly true with very young pups that sometimes fade before diagnosis and treatment can be started.

Considering the relevance of the problem in canine practice, the present study was undertaken with the objectives:

1. To identify the bacterial agents associated with neonatal puppy mortality.
2. To conduct antibiogram of the isolates.
3. To assess the role of parvoviral infection in neonatal mortality of pups and
4. To evaluate non-specific factors associated with neonatal puppy mortality.

2. REVIEW OF LITERATURE

The rate of stillbirth and neonatal death upto 4 weeks of age is highly variable and related to many factors including the quality of labour, congenital abnormalities and acquired disorders. Mortality is greatest during the first week of life. The peri-natal period might be defined as the time from the last two weeks of gestation until weaning and can be divided into pre-partum period (pre-natal), parturition and post-partum (neonatal) period (Davidson, 2003).

2.1 ETIOLOGY

2.1.1 Infectious causes

2.1.1.1 Bacterial causes

Stafseth *et al.* (1937) after clinical and pathological studies concluded that the main cause of puppy mortality was not the so-called "acid milk", but a bacterial infection, whose principal cause was *Streptococcus canis*. They isolated this organism from the organs of dead puppies, from the genital tracts of the bitches whose pups had died and from the prepuce of a dog that had served an infected bitch.

Hare and Fry (1938) isolated beta haemolytic streptococci from 128 bitches, dogs and puppies. They belonged to group G, C, A, D, E, L and M. while group G and L streptococci were often associated with pathological lesions, group M were non pathogenic.

Determination of the Lancefield group to which *Streptococcus* sp. belongs, was found to be useful for studying the pathogenicity of the organisms (Laughton, 1948).

Davies and Skulski (1956) isolated beta haemolytic streptococci from 13 per cent of 138 less than eight day old pups and 26 per cent of vaginal swabs from bitches with fading litters. They also found dual infection with streptococci and

hepatitis virus in four cases and streptococci alone in 22 out of 138 cases of neonatal death.

A beta haemolytic *Streptococcus* (Type L) was isolated from a natural outbreak of a disease whose predominant symptoms were abortion and sterility in bitches. Streptococci were also isolated from cases of mastitis in bitches associated with puppy death. Attempts to reproduce mastitis in the bitch associated with puppy death by injection of dogs with streptococci either in pure or mixed culture failed and suggested that viruses or other factors might be the primary cause or predisposing factors (Mantovani *et al.*, 1961).

Bowden *et al.* (1963) isolated beta haemolytic streptococci and *Escherichia coli* from 21.5 per cent and 5.2 per cent of 385 less than seven days old puppies respectively and suggested that only haemolytic strains of *Escherichia coli* should be considered significant. They isolated *Bordetella bronchiseptica* in a mixed growth from dead puppies.

Drake and McCarthy (1964) isolated beta haemolytic streptococci and *Escherichia coli* from 10.2 per cent and 6.8 per cent of 147 pups respectively and concluded that many of the strains of *Escherichia coli* isolated from puppies which died might be only post-mortem invaders and not of pathological significance.However, strains of *Escherichia coli* which are normally saprophytic may become pathogenic in puppies which have not received colostrum.

The most common causes of neonatal pup death were septicaemia, of which bacterial causes were more common than viral ones. The organisms commonly found were *Staphylococcus* spp., *Streptococcus* spp.,*Escherichia coli* or *Pseudomonas* spp (Kirk, 1965).

Carmichael (1966) isolated an organism belonging to the *Brucella* species from more than 200 cases of abortions in bitches. The organism was recovered from foetuses, vaginal discharges and lymph nodes of infected dogs.

Moore and Bennett (1967) isolated *Brucella canis* from 27 foetuses and neonates, and 17 bitches which had aborted.

Evans (1968) reported that the organisms that are important in the etiology of fading puppy complex are haemolytic streptococci, *Escherichia coli* and *Brucella* species.

Wright and Cornwell (1968) found that the presence of hepatitis virus in the tissues aggravated streptococcal infection in puppies.

Brucella canis infection is a contagious disease of dogs characterized by bacteremia of extended duration, abortion infertility and whelping of stillborn pups or weak pups with later mortality (Moore,1969).

Olson (1975) isolated coagulase positive staphylococci from the urine from the bladder, peritoneal fluid and lungs of two day old puppies died with signs of cyanosis, dyspnoea and recumbency. She obtained a mixed culture of beta haemolytic streptococci, coagulase positive *Staphylococcus*, *Escherichia coli* and a pure culture of *Streptococcus canis* from the vagina and uterus respectively of a bitch with vaginitis and infertility.

The aerobic bacterial flora of the vagina of clinically normal bitches and those with vaginitis were identified as *Escherichia coli,Streptococcus viridans,Staphylococcus aureus and Streptococcus canis.*The only consistent difference between the bacterial flora from the two sources was the relatively high numbers of bacteria in the exudates from cases of vaginitis (Hirsh and Wiger,1977).

Clinical and bacteriological examination of 62 bitches with vaginitis was conducted by Okkens *et al.*(1977) and revealed that ninety per cent showed normal haemograms. *Streptococci*, *Escherichia coli* and *Pasteurellae* spp. were often isolated.

Askaa *et al.*(1978) described three cases of neonatal *Escherichia coli* infection in Newfoundland. The serological examination showed that the implicated *Escherichia coli* belonged to sero group O4 in two outbreaks while sero group O25 was responsible for one outbreak. In one of the kennels with implication of O4, this O group was also isolated from adult dogs with diarrhoeal conditions.

Olson and Mather (1978) cultured vaginal and uterine swab specimens from pre-puberal and post-puberal bitches for aerobic bacteria.Coagulase positive staphylococci were isolated more frequently from pre-puberal bitches than from post-puberal bitches and no bacterial growth was obtained in 37 per cent of vaginal swab specimens.

Twenty one isolates of beta haemolytic streptococci from vagina of 125 healthy bitches were characterised and grouped serologically into Lancefield's groups. Group G, C and L streptococci were pathogenic while group M were not (Olson *et al.*, 1979).

Beta haemolytic streptococci,(groupG) *Staphylococcus aureus* and *Escherichia coli* were among the organisms isolated from normal bitches in heat, abnormal bitches and normal dogs. It was also shown that bacteria were transmitted from bitch to dog. (Allen and Dagnall, 1982)

Aerobic and anaerobic microflora were identified and quantitated in vaginal and uterine samples obtained from mature bitches during different stages of the estrous cycle. The common organisms isolated from the vaginas were *Bacteroides, Streptococcus, Pasteurella* and *Mycoplasma. Staphylococcus and Mycoplasma* were

frequently isolated from the uterine contents. Although many uterine microfloras were similar to vaginal microfloras, some uterine culture had a single isolate identified. There were no pathologic findings in most of the uteri (Baba *et al.,* 1983).

Fastard (1983) attributed infectious causes to 61 per cent of puppy deaths.

Kornblatt *et al.* (1983) isolated *Streptococcus agalactiae* from the internal organs of puppies from two different litters that had died in the neonatal period as well as from the vagina of two bitches.

Staphylococcus intermedius, considered as one of the most common genital bacteria in healthy dogs as well as in dogs with reproductive problems, was isolated significantly more often in specimens from bitches with signs of genital tract disease than from those without (Skaar, 1989).

Sager and Remmers (1990) opined that *Staphylococcus aureus, Streptococcus* (groupG) and beta haemolytic *Escherichia coli* were transmitted intra-uterine or by the infected genital tract to the puppies and were the cause of septicaemic death of puppies.A second important cause of infection was sub clinical mastitis of the bitch,leading to septicaemic death of newborn puppies.

Kuhn *et al.* (1991) conducted bacteriological investigation of milk from 44 clinically healthy post parturient bitches and isolated staphylococci from 30 animals, of which 30.3 per cent (40 samples) of the samples yielded pure cultures and 6.8 per cent (nine samples) anacultures. Small numbers of bacteria were isolated in most of the samples; 67.4 per cent showed moderate bacterial growth (less than or equal to 10(4)/ml sample). A direct influence of lactiferous gland colonization with bacteria on mortality of puppies was not observed.

In a study of the composition of the normal staphylococcal flora of bitches and their litters by Allaker *et al.* (1992), *Staphylococcus intermedius* formed the predominant staphylococcal isolate. *Staphylococcus intermedius* counts at the oral and nasal sites on the bitches did not change markedly before whelping and

remained low. Significant rises in the oral counts on both the bitches and puppies were observed after whelping (days 11 to18). Abdominal counts on both the bitches and puppies also rose after whelping. *Staphylococcus intermedius* counts at the vaginal vestibulum of the pregnant bitches were found to be higher than at any other site sampled and did not alter markedly until whelping when a decrease was observed. *Staphylococcus intermedius* was not found at the anal site in any of the six bitches and only transiently colonized five of the puppies.

Bjurstrom and Forsberg (1992) studied on the aerobic bacterial flora of the genital tract in normal breeding bitches for an 18 month period and found that *Pasteurella multocida*, beta hemolytic streptococci group G and *Escherichia coli* were the most common bacteria isolated.. The flora varied during the reproductive cycle and with breed. *Pasteurella multocida* was isolated significantly more often during proestrus, estrus, metestrus, and pregnancy, than during anoestrus and the post-partum period, and beta-haemolytic streptococci were isolated significantly more often during proestrus period. *Staphylococcus intermedius* was almost exclusively found after parturition. Culture results were negative for 5.2 per cent of specimens cultured.

Duijkeren (1992) opined that many aerobic and anaerobic bacteria normally inhabit the vagina of bitches and they might become pathogens if a break down in local immunity occurred. *Brucella canis* is the only bacterium known to be a specific cause of infertility in bitches.

Gandotra *etal.*(1992) attributed *Escherichia coli*, haemolytic streptococci, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Klebiella* spp. and *Bacillus cereus* as the causes of infectious infertility in bitches. Majority of the isolates were sensitive to gentamicin and chloramphenicol.

A retrospective survey was performed of aerobic bacterial species found in the vagina of 203 bitches with infertility, vaginitis, pyometra and puppy death. *Escherichia coli*, beta-haemolytic streptococci, *Staphylococcus intermedius* and *Pasteurella multocida* were the species most often isolated. *Escherichia coli* was the

most frequently isolated bacterial species from bitches with dead puppies. However, in such cases it is important to relate the vaginal bacterial findings to autopsy findings and the results of bacteriological cultures of the pups (Bjurstrom, 1993).

A total of 70 puppies and their dams, distributed in 14 litters, were submitted to weekly fecal examinations for *Clostridium difficile* during the first 10 weeks after birth by Perrin *et al.*(1993) .During the study, 94.3 per cent of the puppies and 42.9 per cent of the dams harboured *Clostridium difficile* at least once in their feces. No pathogenicity of the organism for neonate dogs could be demonstrated.

Caudle *et al.*(1994) investigated the bacterial flora of vagina and uterus at different stages of reproductive cycle using paracervical cultures and isolated streptococci, (64 per cent)*Escherichia coli*,(54per cent)*Staphylococcus epidermidis*(32per cent)and *Staphylococcus aureus* (28per cent)from 418 healthy bitches.The organisms isolated from normal bitches in estrus were streptococci, (52 per cent) *Escherichia coli* (51per cent) and *Pasteurella* sp(29 per cent). Paracervical cultures from normal diestrus or pregnant bitches revealed *Escherichia coli*,(16 per cent), non haemolytic streptococci,(11 per cent) beta haemolytic streptococci,(11per cent) *Staphylococcus aureus*, *Pasteurella* and *Proteus* spp.(11 per cent) whereas 32 per cent of samples showed no growth. In 34 healthy anoestrus bitches, no growth occurred in 53 per cent, *Escherichia coli* was found in 18 per cent, beta haemolytic streptococci in 15 per cent, alpha haemolytic streptococci in nine per cent, *Staphylococcus aureus*, *Bacillus* spp., *Corynebacterium* spp., *Haemophilus* spp. ,*Pasteurella* spp. and *Proteus* species from three per cent of cases. The predominant ones in vaginitis were streptococci, *Staphylococcus aureus*, *Pasteurella* spp. or *Escherichia coli. Escherichia coli* was isolated from 70 per cent of uteri of 50 bitches with pyometra. The organism has been incriminated to cause infertility by producing conception failure and puppy death.

Floss and Hardin (1994) reviewed the infectious causes of abortion in bitches and neonatal mortality of pups and stated that bacterial agents such as *Mycoplasma*,

Ureaplasma, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus* spp. and *Brucella canis* were associated with abortion and neonatal mortality.

Common causes of neonatal sepsis include *Staphylococcus, Escherichia, Klebsiella, Enterobacter, Pseudomonas, Streptococcus, Enterococcus, Clostridium, Bacteroides, Fusobacterium, Pasteurella, Brucella and Salmonella* (Hoskins, 1995).

Watts and Wright (1995) isolated the following bacteria from uterus of bitches in the order of frequency such as *Escherichia coli*, *Haemophilus* spp., alpha haemolytic streptococci, *Corynebacterium* spp., *Streptococcus canis*, *Alkaligenes faecalis*,*Bacteroides* spp., *Pastuerella* spp. and *Proteus mirabilis* and the mean number of isolates and bacterial growth were greater during estrus than at other stages.

The results of gynaecological investigations in 142 bitches were evaluated by Wendt and Stellmacher (1996). High proportions of infectious cases were found in cases of limitation of fertility (67.5 per cent), in vaginal discharge in the estrus (60.8 per cent), in cases of mastitis/pseudopregnancy (61.5 per cent) and in mortality of newborn puppies. *Staphylococcus aureus* and *Escherichia coli* were often isolated.

Nielen *et al.* (1998) reported that 32 per cent of puppy deaths was due to infectious cause and that the most frequent isolate from dead puppies was *Escherichia coli.*

In a study of bacteriological investigation of canine colostrum of dams with a complaint of neonatal pup mortality, Dakshinkar *et al.* (2001)detected coagulase positive *Staphylococcus*, coagulase negative *Staphylococcus*, *Streptococcus*, *Proteus*, *Pseudomonas*, *Klebsiella* and *Escherichia coli.*

In the domestic environment *Staphylococcus intermedius* establishes itself soon after birth of the pup. The level of colonization by *Staphylococcus intermedius*

in the bitches seems to influence the colonization by pathogenic staphylococci in puppies. (Koulumies and Lloyd, 2002)

Somi *et al.* (2003) reported that in most cases of neonate septicaemia, bacteria from the bitches' milk were not the primary cause. Out of a broad spectrum of bacteria isolated from the milk of clinically healthy and diseased bitches, only *Escherichia coli, Klebsiella pneumoniae* and haemolytic *Streptococcus* species could be isolated from organs of their septicaemic puppies. *Staphylococcus intermedius*, although frequently isolated from canine milk, did not seem to be a cause of septicaemia of neonates.

2.1.1.2 VIRAL CAUSES

Ablett *et al.* (1961) demonstrated the susceptibility of the sucking puppy to the canine hepatitis virus by the inoculation of liver material from a fatal case of the disease into sucking puppies, which resulted in variable morbidity and mortality. They isolated the organism from fading puppy and concluded that there was an association between the carrier bitch and the mortality in young puppies.

No single aetiological agent is the sole cause of neonatal mortality in dogs (Fox,1963).

Direct isolation of infectious canine hepatitis virus from infant puppies in tissue cultures was reported in Great Britain (Spalding *et al.*,1964)

Carmichael *et al.* (1965) reported on the clinical and pathologic manifestations of a viral disease of young pups characterised by their death after a brief illness. The disease could be transmitted by inoculation into pups 24 to 36 hours old. Under electron microscope, the viral particles were morphologically like herpes virus.

Stewart *et al.* (1965) suggested that pups known to be infected with canine herpes virus and recovered might become carriers.

Infectious hepatitis virus may cause death in neonatal pups. Distemper virus causes death in older pups (Kirk, 1965).

Isolation of canine herpes virus from infant puppies was reported in Great Britain by Prydie *et al.* (1966).

Geary (1966) reported that in a study of unexplained deaths of pups in Central NewYork, a septicaemic disease not attributable to recognized bacterial, protozoan or viral infections appeared to be the cause in some cases. In all instances, the virus isolates had the characteristics of herpes virus group.

Herpes virus was isolated from the brain, lung, liver, spleen, kidney and intestine of one of the affected litter that died on day 10 after whelping. The virus was isolated also from the anterior vagina of the bitch 18 days after whelping (Love and Huxtable, 1976).

Levin *et al.* (1979) reviewed on the worldwide occurrence of fatal myocarditis due to canine parvovirus infection in dogs of three weeks to three months old.

Carpenter *et al.* (1980) gave a clinical report of intestinal and cardio-pulmonary forms of parvovirus infection in a litter of pups. The intestinal illness manifested at 12 to19 days of age with death in six out of 12 pups. Five pups out of the remaining six died with signs of cardio-pulmonary disease.

A case of generalised canine parvo viral disease in a litter of pups that died when three to nine days old was reported by Lenghaus and Studdert (1982) and they isolated the virus from heart, lungs, liver, kidneys and small intestine.

Hashimoto *et al.* (1982) reported that transplacental transmission of canine herpes virus might occur in pregnant dogs at a late stage of gestation .In a study,

infection with the virus was recognised in 13 of 18 neonatal pups obtained from four bitches by spontaneous delivery at full term. Eleven pups died within a week of birth and all had characteristic lesions of canine herpes virus infection.

Canine herpes virus might establish a latent infection in the nervous system of a bitch previously infected with the virus (Hashimoto *et al.*, 1983).

Mac Cartney *et al.* (1988) demonstrated that the minute virus of canines replicated in dogs and was capable of producing pathologic changes that were most prominent in oronasally exposed neonatal pups. Macroscopic and microscopic lesions were most prominent in the thymus and lymph nodes. Minor changes were found in the duodenal crypts.

Yates and Weller (1988) noted that severe canine enteric disease caused by canine parvovirus was also associated with a sudden death syndrome in pups between two and eight weeks in which the major changes were cardiac rather than enteric.

Parvovirus infection was confirmed by fluorescent antibody staining or viral culturing in 137 (22 per cent) of 615 necropsy accessions from dogs at Missouri. Septicemic colibacillosis was diagnosed in 88 (90 per cent) of the 98 canine parvovirus positive accessions in which liver or lung was cultured bacteriologically. Pulmonary edema or alveolitis similar to that seen in the human adult respiratory distress syndrome was observed in 63 (69 per cent) of the 91 canine parvovirus positive accessions in which the lungs were examined histologically (Turk *et al.*, 1990).

Canine herpes virus was first recognised in 1965 as the agent responsible for a fatal disease of neonatal pups and it was also found to reduce the reproductive success of the breeding animals (Anvik ,1991)

Harrison *et al.* (1992) isolated minute virus of canines from puppies aged five to 21 days, which were originally thought to be affected with an unusual

pathologic manifestation of canine parvovirus 2 infection, by using Walter Reed canine cell line.

In dogs with latent canine herpes virus infections, the virus may be reactivated without clinical signs (Okuda *et al.*, 1993).

Carmichael *et al.* (1994) reported that minute virus of canines caused inapparent to severe illness in neonatal specific-pathogen free pups exposed by the oronasal route.

Floss and Hardin (1994) reviewed the infectious causes of abortion and neonatal mortality of pups and stated that viral agents such as canine herpes virus, canine distemper virus and canine adeno virus were associated with abortion and neonatal mortality.

Canine parvovirus 2 myocarditis can develop from infection *in utero* or in puppies less than eight weeks old. Usually all pups in a litter are affected. Puppies with canine parvovirus 2 myocarditis are found dead or succumb within 24 hours after the appearance of clinical signs (Hoskins, 1997).

Canine herpes virus, minute virus of canines, canine adeno virus 1, distemper and canine corona virus may cause neonatal puppy deaths (Carmichael, 1999).

Canine herpes virus 1 was first recognized in the United States in 1965 as the cause of a highly fatal, generalised haemorrhagic disease of pups less than four weeks of age. Canine parvovirus 2 caused generalized neonatal disease in the age group two to 12 days, but was usually rare (Murphy *et al.*, 1999).

Gucht *et al.* (2001) reported that kennels where canine herpes viruses were present enzootically experience neonatal death and infertility more frequently.

Minute virus of canines (MVC), also known as canine parvovirus type 1, was initially believed to be a nonpathogenic agent, since it was first isolated from canine faecal specimens in the late 1960s. However, subsequent pathological as well as

epidemiological studies suggested that MVC was a pathogen of neonatal puppies .The virus also has been shown to cause fetal deaths (Mochizuki *et al.*, 2002).

Nandi and Sinha (2002) reported that the myocardial form of canine parvovirus, which was common in four to eight week old pups characterised by sudden death without any clinical signs, was not common nowadays. This has been attributed to large scale immunisation programme.

2.1.1. 3 PARASITIC CAUSES

Toxoplasma gondii causes death of pups past the neonatal age. Parasites may pose a serious problem in neonates. Hookworms may kill two week old pups. Ascarids may kill three to four week old pups and coccidia those a little older (Kirk, 1965).

Evans (1968) reported *Toxoplasma gondii* as an important aetiology of fading puppy complex and opined that puppies can become infected with *Toxoplasma gondii in utero* and that milk of infected bitches contains the organism. *Toxoplasma gondii* infection in dogs can lead to severe and widespread lesions, which can prove fatal.

Floss and Hardin (1994) reviewed the causes of abortion in bitches and neonatal mortality of pups and stated that protozoan agents such as *Toxoplasma gondii* and *Neosporum caninum* were associated with abortion and neonatal mortality.

2.1.2 MANAGEMENTAL CAUSES

Fox (1963) investigated on managemental causes of death in the newborn pups even without infectious disease. He explained the death on the basis of

agalactia, behavioural inadequacies due to maternal inexperience, placental competition for uterine space and physical inability to compete for feeding space around the nipples in very large litters, inbreeding and environmental factors such as low environmental temperatures. Accidental deaths by crushing by the bitch also may happen.

Fox (1965) opined that the term cardio-pulmonary syndrome should be used to describe cases of puppy deaths characterised by venous congestion and hypothermia and caused by physical factors. Chilling and exposure were incriminated as important contributory factors.

Bitches may contribute to a pup's death by cannibalism, by inanition (lack of adequate milk) or by culling. In culling, the bitch disowns one of her litter. Kennel mismanagement can also cause losses due to chilling or overheating, carelessness in handling or aspiration pneumonia when formula was forcibly fed (Kirk, 1965).

A frequent clinical observation in newborn puppies was hypothermia, which was often associated with gradual loss of vigour and death (Crighton, 1968).

Wilsman and Sickle (1973) incriminated pre and post-natal malnutrition, cardio-pulmonary factors and inadequate thermoregulation as the cause of high mortality among newborn pups.

Lanting and Carmichael (1988) noted that chilling ranks as the leading cause of early death. Poor mothering was another important cause.

In a birth cohort of boxer puppies in Netherlands, Nielen *et al.*(1998) attributed 21.9per cent of 302 puppy deaths to malnutrition and asphyxia.

2.13 MISCELLANEOUS CAUSES

The haemolytic disease syndrome that is easily produced in neonates experimentally has not been reported as a naturally occurring problem. A Von Gierke like syndrome in pups has been reported wherein at times of stress, affected pups become hypoglycaemic(Kirk, 1965).

Wilsman and Sickle (1973) associated the growth pattern of pups with their chances of survival. A pattern wherein the pups gained weight from the onset of nursing or wherein weight loss during the first two days of life did not exceed 10 per cent of their birth weight after which the pup began and continued to gain carried a favourable prognosis for survival. A pattern wherein weight loss exceeded 10 per cent of the birth weight had a poor prognosis for survival.

Mosier (1978) pointed out various causes for puppy mortality with their cause per cent as chilling,(16) stillbirth,(15) trauma by bitch,(13) dystocia,(11) undetermined,(9) accidents (6)"weak pups",(5)cannibalism,(3)and others,(lactation failure, deformities...) (12)

Congenital anomalies, teratogenic effects, malnutrition, low birth weight, traumatic insult and neonatal isoerythrolysis were reported to cause neonatal mortality in pups (Hoskins, 1995).

Nielen *et al.*(1998) presented puppy mortality and post-mortem findings for a birth cohort of boxer puppies born in Netherlands between 1994-1995. Post-mortem examination of 302 pups revealed the important death causes as asphyxia and malnutrition, (21.9 per cent) euthanasia because of white colour (16.9 per cent) and congenital abnormalities (14.9 per cent). No cause could be found for 12.6 per cent of deaths. Asphyxia sometimes referred to as anoxaemia or hypoxia is an important cause of death during the first three days of life

Beek *et al.* (1999) reported that out of 571 neonatal boxer puppy deaths, 4.9 per cent were due to asphyxia, 1.5 per cent due to atresia ani, 10.68 per cent cheiloschisis, palatoschisis or cheilopalatoschisis, 25.39 per cent were stillborn, 18.1 per cent were euthanised due to white colour, 3.5 per cent had malnutrition, 21.7 per cent went undiagnosed and 2.1 per cent due to other miscellaneous causes.

2.2.EPIDEMIOLOGY

2.2.1.INCIDENCE

Andersen *et al.* (1962) reported of 17 per cent pup mortality out of 200 Beagle pups whelped from 11 dams.

Mortality of young pups between birth and weaning approximates 30 per cent (Carmichael *et al.*, 1965).

Kirk (1965) reported 29 per cent mortality of pups through the weaning period as an average normal loss, which however may be reduced to 10-15 per cent by intensive care and ideal management.

Appel *et al.* (1978) reviewed what appeared to be a newly recognised disease entity in four to eight week old pups, first observed in August (Queensland, Australia and Britain) and September 1978. (United States and Canada) Myocarditis was found in 59 per cent pups in 10 litters and was characterised by sudden death.

A serologic study in Switzerland during 1980 revealed an overall canine herpes virus infection rate of six per cent in a population of 745 dogs. Herpes virus tends to have poor immunogenicity and detectable serum antibody levels that rarely last more than two months. Thus any study would identify only dogs with active canine herpes virus infections or those that were recently exposed (Anvik,1991).

In a nationwide survey in the United States from January 1978 through September 1979, Mulvey *et al.* (1980) observed acute parvovirus induced myocarditis in 76 of 147(52 per cent) pups in 19 litters.

Jones (1984) reported a seroprevalence rate of 21 per cent of *Brucella canis* out of 160 dogs in a kennel in El Paso County, Colorado wherein infertility, abortions and weak and dead newborn litters were noted.

Lanting and Carmichael (1988) pointed out that breeders have recognised a mortality rate of 20 to 25per cent before weaning of pups.

Yates and Weller (1988) reported that the cardio-pulmonary form of canine parvovirus infection caused mortality rates of 20-100 per cent in the late 1970s.

Neonatal puppy mortality may occur at a rate of six to 34 per cent (Beek *et al.* 1999)

A death rate of neonatal pups of 15 to 25 per cent was reported by Carmichael (1999). Serologic evidence indicated that canine parvovirus 1 was widespread in dog population, with 50 to70 per cent seroprevalence rates in USA, Japan and Switzerland. For canine herpes virus, serpositive rates of more than 30 per cent were common in field dogs.

Gucht *et al.* (2001) reported a prevalence of 49.5per cent of canine herpes virus infection in Belgian kennels by using a serum neutralisation test with complement to detect anti canine herpes virus antibodies.

Mochizuki *et al.* (2002) examined 470 clinical specimens from 346 dogs in Japan by PCR and detected minute virus of canine-specific gene fragments from 1.2 per cent diseased puppies and also obtained seroepidemiological evidence of anti minute virus of canine antibodies in 5.0 per cent of dogs.

The average incidence of stillbirths during both complicated and uncomplicated vaginal deliveries were reported to be 33 per cent and that of

neonatal mortality in uncomplicated whelping ranged from 9.23 per cent to 26per cent (Davidson, 2003).

2.2.2 AGE

Andersen (1957) reported high death rates during the first week of life. After 21 days of age, mortality decreased.

Up to 30 per cent of all newborn pups died within the first 21 days of life, and the majority of these deaths occurred during the first seven days post-partum. (McKelvie and Andersen, 1963;Bowden *et al.*, 1963; Fox, 1963;Drake and McCarthy, 1964).

More than 80 per cent of neonatal deaths occurred within the first week after birth (Carmichael *et al.*, 1965).

Kirk (1965) reported that the peak mortality occurred at two periods of transition, birth and weaning. Of all the deaths, 65 per cent occurred during the first week and 15 per cent at weaning.

Lenghaus and Studdert (1982) reported generalised canine parvovirus infection in three to nine day old pups.

Lanting and Carmichael (1988) noted that approximately 75per cent of puppy deaths occurred during the first three weeks of life.

Hoskins (1995) reported that puppy losses during the first 12 weeks of life usually approximated 15 to 40 per cent.

Neonatal puppies experimentally infected when they were younger than one week were particularly susceptible to fatal generalized infections of canine herpes virus. Dogs older than two weeks at the time of infection were relatively resistant

and generally developed mild or in apparent clinical illness (Carmichael and Greene, 1998;Carmichael, 1999).

Nielen *et al.* (1998) opined that the puppy's first seven days were the most susceptible period for mortality, which decreased by three weeks of age.

Deaths of one to four week old pups were most common due to canine herpes virus and canine parvovirus 1 infections (Carmichael, 1999).

Davidson (2003) reported that neonatal mortality of pups was greatest during the first week of life.

2.2.3 RISK FACTORS

A pup mortality of 72.8per cent was reported when the whelping was by caesarean section. Dams requiring caesarean section were frequently repeaters and there was high pup mortality (Andersen *et al.*, 1962).

Fox (1963) reported on the neonatal mortality in purebred and hybrid dogs. In the Basenji breed there was 3.5 per cent mortality out of 57 pups born, 13.2 per cent of 83 for the Beagle, 8.9 per cent of 90 for the Cocker spaniel, 20.9 per cent of 43 Sheltie pups and 14.8 per cent of 54 Fox terrier pups. Out of 147 pups, 12.9 per cent died at the neonatal age.

Though the climatic conditions such as cold weather may affect puppy losses, there was little difference due to season in the Cornell University kennel (McCay and Stevens, 1963). They also reported that the age of the dam influenced the mortality rates of pups. Litter size was smaller and pup losses greater in the dam's first year than during the second, third or fourth. Poor breeders are usually discarded before that age.

The Beagle breed was involved in the great majority of cases of infection with *Brucella canis* but a few cases had involved Weimaraners, Foxhounds, Old English sheepdogs, Pointers and Greyhounds (Moore, 1969).

Nielen *et al* .(1998) suggested that there might be a relationship between brachycephaly and a high incidence of stillbirths because of dystocia and problems with removing the amnion.

Yates and Weller (1988) opined that there was no apparent hereditary, breed, or sex predisposition for the cardio-pulmonary form of canine parvovirus infection.

Anvik (1991) reported that the primary targets of canine herpes virus were patients with compromised or poorly developed immune responses. Neonates and pregnant females were most susceptible but all stressed dogs were susceptible too. Immunosuppressive therapy, concurrent infections with canine parvovirus, corona virus and enteric *Clostridium perfringens* were the predisposing factors.

Bjurstrom and Forsberg (1992) gave the breed distribution in pup mortality as 30.4 per cent of 46 Airedale terrier pups, 14.9 per cent of 338 Beagle pups, 10.52 per cent of 38 Great Dane pups and 6.4 per cent of 31 Miniature poodle pups. Breed differences existed in the frequency of isolation of bacterial species from vaginal species also. *Pasteurella multocida* was isolated from 83 per cent of Airedale terriers while beta haemolytic streptococci and *Escherichia coli* were isolated from 30 and 12 per cent of the breed respectively; the three organisms were isolated at 59, 55 and 38 per cent respectively from the Beagle; 49 per cent of Great Danes yielded *Pasteurella multocida* while beta haemolytic streptococci was obtained from 75 per cent of the breed and *Escherichia coli* from 40 per cent.

According to Beek *et al.* (1999) additive genetic factors had less impact on the preweaning mortality than common-litter factors, which in turn had less impact than within litter factors. Mortality attributable to infection increased significantly with increase in inbreeding.

Transmission of canine herpes virus was described by Lanting and Carmichael (1988) to be from saliva or vaginal discharge of dams infected up to three weeks before whelping and nasal in reactivated dormant carriers. They also opined that in case of canine parvovirus, transmission was by direct contact with feces produced between fifth and sixth day of infection and contamination of hands, feet, clothing and utensils could transmit the infection. Oocysts of *Toxoplasma gondii* infected dogs and bradyzoites could be transplacentally transmitted.

Anvik (1991) reported on the various forms of canine herpes virus infection and noted that the acute neonatal viraemic form could be transmitted *in utero* just before whelping, by contact with infected vaginal or cervical secretions during whelping, by contact with secretions from a recently infected post partum female, from secretions from neighbouring infected dogs, or from fomites or transmission by animal handlers.

Minute virus of canines (MVC) was shown to cause transplacental infections with embryo resorptions (Carmichael *et al.*,1991).

Cole, *et al.*(1995) reported experimental evidence of the transplacental and/or transmammary transmission of *Neospora caninum* causing abortion in bitches. *Neospora caninum* was isolated from tissues of the experimentally infected bitches and pups from the miscarried litters. Experimental infections of two litters of five day old nursing pups produced variable results.

Barber and Trees (1998) reported that the frequency of vertical transmission of naturally acquired neospora infection in dogs is variable, but much too low to sustain infection alone and that post-natal infection must occur to maintain infection at seroprevalence rates reported in dog populations.

Minute virus of canines naturally infected susceptible pups via the oronasal route. Transplacental infections occurred most commonly when dams are infected between 20 and 35 days of gestation. For canine herpes virus, transmission was by direct contact with infectious body fluids. The virus became latent after a primary

infection and was shed periodically, primarily in nasal or rarely in genital secretions (Carmichael, 1999).

2.3 CLINICAL SIGNS

2.3.1 CANINE HERPES VIRUS INFECTION

Lanting and Carmichael (1988) reported on the various clinical signs of puppies dying at the neonatal stage. With canine herpes virus infection, symptoms may be either foetal deaths or in neonates, general illness, dullness cessation of nursing and incessant crying.

Anvik (1991) gave an account of the various forms of canine herpes virus infection as the acute neonatal viraemia characterised by high mortality and the occular form characterised by panuveitis, retinitis, cataracts, keratitis and peripheral anterior synechia. The acute form generally struck within first three weeks of life. They showed abdominal pain and persistent crying.

Canine herpes virus caused asymptomatic infection in dogs older than two weeks of age. In younger pups, the duration of illness was one to three days and the signs consisted of anorexia dyspnoea, pain upon abdominal palpation, incordination and often, soft yellow green faeces. There may be serous or haemorrhagic nasal discharge and petichae on mucous membranes. They cried persistently, and despite the muscular activity associated with crying, there was no elevation in body temperature. An erythematous rash consisting of a vesicle or papule and subcutaneous oedema of the ventral abdomen was noted. Animals that survive were likely to have persistent neurologic signs characterised by ataxia, blindness and cerebellar vestibular defects (Geary, 1966;Carmichael and Greene, 1998;Carmichael, 1999).

2.3.2 INFECTIOUS CANINE HEPATITIS

Spalding *et al.* (1964) isolated canine hepatitis virus from puppies that showed the fading puppy syndrome and also from their bitches. The affected bitches had enormously distended abdomens, temperature of around 104°F, pale mucous membranes and weak pulses. Majority of their pups died.

Puppies infected with the virus often died suddenly shortly after birth without previous signs of illness. Other puppies were vigorous at birth but soon developed progressive weakness, stopped sucking, and eventually became comatose before death. (Wright and Cornell, 1968)

2.3.3 CANINE PARVO VIRAL INFECTION

Levin *et al.* (1979) reviewed on canine myocarditis due to canine parvovirus. Affected pups were robust and healthy until a few minutes or hours before their deaths. Signs included hyperpnoea, dyspnoea, crying and cyanosis.

Parvovirus infection caused signs of gastrointestinal illness in a litter of 12 and death in six of them when 12 to 19 days old. The signs observed were retching, vomiting, diarrhoea, lethargy and dehydration. The remaining pups recovered from the gastrointestinal infection, but five of the six died before they were nine weeks old, after showing symptoms of arrhythmia, severe dyspnoea and pulmonary oedema (Carpenter *et al.*, 1980).

The following signs of illness were reported by Mulvey *et al.* (1980) in 76 pups affected with canine parvo viral induced myocarditis. Acute respiratory distress (97 per cent); anorexia (46 per cent); lassitude (33 per cent); frothing from mouth and nostrils (25 per cent); fever (21 per cent) vomiting (8 per cent); convulsions (seven per cent); diarrhoea (five per cent) and loss of consciousness (four per cent).

Many of the pups affected with minute virus of canines had vague symptoms. Affected pups had diarrhoea, vomiting, and dyspnoea and cried incessantly. Sudden death also had been observed (Hoskins,1998).

2.3.4 BACTERIAL INFECTION

Mantovani *et al.* (1961) reported prostration and unwillingness to suckle in newborn puppies experimentally infected with *Streptococcus* L.In the infected bitch, fever, white vaginal disharge, leucocytosis and posterior paresis developed. The signs in the bitch were classified as a localised genital infection characterised by abortion, metritis, sterility and enlargement of lymph nodes and a generalized form with progressive loss of weight in addition to the former symptoms.

The pups affected by bacterial septicaemia appeared vigorous and healthy at birth and sucked the colostrum avidly for the first 24 hours. Thereafter they became progressively weaker, made no further attempt to suck and lost weight rapidly (Fox, 1963).

Evans (1968) noted that in puppies that died due to streptococcal infection, their heads swayed from side to side, they paddled feebly with the forelimbs at the mammary glands, and lacked the strength to find or hold on to a teat. The puppies became inactive, their abdomen became distended and they strained spasmodically. Tetanic rigors with hyperextension of the forelimbs and spine occurred just before death. They became restless and cried incessantly. Eventually respiration became laboured with prolonged spells of apnoea, followed by death.

The cardinal sign of *Brucella canis* infection was abortion during the last trimester of pregnancy, with the majority aborting between gestation days 48 and 52. Whelping of stillborn or weak pups with later mortality also occurred (Moore, 1969).

2.3.5 MANAGEMENTAL CAUSES

Fox (1963) reported the following signs in litters with fading puppy syndrome suspected to be due to postnatal and prenatal malnutrition, genetic factors and chilling. Rapid decrease in weight after birth with persistent hypothermia, slowing down of heart rate to 24 per minute and tetanic rigors with hyperextension of forelimbs and spine just before death. Fluid in the nostrils, audible bronchial rales and gagging suggested respiratory distress. Vocalisation, locomotion, righting ability and progression on all fours gradually weakened until the pup lay immobile on its side.

Fox (1963) and Kirk (1965) described the signs in the normal newborn animal. Increase in both heart rate and body temperature occurred in pups during the first 24 hours after birth. Increase in body weight occurred after first feeding and there was no postnatal drop in body weight. The pups were plump quiet, warm and sleepy .By six days of age, temperature did not range much below 96°F and the first shivering reactions were seen. Muscle tone increased and the nature of the tonus were of flexor dominance.

Kirk (1965) noted that whatever be the cause of death, pups that die within a few days of birth had extreme weight loss, hypothermia, bradycardia and that they salivated and gagged, cried incessantly and became comatose before death.

Crighton (1968) classified the clinical signs of hypothermia into mild, moderate and deep. In mild hypothermia, the puppy became active and cried with a high-pitched note. Respiratory rate increased to 40 per minute and cardiac rate declined. The ability to suck and ingest milk became impaired eventually. The mucous membranes remained bright red as of normal. Rectal temperature falls to 70°F.In moderate hypothermia, the temperature falls to 60°F.The pup became torpid and crying plaintive. Inability to suck milk was noted. Respiratory rates were 20 to 25 per minute and cardiac rates less than 50 per minute. Incordination developed. In the deep hypothermia, the rectal temperature fell to between 50 and 60°F.The pup lay motionless in lateral recumbency. Crying stopped. The normal bright red mucosa

became bronze coloured. Respiration and heart rates became imperceptible. Reflexes were eventually lost.

2.4 PATHOGENESIS

2.4.1 CANINE PARVO VIRUS INFECTION

Lenghaus and Studdert (1982) and Yates and Weller (1988) reported on the cardiac effects of canine parvovirus infection. Parvovirus multiplies in the nuclei of cells that actively synthesise DNA and usually infect rapidly dividing cells. Autoradiographic studies of one to 45 day old pups have indicated that uptake of tritiated thymidine, a measure of cell DNA synthesis was high in tissues like heart, lung, liver, kidney, intestine, lymphoid and haematopoietic tissues. The distribution of HT-labelled cells paralleled the sites of parvoviral infection in neonatal pups examined.

In the neonate less than two weeks of age, the generalised form occurred rarely wherein the intestinal illness was seen. Virus replicated in the regional lymph nodes, tonsils and pharynx after oronasal exposure, followed by plasma associated viraemia develops and the virus later localises in the gastrointestinal epithelium. Parvovirus infects the germinal epithelium of the intestinal crypts causing destruction and collapse of the epithelium, which causes increase in permeability and decrease in absorption. The enteritis can be complicated by Gram negative sepsis. Disseminated intravascular coagulation and death due to dehydration follows (Hoskins, 1998).

Decaro *et al.* (2002) evaluated the innate immune response in pups with canine parvovirus 1 infection and reported that CPV 1 infection led to a marked reduction in the monocyte phagocytosis. Also monocyte killing was impaired or completely affected. These suggested possible mechanisms of viral evasion.

Suikkanen(2002)reported that lysosomotropic agents and low temperature are known to prevent CPV infection, indicating that the virus enters its host cells by endocytosis and requires an acidic intracellular compartment for penetration into the cytoplasm. After escape from the endocytotic vesicles, CPV is transported to the nucleus for replication. CPV or its DNA was released from the lysosomal compartment to the cytoplasm to be then transported to the nucleus. Electron microscopy analysis revealed endosomal vesicles containing CPV to be associated with microtubules. Some steps of the entry process were dependent on microtubules. Microinjection of antibodies to dynein caused CPV to remain in pericellular vesicles. This suggests an important role for the motor protein dynein in transporting vesicles containing CPV along the microtubule network.

2.4.2 CANINE HERPES VIRUS INFECTION

After oronasal exposure, the primary replication occurred in the nasal epithelium and pharyngeal tonsils leading to macrophage associated viraemia and localisation in the mononuclear cells of lymph nodes and spleen results in cell to cell spread, lymphoid hyperplasia and progressive multifocal haemorrhagic necrosis in several organs. Thrombocytopaenia may result from disseminated intravascular coagulation. Ganglioneuritis occurred by the travel through the nerve axons to central nervous system. Temperature regulation and immune status were involved in the abrupt development of resistance to infection that occurs between one to two weeks of age (Carmichael and Greene, 1998).

2.4.3 BACTERIAL

On mucosal contamination with *Brucella canis*, the organism was taken up by the phagocytic cells in the regional lymph nodes and the ensuing bacteremia spreads the infection to spleen, liver, lymph nodes, intervertebral disc, eyes and

kidneys. In the pregnant female, transplacental transmission occurred, causing abortion of partially autolysed pups, or birth of weak pups that died subsequently (Moore,1969).

Gyles (1994) reviewed on the pathogenesis of *Pseudomonas aeruginosa* and considered it in two aspects, establishment of infection and production of toxic metabolites.Colonisation in the damaged epithelium was mediated by pili,exoenzyme S and other surface proteins. The organisms multiplied at the site aided by the antiphagocytic effects of lipopolysaccharide and slime, resistance to killing by complement and the ability to obtain iron from the low environment of the tissue. The infected tissue was damaged by extra cellular enzymes, phospholipase C and exotoxin A and exoenzyme S caused dissemination throughout the body.

Staphylococcus aureus used multiple virulent factors to cause infection. At the cellular level, infection began with the prokaryotic bacterial cell manipulating the eukaryotic host cell through its virulent factors (Villavicencio and Wall, 1996).

Staphylococcus aureus pathogenesis depends upon complex interaction among the pathogen, platelets, plasma proteins, and vascular endothelial cells.. Platelets, now appear to play an important role in antimicrobial host defense against *Staphylococcus aureus* infections. Platelet microbicidal proteins are believed to significantly contribute to the antimicrobial properties of platelets (Yeaman and Bayer, 2000).

Balaban *et al.* (2001) and Gov *et al.* (2004) reported that *Staphylococcus aureus* caused infections by producing toxins, a process regulated by cell to cell communication (quorum sensing) through the histidine-phosphorylation of the target of RNAIII-activating protein (TRAP). They also reported that TRAP was highly conserved in *Staphylococci* and contained three completely conserved histidine residues (His-66, His-79, His-154) that were phosphorylated and essential for its activity, and RNAIII was not expressed in the TRAP(-) strain, it was non hemolytic, and that it did not cause disease in vivo.

Mazmanian *et al.* (2002) identified a sortase (SrtB) enzyme in the Gram positive pathogen *Staphylococcus aureus* that is required for anchoring of a surface protein with a NPQTN motif. Purified SrtB cleaves NPQTN-bearing peptides in vitro, and a srtB mutant is defective in the persistence of animal infections. srtB is part of an iron-regulated locus called iron-responsive surface determinants (isd), which also contains a ferrichrome transporter and surface proteins with NPQTN and LPXTG motifs. Cell wall-anchored surface proteins and the isd locus seem involved in a novel mechanism of iron acquisition that is important for bacterial pathogenesis.

Sifri *et al.* (2003) recognized several *Staphylococcus aureus* virulence determinants to be important in mammalian pathogenesis, including the quorum-sensing global virulence regulatory system agr and the global virulence regulator sarA, the alternative sigma factor sigma(B), alpha-hemolysin, and V8 serine protease.

Known virulence factors of *Proteus mirabilis* besides urease, are hemolysin, fimbriae, metalloproteases, and flagella (Burall *et al.*,2004).

2.5 DIAGNOSIS

2.5.1 CANINE HERPES INFECTION

Carmichael *et al.*(1965) and Wright and Cornwell (1968) noted that the post mortem and histopathological picture of canine herpes virus infection was quite characteristic, but the presence of herpes inclusions could not be relied on.The virus could be cultivated in canine kidney tissue culture and identified by immunofluorescence or specific neutralization with hyperimmune serum.Electronmicrographs of thin sections of infected dog kidney cells revealed viral particles.

Herpes virus infection should be suspected when necrotic and haemorrhagic lesions especially in the kidneys,liver and lungs were found at necropsy.Virus neutralisation tests could also be used for diagnosis (Geary,1966).

Necropsy of cases of acute neonatal canine herpes viraemia revealed petichae on kidneys, liver, lungs and intestinal mucosa. The spleen was often enlarged and excessive pleural and abdominal fluid might be present. Histopathologic evaluation revealed highly diagnostic inclusion bodies in hepatic tissue and other affected tissues. Virus isolation from these tissues also served as a reliable method of confirming the diagnosis (Anvik, 1991).

Carmichael and Greene (1998) have reviewed on the diagnosis of canine herpes virus infection and listed various tests for diagnosis such as virus isolation, serologic testing using virus neutralization tests, Enzyme linked immunosorbent assay and Haemagglutination inhibition assay, gross pathological and histopathological findings.

2.5.2 INFECTIOUS CANINE HEPATITIS

Ablett *et al.*(1961) and Spalding *et al.* (1964) described diagnosis of infectious canine hepatitis infection based on isolation of the virus from tissues of dead puppy, autopsy findings and specific neutralisation by antiserum.

As the post-mortem findings were not conclusive,a definite diagnosis of infectious canine hepatitis could not be made at autopsy.A diagnosis could be readily made by tissue culture isolation. Immunofluoresence was more sensitive than histopathological examination for inclusion bodies (Wright and Cornwell, 1968).

2.5.3 CANINE PARVO VIRUS INFECTION:

The infection could be diagnosed by gross and histopathological examination, identification of the viral particles in the inclusion bodies, by electron microscopy and the fluorescent antibody technique (Levin *et al.*, 1979).

Pollock and Carmichael (1979) observed significant amounts of canine parvovirus antibodies in the serum of affected dogs, even five days following infection and recommended HI test for routine serological diagnosis of CPV infection.

Carmichael *et al.* (1980) diagnosed parvo viral infection by conducting haemagglutination test using piglet RBC and suggested that a haemagglutinating titre of 64 and above be taken as positive.They considered HI titres of >320 and SN titre of >90 as positive.CPV antibodies in sera were reported to persist at high levels for at least one year.

Mulvey *et al.* (1980) reported of haemagglutination inhibition test in the diagnosis of myocarditis induced by parvoviral infection in weanling pups in U.S.

A case of generalised canine parvovirus disease in a litter of pups that died when 3-9 days old was diagnosed by virus isolation from the heart, lungs, liver, kidneys, and small intestine. Histopathological lesions included haemorrhage and necrosis in brain, liver, lungs, kidneys, lymphoid tissues, and intestinal mucosa. Intranuclear inclusions were found in the organs affected. (Lenghaus and Studdert, 1982).

A reciprocal HI titre of 80 was considered by some laboratories sufficient enough to protect against virulent CPV infection (Pollock and Carmichael,1982;Olson *et al.*,1988).

Studdert *et al.* (1983) reported that the most sensitive and rapid method for parvo viral diagnosis was the haemagglutination test.

A case of cardio-pulmonary form of parvoviral infection was diagnosed by physical examination, radiography, electrocardiogram and post-mortem lesions (Yates and Weller,1988).

Sherikar *et al.*(1989) reported that faecal HA and HI test were more rapid and economic than serum HI,electron microscopy and FAT.

Carmichael (1999)observed that minute virus of canines infection is difficult to diagnose because of the lack of commercially available reagents. The virus could be isolated only in WR 3873 D cells.Immunofluorescence and immunocytochemistry could be used.Histopathologic examination also was helpful.

Ok *et al.*(2000) reported that both faecal ELISA antigen tests and haemagglutination inhibition tests are valuable diagnostic tools in the diagnosis of canine parvo virus infection.

Savic-Jevdenic *et al.*(2001) compared the haemagglutination, immunofluorescence and immunochromatographic tests in the diagnosis of canine parvo virus infection and suggested greater sensitivity for the haemagglutination test than the immunochromatography in live animals while the immunofluorescence had 100 per cent sensitivity.

Canine parvovirus1were recovered from three PCR-positive rectal specimens by using WRCC/3873D and MDCK cells (Mochizuki *et al.*, 2002).

Rapid immunomigration on a membrane was applied for the diagnosis of canine parvovirus (CPV) in 128 samples of faeces containing four strains of parvovirus The results were compared with the results of haemagglutination and ELISA sandwich techniques. The new test was quick and easy to use, and made it possible to identify both the CPV-2a and CPV-2b strains (Lacheretz *et al.*,2003).

Praveen kumar *et al.* (2003) standardised the haemagglutination test for the diagnosis of canine parvovirus infection in dogs and suggested that the test could be used easily and successfully for the viral detection.

Parvoviral DNA can be amplified from feline and canine archival brain tissue and cerebellar hypoplasia in dogs might be associated with *in utero* parvovirus infection (Schatzberg *et al.*, 2003).

Waner *et al.*(2003) evaluated a dot ELISA kit for thedetection of immunoglobulin M and G to canine parvovirus by comparing with results derived from the immunofluorescence assay and reported that a strong correlation exists between the two techniques.

2.5.4 BACTERIAL

Diagnosis of bacterial infections can be made only by isolation of the organism concerned, and by detailed post-mortem and histopathological examination. Serological and biochemical examination of the organism and antibiotic sensitivity tests should also be carried out. (Evans, 1968)

Moore (1969) opined that canine brucellosis should be suspected whenever spontaneous abortions occurred during the last trimester of pregnancy. Tube agglutination tests detected antibodies, which but might be due to previous or active infection. Isolation of the organism was the best method.

Lucero *et al.* (2002) described an indirect enzyme linked immunosorbent assay that detected immunoglobulin G and A antibodies against *Brucella canis* that were useful for evaluating the clinical status of dogs. They suggested that although the rapid slide agglutination test (RSAT) was a practical screening test, a supplementary technique such as indirect ELISA should be used on all positive RSAT samples to ensure diagnostic specificity.

2.6 TREATMENT

2.6.1 CANINE HERPES VIRUS INFECTION

Treatment of neonatal viraemia with intraperitoneal or subcutaneous injection of hyperimmune serum should be considered. In case of neonatal death,the remaining puppies should be tube fed with a milk replacer.These should be given a broad spectrum antibiotic therapy and fluid and electrolyte replacement.They should be placed under a heat lamp source that keeps the ambient temperature at 102.2°F (Anvik,1991).

Treatment of puppies suffering from systemic illness of the infection was unrewarding because of the rapidly fatal progression of the disease.Mortality might be reduced by injecting each pup with one to two ml of immune sera.Elevating body temperature of already infected puppies was ineffective.Treatment with a course of VIDARABINE as soon as the cause was identified and met with survival of all infected pups (Carmichael and Greene,1998).

Carmichael, (1999) opined that antiviral drugs have been generally unsuccessful in the treatment of herpes virus infection.

2.6.2 CANINE PARVO VIRUS INFECTION

Martin *et al.* (2002) and De Mari *et al.* (2003) investigated the clinical efficacy of a recombinant feline interferon (IFN) (type omega) under field conditions for the treatment of dogs with parvoviral enteritis . Ninety four dogs from one to 28 months old were treated intravenously with IFN (2.5 million units/kg) once a day for three consecutive days, and monitored for clinical signs and mortality for 10 days. A significant improvement in clinical signs and 6.4 fold reduction in mortality was reported.

As there were no specific treatments for the disease ,symptomatic treatments were to be provided.The affected dogs should be put under broad spectrum antibiotics to check secondary bacterial,infection.Fluid and electrolytes were to be given to restore the losses.The use of hyperimmune serum raised in heterologous animals may give promising results (Nandi and Sinha,2002).

2.6.3 BACTERIAL

Mantovani *et al.* (1961) reported that the only antibiotic found to be sensitive to *Streptococcus* L isolated from the bitches' milk and from the dead pups was chloramphenicol.

Fox (1965) opined that treatment of the fading puppy was generally unrewarding. However, if recognised at an early stage, appropriate antibiotic might be of help.

Evans (1968) suggested that treatment with antibiotics should be a standard procedure on isolation of pathogenic organisms from the bitches'milk.

RNAIII-inhibiting peptide (RIP) is a heptapeptide that inhibits *Staphylococcus aureus* pathogenesis by disrupting quorum-sensing mechanisms. RIP inhibited drug-resistant *Staphylococcus epidermidis* biofilm formation through a mechanism similar to that evidenced for *S. aureus*. RIP is synergistic with antibiotics in eliminating 100 per cent of graft-associated in vivo *Staphylococcus epidermidis* infections, which suggests that RIP may be used to coat medical devices to prevent staphylococcal infections. Disruption of cell to cell communication can prevent infections associated with antibiotic resistant strains. (Balaban *et al.*,2001)

2.7 CONTROL AND PREVENTION

2.7.1 CANINE HERPES VIRUS INFECTION

Anvik (1991) observed that the best safeguard against the spread of herpes infection is for the dog's owner to impose a quarantine on whelping bitches and their litters during the period of maximum susceptibility, three weeks before to three weeks after whelping.

The environmental temperatures of the newborn puppies should be kept warm. This can be achieved by heating whelping boxes with heat lamps or other warming devices that do not cause excessive dehydration. There is no truly effective vaccine at present (Carmichael and Greene, 1998).

Carmichael (1999) reported that a 'cold adapted' canine herpes virus mutant has been developed but it is not commercially available. Administration of avian pox virus ,an interferon inducer to dams prior to breeding and whelping has been claimed to provide non specific protection against fatal infection.

2.7.2.INFECTIOUS CANINE HEPATITIS

In a breeding kennel, all animals should be immunised against the virus. Pregnant bitches should be given inactivated vaccine. Recovered animals should be housed separately as they may excrete virus through their urine. Administration of immunoglobulins to puppies immediately after birth is advised (Wright and Cornwell,1968).

2.7.3 CANINE PARVO VIRUS INFECTION

Once a diagnosis of canine parvovirus 1 had been made, treatment was unrewarding. Ensuring that the environmental temperature of newborn pups was

kept warm and adequate nutrition and hydration was provided may reduce mortality. No vaccine is available at present.For caine parvovirus 2,commercially prepared attenuated live and inactivated CPV 2 vaccines are available.Interfering levels of maternal antibodies to the virus and lack of sufficient seroconversion to the CPV-2 vaccine administered may cause failure of vaccines. To solve this, an approach is the vaccine that contains highly immunogenic, attenuated live CPV-2 strain produced at a high titre. (Carmichael and Greene, 1998).

2.7.4 BACTERIAL

The susceptible bitches in a kennel infected with *Streptococcus* L were successfully treated with chloramphenicol at 10 mg/kg body weight twice daily for three consecutive days around the 30th, 40th,and 50th days of pregnancy (Mantovani *et al.*, 1961).

The administration of antibiotics during pregnancy to bitches, which have previously produced fading puppies, should be considered (Evans, 1968).

2.7.5 MANAGEMENTAL:

The introduction of insulated whelping boxes led to a marked reduction in unexplained deaths in puppies during first 4 days of life .If open boxes were used, the room temperatures should be at least 70°F day and night (Crighton, 1968).

2.8 ANTIBIOGRAM

Gandotra *et al.* (1992) identified *Escherichia coli*, haemolytic streptococci, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Klebsiella* spp. and *Bacillus cereus* as the causes of infectious infertility in bitches.Majority of the

isolates were sensitive to gentamicin (86per cent) and chloramphenicol (70 per cent). Penicillin (6 per cent) was least effective.

A total of 47 isolates of *Pseudomonas aeruginosa* obtained from animals and humans over a period of three years were subjected to antibiogram studies by Singh *et al.* (1993) and they found highest sensitivity to gentamicin and carbenicillin.None of the isolates showed sensitivity to penicillin and tetracycline.

Mohan *et al.* (1994) obtained bacterial isolates such as *Staphylococcus* spp., *Proteus* spp., *Streptococcus* spp., *Pseudomonas* spp., *Klebiella* spp. and *Escherichia coli* from canine colostrum and reported sensitivity of cultural isolates to erythromycin, cephalexin, chloramphenicol, cephaloridine and cloxacillin.

Hariharan *et al.* (1995) obtained 113 *Pseudomonas aeruginosa* isolates from dogs and found them to be 100 per cent sensitive to tobramycin, netilmycin and polymixinB. Other results were Amikacin (99 per cent), Gentamicin (95 per cent), Carbenicillin (69 per cent), Neomycin (61 per cent), Enrofloxacin (36 per cent) and tetracycline and chloramphenicol (4 per cent). Amoxycillin clavulanicacid combination, cephalothin, cephalexin and ceftiofur were completely resistant.

Antibiotic sensitivity pattern of 39 *Staphylococcus epidermidis* strains isolated from animals was determined using disc diffusion method. Most isolates were sensitive to vancomycin (84.61 per cent), followed by cephalothin (74.36 per cent), clindamycin (66.66 per cent), gentamicin (61.53 per cent) and resistant to ampicillin and penicillin G (100 per cent) followed by tetracycline (92.3 per cent), methicillin (76.92 per cent) and ciprofloxacin (43.58 per cent) (Das *et al.* 2000).

In a study of bacteriological investigation of canine colostrum of dams with a complaint of neonatal pup mortality, Dakshinkar *et al.* (2001) detected coagulase positive *Staphylococcus*, coagulase negative *Staphylococcus*, *Streptococcus, Proteus, Pseudomonas, Klebiella* and *Escherichia coli.* All the organisms were sensitive to chloramphenicol and gentamicin followed by ciprofloxacin (91.3 per

cent), amoxycillin (86.95 per cent), oxytetracycline and Streptomycin (47.82 per cent), and nalidixic acid (39.13 per cent).

Ganiere *et al.* (2001) reported that resistance to enrofloxacin was rare in *Staphylococcus intermedius* isolates from dogs in France over a period of four years.

Antibiotic sensitivity test was done on bacterial isolates from nosocomial infections. *Klebsiella pneumoniae* were the most prevalent isolates from respiratory tract infections followed by *Proteus* spp., *Escherichia coli*, *Staphylococci* spp. and *Acinetobacter* spp. The Gram negative enteric bacilli were uniformly resistant to betalactam antibiotics as well as betalactam-betalactamase inhibitors. Resistance to Ciprofloxacin and Ceftriaxone ranged from 50 to100 per cent and 25 to 83.3 per cent respectively. *Staphylococci* were 100 per cent resistant to penicillin and tetracycline, 80 per cent to cotrimoxazole, 60 per cent to erythromycin and gentamicin and 40 per cent to amikacin. *Acinetobacter* spp. were highly resistant to most of the antibacterial agents except gentamicin while *Pseudomonas* spp. showed 75 per cent resistance to it (Singh *et al.*, 2002).

3. MATERIALS AND METHODS

The present study was carried out in the Department of Veterinary Epidemiology and Preventive Medicine during the period June 2003- June 2004.

Bitches with a history of neonatal mortality presented at the University Veterinary Hospitals Mannuthy and Kokkalai, and those at the various Veterinary Hospitals in Thrissur district were subjected to the study. Detailed history was taken from each case using a format (Appendix).

3.1 MATERIALS

3.1.1 Glassware and reagents.

In this study, Borosil brand of glassware, Laxbro plastics, analytical or guaranteed reagent grade chemicals and culture media from Hi-media were used.

3.1.2 Preparation of glassware.

The Petriplates and test tubes were kept in 0.1 per cent hydrochloric acid overnight. They were washed in tap water and immersed in detergent solution for one day. Then the Petriplates and test tubes were again washed thoroughly in tap water. The glassware were afterwards washed in distilled water, dried and sterilised in hot air oven at a temperature of 160°C for one hour (Hoskins,1967).

3.1.3 Preparation of blood agar.

Blood was collected under sterile conditions from cattle of the University Livestock Farm, Mannuthy in sterilised round bottom flasks with glass beads. For the preparation of the culture media, reconstitution of dehydrated blood agar base was done in double distilled water and sterilised by autoclaving at 121°C and 15 pounds pressure for 15 minutes. The media was allowed to cool to 45°C and to this, the collected blood was added at five per cent level under sterile conditions. Thiswas poured into sterile Petridishes, allowed to set and incubated at 37°C for 24 hours

for sterility check. The prepared culture media plates were stored at 4°C till use. (Quinn *et al.*,1994)

3.1.4 Collection of clinical materials.

The clinical materials for study were milk, blood, vaginal swabs and serum samples collected aseptically from nineteen bitches postpartum and tissues from twelve dead pups. The same set of materials collected in a similar manner from six bitches with normal, healthy pups formed the control of the study.

3.1.4.1 Collection of samples from the bitch.

Milk: The teat was sterilised with 70 per cent alcohol and allowed to dry for one minute. Milk was first stripped off and then collected in a sterile glass vial.

Blood: After applying 70 per cent alcohol over the skin, about five millilitre blood was collected from the cephalic vein in a sterile syringe .Two millilitre was transferred into a sterile glass vial containing glass beads and rotated uniformly so as to defibrinate the blood for culture. The remaining blood was used for serum separation for canine parvovirus antibody detection. The sera samples were inactivated at 56°c for 30 minutes and stored at –20°C.

Vaginal swab: A sterile swab was used for collection of the discharge from the vagina.

3.1.4.2 Collection of samples from the dead pup.

Post-mortem of the dead fetuses were done and samples were collected aseptically from tissues for bacterial isolation and detection of parvoviral antigen.

3.1.4.2.1 For bacterial isolation:

After taking all aseptic precautions, the organs of the pups including heart, lung and liver were singed, sterile inoculation loop was introduced and the tissue samples were streaked on blood agar.

3.1.4.2.2 For parvo viral antigen detection:

Tissue samples were cut using a sterile scissors from the heart, lung, liver, spleen and kidney and collected into phosphate buffered saline in sterile vials. The tissue specimens were then macerated in a sterile mortar and pestle, strained and centrifuged at 5000 rpm for 20 minutes in a refrigerator centrifuge at 4°C (Remi, c-24). The supernatants of the samples were frozen at –20°C till use.

3.1.4 Agar gel immunodiffusion test.

Reagents.

a. Noble agar - 1.2 g
Sodium chloride - 0.85 g
Sodium azide - 0.01 g
Distilled water - 100 ml

b Microscopic slides were cleaned with methylated spirit. The dried slides were stored at room temperature until use.

c. Staining solution.
Amidoblack IOB - 1 g
Sodium chloride - 8.5 g
Distilled water - 1000 ml

d. Destaining solution
Hypertonic saline (1.5 per cent)
Glacial acetic acid (7.0 per cent)

3.1.6 Haemagglutination and haemagglutination inhibition test.

Reagents.

a. Alsever's solution

Dextrose - 10.25 g

Trisodium citrate	-	4.00 g
Sodium chloride	-	2.10 g
Citric acid	-	0.275 g
Distilled water to make	-	500 ml

The pH of the solution was adjusted to 7.2 and autoclaved at 115°C for 30 minutes and stored at 4°C till use.

b. Phosphate buffered saline (PBS)

PBS stock solution (10x)

Sodium chloride	-	80 g
Potassium chloride	-	2 g
Disodium hydrogen phosphate	-	11.33 g
Potassiumdihydrogen phospate	-	2 g
Distilled water to make	-	1000 ml

The pH of the solution was adjusted to 7.2 and autoclaved at 121°C for 15 minutes.

PBS working solution (pH 7.2)

PBS (10x)	-100 ml
Distilled water to make	-1000 ml.

c. Diluent for haemagglutination test (PBS-BSA)

Bovine serum albumin, fraction V (Sigma) – 0.1 g

Phosphate buffered saline – 100 ml

Diluent was maintained at 4°C till use.

3.2. METHODS.

3.2.1 Clinical cases undertaken for study.

Nineteen bitches and twelve dead pups from ten of these bitches were subjected to the study. Out of these, three were cases of stillbirth and the rest were that of pup mortality within the first three weeks of life. Nine bitches had previous reproductive history of neonatal mortality /stillbirth. Out of the ten bitches without any such previous history, five were whelping for the first time.

3.2.2 Isolation and identification of bacteria.

3.2.2.1 Cultural examination

The samples of milk ,blood and vaginal discharge collected from the bitches for bacterial culture were streaked on blood agar under sterile conditions, incubated at 37°C for 24 to 48 hours and then examined.

The isolated colonies were then streaked on nutrient agar slants in sterile test tubes, sealed with paraffin wax and stored at 4°C.

3.2.2.2 Identification of bacterial culture.

The isolates were stained using Gram's method. The preliminary tests to identify the family of the bacteria were done as per Barrow and Feltham (1993)
Selective media used were

1. Mannitol salt agar.
2. Edwards's medium
3. Mac Conkey agar
4. Brilliant green agar.

For further identification, a rapid biochemical test kit (Hi-media) was used.

The tests in the kit were

1. Citrate utilization.
2. Lysine and ornithine decarboxylation.
3. Urease.
4. Tryptophan deamination .
5. Nitrate reduction test.
6. Hydrogen sulphide production.
7. Sugar utilization tests.
    a. Glucose.
    b. Adonitol.
    c. Lactose.
    d. Arabinose.
    e. Sorbitol.

Other tests employed as per Barrow and Feltham (1993)

1. Catalase.
2. Oxidase.
3. Oxidation /fermentation
4. Carbohydrate utilization
5. Coagulase.
6. Indole production.
7. Methyl red production.
8. TSI agar.
9. Voges Proskauer test.
10. Citrate test.
11. Arginine dihydrolase.
12. Phosphatase.

3.2.3 Antibiogram

3.2.3.1 Procedure

The disc diffusion technique (Bauer *et al.*, 1966) was used for testing the antibiotic sensitivity *in vitro*. Five colonies of each pure culture were picked up with sterile platinum loop and inoculated in four milliliters of peptone broth. Inoculum was applied on the surface of a Mueller Hinton Agar using a sterile swab .The plate was kept covered for 15 minutes at room temperature for drying. The antibiotic discs were then placed 20 millimeters apart and they were gently pressed on the agar surface. The plates were incubated at 37°c for 18 to 24 hours.

The zone of inhibition of bacterial growth was measured and interpreted as sensitive, moderately sensitive or resistant by comparing with the ranges given by the manufacturer.

3.2.3.2 Antibiotic discs

The antibiotics used in the present study were

1.Amoxycillin (Am) - 30 mcg/disc
2.Chloramphenicol (C) - 30 mcg/disc
3.Ciprofloxacin (RC) - 5 mcg/disc
4.Enrofloxacin (Ex) - 5 mcg/disc
5.Gentamicin (GM) - 10 mcg/disc
6.Norfloxacin (Nx) - 10 mcg/disc
7.Amoxycillin/Clavulanic acid (Ac) - 30 mcg/disc

3.2.4 Preparation of hyper immune serum against canine parvovirus

Inactivated canine parvovirus antigen (Indian Immunologicals) was used to produce anti-canine parvovirus serum in rabbits as per Ramdass and Khader (1982).

Two healthy male rabbits aged six months procured from Small Animal Breeding Station of Kerala Agricultural University were used for this purpose.

Rabbits were injected intramuscularly with one milliliter of antigen emulsified in Freund's adjuvant (DIFCO). Totally four injections were given at an

interval of 10 days. Freund's complete adjuvant (FCA) was used for the first injection and Freund's incomplete adjuvant (FICA) was used for subsequent injections. Ten days after the last injection, the rabbits were test bled from ear vein and sera samples tested by AGID for the antibody. When the results were found satisfactory the rabbits were bled and the serum was separated, inactivated at 56°C for 30 minutes and stored in small aliquots of two millilitre each at –20°C.

3.2.4.1 Positive control sera.

Anti-canine parvovirus hyper immune sera prepared in rabbits were employed in the tests, as known positive sera, for screening the tissue samples for the detection of antigen.

3.2.4.2 Negative sera.

Sera from healthy dogs, which were not immunised and were not infected with canine parvovirus, were taken as negative controls.

3.2.4.3 Positive canine parvovirus antigen.

Inactivated canine parvovirus antigen supplied by M/s.Indian Immunonologicals Hyderabad was used.

3.2.5 Agar gel immuno diffusion test.

The test was carried out as per the method described by Williams and Chase (1971) with some modifications.

3.2.5.1 Preparation of agar gel slides

Noble agar (DIFCO) 1.2 g was melted with 100 millilitre of normal saline solution, cooled to 60°C and 0.01 per cent sodium azide was added. Four millilitre of melted agar was poured on to a clean microscope slide and allowed to solidify and then kept in a moist chamber at 4°C till use.

3.2.5.2 Test proper

3.2.5.2.1 Bitches

Wells of five millimetres were punched out on the solidified agar over the slide at a distance of three to four millimetres. The wells were charged with the positive control serum, suspected serum sample, and positive control antigen. The slides were then incubated in a moist chamber at room temperature for about 24 to 48 hours. An opaque white precipitate line between the antigen and suspected serum sample was taken as a positive result.

3.2.5.2.2 Pups

Wells of five millimetres were punched out on the solidified agar over the slide at a distance of 3 to 4 millimetres. The wells were charged with tissue sample of pup, positive serum control and positive antigen control. The slides were then incubated in a moist chamber at room temperature for about 24 to 48 hours. An opaque white precipitate line between the tissue sample and positive serum sample was taken as a positive result.

3.2.5.3 Washing and drying of slides

The slides were kept in 1.5 per cent hypertonic saline solution with three changes at eight hours interval, to remove the soluble non- reacting constituents. Drying was done by placing a strip of good quality filter paper over the surface of the gel and kept at 37°C for 24 hours. The filter paper was removed and the slide was cleaned for a few seconds in running water to remove the adhering particles of filter paper.

3.2.5.4 Staining procedure

The method followed by Williams and Chase (1971) was adopted for staining the dried gels. The dried slides were immersed in 0.1 per cent Amidoblack stain

solution for about 10 minutes. Destaining was carried out using 7 per cent aqueous glacial acetic acid till the background was decolourised. When complete decolouration was obtained, the slides were dried in air and the results obtained recorded.

3.2.6 Haemagglutination test

The haemagglutination test was carried out as per the method of Carmichael *et al.* (1980) with few modifications in a 96 well U bottom microtiter plates (Laxbro).

3.2.6.1 Preparation of piglet red blood cell suspension

Swine blood was collected in Alsever's solution to prepare piglet red blood cell (PRBC) suspension. It was centrifuged at 3000 rpm for 10 minutes to remove the plasma portions and the buffy coat layers. They were then washed three times in ice cold PBS- BSA and packed at 3000 rpm in a refrigerated centrifuge for 10 minutes. After the final wash, a one per cent PRBC suspension was made in PBS-BSA.

3.2.6.2 Test proper

A two-fold dilution (0.05millilitre) of the test tissue samples starting from an initial 1:2 dilution was made in ice cold PBS –BSA and 0.05 milliliter cold one per cent PRBC were added to all wells. The plate was incubated at 4°c for 2 to 4 hours. Controls were included with positive and negative samples and all controls in separate rows of the microtiter plates. The results were read when buttons were formed in the RBC control wells. The highest dilution forming a uniform mat was considered as the end point.

3.2.7 Haemagglutination inhibition test

This test was carried out as per the method of Carmichael *et al.* (1980) with few modifications using 96 well U-microtitre plates. A 1:10 dilution of sera samples in PBS was made. This was treated with 0.1 milliliter of 50 per cent PRBC to

remove non-specific inhibitors of HA and allowed to stand overnight at 4°C. The sera were then centrifuged at 1500 rpm for 15 minutes to remove pig RBC.The supernatant that was inactivated and PRBC treated was used for serology.

3.2.7.1 Test proper

A two fold dilution (0.025 millilitre) of the test serum was made starting with a 1 in 20 through 1 in 40,960 in ice cold PBS-BSA.After adding 0.025 millilitre of antigen (titrated to 8 HA units/0.025 ml) to the serum, and plates kept at room temperature for one hour. Then 0.05 millilitre of one per cent PRBC was added to all wells and plates were incubated at 4°C, haemagglutination inhibition end points were determined after an overnight incubation.

The controls consisted of PRBC, postive serum sample and parvo virus antigen. The haemagglutination inhibition (HI) end points were determined after the incubation period and read as reciprocals of serum dilutions that completely inhibited 4 to 8 units of HA antigens and were expressed as HI titres.

Results

4. RESULTS

Nineteen bitches with a history of neonatal mortality presented at the University Veterinary Hospitals at Mannuthy and Kokkalai and Veterinary Hospitals in Thrissur district constituted the materials for the present study. The control group consisted of six apparently healthy bitches with normal pups and without any previous history of neonatal mortality.

4.1 CULTURAL EXAMINATION

4.1.1 Bitches

4.1.1.1 Milk

On cultural examinations of milk samples from19 bitches with a history of neonatal mortality, only 17 yielded bacterial growth. Out of the 17 bacterial isolates obtained six (35 per cent) were Gram positive organisms and 11 (65 per cent) were Gram negative organisms. Among the 17 milk samples eight samples yielded *Klebsiella pneumoniae*(47 per cent),five yielded *Staphylococcus aureus* (29 per cent) and two yielded *Proteus vulgaris* (12 per cent). *Staphylococcus intermedius and Pseudomonas aeruginosa* were yielded from one sample of milk each (six per cent). The number of isolates obtained is shown in Table (1) and Fig (1).

4.1.1.2 Blood

From 19 blood samples tested only two samples were positive for bacterial growth among which one was Gram positive and other Gram negative. Isolates were identified as *Staphylococcus aureus* (50 per cent) and *Klebsiella pneumoniae* (50 per cent). (Table 1 and Fig 2).

4.1.1.3 Vaginal swabs

Vaginal swabs taken from 19 bitches were subjected to isolation of bacteria, of which 17 yielded growth, 14 being Gram negative and three Gram positive. Among 17 positive samples, *Klebsiella pneumoniae* was isolated from six samples (35 per cent), *Proteus vulgaris* from four (23 per cent), *Staphylococcus aureus* and *Escherichia coli* from two each (12 per cent) and *Staphylococcus intermedius, Pseudomonas aeruginosa* and *Hafnia alvei* from one each (six per cent) (Table 1 and Fig 3).

4.1.2 Control group

Milk, blood and vaginal swabs collected from six bitches of control group were subjected to cultural examination. None of the samples yielded any bacterial growth.

4.1.3 Pups

Tissues from 12 pups were cultured and only five yielded growth. Isolates were obtained from heart blood and from liver and lung. Among these, two pups were of the same litter and *Pseudomonas aeruginosa* was isolated from the heart blood of each (40 per cent). The other three pups of different litters yielded one *Proteus vulgaris* isolate from the liver (20 per cent) and two *Klebsiella pneumoniae* isolates from the heart blood and lung (40 per cent each) (Table 2 and Fig 4) .

Staphylococcus aureus was grown in Mannitol salt agar (Fig. 5). Mac Conkey agar was used for differentiating lactose fermenting and lactose non - fermenting colonies (Fig 6). *Klebsiella pneumoniae* yielded mucoid colonies in Brilliant green agar (Fig 7). The rapid biochemical test kit (Hi-media) was used for identification of bacteria (Fig 8). The biochemical tests used to differentiate *Escherichia coli and Klebsiella pneumoniae* are shown in (Fig 9,10,11,12).

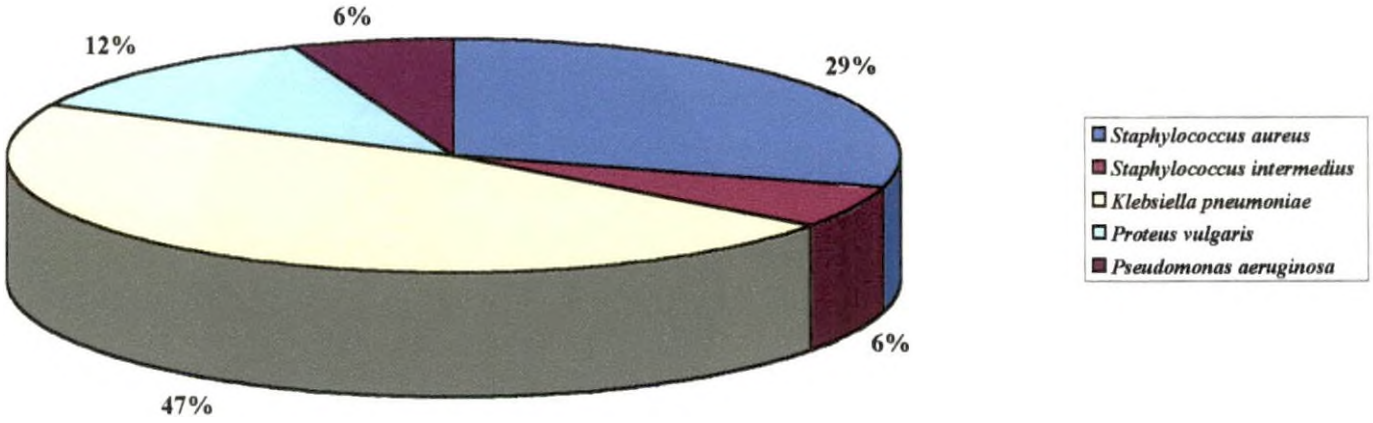


Fig 1. Bacterial isolates from milk of 17 bitches with neonatal pup mortality

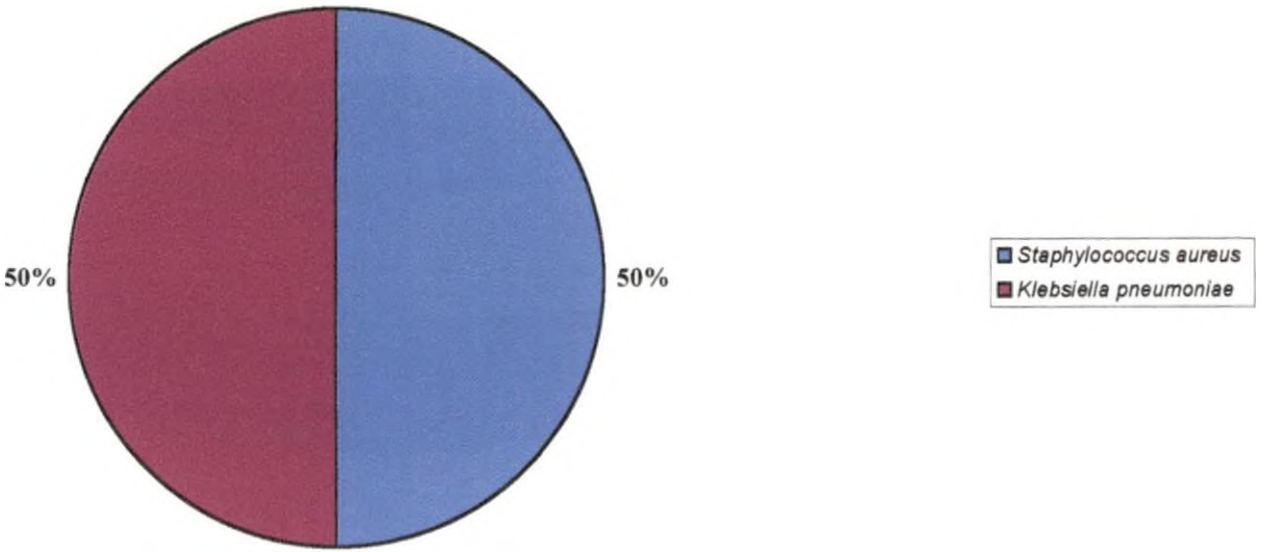
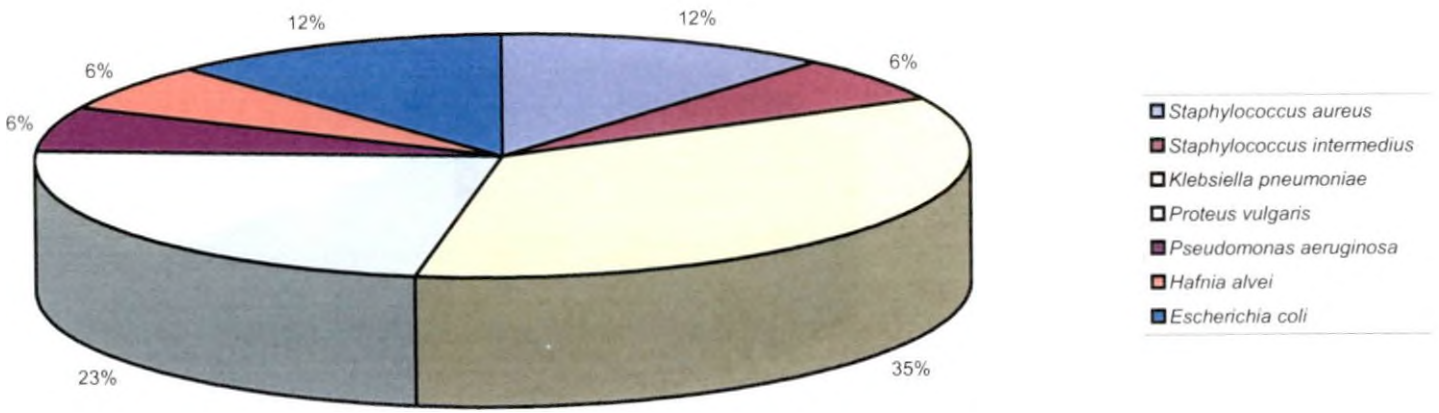


Fig 2. Bacterial isolates from blood of two bitches with neonatal pup mortality

Fig.3Bacterial isolates from vagina of bitches with neonatal pup mortality



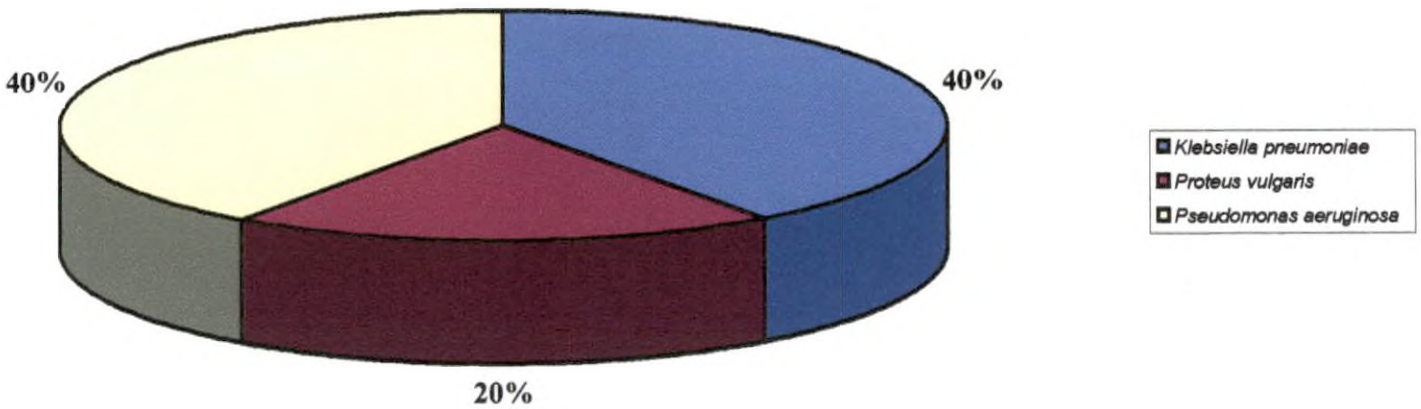


Fig 4. Bacterial isolates from tissues of dead pups

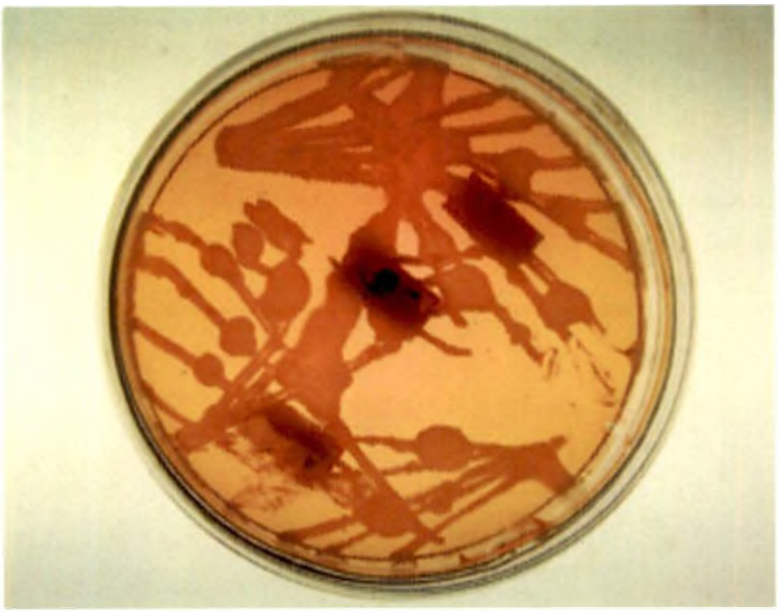


Fig 5 *Staphylococcus aureus* grown on Mannitol salt agar yielding yellow colonies and medium changing from pink to yellow

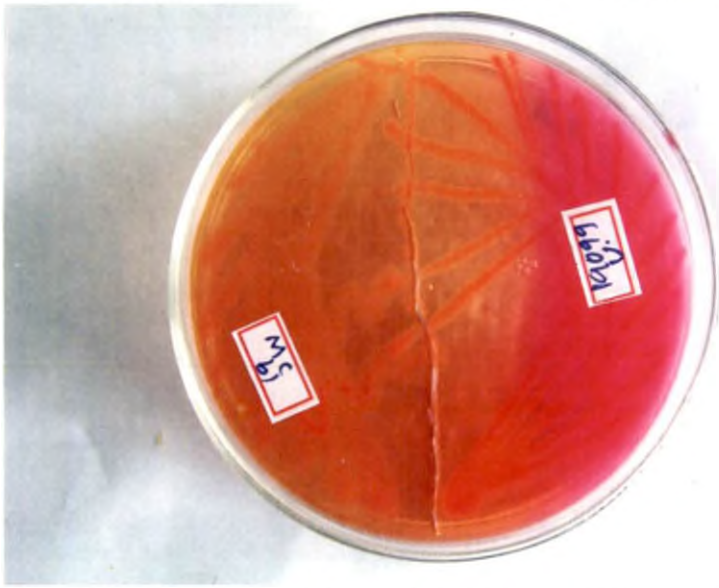


Fig 6 Mac Conkey agar differentiating lactose fermenting and lactose non-fermenting colonies(A & B)

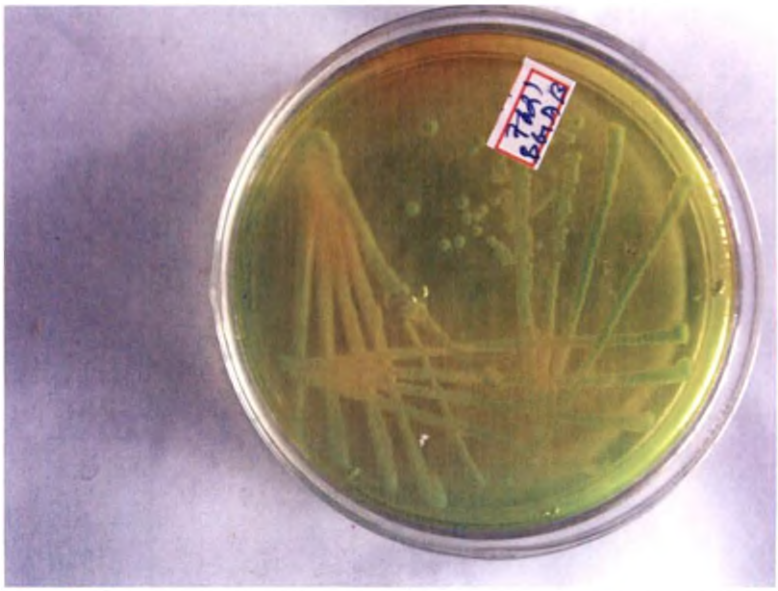


Fig 7 Mucoid colonies of *Klebsiella pneumoniae* in Brilliant Green agar

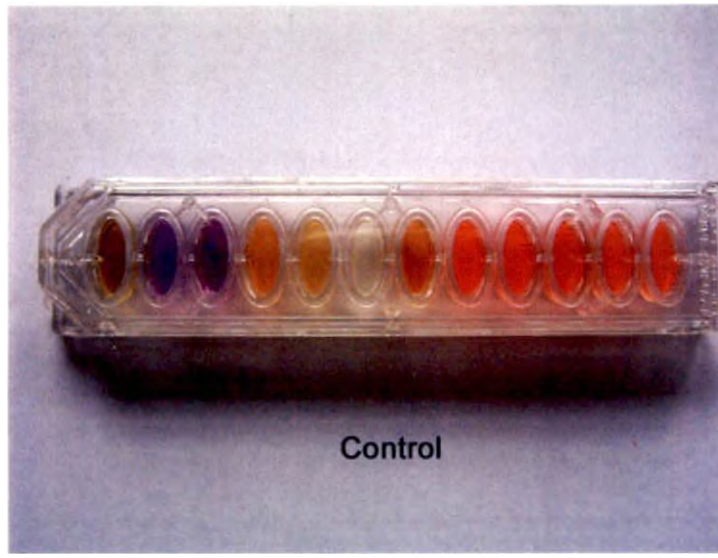
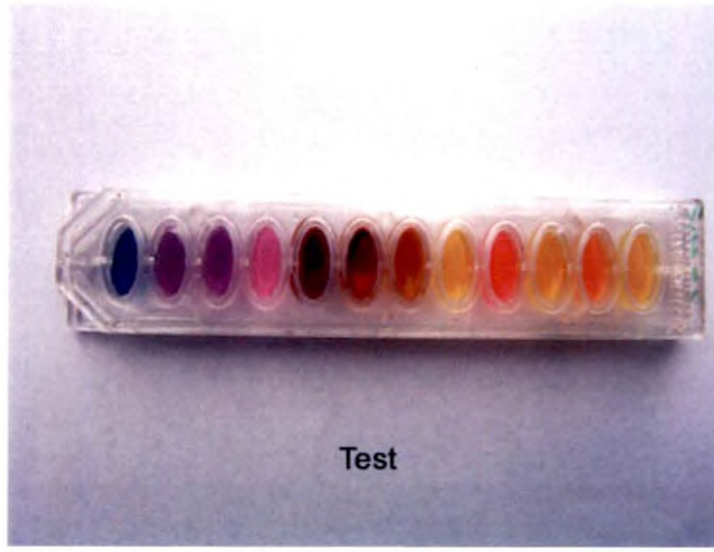


Fig 8 Rapid biochemical test kit used for identification of bacteria



Test used :

1.Citrate
2. Lysine decarboxylation
3. Ornithine decarboxylation
4. Urease
5. Tryptophan deamination
6. Nitrate
7. H_2S
8.Glucose
9. Adonitol
10. Lactose
11. Arabinose
12. Sorbitol

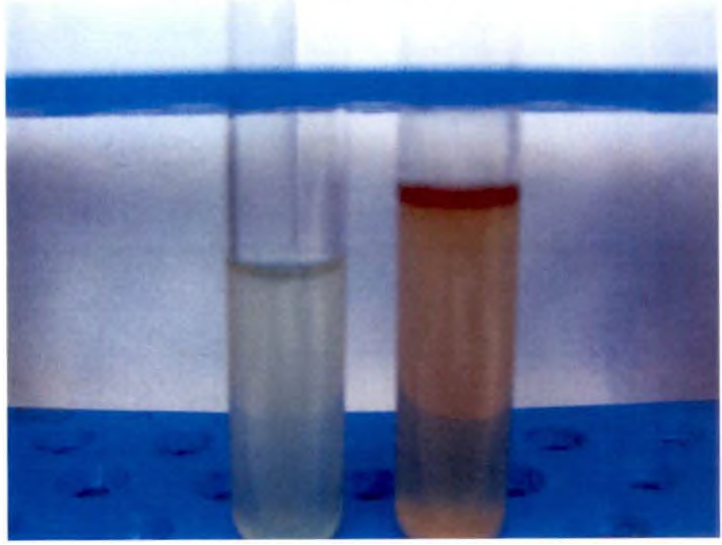


Fig 9 Indole test - negative and positive

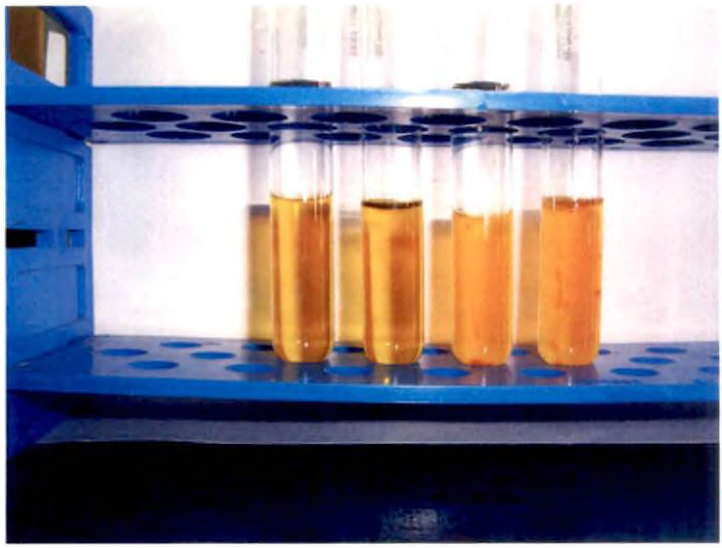


Fig 10 Oxidation - fermentation test - Fermentation

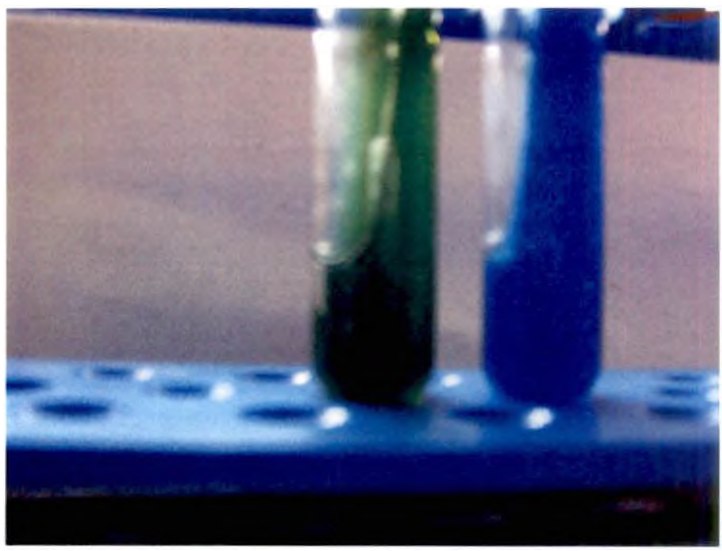


Fig 11 Citrate test - negative and positive

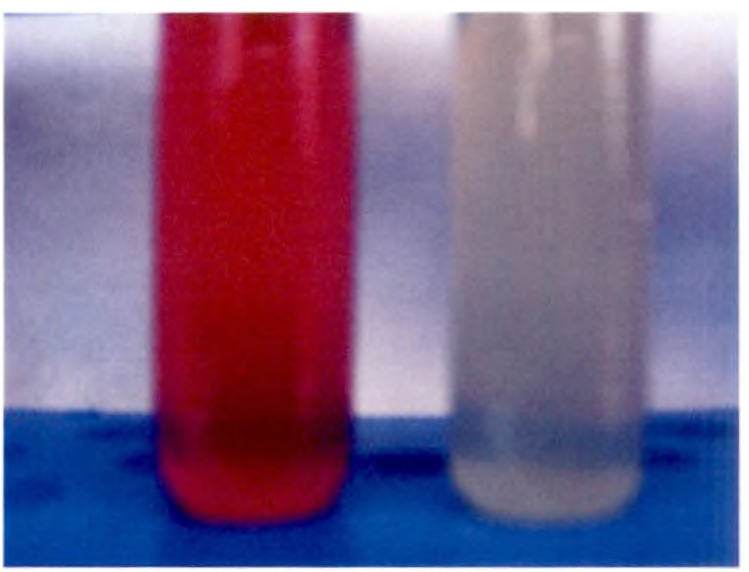


Fig 12 Methyl red test - positive and negative

4.2 ANTIBIOGRAM

4.2.1 Bitch

4.2.1.1 Milk

In vitro antibiotic sensitivity studies of bacterial isolates from 17 milk samples of bitches showed that *Staphylococcus aureus* isolates were mostly sensitive to ciprofloxacin (80 per cent) followed by amoxycillin (60 per cent) and gentamicin and enrofloxacin (40 per cent each). All the isolates were resistant to norfloxacin and chloramphenicol.

The *Staphylococcus intermedius* isolate obtained from milk was sensitive to ciprofloxacin and amoxycillin and was resistant to enrofloxacin, norfloxacin, gentamicin and chloramphenicol. Antibiogram pattern of *Staphylococcus intermedius* is shown in Fig 13.

Sixty three per cent of the *Klebsiella pneumoniae* isolates obtained were sensitive to ciprofloxacin, enrofloxacin and amoxycillin. Twenty five per cent were sensitive to gentamicin and norfloxacin, 13 per cent to amoxycillin clavulanic acid while none of the isolates were sensitive to chloramphenicol.

All the *Proteus vulgaris* isolates from milk were sensitive to ciprofloxacin, gentamicin and amoxycillin and 50 per cent of isolates to enrofloxacin, chloramphenicol and norfloxacin.

The *Pseudomonas aeruginosa* isolated was sensitive to gentamicin, chloramphenicol and enrofloxacin and resistant to amoxycillin, ciprofloxacin and norfloxacin (Table 3).

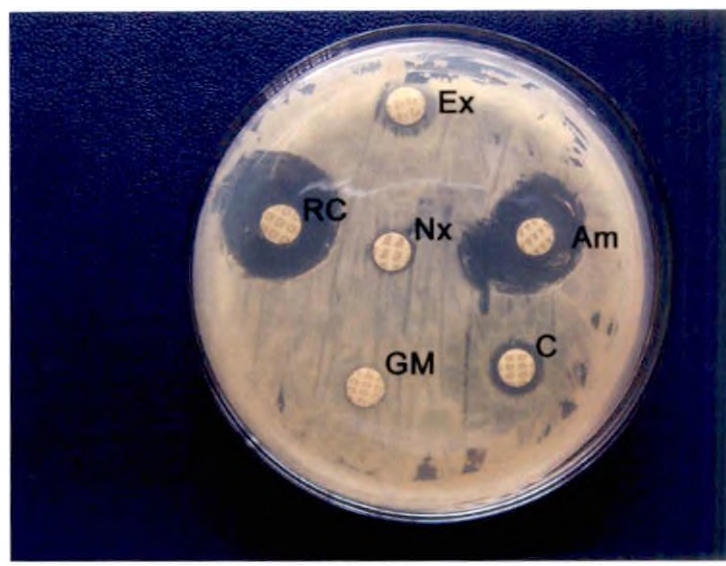


Fig 13 Antibiogram of *Staphylococcus intermedius*

Am : Amoxycillin
C : Chloramphenicol
RC : Ciprofloxacin
Ex : Enrofloxacin
GM : Gentamicin
Nx : Norfloxacin

4.2.1.2 Blood

The *Staphylococcus aureus* isolated from blood was sensitive to ciprofloxacin and enrofloxacin and resistant to amoxycillin, gentamicin, chloramphenicol and norfloxacin while *Klebsiella pneumoniae* was sensitive to gentamicin, ciprofloxacin, amoxycillin and enrofloxacin and resistant to norfloxacin and chloramphenicol (Table 4).

4.2.1.3 Vagina

In vitro antibiotic studies of *Hafnia alvei* showed sensitivity to ciprofloxacin and enrofloxacin and resistance to amoxycillin, chloramphenicol, gentamicin and norfloxacin.

Among the *Klebsiella pneumoniae* isolates, 83 per cent were sensitive to ciprofloxacin, 67 per cent to amoxycillin and enrofloxacin each, 50 per cent to chloramphenicol, 33 per cent to gentamicin and 17 per cent to norfloxacin.

Hundred per cent of the *Staphylococcus aureus* isolates were sensitive to ciprofloxacin, amoxycillin and enrofloxacin and resistant to norfloxacin, chloramphenicol and gentamicin.

The *Staphylococcus intermedius* isolated was sensitive to ciprofloxacin and enrofloxacin and resistant to gentamicin, amoxycillin, chloramphenicol and norfloxacin.

The *Escherichia coli* isolates were sensitive to gentamicin and ciprofloxacin(100 per cent each) and amoxycillin and norfloxacin(50 per cent each). None were sensitive to enrofloxacin and chloramphenicol.

The *Pseudomonas* isolate obtained from vaginal swab was sensitive to gentamicin, enrofloxacin and chloramphenicol, and resistant to ciprofloxacin, amoxycillin and norfloxacin.

All the four *Proteus vulgaris* isolates were sensitive to amoxycillin, ciprofloxacin, enrofloxacin, gentamicin, chloramphenicol and norfloxacin. (Table 5).

4.2.2.Pups

The *Klebsiella pneumoniae* isolates from the heart blood and lung of pups showed sensitivity to ciprofloxacin (100 per cent) and amoxycillin (50 per cent), while they were resistant to gentamicin, enrofloxacin, chloramphenicol and norfloxacin.

The proteus isolate was sensitive to ciprofloxacin, amoxycillin, gentamicin, norfloxacin and enrofloxacin and resistant to chloramphenicol.

Hundred per cent of the *Pseudomonas* isolates showed sensitivity to chloramphenicol, followed by gentamicin (50 per cent) and ciprofloxacin (50 per cent).All the isolates were resistant to amoxycillin, enrofloxacin and norfloxacin.(Table 6)

Majority of the isolates from milk were sensitive to ciprofloxacin (71 per cent), followed by amoxycillin (65 per cent), gentamicin (41 per cent), enrofloxacin (59 per cent), norfloxacin (18 per cent) chloramphenicol (12 per cent) and amoxy-clav (6 per cent).The bacterial isolates from blood were 100 per cent sensitive to ciprofloxacin and chloramphenicol, 50 per cent to gentamicin, 50 per cent to amoxycillin and resistant to enrofloxacin and norfloxacin. The vaginal isolates were sensitive to ciprofloxacin (88 per cent), enrofloxacin (71 per cent) amoxycillin (59 per cent), chloramphenicol (59 per cent), gentamicin (53 per cent) and norfloxacin (35 per cent).(Fig 14,15,16,17).

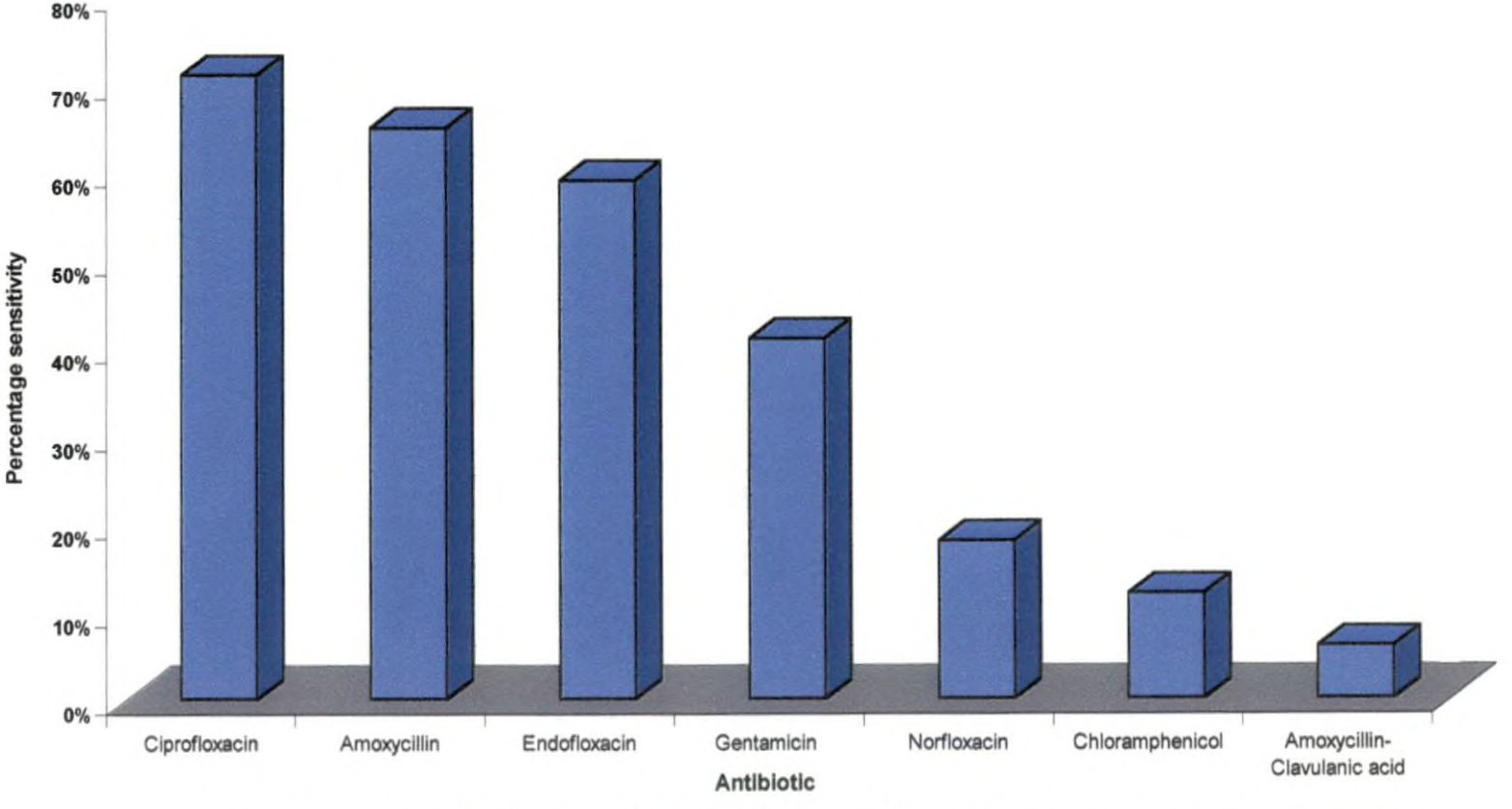


Fig 14. Percentage distribution of antimicrobial sensitivity pattern of bacterial isolates from milk

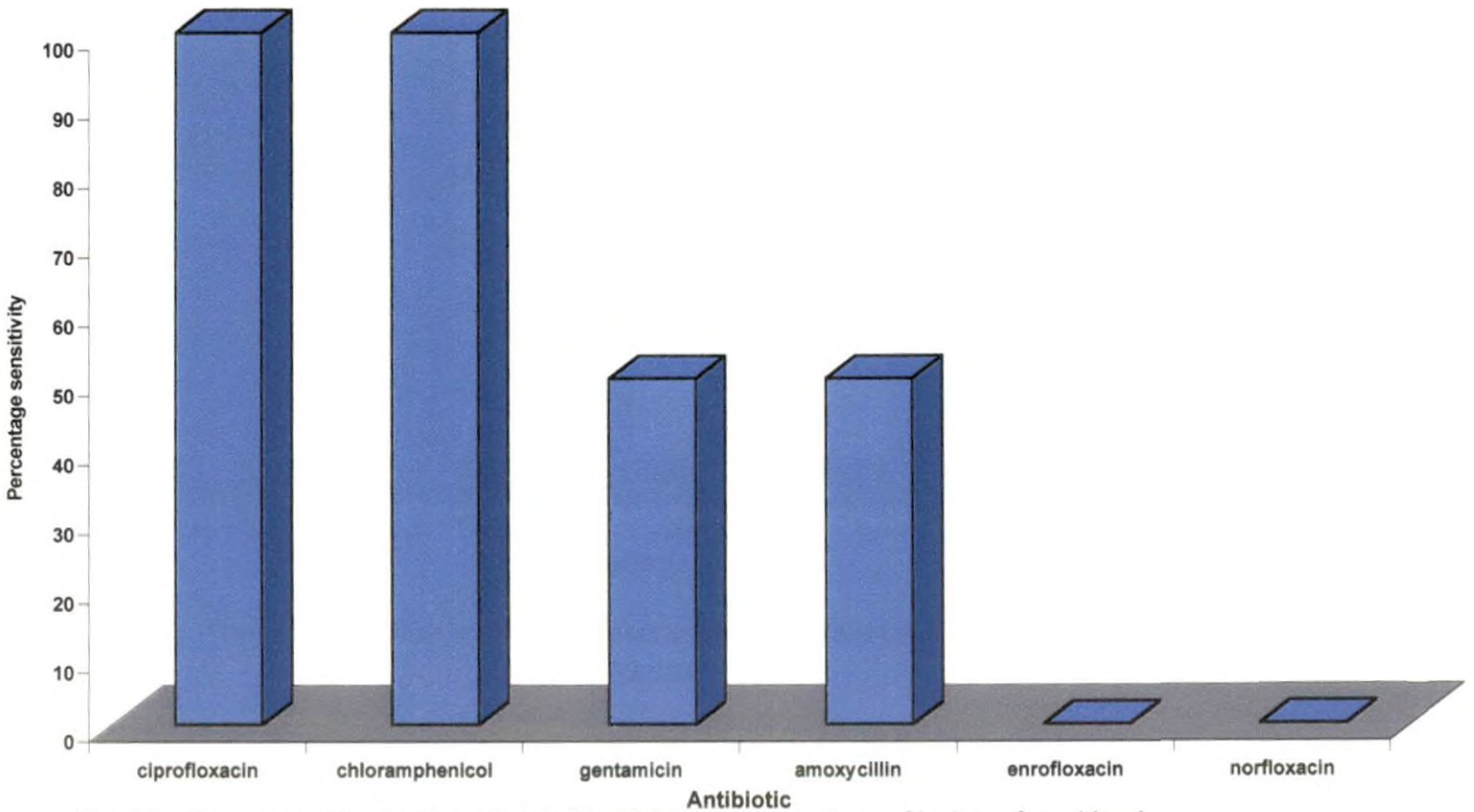


Fig. 15. Percentage distribution of antimicrobial sensitivity pattern of isolates from blood

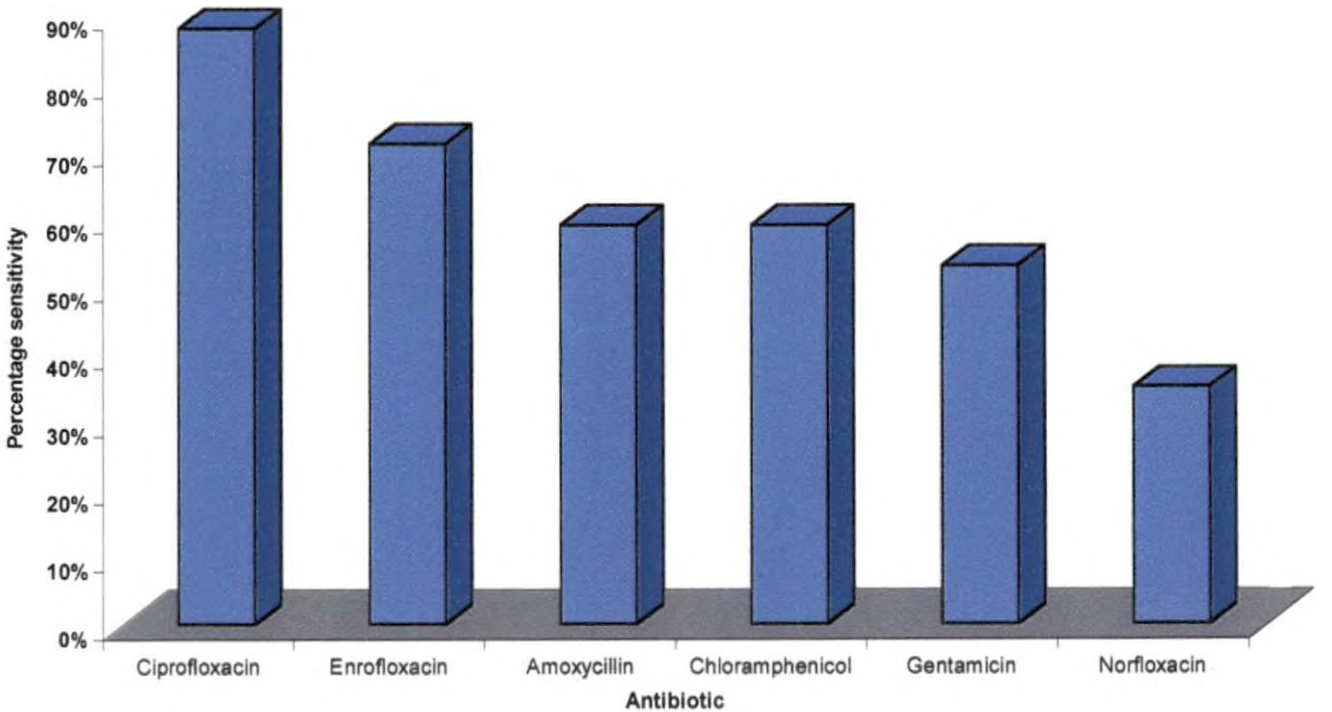
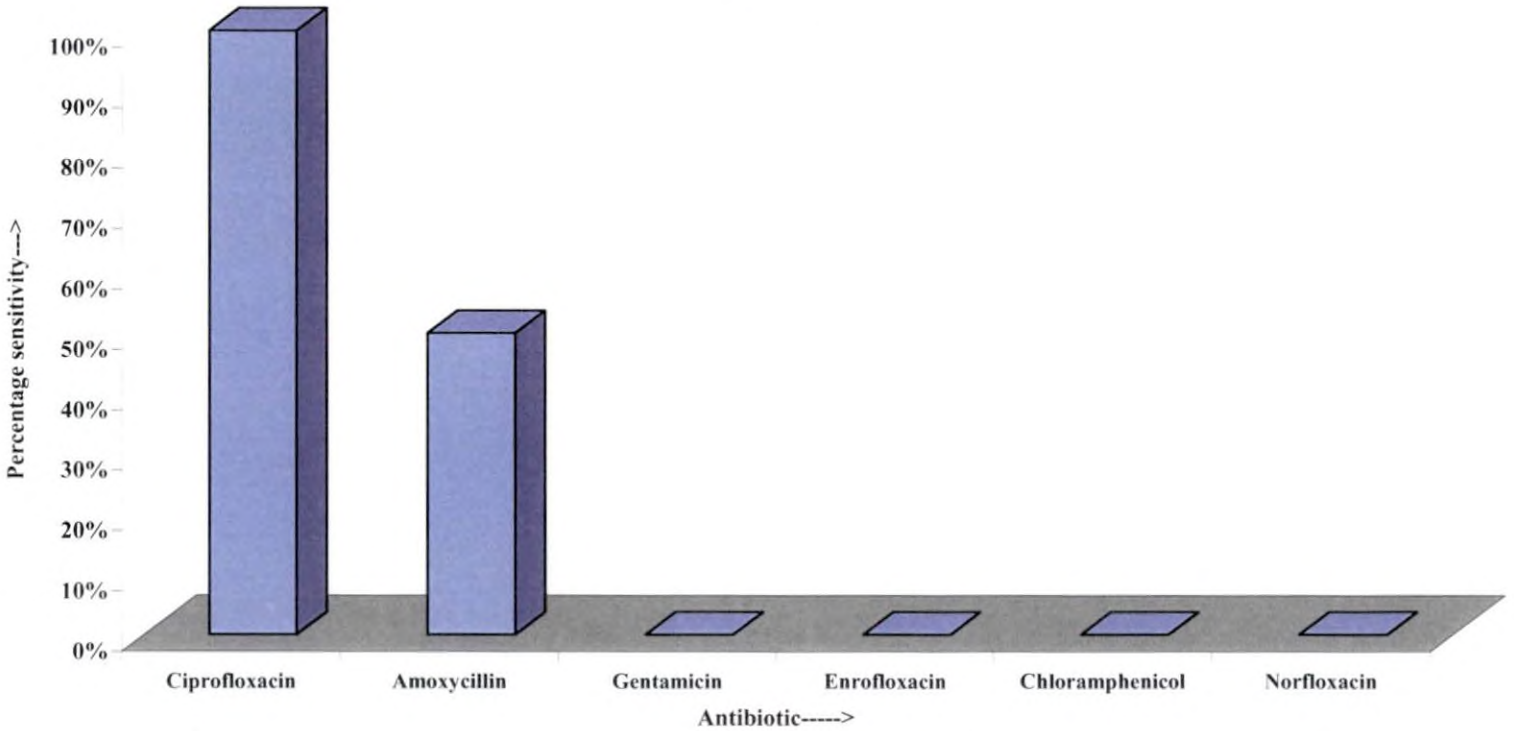


Fig 16. Percentage distribution of antimicrobial sensitivity pattern of bacterial isolates from vagina.

Fig. 17. Percentage distribution of antimicrobial sensitivity pattern of isolates from pup



4.3 AGAR GEL IMMUNODIFFUSION TEST

4.3.1 Bitches

Nineteen serum samples from bitches were subjected to AGID using known parvoviral antigen. None of the samples gave positive result for antibodies against the antigen.(Fig 18)

4.3.2 Pups

AGID was performed using tissue suspensions from liver, lung, intestine, heart and kidney of dead pups for detection of parvoviral antigen using known hyperimmune serum against parvovirus 2 antigen. All the samples were negative for parvovirus.

4.4 HAEMAGGLUTINATION TEST-PUPS

Haemagglutination test was conducted using tissue suspensions of liver, lung, intestine, heart and kidney of the 12 dead pups .Of these the liver and lung tissue of one dead pup gave 1:64 titre in haemagglutination test for parvoviral disease. This was confirmed by haemagglutination inhibition test using known hyper immune serum against canine parvo virus2 antigen.

4.5 HAEMAGGLUTINATION INHIBITION TEST-BITCHES

The serum samples from the bitches were screened for antibodies against parvoviral antigen. The haemagglutination inhibition titers of 64 and above were taken as protective. Among 19 bitches tested, 13 were having a history of prophylactic vaccination against parvo, and six were without any prophylactic vaccination history against parvo. HI titer of the vaccinated bitches ranged from 8 to 64.Only one bitch was having the protective titer of 64.All the unvaccinated bitches were not having any titer of antibodies against parvo by HI test. (Fig 19)

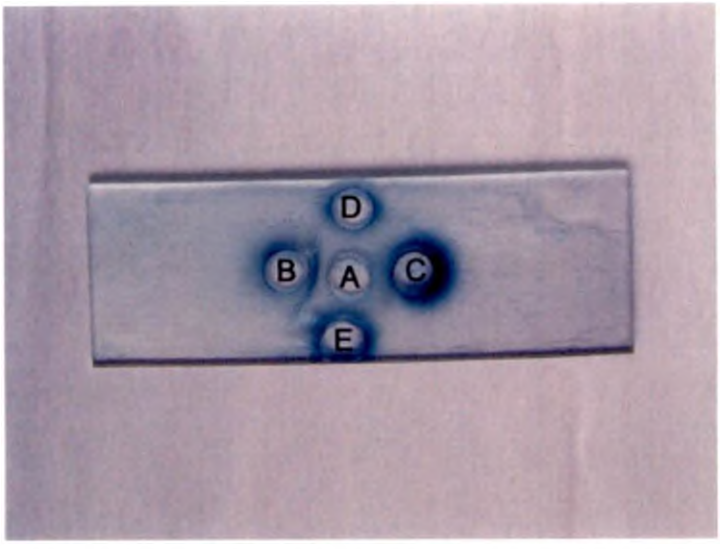


Fig 18 Agar gel immuno diffusion test-precipitation pattern of canine parvo antigen with sera samples

A : Known positive antigen
B : Known positive serum
C : Known negative serum
D&E : Test serum

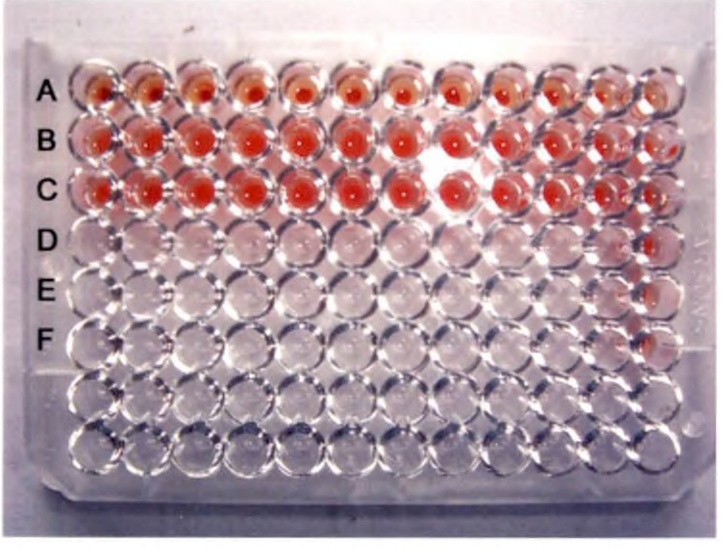


Fig 19 Haemagglutination inhibition assay for canine parvo virus in tissue of pup

Rows A to C : Test tissue samples
Row D : Positive serum control
Row E : Virus control
Row F : Red blood cell control

4.6 NON SPECIFIC CAUSES

4.6.1 Age

The age group in bitches with occurrence in neonatal mortality is given in Table(7). Fifty eight per cent of cases were in bitches aged one to two years of age, 26 per cent of cases in two to three years and 16 per cent in four to five years of age.Among the 19 cases presented, maximum incidence was observed between one and two years of age.

4.6.2 Parity

Five of the bitches were primiparous. Of the remaining 14 bitches, six were at their second whelping, six at their third and two bitches were at their fourth whelping.Highest incidence of neonatal mortality of pups was noticed in bitches of second and third parity among those presented (32 per cent each) (Table 7).

4.6.3 Breed

The breeds of the bitches presented with a history of neonatal mortality included German shepherd (six), Rottweiler(5) Boxer (4) Spitz, (2)and one each of Dobermann pinscher and Labrador retriever (Table 7).

4.6.4 Previous history of neonatal mortality

Five of the bitches were primiparous. Of the remaining 14,nine had a history of neonatal mortality in previous whelpings.

4.6.5 Vaccination history

Routine vaccination with multicomponent vaccine against parvoviral disease was reported in 13 bitches and for the remaining six bitches, the vaccinations were not done properly.

4.6.6 Previous history of illness

One bitch had a history of parvoviral enteritis at 10 months of age.

4.6.7 Other reproductive problems

Four of the bitches were treated for infertility with antibiotics at the time of estrus. One bitch had dystocia and was given oxytocin at the time of whelping.

4.6.8 Mothering ability

The owners of two bitches complained of insufficient milk for the litters. Both the bitches were primiparous. Otherwise, the bitches showed good mothering ability with utmost care for their pups.

4.6.9 Mating history

Of the nine bitches with previous history of neonatal mortality, two were crossed with the same male. The mating history was not clear in one case. In one bitch with a parity of 3,the same male was used in the first and third crossing with neonatal death occurring in second and third whelpings. The other five bitches were crossed with different males.

4.7 TREATMENT

Based on antibiogram results obtained, treatment was given to bitches and the pups using suitable antibiotics and in cases where bacterial growth was obtained from milk, directions were given to separate the pups from bitches and to feed them with milk replacements. Also, the owners were advised to provide proper warmth to the pups for preventing chilling. Supportive treatment was given using fluids and vitamins. With these treatments adopted, only six cases showed response with absence of further pup mortality in the litter.

In three cases of isolation of *Staphylococcus aureus* from the milk, Amoxycillin cloxacillin combination was given at the dose rate of 10mg/kg body weight intramuscularly daily for five days for the bitch and in pups, orally at 20 mg/kg body weight thrice daily. In two other cases eventhough treated with amoxycillin cloxacillin combination initially for two days,no response was obtained.Hence, on getting antibiogram results the antibiotic was changed to ciprofloxacin at the dose rate of 15mg/kg body weight orally for bitch and pups. One bitch was given supportive fluid therapy –300 ml of five per cent dextrose normal saline for the first two days of treatment. It was also given diclofenac sodium injection at 1mg/kg body weight intramuscularly. There was improvement in the health of the bitch. The pups survived in the last two cases only.

In one case where *Staphylococcus intermedius* was isolated from the milk of bitch ,it was treated with ciprofloxacin orally at dose rate of 15mg/kg body weight for 5 days.This was a case wherein there was history of puppy mortality in the previous whelping. The treatment was done on the basis of isolation from milk and vagina in the present case and none of the pups died in the case.

Among the eight cases from which *Klebsiella pneumoniae* was isolated, four of the cases were treated with ciprofloxacin at 15mg/kg body weight intramuscularly both for bitches and pups.In addition, betamethasone sodium was given at dose rate of 1mg/kg body weight for the pups in two cases. Both these cases did not respond and the pups could not be saved.The other two responded and one among these was initially treated with amoxycillin, which was later changed to ciprofloxacin on antibiogram results of bitche's milk.

Three other cases (where *Klebsiella* was isolated) were treated with amoxycillin cloxacillin antibiotic to the bitches and pups. There was response in pups of one case only,where there was no further mortality.Among the other two cases ,one lost two additional pups during course of treatment leaving behind three

pups healthy out of a litter of seven.In the other,all the pups of the litter died within the treatment course itself.

In one case where *Klebsiella pneumoniae* was isolated from milk,the organism was sensitive to amoxycillin clavulanic acid combination only. This bitch's milk was cultured one day before the whelping on the grounds of the owner's complaint of previous history of neonatal puppy mortality and no growth was obtained, but puppy mortality was observed subsequent to this whelping too. The treatment of the bitch with amoxyclavulanic acid did not prove successful; parvoviral antigen could be detected from the liver and lungs of the dead pup.

In the two cases where *Proteus vulgaris* was isolated, the bitches and pups were treated with amoxycillin cloxacillin combination. The remaining pups in the litter died in both the cases.The milk of the bitch was insufficient for the pups in one case.

The bitch and pups were treated with gentamicin parenterally at 4mg/kg body weight for 5 days in the case where *Pseudomonas aeruginosa* was isolated. The treatment was initially started with ciprofloxacin, which was later changed to gentamicin on antibiogram results. There was no response in this case also.

In two cases no bacterial isolate was obtained from the milk and the antibiogram based on the vaginal isolates(two *Klebsiella pneumoniae*) was used for treatment.Ciprofloxacin at 15 mg/kg body weight was used for the bitch and pups.Both the cases ended in mortality of the entire litter.

Table1. Organisms isolated from milk, blood and vagina of bitches

Organism	Milk No: (per cent)	Blood No: (per cent)	Vagina No: (per cent)
Staphylococcus aureus	5 (29)	1 (50)	2 (12)
Staphylococcus intermedius	1 (06)	0 (0)	1 (06)
Klebsiella pneumoniae	8 (47)	1 (50)	6 (35)
Proteus vulgaris	2 (12)	0 (00)	4 (23)
Pseudomonas aeruginosa	1 (06)	0 (00)	1 (06)
Hafnia alvei	0 (00)	0 (00)	1 (06)
Escherichia coli	0 (00)	0 (00)	2 (12)

Table 2. Organisms isolated from the liver, lung and heart blood of pups

Organism	Number of isolates			
	Heart blood	Lung	Liver	Total (per cent)
Klebsiella Pneumoniae	1	1	0	2(40)
Proteus vulgaris	0	0	1	1(20)
Pseudomonas aeruginosa	2	0	0	2(40)

Table 3. Antibiogram of isolates from milk

Organism	Number of Isolates	Sensitive antibiotic (per cent)						
		GM	RC	Am	Ex	C	Nx	Ac
Staphylococcus aureus	5	2(40)	4(80)	3(60)	2(40)	0(0)	0(0)	
Staphylococcus intermedius	1	0(0)	1(100)	1(100)	0(0)	0(0)	0(0)	
Klebsiella pneumoniae	8	2(25)	5(63)	5(63)	5(63)	0(0)	2(25)	1(13)*
Proteus vulgaris	2	2(100)	2(100)	2(100)	1(50)	1(50)	1(50)	
Pseudomonas aeruginosa	1	1(100)	0(0)	0(0)	1(100)	1(100)	-	

*The antibiotic was used on resistance to all other six.

GM-Gentamicin RC-Ciprofloxacin Am-Amoxycillin

Ex-Enrofloxacin C-Chloramphenicol Nx-Norfloxacin

Ac-Amoxycillin clavulanic acid

Table 4. Antibiogram of isolates from blood

Organism	No.	Sensitive antibiotics (per cent)					
		GM	RC	Am	Ex	C	Nx
Staphylococcus aureus	1	0(0)	1(100)	0(0)	1(100)	0(0)	0(0)
Klebsiella pneumoniae	1	1(100)	1(100)	1(100)	1(100)	0(0)	0(0)

Table 5. Antibiogram of vaginal isolates

Organism	No. of isolates	Sensitive antibiotics (per cent)					
		GM	RC	Am	Ex	C	Nx
Hafnia alvei	1	0 (0)	1(100)	0(0)	1(100)	0(0)	0(0)
Klebsiella pneumoniae	6	2 (33)	5(83)	4(67)	4(67)	3(50)	1(17)
Staphylococcus aureus	2	0 (0)	2(100)	2(100)	2(100)	0(0)	0(0)
Staphylococcus intermedius	1	0(0)	1(100)	0(0)	1(100)	0(0)	0(0)
Escherichia coli	2	2 (100)	2(100)	1(50)	0(0)	0(0)	1(50)
Pseudomonas aeruginosa	1	1(100)	0(0)	0(0)	1(100)	1(100)	0(0)
Proteus vulgaris	4	4(100)	4(100)	4(100)	4(100)	4(100)	4(100)

GM- Gentamicin RC- Ciprofloxacin Am- Amoxycillin

Ex- Enrofloxacin C- Chloramphenicol Nx- Norfloxacin

Table 6. Antibiogram of isolates from pups

Organisms	No: of isolates	Sensitive antibiotics (per cent)					
		GM	RC	Am	Ex	C	Nx
Klebsiella pneumoniae	2	0(0)	2(100)	1(50)	0(0)	0(0)	0(0)
Pseudomonas aeruginosa	2	1(50)	1(50)	0(0)	0(0)	2(100)	0(0)
Proteus vulgaris	1	1(100)	1(100)	1(100)	1(100)	0(0)	1(100)

GM-Gentamicin RC-Ciprofloxacin Am-Amoxycillin Ex-Enrofloxacin C-Chloramphenicol Nx-Norfloxacin

Table 7. Distribution of cases based on age, parity and breed of bitches.

Age group	Number of cases	Percentage
1-2	11	58
2-3	5	26
3-4	0	0
4-5	3	16
Parity		
1	5	26
2	6	32
3	6	32
4	2	10
Breed		
Rottweiler	5	26
Boxer	4	21
Labrador retriever	1	5
German shepherd	6	32
Doberman	1	5
Spitz	2	11

Discussion

5. DISCUSSION

Puppy mortality not only has financial and emotional consequences for breeders and pet owners, but may also have an impact on a breeding programme. Also, records of neonatal pup mortality can be considered to provide a realistic indication of success in dog breeding. The present study undertaken to explore the causes of the problem has given an insight into the same.

5.1 CULTURAL EXAMINATION

5.1.1 Bitches

5.1.1.1 Milk

The organisms obtained from milk of bitches included *Staphylococcus aureus, Staphylococcus intermedius, Klebsiella pneumoniae, Proteus vulgaris* and *Pseudomonas aeruginosa.* This is in accordance with the finding of Dakshinkar *et al.* (2001) who isolated *Escherichia coli* along with the above and in partial accordance with the findings of Sager and Remmers (1990) who identified along with *Staphylococcus aureus*, streptococcus G and *Escherichia coli* also as cause of sub clinical mastitis of bitches leading to septicaemic death of newborn puppies. Somi *et al.* (2003) on the other hand, did not consider bacteria from the bitch's milk like *Staphylococccus intermedius* to be a primary cause of neonatal septicaemia and only considered the importance of *Escherichia coli, Klebsiella pneumoniae* and haemolytic streptococcal isolates from canine milk in puppy mortality. However, *Staphylococcus intermedius* isolated from the bitch may have the importance of influencing the colonization by pathogenic staphylococci in puppies (Koulumies and Lloyd, 2002).

None of the samples in the study yielded *Streptococcus* spp., which was considered as an important cause of mastitis in bitches associated with puppy death

by Evans (1968). The role of streptococcal species was also highlighted by Sager and Remmers (1990), Dakshinkar *et al.* (2001) and Somi *et al.* (2003).

In the present study, no bacteria was isolated from the milk of bitches of the control group. This does not confirm with the findings of Kuhn *et al.* (1991) who had obtained *Staphylococcus* spp. isolates from 68 per cent of the milk samples from healthy post parturient bitches.

The role of bacterial infection in pups from the milk of bitches was proved in only two cases in the present study as the same organisms could be isolated from the milk of bitch and tissues of pups in two cases only. This is in accordance with the observation made by Kuhn *et al.* (1991) that there is no direct influence of lactiferous gland colonisation with bacteria on mortality of puppies. However, according to Sager and Remmers (1990), sub clinical mastitis of bitches can lead to septicaemic death of newborn puppies.

5.1.1.2 Blood

Two of the 19 bitches yielded bacterial growth from their blood, which was identified as *Staphylococcus aureus* and *Klebsiella pneumoniae.* The organisms were isolated from milk too in the cases. The isolation of *Brucella canis* in a similar manner, that is from the blood and milk, along with vaginal and fetal tissues have been reported by Moore(1969)

5.1.1.3 Vagina

Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa, Hafnia alvei and *Staphylococcus intermedius* were isolated from the vaginal swab specimens. This correlates partially with the findings of Olson (1975) who isolated *Escherichia coli* and colagulase positive staphylococci from the vagina of bitches with vaginitis, infertility and mortality in their neonates; Hirsh and Wiger(1977) who isolated *Escherichia coli,Streptococcus viridans ,Streptococcus canis* and *Staphylococcus aureus* from the vagina of

clinically normal bitches and those with vaginitis ; Sager and Remmers (1990) who opined that *Staphylococcus aureus*, streptococcus G and *Escherichia coli* were transmitted intrauterine or by the infected genital tract to the puppies and were the cause of septicaemic death of puppies; Gandotra *et al.* (1992) who attributed *Escherichia coli*,haemolytic streptococci, *Staphylococcus aureus*,*Pseudomonas aeruginosa, Proteus vulgaris, Klebsiella spp.* and *Bacillus cereus* to the causes of infectious infertility in bitches;Bjurstrom(1993) who isolated *Escherichia coli*,beta hemolytic streptococci, *Staphylococcus intermedius* and *Pasteurella multocida* from the vagina of bitches with infertility,vaginitis,pyometra and puppy death; Floss and Hardin(1994) who considered *Escherichia coli, Staphylococcus aureus*, *Streptococcus* spp. *Mycoplasma* spp. ,*Ureaplasma* spp. and *Brucella canis* to be associated with abortion and neonatal mortality.

Streptococcus spp., which was not isolated in the present study, was isolated by Davies and Skulski (1956), Mantovani *et al.* (1961), Kornblatt *et al.* (1983), Sager and Remmers (1990), Gandotra *et al.* (1992) and Bjurstrom (1993) along other bacteria from the vagina of bitches associated with neonatal mortality.

None of the vaginal swab specimens from control group yielded any bacterial growth on culture in this study. Negative vaginal culture reports were made by Olson and Mather (1978) and Bjurstrom and Forsberg (1992) in 37 per cent and in 5.2 per cent specimens respectively. The majority of workers have isolated bacteria such as *Escherichia coli*, *Streptococcus* spp., *Staphylococcus aureus*, *Bacteroides* spp., *Pasteurella* spp., *Staphylococcus intermedius* and *Staphylococcus epidermidis* from the vaginal specimens of normal healthy bitches at different stages of the reproductive cycle, including that at the post-partum phase (Hirsh and Wiger, 1977;Olson *et al.*, 1979;Allen and Dagnall, 1982;Baba *et al.*, 1983;Skaar, 1989;Bjurstrom and Forsberg, 1992;Caudle *et al.*, 1994). Bjurstrom and Forsberg (1992) isolated *Staphylococcus intermedius* exclusively at the post-partum period.

The organisms isolated from the post-partum vagina of bitches associated with neonatal mortality in this study are the normal inhabitants of the genital tract in the species. However, the only consistent difference between the isolates from

healthy and diseased bitches are the number of the bacteria in the exudates (Hirsh and Wiger, 1977). Duijkeren (1992) opined that the many aerobic and anaerobic bacteria that inhabit the normal vagina might become pathogens if a breakdown in local immunity occurred.

5.1.2 Pups

The bacteria isolated from tissues of dead pups in this study were *Pseudomonas aeruginosa, Klebsiella pneumoniae and Proteus vulgaris.* This does not conform to the findings of Drake and McCarthy (1964) who obtained *Escherichia coli* on culture of the organs of dead pups and concluded that these might be only postmortem invaders and without any pathological significance. Some similation of results could be observed with that of Kirk (1965) who opined that *Escherichia coli* and *Pseudomonas* spp. isolated from dead pups are important causes of death due to septicaemia. Askaa *et al.* (1978) identified *Escherichia coli* to be the most frequent isolate from dead puppies. Hoskins (1995) considered along with *Klebsiella* and *Pseudomonas* species, *Staphylococcus, Escherichia, Enterobacter, Enterococcus, Clostridium, Bacteroides, Streptococcus, Fusobacterium, Pasteurella, Brucella* and *Salmonella* species as common causes of neonatal sepsis.

Streptococcus spp., which was not isolated from any of the dead puppies in the this study was considered as an important etiology in the fading puppy syndrome by Stafseth *et al.* (1937), Hare and Fry (1938), Davies and Skulski (1956), Mantovani *et al.* (1961), Bowden *et al.* (1963) and Hoskins (1995).They have isolated *Streptococcus* spp. from dead puppies in neonatal period.

Brucella canis, which was not isolated in the present study, was reported as a cause of neonatal mortality by Carmichael (1966) and Moore and Bennett (1967) who have isolated the organism from the aborted fetuses. The role of the bacterium has been elaborated in abortion and stillbirth cases.

5.2 ANTIBIOGRAM

5.2.1 Bitches

5.2.1.1 Milk

Majority of the isolates were sensitive to ciprofloxacin (71 per cent), followed by amoxycillin (65 per cent), gentamicin (41 per cent), enrofloxacin (59 per cent), norfloxacin (18 per cent) chloramphenicol (12 per cent) and amoxy-clav (6 per cent). The study conducted by Dakshinkar *et al.* (2001) differed in showing 100 per cent sensitivity to chloramphenicol and gentamicin followed by ciprofloxacin (91.3 per cent), amoxycillin (86.95 per cent), oxytetracycline and streptomycin (47.82per cent) and nalidixic acid (39.13 per cent). Mohan *et al.* (1994) obtained similar isolates and reported sensitivity of these to erythromycin, cephalexin, chloramphenicol, cephaloridine and cloxacillin.

5.2.1.2 Blood

The bacterial isolates from blood were 100 per cent sensitive to ciprofloxacin and chloramphenicol, 50 per cent to gentamicin, 50 per cent to amoxycillin and resistant to enrofloxacin and norfloxacin. The organisms isolated were the same as isolated from milk in the respective cases. The general pattern of the antimicrobial sensitivity of the isolates from milk could be observed.

5.2.1.3 Vagina

The vaginal isolates were sensitive to ciprofloxacin (88 per cent), enrofloxacin (71 per cent), amoxycillin (59 per cent), chloramphenicol (59 per cent), gentamicin (53 per cent) and norfloxacin (35 per cent). The antibiogram of similar isolates from the vagina showed highest sensitivity to gentamicin (86 per cent) and chloramphenicol (70 per cent) and least sensitivity to penicillin (6 per cent) (Gandotra *et al.*, 1992).

The use of *in vitro* antibiotic studies is an indicator of the emergence of resistant bacterial strains. Changes in the pattern of susceptibility might be due to the changes in the genetic make up of local strains. The administration of drugs to the neonate should also take into consideration the development of the organ systems and mechanisms that affect the drug concentration in target tissues and at receptors.

5.3 AGAR GEL IMMUNODIFFUSION TEST

The serum samples from the bitches could not identify any positive cases for parvoviral antibody and none of the tissue samples of the pups yielded positive in AGID for parvovirus detection. Gunaseelan(1993) observed CPV antigen in the faecal samples of only 11.2 per cent of clinically suspected dogs.

5.4 HAEMAGGLUTINATION AND HAEMAGGLUTINATION INHIBITION

The presence of parvovirus was detected and confirmed by haemagglutination and haemagglutination inhibition test in one dead pup. The antigen was detected in the lungs and liver of the affected pup. Studdert *et al.* (1983) reported that the most sensitive and rapid method of parvoviral diagnosis was the haemagglutination test. A similar case of generalized canine parvovirus disease in a litter of pups that died when three to nine days old was diagnosed by virus isolation from the heart, lungs, liver, kidneys, and small intestine by Lenghaus and Studdert (1982).

Among the vaccinated bitches, only one had protective HI titre against parvo. This points to the lack of proper vaccination schedules in the bitches and suggests the need for a booster vaccination at the time of pregnancy in addition to routine vaccination especially in the face of an impending plan of breeding.

5.5 NON SPECIFIC FACTORS

5.5.1 Age

Maximum incidence of neonatal mortality was observed in the age group of one to two years of the bitch. This is in accordance with Mc Cay and Stevens (1963) who reported that the age of the dam influenced the mortality rates of pups. Litter size was smaller and pup losses greater in the dam's first year than during the second, third or fourth.

5.5.2 Parity

There was no relation with the occurrence of the problem with the parity of the bitch. Majority of the bitches were at their second and third whelpings (32 per cent each) while 26 per cent were primiparous and 11 per cent were at their fourth .

5.5.3 Breed

German shepherd, Rottweiler and Boxer breeds showed highest incidence in the present study. Nielen(1998) suggested that there might be a relationship between brachycephaly and a high incidence of stillbirths because of dystocia and problems with removing the amnion.

5.5.4 Previous history of neonatal mortality

Among the multiparous bitches, 64 per cent showed repeat occurrence of the problem.

5.5.5 Vaccination history

Routine vaccination with multicomponent vaccine against parvoviral disease was reported in 13 bitches and for the remaining six bitches the vaccinations were not done properly.

5.5.6 Previous history of illness

One bitch had a history of parvoviral enteritis at 10 months of age.

5.5.7 Other reproductive problems

In the present study, one bitch had dystocia and had to be treated with oxytocin at the time of whelping. According to Davidson (2003), the average incidence of stillbirths during both complicated and uncomplicated vaginal deliveries were reported to be 33 per cent and that of neonatal mortality in uncomplicated whelping ranged from 9.23 per cent to 26 per cent.

5.5.8 Mothering ability

As against Fox (1963), Kirk (1965) and Lanting and Carmichael (1988) who explained the causes of neonatal mortality in dogs with the poor mothering ability and agalactia on the part of the bitch, the present study showed that majority of the bitches (89 per cent) performed good on this part. Lack of sufficient milk and insufficient mothering instinct was shown in only 11 per cent of the bitches.

5.5.9 Mating history

The mating history shows that the same male was used for crossing in only three of the nine repeat cases. One among this was crossed with the same male in the first and third whelping with mortality occurring in the second and third. This shows the relative insignificance of the role of the male in the occurrence of puppy mortality.

5.6 TREATMENT

Based on antibiogram results obtained, treatment was given to bitches and the pups using suitable antibiotics and supportive treatment was given using fluids and vitamins and directions to maintain the pups in a warm drought free environment. Inspite of the treatment efforts, a general failure in response was observed in 13 cases, which might be attributed to the multiple etiology of the problem. Viral diseases including canine herpes virus and canine parvovirus 1 have not been investigated in this study, both being incriminated as important causes. It is important to note that there is not just one simple cause of neonatal mortality in dogs. That is why the situation in which a number of puppies apparently healthy at

birth but which fail to thrive and die before they reach 21days of age are best referred to as the " FADING PUPPY COMPLEX " (FPC).

Summary

None of the milk, blood or vaginal swabs taken from the control group yielded any bacterial growth.

Of the 12 pups, only five yielded growth on culture. Isolates were obtained from heart blood and from liver and lung. Among these, two pups were of the same litter and *Pseudomonas aeruginosa* was isolated from them(40 per cent). The other three pups of different litters yielded *Proteus vulgaris* (20 per cent) and *Klebsiella pneumoniae* (40 per cent).

Antibiogram of the isolates was conducted. Majority of the isolates from milk were sensitive to ciprofloxacin (71 per cent), followed by amoxycillin (65 per cent), gentamicin (41 per cent), enrofloxacin (59 per cent), norfloxacin (18 per cent), chloramphenicol (12 per cent) and amoxy-clav (6 per cent). The bacterial isolates from blood were 100 per cent sensitive to ciprofloxacin and chloramphenicol, 50 per cent to gentamicin, 50 per cent to amoxycillin and resistant to enrofloxacin and norfloxacin. The vaginal isolates were sensitive to ciprofloxacin (88 per cent), gentamicin (53 per cent), amoxycillin (59 per cent), enrofloxacin (71 per cent), chloramphenicol (59 per cent) and norfloxacin (35 per cent). In pups, 40 per cent of the bacterial isolates were sensitive to gentamicin and chloramphenicol, 80 per cent to ciprofloxacin, 60 per cent to amoxycillin and 20 per cent to norfloxacin and enrofloxacin.

The role of parvoviral infection in neonatal pup mortality was studied by subjecting the lung, liver, kidney, heart and intestine of the pups to agar gel immunodiffusion test, haemagglutination test. Parvoviral antigen could be detected from tissue samples of one pup using HA. This was confirmed by haemagglutination inhibition test using known hyper immune serum against canine parvo virus 2 antigen.

The serum samples of bitches were screened for antibodies against parvo using agar gel immunodiffusion and haemagglutunation inhibition test. None of the

serum from the bitches gave positive result for antibodies against parvovirus in agar gel immunodiffusion test.HI titre of the vaccinated bitches ranged from 8 to 64.Only one bitch was having the protective titre of 64.All the unvaccinated bitches were not having any titre of antibodies against parvo by HI test.

Among the 19 cases presented, maximum incidence was observed between one and two years of age.Five of the bitches were primiparous. Of the remaining thirteen bitches, six were at their second whelping, six at their third and two bitches were at their fourth whelping. The breeds of the bitches included German shepherd (32 per cent), Rottweiler (26 per cent), Boxer (21 per cent), Spitz (11 per cent) and one each of Doberman and Labrador retriever(5 per cent each). Five of the bitches were primiparous. Of the remaining 14, nine had a history of neonatal mortality in previous whelping. Routine vaccination with multicomponent vaccine against parvoviral disease was reported in 13 bitches and for the remaining six bitches, the vaccinations were not done properly. The owners of two bitches complained of insufficient milk for the litters. Both the bitches were primiparous. Otherwise, the bitches showed good mothering ability with utmost care for their pups.

Based on the antibiogram results, treatment with antibiotic was done to bitches and pups with response as no further puppy mortality seen in only six cases.

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Abstract

EPIDEMIOLOGY OF CERTAIN BACTERIAL AND VIRAL DISEASES CAUSING NEONATAL MORTALITY IN PUPS

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ABSTRACT

The present study was conducted to assess the role of bacterial and certain viral etiology, to conduct antibiogram and to estimate the nonspecific factors associated with neonatal mortality in pups.The bacterial isolates from milk of bitches were identified as *Staphylococcus aureus* (29 per cent) *Staphylococcus intermedius* (six per cent), *Klebsiella pneumoniae* (47 per cent), *Proteus vulgaris* (12 per cent) *and Pseudomonas aeruginosa* (six per cent). Antibiogram of the isolates showed that majority of the isolates from milk were sensitive to ciprofloxacin (71 per cent), followed by amoxycillin (65per cent), gentamicin (41per cent), enrofloxacin (59 per cent), norfloxacin (18 per cent) chloramphenicol (12 per cent) and amoxy-clav (6 per cent).Blood samples taken from two bitches yielded bacterial growth, which were identified as *Staphylococcus aureus* (50 per cent) *and Klebsiella pneumoniae* (50 per cent). The bacterial isolates from blood were 100per cent sensitive to ciprofloxacin and chloramphenicol, 50 per cent to gentamicin, 50 per cent to amoxycillin and resistant to enrofloxacin and norfloxacin. The bacteria isolated from the vaginal samples were identified as Hafnia alvei (*Enterobacter alvei*)(six per cent), *Klebsiella pneumoniae* (35 per cent), *Staphylococcus aureus* (12 per cent), *Escherichia coli* (12 per cent), *Proteus vulgaris* (23 per cent), *Pseudomonas aeruginosa* (six per cent) and *Staphylococcus intermedius* (six per cent). The vaginal isolates were sensitive to ciprofloxacin (88 per cent), gentamicin (53 per cent), amoxycillin (59 per cent), enrofloxacin (71 per cent), chloramphenicol (59 per cent) and norfloxacin (35 per cent).

None of the milk, blood or vaginal swabs taken from the control group yielded any bacterial growth. Isolates were obtained from heart blood and from liver and lung of pups. These included *Proteus vulgaris*(20 per cent),*Pseudomonas aeruginosa*(40 per cent) and *Klebsiella pneumoniae*(40 per cent). Fourty per cent of the bacterial isolates from pups were sensitive to gentamicin and chloramphenicol, 80 per cent to ciprofloxacin, 60 per cent to amoxycillin and 20 per cent to norfloxacin and enrofloxacin.

The role of parvovirus as a causative agent for neonatal mortality was studied by subjecting the liver, lung,intestine spleen and kidney tissue of dead pups to AGID and HA for detection of parvoviral antigen and one sample was positive by HA. This was confirmed by haemagglutination inhibition test. None of the serum from the bitches gave positive result for antibodies against parvoviral antigen in agar gel immunodiffusion test.

The titre of antibodies of bitches were assessed by HI. HI titre of the vaccinated bitches ranged from eight to 64.Only one bitch was having the protective titre. All the unvaccinated bitches were not having any titre of antibodies against parvo by HI test.

Appendix

APPENDIX

PROFORMA

I. Date: Case No.: Hospital:

II. Owner's name and address:

III. Patient Data

Name: Breed: Age: Parity:

Date of crossing: Whether AI/Natural service:

Date of whelping: No. of litters:

No. of puppies died: Age of death (days):

IV. Previous history

A. 1. History of neonatal deaths

Parity Litter size No. of deaths Age at death

2. Laboratory tests performed if any

3. Treatment given if any

B. Vaccination history

Disease Date of last vaccination

- ☐ Rabies
- ☐ Leptospirosis
- ☐ ICH
- ☐ Parvo
- ☐ Distemper

C. History of previous illness

Disease Treatment given

D. History of reproductive problems

Disease	Treatment given

E. Mothering ability

V. Materials collected — Result

1. Blood for culture
2. Milk for culture
3. Serum for AGID for parvo
4. Tissue from dead pups for parvo antigen detection

VI. Any other relevant information