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A LONGITUDINAL STUDY OF SELECT BOVINE PATHOGENS AND OTHER FACTORS WHICH AFFECT CALF HEALTH ON 5 MICHIGAN DAIRY FARMS

By

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ABSTRACT

A LONGITUDINAL STUDY OF SELECT BOVINE PATHOGENS AND OTHER FACTORS WHICH AFFECT CALF HEALTH ON 5 MICHIGAN DAIRY FARMS

By

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The objectives of this study were to assess shedding patterns of select enteric and respiratory pathogens of 145 calves from 5 Michigan dairy farms and to assess shedding of attaching and effacing *E. coli*. Blood, fecal and tracheal swab specimens were obtained from calves beginning at less than 5 days of age (T1) and then at 2 week intervals up to weaning (T4). Enteric pathogens evaluated at each collection included *Salmonella spp*, *C. parvum*, rotavirus, attaching and effacing *E. coli* (AEEC), and enterotoxigenic *E. coli* (ETEC). *Mycoplasma spp.*, *Pasteurella spp.*, and bovine viral diarrhea virus were screened at each collection period. *Salmonella spp.* and *Manheimia haemolytica* were not isolated from calves. Bovine viral diarrhea virus and ETEC were isolated from 2.8% and 4% of calves, respectively. Seventy percent of calves were shedding *C. parvum* during T2. Rotavirus, AEEC, *Pasteurella multocida* and *Mycoplasma spp* were detected predominately at T4 (40%, 43%, 23% and 45%, respectively). *Mycoplasma spp.* and rotavirus were most often detected in calves carrying *P. multocida*. Vitamin A deficiency was significantly associated with the presence of *P. multocida* ($p<0.001$) and *Mycoplasma spp.* ($p<0.5$). Among AEEC, serogroups O26 and O118 were the most frequently typeable serogroups isolated. Antimicrobial susceptibility profiles among *E. coli* strains revealed resistance to ampicillin (32%), cefoxitin (2%), gentamicin (24%), tetracycline (72%), trimethoprim/sulfamethazole (8%) and ceftiofur (0.4%).

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INTRODUCTION

Neonatal diarrhea and enzootic pneumonia remain major disease complexes affecting young calves. Diarrhea and pneumonia are significantly associated with each other, at both the farm and calf level (Waltner-Toews *et al*, 1986). As co-diseases, diarrhea and pneumonia account for up to 80% of all calf mortality. In addition, treatment costs and decreased weight gain results in economic loss. Extensive studies have been performed to assess management practices associated with the occurrence of calf diarrhea and pneumonia (Curtis *et al*, 1988; Radostits, 1991; Frank and Kaneene, 1992; Sivula *et al*, 1996). Hartman, *et al* (1974) and Jenny, *et al* (1981) documented housing, feeding, weaning age and personnel caring for calves as some factors associated with increased mortality.

The influence of farm size on calf mortality has not been clarified, with farm size having been associated with both an increase and decrease in calf mortality (Oxender *et al*, 1973; Speicher and Hepp, 1973; Hartman *et al*, 1974; Jenny *et al*, 1981). Furthermore, calf mortality when associated with herd size, was shown to be significant during the winter period (Jenny, 1981). Waltner-Toews, *et al* (1986b) showed a relationship between age and the season at which calves were most susceptible to diarrhea and pneumonia. In their Ontario herds, calves at risk for disease were first treated for scours during the second week of life, with risk declining until no calves were treated by 6 weeks of age. Treatment for pneumonia peaked by the sixth week of life and pneumonia was more persistant than scours. They also showed that calves were at a greater risk of dying during the first week of life - with calf mortality not exceeding 4%, and treatment for scours and pneumonia was lower in the spring and summer than in

autumn and winter (<5% mortality). In a two year study, Waltner-Toews *et al* (1986a) looked at the association of management and morbidity in 104 dairy farms, and reported that of the 116 calves which developed diarrhea and pneumonia, 63.8% of the calves were treated for scours before pneumonia and 22.4% were treated for scours and pneumonia simultaneously. In their study, prophylactic treatment with antimicrobials were shown to have significantly increased the chance of calves developing scours and pneumonia. They concluded that there may be some type of pathophysiological mechanism involved in which calves with diarrhea (or treated for diarrhea) later develop pneumonia. The literature is replete with studies emphasizing management factors as major contributors to calf health and how the presence and treatment of an enteric disease may potentiate the occurrence of a respiratory disease. It is not known, however, how therapeutic intervention for one disease may exacerbate pathogen shedding and antimicrobial susceptibility of potential pathogens isolated from either the intestinal or respiratory system in dairy calves.

Recently, infected animals, food products and contaminated beef and dairy products have gained much attention as a source of hazardous pathogens for humans (West *et al*, 1988; Fone and Barker, 1994; Wall *et al*, 1994; Wall *et al*, 1995). Several pathogens, including various *Salmonella spp.* and *E. coli* strains, have been isolated from contaminated food products and shown to cause severe and often fatal disease in humans who consume these products (Riley *et al*, 1983; Ostroff *et al*, 1990; Belongia *et al*, 1991; Centers for Disease Control, 1991; Bell *et al*, 1994; Tarr, 1994; Whipp *et al*, 1994; Rodrigue *et al*, 1995; Keene *et al*, 1997). With many antimicrobials approved for cattle, (available as feed additives or over- the - counter medications), their use in the

treatment and control of specific diseases may be compromised. While the ease in availability and application of antimicrobials may be of benefit to the producer, potential hazards - such as the presence of antimicrobial residues in milk and meat products and the emergence of resistant strains of bacteria - have all been associated with the non-judicious use of antimicrobials (Holmberg *et al*, 1984; Levy, 1987). While much information is available on antimicrobial resistance and isolation of resistant microbes from cattle, little information is available on the shedding patterns of *E. coli* in calves and possible resistant strains shed due to antimicrobial therapy given to calves by producers.

The objectives of this study were to evaluate the association between the presence of select enteric and respiratory pathogens in calves and determinine if the presence of one pathogen may exacerbate the presence of another pathogen. Factors such as antimicrobial use, health and nutritional status of calves used in the study were also evaluated as they related to the presence of these pathogens. Second, we assessed shedding patterns of attaching and effacing *E. coli* among the calves, the predominant serogroups of *E. coli* isolates, and performed antimicrobial susceptibility profiles among isolated *E. coli* strains. Based on previous studies which show a relationship between the presence of infectious diarrhea and pneumonia in calves, it is hypothesized that a relationship exists between enteric and respiratory pathogens in pre-weaned dairy calves.

LITERATURE REVIEW

CALF IMMUNITY AND NUTRITION

An important aspect in calf disease prevention is the passive immunity that calves receive by maternal immunoglobulin transfer or colostrum. Early and adequate ingestion of colosrum containing large amounts of immunoglobulin is one of the most important factors influencing morbidity and mortality in neonatal calves (McGuire, *et al.*, 1976; Roy, 1980; Gay, 1983; Besser and Gay, 1985). Immunoglobulin G (IgG) is the isotype commonly used in diagnostic test as the reference immunogloblin in assessing failure of passive transfer (FPT) (Jenson, 1978; Gay and Besser, 1985). The major immunoglobulin in colostrum is IgG_1 (Opdebeeck, 1982), with IgM, IgA, and IgG_2 present, but in smaller amounts (Butler, 1973). IgG_1 is concentrated in the colostrum by a receptor mediated transfer from the maternal bloodstream, crossing the vascular endothelium, and binding to receptors on the membrane of mammary secretory epithelium (Butler, 1983). IgG_1 is taken up by micro-pinocytotic vesicles, crosses the epithelial cells, and is secreted into colostrum. The transfer of colostral immunoglobulins to the calf is a transient nonselective mechanism in which macromolecules cross the absorptive epithelium of the jejunum (Pierce, 1961; Selman, 1973; Bush and Staley, 1980). Calves will begin to suckle and ingest colostrum shortly after birth. The absorbed immunoglobulins then enters the calf's bloodstream via the thoracic duct. By 24 hours after birth, the absorptive cells in the small intestine close and immunoglobulin absorption ceases (Stott *et al*, 1979). At this time, serum immunoglobulin concentrations will be comparable to maternal concentrations, and therefore, effective in preventing

many systemic infections (Besser and Gay, 1985). Calves with serum IgG concentrations less than 800 mg/dl are considered to have failure of passive transfer and calves with greater than 1000 mg/dl have complete antibody transfer (Gay and Besser, 1985; Naylor, 1986).

Colostrum is also a valuable source of nutrients - such as vitamins, minerals, fat, and protein (Fishwick and Clifford, 1975; Swecker *et al*, 1995; Lacetera *et al*, 1996). In regards to vitamins, little reserves of vitamins A and E are present in the neonatal calf. To ensure adequate absorption of these vitamins, good quality colostrum should be ingested within 24 hours after birth. (Abdelrahman and Kincaid, 1995 Swecker *et al*, 1995; Lacetera *et al*, 1996; Blum *et al*, 1997).

Following intake, vitamin A in colostrum is in the form of a retinyl ester which is hydrolyzed to retinyl palmitate and absorbed by epithelial cells into the intestinal tract. Hydrolysis of retinyl esters occurs during digestion and retinol is formed. Retinol is transported to the intestinal absorptive surface, absorbed into enterocytes by a carrier mediated process (Hollander, 1981), transported to the liver and stored in an inactive form (Goodman, 1984a). Retinyl palmitate is secreted from the liver as the active form, holo-retinol binding protein (holo-RBP), transported, and bound to cell surfaces. Once bound, holo-RBP is then released into circulation as apo-RBP and taken up by cells (Goodman, 1984b). Retinoic acid and retinol bind to intracellular binding proteins which are transferred to binding sites on chromosomes. Vitamin A exerts its effect on cell proliferation and differentiation by binding to chromosomes (Eckert and Rorke, 1989).

Vitamin A is important in the maintenance of rapidly dividing cells, cells involved in immunity and decreasing the occurrence of disease (DeLuca and Wolf,

1972; Bondi and Sklan, 1984). Vitamin A deficient animals have impaired intestinal and respiratory immune systems (Smith and Church, 1971). An increase in cell death may lead to an increase in cell proliferation and, therefore, an increase in the utilization of vitamin A. Once cell death occurs, the normal epithelium is replaced with keratinized cells which are insufficient at secreting mucus. In calves with respiratory infections, the decrease in mucociliary clearance enhances the adherence of bacteria to the respiratory epithelium and leads to further damage of the host immune system.

Liver tisssue is an ideal means of assessing vitamin A status, however, serum retinol levels is a practical way to measure vitamin A status (Herdt and Stowe, 1991). Adequate supplementation of vitamin A, which is provided in milk and milk replacer, is a means of obtaining adequate stores of vitamin A. However in calves with an intestinal or respiratory disease, vitamin A may not be readily absorbed and supplementation using injectible compounds will benefit those calves unable to adequately absorb vitamin A.

In selenium deficient areas of the country (Allaway, 1972), supplementation of cows and calves with this mineral is essential. Selenium and/or vitamin E deficiency has been implicated in various calf diseases; including sudden death, neonatal weakness, and nutritional myodegeneration (Muth, 1963; Maas, 1983; Logan *et al*, 1990). Nutritional myodegeneration (white muscle disease) is a peracute disease involving skeletal and cardiac muscle and is common in rapid growing young farm animals whose dams were insufficiently supplemented with selenium during gestation. The disease is charachterized by skeletal and cardiac myonecrosis and my result in lameness or sudden death. By supplementing dam and calf with vitamin E and selenium, white muscle disease can be prevented (McMurray *et al*, 1977; Kennedy *et al*, 1987). Sufficient

amounts of selenium is transported across the placenta to the fetus, and in early postnatal period the selenium status of calves is influence by the selenium concentration in maternal colostrum and milk (Koller et al, 1984; Bostedt and Schramel, 1990).

Deficiency of selenium in calves has been related to a lack of colostral immunoglobulin intake or transfer (Koller *et al*, 1984; McDowell *et al*, 1990; Swecker *et al*, 1995). Calves born to cows supplemented with selenium and vitamin E during pregnancy, were more likely to have a storage pool of selenium and synthesize more glutathione peroxidase during intrauterine development (Lacetera *et al*, 1996). Furthermore, it has been suggested that selenium and vitamin E enhance the ability of immune cells to produce immunoglobulins at the mammary gland level (Swecker *et al*, 1995). Vitamin E functions as an antioxidant and a free radical scavenger. Based on a study in sheep (Hidiroglou *et al*, 1970), the main site of absorption of vitamin E was the jejunum and that pancreatic and bile secretions were needed for the absorption and transport of vitamin E Analysis of vitamin E (α- tocopherol) is performed by assessing the lipoprotein particles in the blood. The lipoprotein concentration is estimated by cholesterol, a lipoprotein constituent.

SELECT PATHOGENS IN CALF DIARRHEA

Infectious diarrhea of neonatal farm animals is one of the most common and economically devastating conditions encountered in the animal agriculture industry. The USDA estimates that 6.6% of preweaned dairy heifers die annually, with diarrhea being the leading cause of death (USDA - APHIS - VS, 1996). Extensive studies on neonatal

calf diarrhea have been performed to describe the herd management practices and risk factors associated with disease (Tennant *et al*, 1978; Heath, 1992; Frank and Kaneene, 1993; Quigley *et al,* 1994). In addition to the economic losses, some of the common calf diarrheal pathogens have increasingly been a major issue in public health. Numerous pathogens commonly associated with calf diarrhea are zoonotic and are also associated with foodborne diseases. Attempts by producers to control these infectious bacterial agents with antimicrobials therapy may lead to the occurrence of antibiotic resistance.

ESCHERICHIA COLI

Escherichia coli is a gram negative, facultative anaerobic, non-spore forming rod. The genus was named after Thiodor Escherich who first isolated the species in 1884. Its distinct chemical properties include lactose fermentation and hydrolysis of tryptophan to indole. *E. coli* strains are defined by their antigenic composition. There are over 170 serologic types (Ørskov and Ørskov, 1983) of polysaccharide antigens (O antigens), 50 flagellar antigens (H antigens) and 80 types of capsular antigens (K antigens). The O antigen is used for serogroup determination and is the foundation for serogroup and serotype designations. An example of this would be enterotoxigenic *E. coli* serogroups O8, O9, and O101 which are frequently isolated from calves and lambs with diarrhea. O and H or K antigens represent the O:H and O:K systems, respectively, and denote serotypes.

Various syndromes are seen with certain strains of *E. coli* due to their ability to express certain virulence attributes. In humans, there are five important diarrheagenic *E.*

coli types: enteroaggregative *E. coli* (EaggEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC). EHEC, EPEC, and ETEC are the strains seen in animals.

Epidemiologic investigations have shown that enterotoxigenic *E. coli* (ETEC) are common causative agent of diarrhea among calves. There are two virulence factors associated with ETEC; fimbrial antigens and production of enterotoxins. A surface protein, fimbrin, enables ETEC to colonize in the intestinal epithelium. *E. coli* express multiple and distinct fimbriae. Fimbrial antigens K99 and F41 are commonly seen on *E. coli* strains and belong to serogroups O9 and O101 (Gaastra and deGraaf, 1992). The interaction of fimbriae to specific intestinal epithelial crypt cells increases the exposure to heat stable enterotoxin (ST). This enterotoxin acts on the epithelial cells of the small intestine and activates cyclic GMP, leading to a secretory diarrhea (Ooms and Degryse, 1986). The ST enterotoxins are produced by all common ETEC strains in farm animals (Klaasen *et al*, 1990), and two type exist; STa and STb. The STa is the enterotoxin produced by the K99 ETEC strain isolated from calves. Futhermore, there are two distinct genes which code for STa production - *sta*H and *sta*P (Maini *et al*, 1985; Mainil *et al*, 1986). While the *sta*H gene is associated with ETEC causing diarrhea in humans, the *sta*P gene has been shown to be present in those ETEC strains which infect pigs, cattle, and humans (Mainil *et al*, 1985; Mainil *et al* 1986; Moon, Schneider, and Moseley, 1986).

Enterohemorrhagic *E. coli* (EHEC) has been shown to be associated with 3 human syndromes: hemorrhagic colitis, purpura hemorrhagica, and a hemolytic uremic syndrome (Karmali, 1989; Griffin and Tauxe, 1993). In 1977, Konowalchuk *et al*

reported an *E. coli* strain from a diarrheic human producing cytotoxins active on Vero cells. Since that time two distinct verotoxins (VT1 and VT2) have been described and shown to be similar to toxins produced by *Shigella dysenteriae* type 1 and 2 (O'Brien and Holmes, 1987). EHEC produce high levels of two cell associated cytotoxins producing shiga toxin genes (*STX* 1 and 2). *E. coli* possessing the shiga-toxins (STX) may also possess a gene highly homologous to enteropathogenic *E. coli* (EPEC) attaching and effacing genes (Francis, 1986; Tzipori, 1986; Moon, 1997). In calves, the ability to form an intimate attachment and cause the actively effacing lesion and the production of a 60 mDa plasmid which encodes a hemolysin is pathognomonic of AEEC pathogens.

EHEC have been isolated from cattle and associated with disease outbreaks in humans due to consumption of undercooked meat and milk products (Griffin and Tauxe, 1991; Tarr, 1994; Whipp, Rasmussen and Cray, 1994). Human and bovine EHEC serotypes isolated from outbreaks include O157:H7, O26:H11 and 0111:H- (Levine and Edelman, 1984; Mohammad *et al.*, 1986; Smith and Scotland, 1988; Willshaw *et al.*, 1993). In the U.S., the prevalence of *E. coli* O157:H7 in the feces of dairy calves is low (0.3 -22.2%) (Moon, 1997). In a study of dairy calves, Zhao *et al*, (1995) revealed that between 1.5 - 2.9% of calves between the ages of birth and weaning shed *E. coli* O157:H7 in their feces and between 4.9 - 5.3% of calves between the ages of weaning and 4 months shed *E. coli* O157:H7 in their feces.

EPEC causes diarrheal disease in humans and is increasingly being detected in association with *E. coli* strains producing attaching and effacing lesions in the intestine of many animal species (Law, 1994). The term attaching signifies the intimate association of bacteria to the enterocyte and effacing describes the localized destruction of brush border

mircovilli. The chromosomal gene, *eae*, is necessary for the attaching and effacement of associated epithelial cells. Those strains of EPEC which possess the *eae* gene and produce attaching and effacing lesions have been termed attaching and effacing *E. coli* (AEEC) (Moon, 1983).

EPEC has surface adhesins specific for receptors on the intestinal brush border membranes. Intimin is the outer membrane product that permits tight adherence of the bacteria to epithelial cells (Jerse and Kaper, 1991). A three stage model has been proposed as the mechanism by which AEEC cause these lesions (Donnenberg and Kaper, 1992). First, bacteria locally adhere to the epithelial cells via the production of fimbriae. Next, initiation of a signal transduction induce the activity of tyrosine kinase and thereby increasing intracellular calcium. This increase in calcium activates a villin protein which severs the actin cytoskeleton of the microvilli. The third stage of infection results when intimin, a 94kDa outer membrane protein, mediates close attachment to the epithelial cells. The intimin is encoded by the *eaeA* gene. The initial adherence allows cytoskeletal rearrangements and proliferation of filamentous actin beneath the attachment site and damage to the intestinal microvilli (Knutton, 1989). The damaged cells form a broad flat pedestal beneath the attached microorganism and damages the absorptive surface.

AEEC is commonly isolated from neonatal farm animals (Moxley and Francis, 1986; Janke *et al.*, 1989; Zhu *et al.*,1989; Holland, 1995) and has also been associated with EHEC (Holland, 1990; Janke *et al.*, 1990; Mainil *et al.*, 1993; Fisher *et al.*, 1994). However, not all AEEC from cattle produce the *stx* strain (Janke *et al*, 1990; Pearson *et al*, 1989), and strains of EPEC are capable of producing *stx* in various amounts (Neill *et al*, 1994). AEEC has been categorized into those which carry the *eae* and *stx* genes and

those which carry the *eae* gene only. Recent studies suggest that strains of *E. coli* which possess the *eae/stx* genes necessary to cause AE lesions may also be an important factor in development of calf diarrhea (Dorn *et al*, 1993; Mainil *et al*, 1993). Diagnosis of *E. coli* strains is determined on the basis of biochemical and nutritional properties. Several commercial products are available which detect specific toxins or agglutination of specific antiserum produced by *E. coli* strains.

ROTAVIRUS

In 1969, Mebus, *et al* demonstrated the importance of rotavirus as a cause of neonatal calf diarrhea. Others have given the same virus various names: neonatal calf diarrhèa virus, Nebraska calf diarrhea virus, neonatal calf diarrhea reovirus like, and orbivirus (Middleton, 1974; Mebus, 1977; Derbyshire, 1978; Woode, 1978). In 1974, Flewett, *et al* proposed the name rotavirus. Belonging to the Reoviridae Family, rotavirus is a double stranded RNA virus consisting of eleven gene segments enclosed in a double shelled protein capsid (Beards, 1980; Kapikian, 1990). The outer capsid of rotavirus consist of two proteins; viral protein 7 (VP7), a 37-kDa glycoprotein, and VP4, a glycoprotein with a molecular mass of 86 kDa (Estes and Cohn, 1989a). Genetic recombination between gene segments occurs naturally and can be reproduced in vitro (Dodet *et al*, 1997). The classification of Group A rotavirus serotypes is based on the specificity of the VP7 antigen, G serotype, and to a lesser extent, the VP4 antigen, or P serotype (Green, 1987; Estes and Cohn, 1989; Estes and Tanaka, 1989; Matsuda, 1990; Snodgrass, 1992). Both of these proteins have been shown to elicit neutralizing antibodies during an immune response to rotavirus infection (Liu, 1988; Offit, 1986).

Rotavirus has recently been divided into seven groups, A-G, with Group A being the most clinically important and most often associated with diarrhea among infants and young farm animals (Snodgrass,1986; Theil, 1990). The other rotavirus groups - termed nongroup A - have been shown to cause diarrhea in humans and animals, but their occurrence is less common (Bridger, 1985; Brown, 1987). Nongroup A rotavirus, Group B and C, are two emerging forms that appear in humans and has been associated with diarrhea in adults and older children (Parwaini, 1996; Chang, 1997) . In calves, nongroup A rotavirus has been shown to be associated with sporadic cases of diarrhea (Chasey, 1984). The Group B virus is difficult to characterize due to its inability to grow in cell culture and its low numbers shed in feces.

Rotavirus has been found in the feces of healthy calves and in calves with clinical disease of varying severity (Bridger, 1975; McNulty, 1976; DeLeeuw, 1980). The variation in clinical disease may depend on differences in virulence among rotavirus strains, age of animal (Dodet *et al*, 1997), host immune status, dose of inoculum, occurrence of mixed infection, environmental stresses and nutrition (McNulty, 1983; Radostits, 1983). Rotavirus often causes severe disease in calves less than two weeks of age. The virus enters the intestinal tract by fecal-oral contamination and selectively infects mature villous absorptive cells. Replication occurs in the cytoplasm of these mature enterocytes and there is loss of enterocytes with replacement by immature undifferentiated cells. Although asoociated with a high morbidity, most infections are self limiting.

Colostrum may offer some protection against rotavirus infection in calves since colostrum may contain anti-rotavirus antibodies. These anti-rotavirus antibodies may

neutralize rotavirus particles present in the gut of calves during the first days of life (Tsunemitsu, 1989). Calves that have received colostrum containing these antibodies, will have anti-rotavirus antibody titers in their serum once colostral immunity has declined. These antibodies may also provide some protection against disease.

There are several diagnostic methods available to detect rotavirus in feces, including electron microscopy, enzyme linked immunosorbent assay (ELISA), radioimmunoassay and latex agglutination. All produce comparable results and only slightly vary in sensitivity and specificity (Kok and Burrell, 1989).

CRYPTOSPORIDIUM PARVUM

Recognized over 90 years ago and belonging to the Suborder Eimeriina, *Cryptosporidium spp.* is a protozoan parasite. In 1912, E. E. Tyzzer named and described the morphology and life cycle of *Cryptosporidium parvum.* It wasn't until 1971 that *C. parvum* was identified as a cause of bovine diarrhea (Panciera, 1971). Since then, *C. parvum* has been associated with diarrhea and gastroenteritis in other animals and humans (Angus, 1983; Tzipori, 1983; Casemore, 1985; Wolfson, 1985; Fayer, 1986). Oocyst of *C. parvum* are 4-5 um in diameter and are resistant in the environment. The lack of host species specificity supports the fact that this parasite is capable of cross infection among mammalian species (Fayer, 1986; Moon, 1986).

In animals, *C. parvum* infection may occur as a nonclinical, carrier state or as a clinical state characterized by profuse diarrhea. The life cycle for *C. parvum* involves ingestion of sporulated oocyst by the host and release of the sporozoite in the intestine to

infect the microvillus brush border (Navin, 1984; Fayer, 1986). The sporozoites become incorporated into the microvillus membrane and develops into trophozoites which undergo schizogony. Development of Type I and Type II meronts occurs. Type I meronts divide into 6-8 merozoites and invade uninfected cells and can further differentiate into Type I and Type II meronts. Type II meronts, which contain 4 merozoites, undergo gametogony and release more Type II meronts. Type II meronts develop into microgametocytes and macrogametocytes and thus fertilize each other and produce zygotes. Zygotes develop into thin and thick walled oocysts which each have 4 sporozoites. The thick wall oocyst (infective) passes out in the feces and the thin walled oocyst rupture within the host and release sporozoites to invade uninfected cells and continue the cycle. Transmission of *C. parvum* is by fecal-oral ingestion and the incubation period is for 2-7 days. Numerous methods for the detection of *C. parvum* oocysts in feces have been published. Various traditional detection methods employ concentration of oocysts in hypertonic sugar or salt solutions followed by microscopic examination (McNabb, 1985; Baron, 1989; Current, 1990; MacPherson, 1993) or staining of fecal smears with special stains (Garcia, 1983; Casemore, 1985). Commercial antigen detection methods such as immunofluorescence on ELISA are also used in human clinical laboratories (Garcia, 1983; Ungar, 1990; Newman, 1993; Parisi, 1995).

SALMONELLA SPP.

Salmonellae are flagellated gram negative facultative anaerobic bacillus which are capable of causing disease in adult and young cattle. There are over 2200 serotypes, however the majority of cattle isolates are *S. typhimurium* and *S. dublin*. Most

Salmonella strains found in cattle lack host specificity. In humans, *S. dublin* infection has been linked to consumption of dairy and beef products. There has been increasing concern in regards to the multi-antibotic resistant *S. typhimurium* definitive phage type (DT) 104 and DT204c found in cattle and humans. Both DT104 and DT204c have gained much attention in the United Kingdom (Threlfall *et al*, 1986; Wray *et al*, 1993; Low *et al*, 1996), and recently in the United States (Besser *et al*, 1998).

In 1993, DT104 accounted for most of the salmonellosis cases reported in cattle from Europe (Threlfall *et al*, 1994). Furthermore, previous reports have stated that individual adult cattle may be symptomless carriers of *S. typhimurium* DT104 (Sharp and Rawson, 1992; Penny *et al*, 1996). The strains are resistant to ampicillin, chloramphenicol, streptomycin, sulphonamides, tetracyclines, and more recently trimethoprim (Anon, 1996; Threlfall *et al,* 1996). Human infections have been associated with the consumption of chicken, beef, and pork (Walls *et al*, 1994), contact with other farm animals (Fone and Barker, 1994; Walls *et al*, 1995), and domestic pets (Walls *et al*, 1996). In the United Kingdom, the prevalence of *S. typhimurium* DT104 resistant types has increased from 7% in 1990 to 33% in 1996 (Threlfall *et al*, 1997). There is increasing concern over *S. typhimurium* DT104 resistance to fluoroquinolones (Threlfall *et al*, 1997) since its licensing for use in livestock.

S. typhimurium DT204c infection appeared in cattle in Britain in 1979, and by 1985, 59% of *S. typhimurium* from cattle and 4% of strains from humans were of this phage type (Hinton *et al*, 1984; McLaren and Wray, 1991; Wray *et al*, 1993).DT204c can persist in the environment an average of 14 months (McLaren and Wray, 1991) and is one of the types derived from a progenitor strain of DT204 resistant to sulphonamides

and tetracyclines (SuT). Initially, this phage type was resistant to at least 3 antimicrobials, but has become more resistant in recent years (Wray *et al*, 1993; Threlfall *et al*, 1985; Threlfall *et al*, 1983). All strains of this type have shown resistance to at least four antimicrobial drugs; with the most common resistance pattern of that of chloramphenicol, streptomycin, sulphonamides, tetracyclines and trimethoprim (CSSuTTm). However, since 1981, strains have been additionally resistant to ampicillin and neomycin-kanamycin. Gentamicin resistance has also emerged and in 1985, 25% of DT204c from cattle and 12% of DT204c from humans were gentamicin resistant. At least three resistant plasmids have been identified in DT204c CSSuTTm; an *Inc*H_2 plasmid of 120 Md coding for CSSuTTm, an unidentified plasmid of 63 Md coding for tetracyline resistance and a plasmid of 4.2 Md coding for sulphonamide resistance (Threlfall *et al*, 1986). Antibiotic resistant bacteria can occur naturally in the environment, or may also occur by contamination with human and animal waste (in water or environment) which can potentiate zoonotic spread (Linton, 1986).

Three forms of Salmonella infections exist in cattle: peracute (septicemic), acute (enteric), and chronic. The peracute form causes few clinical signs and death in calves occurs within hours. Diarrhea may or may not be present. The acute enteric infection, characterized by diarrhea, is the most common seen form in cattle and *S. typhimurium* and *S. dublin* are most often incriminated as the cause of diarrhea. In *S. dublin* infection, septicemia and sudden death are often observed. Other clinical signs include arthritis and respiratory symptoms. *S. typhimurium* causes acute septicemia and enteric lesions with a secondary bacteremia. In older animals, intermittent diarrhea characterizes chronic salmonellosis. The prevalence of asymptomatic salmonellosis in calves ranges

from 1 to 36%, and susceptibility to becoming infected is increased by malnutrition, overcrowding, and shipping. Peak mortality due to salmonellosis in calves occurs at 3-4 weeks of age, however, in a severely contaminated environment, calves can become infected within 72 hours after birth. Diagnosis is by isolation of bacteria in feces using enrichment broths and then subculturing onto selective media plates. Enzyme linked immunosorbent assay, polymerase chain reaction and slide agglutination techniques are also available.

SELECT PATHOGENS IN CALF PNEUMONIA

Over 40% of all episodes of cattle disease in the United States involve the respiratory system (Lillie, 1974). Enzootic pneumonia, or pneumonia of dairy calves, can be seen as an endemic disease and also as a major cause of a respiratory disease outbreak (Sivula *et al*, 1996; Baker *et al*, 1986). Calves can become colonized by respiratory pathogens which are endemic to their population shortly after birth. This makes inadequate ventilation an important environmental factor in influencing respiratory disease (Roe, 1982); however, good ventilation does not prevent colonization. Calf pneumonia has commonly been associated with housed dairy animals (Bryson, 1985).

In Michigan, it was estimated that respiratory disease in calves cost the dairy producer $14.71 per calf/year (Kaneene and Hurd, 1990). Calves which have experienced respiratory disease, have been shown to be at an increased risk of being culled when compared with herdmates of the same age (Curtis *et al*, 1989). Many viruses, bacteria, and mycoplasmas have been recovered from the respiratory tract of

diseased calves (Bryson, 1985). However, bacteria do not generally serve as primary pathogens of respiratory disease in healthy, unstressed cattle (Bryson *et al*, 1978; Bryson, 1985). A fever, nasal discharge, cough, poor weight gain, and increased respiratory sounds are common clinical signs seen. Environmental factors, such as poor ventilation in enclosed facilities, calf stresses, and presence of various etiologic agents may also play a crucial role in the development of disease.

PASTEURELLA SPP.

In young dairy calves, *Pasteurella multocida* serogroup A is the bacterium frequently isolated in respiratory diseases (Confer, 1993), especially in housed animals. *Pasteurella spp.* are gram negative coccobacilli which cause a fulminating respiratory disease. Large numbers of the organism are required to initiate infection (Ames *et al*, 1985). *P. multocida* has been documented to occur more often in housed dairy calves than *Manheimia haemolytica* (Frank, 1989). The initial adhesion and colonization of *P. multocida* to the epithelium of the respiratory tract releases lipopolysaccharide (Brigham and Meyrick, 1986), a major component of *P. multocida*. This component produces a fibrinopurulent inflammation in the lower respiratory tract and aggregation of various blood cells within the pulmonary capillaries occurs (Whiteley *et al,* 1992.). The endothelium undergoes toxic changes with an increase in macrophages and generation of inflammatory mediators.

The characteristic lesions of *Manheimia haemolytica*, the most virulent of respiratory bacterial pathogen, is an acute to subacute anterior ventral pleuropneumonia

with fibrin exudation, an influx of neutrophils and macrophages, and necrotic foci surrounded by bacteria (Frank, 1989). Adherence of *M. haemolytica* to endothelium may be due to deficiencies in the mucociliary clearance or an enhanced adhesion of organisms to the mucosa (Whiteley, 1992). *M. haemolytica* also produces neuroaminidase, neutral protease, and other outer membrane proteins (Frank, 1989) which can alter mucus and mucociliary clearance and enhance bacterial adherence to the epithelium. One primarily important virulent factor of *M. haemolytica* are leukotoxins (LKT). LKT act by forming pores in the plasma membrane of target cells and causing degeneration and lysis of bovine neutrophils and macrophages. Both *M. haemolytica* and *P. multocida* have been shown to produce virulent properties which cause resistance to phagocytosis and therefore prevents bacteriolysis (Confer *et al*, 1990; Czuprynski and Sample, 1990).

MYCOPLASMA SPP.

Mycoplasma spp. have been recovered from the upper and lower respiratory tract of calves in early and late stages of pneumonia outbreaks (Howard, 1983; Gourlay *et al*, 1989). Although the mechanism is not understood, synergism between *Mycoplasma bovis* and *M. haemolytica* has been demonstrated (Gourlay and Houghton, 1985; Virtala *et al*, 1996). Mycoplasmal organisms are the smallest procaryote capable of self replication. *Mycoplasma* was first isolated from cattle with contagious bovine pneumonia, thus the name pleuropneumonia organism (PPO). Respiratory mycoplasmosis occurs as a bronchitis, bronchiolitis and a bronchopneumonia (Rodriguez *et al*, 1996).

Mycoplasma spp. adhere to the host mucosa and this close contact with the cell membrane may lead to an immune response that cause damage to the host cells. Several species of mycoplasma have been isolated from the lungs of cattle (Howard, 1983) but only a few have been associated with primary or secondary infection. This organism does have immunosuppressive potential and may play a role in predisposing animals to respiratory disease (Gourlay and Houghton, 1985; Gourlay, Thomas, and Wyld, 1989; Virtala *et al*, 1996). Those mycoplasmal organisms considered pathogenic include *M. bovis*, *M. dispar*, *M. bovirhinis*, and *Ureaplasma* (Welsh, 1993; Walker, 1995).

Recently, *M. bovis* infection has been associated with otitis media in calves (DeChant and Donovan, 1996; Walz *et al*, 1997). Clinical signs associated with this infection include head tilt, ear droop, and epiphora. Pathogenesis of otitis media caused by *M. bovis* is still undetermined. Three sources of entry are possible; entry from extension of otitis external, extension from the auditory tube, or from a bacteremia. It has been reported (Bennett and Jasper, 1977) that ingestion of colostrum or milk from cows subclinically or clinically infected with a *M. bovis* mastitis increases the prevalance for isolation *M. bovis* in the nares of calves receiving the contaminated milk. *M. bovis* from contaminated milk or feces can colonize the nasopharnyx and infection may extend into the auditory tube. Other factors, such as contaminated feeds, may also play a role in the development of otitis media.

BOVINE VIRAL DIARRHEA VIRUS

Bovine viral diarrhea virus (BVDV) is recognized as a contributor to multiple disease processes (Greig *et al.*, 1981; Richer *et al.*, 1988) which includes bovine viral diarrhea, mucosal disease, congenital defects in calves, and reproductive failure. BVDV is a member of the Pestivirus genus in the Flaviviridae family. It is a single stranded RNA virus and considered very mutable (Dubovi, 1990). There are two recognized biotypes of the virus; a noncytopathic type, which replicates in cell culture but does not destroy the cells, and the cytopathic type, which causes destruction of cells in culture (Ames, 1986; Bolin, 1992). Most field isolates (95%) of BVDV are noncytopathic (Ernst, Baird and Butler, 1983) and cytopathic strains of BVDV originate from mutations of noncytopathic isolates. At least two BVDV genotypes, unrelated to the biotype, exist.

Transmission of BVDV is by direct contact with a carrier animal (Roeder and Drew, 1984). Inhalation, nasal secretion, oral ingestion, urine, feces, semen and uterine secretions are all possible ways of spreading the virus (Dufell and Harkness, 1985). Transplacental infection occurs when the fetus is infected by an infected dam (Malmquist, 1968; Kendrick, 1971; Done and Terlecki, 1980). These carrier cows may remain healthy, successfully breed and produce healthy appearing offspring, however, these offsprings are persistently viremic carriers of the virus (Littlejohns, 1982; Binkhorst *et al*, 1983; Straver, Journee and Binkhorst, 1983).

A nonimmune pregnant cow may become infected with BVDV and can therefore affect early fetal development and lead to infections causing fetal death, malformation, or birth of persistently infected immunotolerant calves (Baker, 1987; Radostits and Littlejohns, 1988). Noncytopathic BVDV infection can occur in the fetus of a BVDV

infected dam and may result in calves born immunotolerant and persistently infected with BVDV. The primary postnatal infection may result in a subclinical infection (Bolin and Ridpath, 1992) or show severe disease with high mortality (Pellerin *et al*, 1994). Persistently infected calves are viremic and immunotolerant to BVDV. Immunotolerant calves, persistently infected with the noncytopathic BVDV, develop mucosal disease when they acquire the cytopathic virus which is antigenically similar. In nature, most BVDV isolates are noncytopathic and animals with mucosal disease may be the only natural source of cytopathic isolates (Potgieter, 1997). One report indicates that BVDV was the virus seen most often isolated from the lungs of pneumonic cattle (Reggiardo, 1979). There is evidence that BVDV may exacerbate neonatal diarrhea or be seen concurrently with other infectious agents (Bohac and Yates, 1980; Van Opdenbosch, Wellemans and Oudewater, 1981; Ames, 1986; Moerman *et al*, 1994).

MATERIALS/METHODS

SAMPLE POPULATION/FARM MANAGEMENT

A total of 145 Holstein calves from 5 Michigan dairy farms were used in this study. For participation in the study, calves were randomly selected and farms agreed to make all records pertaining to the calves available to us. Each farm, represented as A, B, C, D, or E, was evaluated for the following - calf housing, cow/calf vaccination, calf feeding, and antibiotic use in calves. Calves on Farm A (n=30) were raised indoors in elevated cages in a temperature controlled barn. The cages were constructed so that urine and feces dropped to the floor, therefore calves did not have direct contact with either. Ventilation of the barn was by forced air through plastic tunnels located directly over the calf cages. The barn was cleaned at least once a day. Calves on Farm B (n=29) were raised in outdoor straw bedded hutches and on straw in hutches under a three sided barn. Calves on Farm C (n=27) were raised in outdoor hutches on straw. Calves on Farm D (n=28) were raised on straw in a four sided wooden covered hutch. Calves were in an indoor facility which utilized wall fans for air movement. Calves on Farm E (n=31) had a combination of living conditions - outdoor hutches, calves tethered indoor on hay and sawdust, and calves in a three-sided barn tethered on sawdust.

Type of calf feed and milk replacers are listed in **Appendix A**. Neonatal calves on Farm A receive vitamin A,D,E, and selenium supplementations, Endovac-Bovi®, and Bovishield 4® within the first week of life; calves on Farm B were supplemented with vitamin A,D,E, and selenium injections and a clostridium C and D antitoxin. Vitamin A,

D, E, and selenium were supplemented to newborn calves on Farm C, as well as a Clostridium C and D antitoxin (Bio-Ceutic) and Impro (a whey blend). Calves on Farm D received vitamins A,D, E and selenium supplementations and an IBR-PI3 vaccine (TSV-2®) within the first week of life, and calves on Farm E receive selenium supplementation, a Clostridium C and D antitoxin, and First Defense™ (Coronavirus-E. coli Antibody) vaccine. Cow vaccinations from each farm are given in **Appendix B.**

Heart girths, an estimated of weight, was used to assess growth of each calf. Measurements were recorded in inches by use of a Nasco weight tape (Nasco, Fort Atkinson, WI), then converted and recorded in centimeters at each sample collection (**Appendix C**).

SPECIMEN COLLECTION

Feces and tracheal swab samples were obtained from each calf selected for study. The first samples were obtained during the first week of life (WK0); with additional samples obtained at 2 week intervals with the fourth collection (WK6) taken at/or just prior to weaning. Samples were collected between February 11, 1997 and January 24, 1998. Feces from calves at each sampling time were evaluated for the following intestinal pathogens – *C. parvum*, rotavirus, *Salmonella spp.* AEEC and ETEC.. Fecal samples were obtained by digital extraction of feces from the rectum, placed in sterile collection cups and transported on ice packs in Styrofoam containers to the laboratory. Fecal samples were immediately cultured for isolation of *Salmonella* and *E. coli.*

Calves were also evaluated for the following respiratory pathogens – *Mycoplasma spp.*, *Manheimia haemolytica* and *Pasteurella multocida*. Tracheal swabs were obtained by manual extension of the calf's tongue and use of a metal tongue depressor to visualize the epiglottis. Once the epiglottis was visualized, a sterile guarded culturette was inserted through the epiglottis and into the trachea. The culturette was manually pushed through the guard and the trachea was swabbed for sample collection and then pulled back into guarded sheath for removal of the culturette from the trachea. Trachea swabs were placed in 0.5 milliliters (ml) of modified Stuart's bacterial transport medium (Culturette, Becton Dickinson Microbiology Systems, Cockeysville, MD) and the swabs were placed on ice packs in a styrofoam cooler and returned to the laboratory for immediate culturing.

ISOLATION OF INTESTINAL PATHOGENS

E. coli Isolation

Colony Selection

For isolation of *E. coli*, 1 gram of feces was vigorously mixed by vortexing in 9 ml of peptone saline. An inoculating loop was used to transfer 0.01 ml of fecal suspension onto MacConkey agar (MAC) plates and the MAC plates were streaked for isolation. Following overnight incubation at 37°C, ten lactose fermenting colonies (LFC) possessing *E. coli* characteristics were randomly selected and harvested with sterile toothpicks from each MAC plate. A portion of each LFC colony from each calf was also placed in individual 500 microliter (µl) microcentrifuge tubes (Elkay Products,

Inc., Shrewsbury, MA) containing 100 µl of lysis buffer (1.0% Triton X-100, 20mM Tris HCl, 2.0 mM EDTA, pH 8.5), vortexed, and heated at 100°C for 3 minutes. The lysates were centrifuged at 12,000 X g for 3 minutes and stored at -20°C for PCR analysis.

Hemolytic Activity

Hemolytic activity of LFC was assessed after overnight growth at 37°C on columbia agar plates supplemented with 5% defibrinated sheep blood, 1% horse serum and 1% yeast extract (EBA plates). Colonies surrounded by a clear halo were defined as exhibiting hemolytic activity. All LFC were also streaked onto trypticase soy agar (TSA) slants for storage.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) was used to detect the genes encoding heat-stable enterotoxin type A(sta), shiga toxins (stx) and the attaching and effacing gene (eaeA). Amplification of total DNA was performed using 4 µl of bacterial DNA in 43 µl of PCR mixture. The PCR mixture contained 30 µl of double distilled water, 5 µl 10X PCR buffer, 2 mM MgCl2, 10mM dNTP mix, 1.0 µM of each primer, and 1.5 U of Taq DNA polymerase (Gibco BRL, Life Technologies, Inc., Grand Island, NY).

The oligonucleotide sequences AE 13 (5'GTG GCG AAT ACT GGC GAG ACT 3') and AE 14 (5'CCC CAT TCT TTT TCA CCG TCG 3') were selected for

amplification of the *eae* gene (Gannon *et al.*, 1993). The expected size of the amplified DNA product was 890-bp.

For amplification of *stx*1 and *stx*2 genes, the MK1 (5′ TTT ACG ATA GAC TTC TCG AC 3′) and MK2 (5′CAC ATA TAA ATT ATT TCG CTC 3′) oligonucleotide primers were used (Karch and Meyer, 1989; Schmidt *et al.*, 1993). The MK1 and MK2 primer pair amplified a 230 bp fragment for both *stx1* and *stx2* loci (Karch and Meyer, 1989).

Each characteristic colony was also screened for the *sta* gene using published oligonucleotide sequences (5′ CC GTC AAA CAA CAT GAC GG 3′) and (5′ATT ACA TCC AGC ACA GGC AG 3′) (Ojeniyi *et al.*, 1994). A 244 bp fragment was amplified.

Amplifications of *eaeA, stx* and *sta* were performed utilizing a PCR thermal cycler (PTC-100, MJ Research, Inc., Watertown, MA) and amplification was performed using the following cycles for 30 cycles - 94°C for 1 minute allowed denaturing, 1 minute at 57°C for annealing (43°C with *sta*), and 1 minute at 72°C for extension. A total of 15 µl of amplified product was analyzed by gel electrophoresis using 1.5% agarose. DNA products were visualized by ethidium bromide (Sigma Chemical, St. Louis, MO) staining and ultraviolet illumination. A 100 base pair (bp) DNA ladder was used as the molecular weight marker (Gibco BRL, Grand Island, NY). For each *eae* and *stx* PCR assay, DNA from EHEC O157:H7 and a calf strain of AEEC – serotype O5:NM – were used as positive controls and came from a strain of *E. coli* and *Salmonella dublin* that lacked *eae, stx*, and *sta* genes were use as negative controls. For each *sta* PCR assay, DNA from bovine ETEC B41 (O101:NM) was used as the positive control.

Premier EHEC Assay

Positive *E. coli stx* samples were further confirmed using the Premier EHEC enzyme immunoassay test (Meridian Diagnostics, Inc., Cincinnati, OH). Strains possessing stx genes were screened for shiga toxin production by using the Premier EHEC assay. Four *eae* only and 47 *stx* only strains were examined by the enzyme immunoassay. After 12 h growth at 37°C on MAC plates, a single conlony of each strain was suspended in 200 µL of sample diluent for testing. The immunoassay was performed according to the manufacturer's recommendation. The reaction mixture was visually read within 15 minutes of the last solution added.

Serogroup Determinations

The presence of O antigens was determined by standard methods used at the *E. coli* Reference Center, Pennsylvania State University (Wilson and Francis, 1986). Prior to testing, *E. coli* strains were grown overnight in tryptic soy broth. Bacterial growth, in suspension, was heated to 100°C for 2 hours. Agglutinations were performed by using preselected dilutions of each of 183 O group antisera in microtitre plates and the positive reactions were confirmed by microtitre titrations against monovalent antisera (Glantz, 1971). If there was no agglutination in the preliminary screening assay, the bacterial suspension was reheated at 121°C for an additional 1 hour and the microtitre agglutination assays were repeated.

Enterohemolysin Activity

Of the *E. coli* strains possessing *eae* and or *stx* genes submitted to The *E. coli* Reference Center, 106 were randomly selected and assessed for enterohemolysin activity on 5% defibrinated washed sheep blood agar plates as described (Beutin *et al*, 1988). Colonies surrounded by clear zones of hemolysis were defined as exhibiting enterohemolysin activity. Additional antimicrobial sensitivity using the standard disk diffusion on Mueller-Hinton agar was also performed on these samples. The disks contained the following amounts of antibiotics: amikacin (30 μg), cephalothin (30 μg), clindamycin (30 μg), enrofloxacin (5 μg), nalidixic acid (30 μg), neomycin (30 μg) and spectinomycin (100 μg).

Cryptosporidium parvum Isolation

For *C. parvum*, one gram of feces was homogenized in 10 ml double distilled water (DDW). The fecal suspension was sieved through 4 layers of cheesecloth to remove large particulate material. Following filteration, the cheesecloth was rinsed with DDW. To remove fat soluble components from the fecal suspension, an equal volume of diethyl ether was vigorously mixed in the fecal suspension for 30 seconds. The ether/fecal suspension was centrifuged for 10 minutes at 1100 X g. The supernatant was discarded and 7 ml of DDW was added to the pellet. The pellet was mixed by vortexing and washed for 10 minutes at 1100 X g. The supernatant was carefully removed and 15 ml of Sheather's sugar solution were added to the pellet and the suspension was vortexed, then centrifuged at 1100 X g for 10 minutes. After centrifugation sufficient Sheather's

sugar solution (1 liter distilled water, 50g sugar, 1g phenol) was added to form a meniscus at the top of the centrifuge tube. A coverslip was placed on the meniscus. The coverslip was kept in place for 5 minutes and then carefully removed and placed onto a glass slide for detection of oocysts by illumination of 40X microscopy.

Salmonella spp. Isolation

For isolation of *Salmonella spp.* , 1 gram of feces was inoculated into a sterile tube containing 5 ml of selenite broth and allowed to incubate at 37°C for 18 -24 hours. Following incubation, a loop of the inoculated selenite broth was streaked onto Hektoen enteric agar plates and incubated for 24 hours at 37°C. Those colonies possessing a characteristic black centered colony were further evaluated by streaking colony onto MAC plates to ensure that they were non-lactose fermenting colonies. Those which possessed non-lactose fermenting colonies were definitively identified using the Analytical Profile Index (API 20E) System. The API 20E (bioMérieux Vitek, Inc., Hazelwood, MO) system is a conventional procedure for the identification of Enterobacteriaceae and other gram - negative bacteria (Washington, 1971). Twenty-three standard biochemical tests are contained in microtubules on a plastic strip. The bacteria to be tested, was mixed with sterile saline, and the suspension was deposited into each microtubule and the microtubules incubated for 24 hours at 37°C. Following overnight incubation, each microtubule was visually read and determined to be positive or negative for that organism based on reactions tested.

Rotavirus Antigen Identification

Group A rotavirus antigen was detected in feces using a direct enzyme immunoassay (Pathfinder – Kallestad, Austin, TX). Thirty microliters of fecal sample was placed in a plastic tube containing rabbit anti-rotavirus IgG (polyclonal antibody). A horseradish peroxidase - conjugated monoclonal antibody to rotavirus (murine monoclonal antibody) was then added to tube and incubated for 1 hour at room temperature. The fecal sample mixture contained in the inoculated tube was aspirated off and the tube washed 5-7 times with DDW. A working reagent, consisting of a peroxidase substrate (citrate buffer, hydrogen peroxide, and pH 4.2) and chromogen (tetramethybenzidene in hydrogen chloride), was then added to the tube and the tube was visually compared to control samples. A blue color change indicated a positive test for rotavirus and no color change indicated a negative reaction.

ISOLATION OF SELECT RESPIRATORY PATHOGENS

Pasteurella spp. Isolation

Presence of *Pasteurella spp.* was determined by inoculating EBA plates with the trachea culture swab obtained from calf. Following a 24 hour incubation of EBA plates at 37°C, plates were evaluated for the presence of characteristic *Pasteurella* colonies. Those colonies considered suspect colonies were identified by Gram stain, reaction to the oxidase Spot Test and the catalase test. The Gram negative, oxidase and catalase positive colonies were further identified using the Analytical Profile Index (API 20 E) System. For a definitive diagnosis of *Pasteurella multocida,* positive reactions using this system

were as follows: indole, sorbitol, sucrose, and mannitol. *Pasteurella* isolates were stored in 3 ml skim milk at -70°C.

Mycoplasma spp. Isolation

For *Mycoplasma spp.* detection, pleuropneumonia – like organism (PPLO) plates (Remel, Lenexa, KS) were inoculated with trachea swabs obtained from each calf and were incubated in a 37°C humidified CO_2 chamber and assessed daily (14 days microscopically) for the presence of microcolonies with a 'fried egg' appearance. Any samples exhibiting signs consistent with the control and having a 'fried egg' appearance were placed in 3 ml of skim milk and stored at –70°C. Select samples diagnosed as a *Mycoplasma spp.*, were sent to Pharmacia UpJohn for antimicrobial sensitivity to the following antibiotics: erythromycin, spectinomycin, lincomycin, premafloxacin, tilmicosin, tetracycline, and lincomycin/ spectinomycin (1:4) combination.

ANTIMICROBIAL SUSCEPTIBILITY

E. coli and *Pasteurella spp.* isolates were further evaluated for antimicrobial sensitivity using standard disk diffusion on Mueller-Hinton agar (Bauer *et al.*, 1966). Disks containing the following amounts of antibiotics were used - ampicillin (10 μg), penicillin G (10 units), gentamicin (10 μg), ceftiofur (30 μg) cefoxitin (30 μg), enrofloxacin (10 μg), trimethoprim/sulfamethoxazole (1.25 μg/23.75 μg), and tetracycline (30 μg). To make a culture suspension, three to five *E. coli* positive colonies from the stored subculture were transferred, using sterile technique, to a test tube

containing 5 ml tryptic soy broth medium. The culture suspension was allowed to incubate for 2-6 hours at 37°C or until the suspension turbidity was comparable to that of a 0.5 McFarland Standard. A sterile cotton swab on a wooden applicator was dipped into the standardized culture suspension and excess fluid was expressed from the cotton swab by rotating the swab firmly against the inside wall of the test tube.

For *Pasteurella spp.*, stored isolates were thawed and then streaked directly onto Mueller - Hinton agar plates with blood. The entire surface of the agar medium was inoculated with isolate and each susceptibility test disk was applied, using sterile forceps, firmly onto the inoculated agar plate. Plates were incubated at 35-37°C for 18 hours. Each inoculated plate was examined and the diameters of the zone of inhibition were measured (in millimeters) and recorded. Zones of inhibition were interpreted by referring to tables provided by Remel and Difco Laboratories (**Appendix D**).

BLOOD SAMPLES

Immunoglobulin G (IgG) Determination

For determination of IgG (1) levels at WK0 collection, whole blood was obtained via jugular venipuncture into a sterile vacutainer tube containing no additives (Vacutainer, Becton Dickinson,Franklin Lakes, NJ). Whole blood was allowed to set at room temperature and form a clot. The clotted blood was centrifuged at 1000 X g within 24 hours after sample collection, the serum was harvested and placed in cryovials, in duplicate, and stored at -20°C. Immunoglobulin G concentrations were determined by use of a bovine single radial immunodiffusion kit (Veterinary Medical Research and

Development, Inc., Pullman, WA) Three microliters of the frozen serum was thawed and placed in separate wells containing class specific anti-bovine immunoglobulin antibodies in buffered agarose. Reference standards, with concentrations consisting of 412 mg/dl, 825 mg/dl, 1650 mg/dl, and 3300 mg/dl, were used to plot diameters and set reference ranges on graph paper (diameter of reference standard versus concentration of reference standard). Plates were allowed to incubate for 24 hours at room temperature and a visible ring was formed on the agarose with a diameter proportional to the amount of immunoglobulin present in serum. From the reference standards, a standard curve was drawn. The diameters of each sample ring was measured (in millimeters), compared with the reference ranges plotted on the graph, and serum samples were plotted against the curve.

Packed Cell Volume/Total Solids

Total solids(TS) and packed cell volume (PCV) were determined using whole blood obtained via jugular venipunture placed in a heparinized vacutainer tube. The sample was gently mixed and place in a ice packed styrofoam container and transported to the laboratory. A heparinized capillary tube was filled with the blood, sealed, and centrifuged for 3 minutes at 1,200 X g. The PCV was measured in percentage. The capillary tubes were then broken and the serum placed on the refractometer cover plate for total solids (g/dl) determination. Total solids was visualized by a dark line going across the total protein scale.

Vitamin A and E Determination

Frozen serum (stored at -20°C) was sent to the Animal Health Diagnostic Laboratory - Nutrition Lab, Michigan State University for Vitamin A and E measures using the high-performance liquid chromatography techniques described by Stowe (1982) and Dennison and Kirk (1979). One milliliter of serum was combined with 1 ml of 200 proof ethanol and .01% butylated hydroxytoluene and vortexed. Two milliliters of hexane was added to the suspension, vortexed for 5 minutes, then centrifuged at 2,200 X g for 10 minutes. The hexane layer was collected and filtered, using a 0.45-μm filter prior to use. One hundred microliters of the extract was separated on a column with a mobile phase of hexane:chloroform (60:40) for vitamin A determination, and a mobile phase of hexane:chloroform (85:15) for vitamin E determination. Detection was done with the aid of a 35 μl flow cell in a spectrofluorometer set at 330 and 470 nm as the excitation and emission wavelengths, respectively for retinol, and 295 nm for vitamin E quantification. An external reference standard was measured against test serum.

Selenium Determination

Whole blood (sterile vacutainer tube containing K_3 EDTA) was sent directly to the Animal Health Diagnostic Laboratory - Nutrition Section, Michigan State University for selenium analysis. Whole blood selenium concentration was determined using the method described by Reaner and Veillon (1983). One milliliter of whole blood was digested by heat with 3 ml nitric acid followed by 2 ml phosphoric acid. Samples were further digested with 2 ml hydrogen peroxide. Samples and standards were quantified

by use of a fluorometer set at 376 nm for excitation and 518 nm for emission wavelengths.

Bovine Viral Diarrhea Virus Isolation

Heparinized whole blood was centrifuged at 2200 X g for 20 minutes. The mononuclear/platelet layer (buffy coat) was harvested and placed in 50 ml conical tubes for further processing. A 50 ml conical tube containing 2 ml of mononuclear and platelet layers (buffy coat) from whole blood was prepared for bovine viral diarrhea virus isolation utilizing the immunoperoxidase monolayer assay (IPMA) method. The buffy coat was diluted in 1 ml of Eagles Minimal Essential Media (EMEM) containing 10% fetal equine serum (FES) and was processed through 3 freeze/thaw cycles in an acetone and dry ice bath. The buffy coat samples were stored at -70°C until virus isolation could be performed. The IPMA technique (Meyling, 1988) was performed by the Virology Section of the Animal Health Diagnostic Laboratory at Michigan State University. Briefly, microtiter plates were inoculated with bovine turbinate cells in EMEM plus 1% glutamine, 1% tylosine and 10% FES. the plates were allowed to incubate for 2 days at 37°C in 90% humidity and 5% CO_2. Frozen buffy coats were thawed and centrifuged for 5 minutes at 2000 X g. Incubated bovine turbinate cells were removed form each well and 1.75 ml of 5% FES and 11.5 ml of the buffy coat to be tested was added to each well. The microtiter plates were incubated for 3 additional days at 37°C in 90% humidity and 5% CO_2. Following incubation, wells were washed in 0.01 phosphate buffered saline (PBS) and fixed for 10 minutes with acetone in 0.02% bovine serum albumin. The fixative was removed and cells were dried for 90 minutes at 37°C.

Polyclonal antibody was added to each well, incubated at room temperature for 30 minutes, and rinsed 3 times. Horseradish peroxidase was then added to each well and allowed to incubate at room temperature for 15 minutes. Microtitration plates were rinsed twice and substrate solution added to each well. The wells were allowed to incubate in the dark at room temperature for 60 minutes. Following incubation, the microtitration plates were rinsed twice with tap water. Samples were visually examined with light microscopy. Those positive for BVDV displayed a reddish brown staining in the cytoplasm similar to positive control and those negative displayed no discoloration (similar to negative control).

Deaths Prior to Last Collection

Calves evaluated in this study that died prior to last collection received post mortem examinations. Dead calves were sent to the Michigan State University Animal Health Diagnostic Laboratory for a full necropsy. If a calf was unavailable for a postmortem examination, samples from the intestinal and respiratory tracts were obtained and saved for our laboratory to check for pathogens.

Definitions

For this study, respiratory disease was defined as the detection of clinical signs related to an abnormal respiratory tract. Clinical signs included depression, elevated rectal temperature (>103.5 °C), and abnormal lung sounds. Lungs were auscultated and considered abnormal if crackles, wheezes, or rales were heard. Likewise, if no air

movement was auscultated, lung sounds were also considered abnormal. If there was recorded evidence of treatment for respiratory disease, each calf received a physical examination and abnormalities were recorded.

Diarrhea was defined as an increase in volume and frequency of defecation, and a change in fecal consistency. A calf was determined to have had a previous history of diarrhea if its tail and/or perineal areas were matted with dried feces or if there was recorded evidence of treatment for diarrhea. Calves with diarrhea received a physical examination and any abnormalities present were noted.

Passive transfer of immunoglobulins, as determined by serum, was considered inadequate if serum levels were less than 1000 mg/dl and total solids were less than 5.0 g/dl. middle ear infection was considered to be present if calves exhibited a head tilt and ear droop. If tracheal isolation revealed the presence of *Mycoplasma* infection, a presumptive diagnosis of otitis media was made if there was ear droop.

STATISTICAL ANALYSIS

Sample size was determined by a significance level (α) set at 0.05 and a power ($1-\beta$) of 0.80. Statistical analysis was performed using the Statistical Analysis System software (SAS), for determination of the total weeks at risk and exposure/nonexposure of infectious agents, and Epi Info 6.04b for analysis of relative risks. This was an observational cohort study in which relative risks where assessed for the incidence of an infectious agent being present in a calf and the likeliness of having another infectious agent present at subsequent, or later, collections.

Exposure in this study was defined as those calves having the presence of an infectious respiratory and/or intestinal agent. Calves used in this study were initially classified as nonexposed, and examined over an 8 week time period for possible exposure. Outcome variables included isolation of infectious agent(s) from calves (exposure) and presence of other infectious agents at later collections (i.e., isolation of intestinal pathogen rotavirus at first collection followed by isolation of respiratory pathogen , *P. multocida*, at second or third collections).

Based on collection time, the total weeks at risk for possible exposure to an infectious agent was 8 weeks. Weeks at risk was defined as the total number of weeks a calf could be exposed to a second infectious agent following isolation of a primary infectious agent. For example, if a calf had agent A isolated at the initial collection period (T1) and agent B isolated at T2, then the total weeks at risk for exposure to agent B, following the isolation of agent A, would be 2 (**Appendix E**).

Incidence density was calculated by defining those animals exposed to an infectious agent and those nonexposed to an infectious agent. The incidence density in the nonexposed group was defined as the total number of cases of an infectious agent being present in the calves tested following the presence of no primary infectious agent divided by the total weeks at risk if no primary infectious agent is isolated (i.e., total number of calves not shedding the *E. coli eae* gene- the primary agent- isolated at any collection time, but being positive for *C. parvum*, rotavirus, *P. multocida*, or *Mycoplasma*- the second agent- at some time in the collection period). The incidence density for those exposed was calculated by identifying the total number of calves having an infectious agent present following the identification of a primary infectious agent divided by the total weeks at risk following identification of the primary agent (i.e., total number of calves having *C. parvum* isolated then having *P. multocida* isolated at another collection time). Finally, relative risk was calculated by dividing the incidence density for the exposed by the incidence density for the nonexposed (**Appendix E**).

RESULTS

Age and Growth of Calves

The mean ages of calves from each farm are listed in **Table 1.** At T1, calves had a mean age of 4.6 ± 1.5 days, at T2 19.1 ± 1.7 days, T3 33.8 ± 1.0 days, and 48.1 ± 1.2 days at T4. The mean weight among the calves on five farms is shown in **Table 2.** Estimated weights was determined by the Nasco Weight by Breed dairy cow tape (**Appendix C**) and recorded for Holstein calves and converted to kilograms. At T1, the mean weight of calves was 49.7 ± 3.3 kg, T2 42.8 ± 5.0 kg, T3 65.0 ± 3.6 kg, and 79.0 ± 4.0 kg at T4.

Clinical Signs of Disease

In this study, 36/145 (24.8%) calves had diarrhea at one or more times during the sampling periods. Most cases of diarrhea occurred at T1 and T2 where 13/145 (9.0%) and 16/142 (11.3%) calves had diarrhea, respectively. Eighteen calves (12.4%) exhibited clinical signs of pneumonia and/or otitis media at least once during the collection period. Of the 145 calves examined, 33% of calves on farm A had pneumonia and/or otitis media, whereas 6.9% of the calves on farms B, C, D, and E combined had pneumonia and /or otitis media. Most pneumonia and otitis media signs were seen at T3 and T4 collections, where 7/145 (5.1%) and 6/145 (4.4%) had clinical signs of pneumonia and/or otitis media, respectively.

TABLE 1

MEAN AGE ± SD (DAYS) OF CALVES AT EACH COLLECTION TIME

FARMS	T1	T2	T3	T4
A (N=30)	5.4	19.1	32.8	46.1
B (N=29)	4.2	19.1	33.6	48.6
C (N=27)	6.5	21.1	34.7	49.1
D (N=28)	2.3	16.3	33.5	48.8
E (N=31)	4.8	19.7	34.2	48.0
MEAN AGES	4.6 ± 1.5	19.1 ± 1.7	33.8 ± 1.0	48.1 ± 1.2

T1 = first collection; T2 = second collection; T3= third collection; T4= fourth collection

TABLE 2

MEAN WEIGHT ± SD (KG) OF CALVES AT EACH COLLECTION TIME

FARM	T1	T2	T3	T4
A (N=30)	46.8	53.8	61.8	74.1
B (N=29)	48.2	51.4	66.4	81.8
C (N=27)	55.5	60.5	69.5	81.8
D (N=28)	46.8	46.8	60.9	74.1
E (N=31)	51.4	51.4	66.4	81.8
WEIGHTS	49.7 ± 3.3	52.8 ± 5.0	65.0 ± 3.6	79.0 ± 4.0

T1 = first collection; T2 = second collection; T3 = third collection; T4 = fourth collection

Presence of Diarrheal Agents

Of the 145 calves, none were positive for *Salmonella spp.* Six of the 145 (4.1%) calves were positive for ETEC. Only one calf ETEC positive had diarrhea. Intestinal pathogens were found at subsequent collections in all 6 calves. Subsequently, 2 calves developed a respiratory infection.

Cryptosporidium parvum was detected in 72 of the 145 (50%) calves in at least one collection time, and in 5 calves at multiple collections. Shedding of *C. parvum* was highest during T2 collection, where 48 (33%) of the calves were shedding *C. parvum.* Calves positive for *C. parvum* (1.4%) decreased by T4 collection.

The shedding of AEEC in calves was consistent on all the farms. One hundred-six (73%) calves had AEEC isolated from their feces at least at one collection time. 45/145 (31%) positive calves were at multiple collection times. Forty percent of the calves were positive for AEEC during T4.

Rotavirus antigen was detected in 107/145 (74%) calves at least one collection time. Forty three percent of the calves were positive for rotavirus antigen at T4. Sixty six (45.5%) calves were positive for rotavirus antigen at multiple sampling times.

Table 3 list enteropathogens isolated from calves at each collection time. The most common enteropathogens isolated were rotavirus and AEEC, which had an increase in number of positive calves between T1 and T4 collection. Rotavirus and AEEC were co-isolated from only 5 (3.4%) calves at T1, however at each collection time an increase in isolates occurred and by T4, 27 (20%) calves were shedding rotavirus and AEEC.

Presence of Respiratory Agents

None of the 145 culture swabs from calves was positive for *M. haemolytica*. However, 33 (23%) of the calves were positive for *P. multocida* at least one of the collection times and 10 (7%) calves positive at multiple collections. The majority of *P. multocida* isolates (17%) were from calves during T4 collection.

Culture swabs from 66 (46%) calves were positive for *Mycoplasma spp.* at all collection times. Although *Mycoplasma spp.* were detected in calves during all 4 collection times, the number of positive calves was highest at T4 (52%).

Detection of respiratory pathogens in all calves used in the study is shown in **Table 4**. *P. multocida* isolates were detected in 2 calves at T1, 5 at T2, 3 at T3, and 2 calves at T4. At T1, only 11 calves had *Mycoplasma spp.* isolates, however, between T2 and T4 collections, 38% - 40% of he calves were culture-positive for *Mycoplasma spp.* Co-infection of calves with *Mycoplasma* and *P. multocida* occurred during T2, T3 and T4 collections.

Presence of BVDV

Buffy coat cells from 4 calves were positive for BVDV. One calf from farm A and one from farm D, were persistently infected, as BVDV was cultured from buffy coat cells at T1 and T4. Two calves, one from farm C and one from farm B, were acutely infected with BVDV, as culture from buffy cells at either T1 or T4. All calves from farm E tested negative for BVDV.

TABLE 3

PERCENTAGE OF CALVES INFECTED WITH POTENTIAL ENTEROPATHOGENS

COLLECTION TIMES

Enteropathogens detected	T1 n=145	T2 n=142	T3 n=136	T4 n=136
none detected	53.8	9.2	28.7	30.9
C. parvum only	8.2	14.8	2.9	0.74
rotavirus only	13.8	21.8	27.2	27.9
AEEC only	12.4	13.4	16.9	19.9
ETEC only	3.4	ND	ND	ND
rotavirus & AEEC	3.4	12.7	13.2	19.9
C. parvum & AEEC	2.1	10.6	4.2	0.74
C. parvum & rotavirus	2.1	12.0	3.7	0
AEEC & ETEC	0.68	ND	ND	ND
C. parvum, rotavirus & AEEC	0	5.6	2.9	0

AEEC – presence of *E. coli eae* and/or *stx* genes; ETEC – enterotoxigenic *E. coli*; ND – not determined

TABLE 4

PERCENTAGE OF CALVES CULTURE - POSITIVE FOR *P. MULTOCIDA* AND *MYCOPLASMA SPP.*

	COLLECTION TIMES			
Agent	T1 n=145	T2 n=142	T3 n=136	T4 n=136
None detected	91.0	54.9	51.5	45.6
P. multocida	1.4	3.5	2.2	1.5
Mycoplasma	7.6	39.4	39.7	37.5
Both	0	2.1	6.6	15.4

Mycoplasma Antimicrobial Susceptibility

Fourteen *Mycoplasma* isolates from calves that displayed clinical signs of otitis media, were sent to Pharmacia for antimicrobial susceptibility assay. Six calves had received antimicrobial treatment with three of the calves having been treated with more than one antimicrobial. All isolates were sensitive to tilmicosin, showing minimum inhibitory concentrations of 0.03 μg/ml. Variable sensitivity was shown for tetracycline, lincomycin, spectinomycin and lincomycin/spectinomycin (**Appendix F**). All isolates were resistant to erythromycin.

Treatment of Infections

Forty-one calves (19%) were recorded as receiving some form of therapy by their owner for a respiratory and/or gastrointestinal infection at least one time during collection. **Table 5** list all antimicrobial agents administered to calves prior to each collection.

TABLE 5

ANTIMICROBIAL AGENTS ADMINISTERED TO CALVES PRIOR TO EACH COLLECTION

	Percentage of Calves Receiving Antimicrobials			
Antimicrobial	T1	T2	T3	T4
Ampicillin	0	0.7	0.7	0
Ceftiofur	1.5	3.5	3.7	1.5
Corid	0	3.0	0	0
Lincmycin/Spectinomycin	0	0	4.4	0
Tilmicosin	0	0	0	1.5
Nuflor	0	0	0.7	0.7
Penicillin	20.0	0.7	0.7	0.7
Trimethoprim/ Sulfamethazole	1.5	8.5	1.5	0

Immunoglobulin G, Packed Cell Volume, Total Solids

IgG, PCV, and TS were evaluated to assess failure of passive transfer and hydration status of animals. Twenty four (17%) calves had IgG concentrations ≤ 825 mg/dl (**Table 6**). Nineteen calves had IgG concentrations ≤ 825 mg/dl and total solid levels ≤ 6.0 g/dl. Failure of passive transfer was determined in two calves that died. Twenty six calves IgG concentrations between 826-1650 mg/dl and 95 calves had IgG concentrations ≥ 1650 mg/dl. Eleven calves had packed cell volumes below 24% or above 45%. Those with a PCV ≥45%, all had IgG concentrations ≤825 mg/dl and total solids <6.0 g/dl.

Vitamin Deficiencies

Normal values for Vitamins A, E and selenium in various age groups of calves are given in **Appendix G**. Vitamin E results were expressed as the vitamin E: cholesterol ratio. Based on the normal values, none of the 145 calves used in this study were deficient in vitamin E and one calf was deficient in selenium. The selenium deficient calf was detected at T1. Forty one (28%) calves were deficient in vitamin A at either T1 or T4. Of these, 7.3% were deficient at T1 and 93.0% were deficient atT4. No calf was deficient at both T1 and T4 collections. Mean vitamin A, vitamin E/cholesterol ratio and selenium concentrations and corresponding mean PCV and TS are given in **Table 7**. At T1 collection, the mean vitamin A and E concentrations were above the normal range for calves for this age group. However, by T4 collection, there was a statistically significant difference ($p<0.05$) in vitamin concentrations between the two collection times. A

staticstically significant difference ($p<0.05$) in vitamin concentrations was also seen between collection times for selenium and vitamin E/cholesterol ratio.

Calves deficient in vitamin A during T1 collection were most likely to have AEEC (RR=3; $p<0.01$), *Mycoplasma* (RR=2; $p<0.5$) and *P. multocida* (RR=3; $p<0.01$) isolated at later collection times. Calves deficient in vitamin A during T4 collection were most likely to have had *P. multocida* isolated at a previous collection ($p<0.001$). **Tables 8a** and **8b** show relationships for vitamin A deficiency and isolation of pathogens.

Occurrence of Subsequent Intestinal and Respiratory Pathogens

Relationships between isolation of an infectious agent (A) and presence of a subsequent infectious agent (B) are listed in **Table 9**. Calves shedding *P. multocida* were at a statistically significant risk (RR=2.2; $p<0.05$) of having rotavirus antigens and *Mycoplasma* (RR=2; $p<0.1$) isolated at subsequent collection times. No significant associations were shown between the isolation of an enteric pathogen and subsequent isolation of respiratory pathogens.

TABLE 6

IGG AND TOTAL SOLIDS AMONG CALVES AT T1 COLLECTION

IgG conc. (mg/dl)	Calves at T1	Mean IgG conc. ± SD(mg/dl)	T.S. ± SD (g/dl)	Unhealthy Calves
0 – 825	24	547±319	5.6±1.2	3
825 – 1650	26	1553±166	6.1±0.4	2
≥1651	95	2551±500	6.2±1.9	4

≤825mg/dl, Failure of Passive Transfer

TABLE 7

MEAN PCV, TS, VIT A, VIT E/CHOLESTEROL AND SELENIUM CONCENTRATIONS AT T1 AND T4

	PCV	T.S.	VIT A*	VIT E/CHOL*	SE*
T1 (N=145)	32.0%	6.8 g/dl	116 ng/ml	11.0	194 ng/ml
T4 (N=136)	35.5%	6.3 g/dl	159 ng/ml	3.0	179 ng/ml

*p value significance <0.05; T4, vit A deficiency < 120 ng/ml; vit E/Chol ratio <0.75; selenium deficiency < 25 ng/ml

TABLE 8a

VITAMIN *A* DEFICIENCY AT T1 AND RATE OF OCCURRENCE OF AN INFECTIOUS AGENT

AGENT	ID FOLLOWING				
	No Agent	Agent	p	RR	CI
CRYPTO	1.256	1.667	0.77873	1.33	[0.18,9.60]
ROTA	1.974	3.611	0.04119	1.83	[1.02,3.30]
AEEC	1.726	3.529	0.00889	3.04	[127,7.30]
MYCO	1.695	3.421	0.01640	2.02	[1.12,3.63]
PAST	0.493	1.500	0.00889	3.04	[1.27,7.30]

Incidence Density (ID) in the absence/presence of an agent in cases per 10 weeks at risk;

p - p value significance; RR - relative risk; CI - confidence interval

TABLE 8b

PRESENCE OF AN INFECTIOUS AGENT AND RATE OF OCCURRENCE OF A VITAMIN *A* DEFICIENCY AT T4

AGENT	ID FOLLOWING				
	No Agent	Agent	p	RR	CI
CRYPTO	1.353	0.625	0.06235	0.46	[0.20,1.06]
ROTA	1.969	1.420	0.16754	0.72	[0.45,1.15]
AEEC	1.733	1.620	0.77906	0.93	[0.58,1.50]
MYCO	1.696	2.600	0.15111	1.53	[0.85,2.76]
PAST	0.650	3.50	0.00003	5.39	[2.43,11.9]

Incidence Density (ID) in the absence/presence of an agent in cases per 10 weeks at risk;

p - p value significance; RR - relative risk; CI - confidence interval

TABLE 9

PRESENCE OF AN INFECTIOUS AGENT (A) AND RISK OF SHEDDING ANOTHER INFECTIOUS AGENT (B)

AGENTS		ID FOLLOWING				
(A)	(B)	No Agent(A)	Agent (A)	p	RR	CI
ROTA	CRYPTO	1.52	0.672	0.0030	0.4	[0.25,0.77]
	AEEC	1.859	1.239	0.07583	0.67	[0.42,1.05]
	MYCO	1.810	1.314	0.14680	0.73	[0.47,1.12]
	PAST	0.625	0.340	0.07305	0.54	[0.28,1.07]
CRYPTO	ROTA	1.852	1.299	0.16714	0.70	[0.42,1.16]
	AEEC	1.472	1.378	0.77859	0.94	[0.59,1.49]
	MYCO	1.616	1.022	0.07648	0.63	[0.38,1.05]
	PAST	0.409	0.377	0.82734	0.92	[0.44,1.91]
AEEC	CRYPTO	1.799	0.413	0.000003	0.23	[0.12,0.45]
	ROTA	0.549	0.387	0.31109	0.70	[0.36,1.39]
	MYCO	1.916	0.850	0.00220	0.44	[0.26,0.76]
	PAST	0.651	0.429	0.20921	0.66	[0.35,1.25]
MYCO	CRYPTO	1.667	0.341	0.00004	0.2	[0.09,0.48]
	ROTA	2.062	2.273	0.6368	1.1	[0.70,1.74]
	AEEC	1.693	1.538	0.70855	0.91	[0.55,1.50]
	PAST	0.605	0.703	0.6485	1.16	[0.65,2.09]
PAST	CRYPTO	1.201	0.250	0.08546	0.21	[0.03,1.50]
	ROTA	1.606	3.462	0.02619	2.16	[1.08,4.32]
	AEEC	1.667	1.667	1.0	1.0	[0.50,2.0]
	MYCO	1.431	2.50	0.18252	1.75	[0.76,4.01]

ID following no agent (A) - Incidence density of agent (B) in the absence of agent (A) in cases per 10 weeks at risk

ID following agent (A) - Incidence density of agent (B) in the presence of agent (A) in cases per 10 weeks at risk

RR - relative risk

CI - confidence interval

P – p value significance

E. coli Results

Hemolysin Activity

Of 5,590 lactose fermenting colonies (LFC), 129 (2.3%) possessed hemolytic activity. These hemolysin active colonies were isolated from 34 calves at sometime during the collection period. Only 5.4% of the hemolysin active LFC possessed the *stx* - only gene and none possesed the *eae* and *eae/stx* genes.

Enterohemolysin Activity

One hundred six AEEC samples were randomly selected for analysis of enterohemolysin activity and antimicrobial susceptibility. The enterohemolytic phenotype was expressed by 50% of these isolates. Enterohemolysin activity was not restricted to one particular serogroup and was detected in both *eae* and *stx* strains from all calves.

PCR Results/Gene Isolation

A total of 5,590 (LFC) having *E. coli* – like growth characteristics were evaluated for AEEC. Of the 5,590 LFC, 818 LFC (14.6%) possessed AEEC genes. **Table 10** list the number of colonies from calves tested on each farm, the total *E. coli* colonies possessing AEEC genes, and the average number of colonies per calf isolated from each farm.

One hundred six calves (73%) were culture-positive for AEEC at least once during the 4 collection times. 95.5% of these AEEC isolates were from calves that did

not have diarrhea. Overall, 40.4% of calves were culture-positive for AEEC at T4, whereas 19.0% of the calves were culture –positive for AEEC at T1.

Among the *E. coli* colonies possessing AEEC genes, the *E. coli eae* gene was deteceted among 63.1% (516/818) of the AEEC isolates, while the *eae/stx* genes (23.6%) and the *stx* gene where isolated less frequently (13.3%). Positive *E. coli* isolates possessing the *stx* - gene by PCR and the Premier EHEC test are shown in **Appendix H.**

TABLE 10

TOTAL NUMBER OF *E. COLI* COLONIES TESTED AND COLONIES POSSESSING AEEC

# of Calves on each farm	AEEC + calves	Total # of colonies tested	Total *E. coli* colonies with AEEC genes	+colonies/+ (colony per calf)
Farm A(30)	21	1,170	176	176/21 (8.4)
Farm B (29)	22	1,130	172	172/22 (7.82)
Farm C(27)	21	1,080	166	166/21 (7.9)
Farm D(28)	23	1,000	189	189/23 (8.22)
Farm E(31)	19	1,210	115	115/19 (6.1)
TOTAL(145)	106	5,590	818	

Serogroups and Antimicrobial Susceptibility

Fifty four different serogroups were identified among the 818 AEEC isolates. Among these, 9 AEEC serogroups have been reported as zoonotic (**TABLE 11**). Zoonotic serogroups were isolated from calves with and without diarrhea. Untypeable or O^- serogroup, accounted for 34.0% of the AEEC isolates.

TABLE 12 summarizes antimicrobial resistance among AEEC at each collection. All strains were sensiive to amikacin, enrofloxacin and nalidixic acid. Resistance to tetracycline, penicillin, clindamycin, neomycin and spectinomycin was detected at each collection time.

TABLE 11

ZOONOTIC STRAINS OF AEEC SEROGROUPS ISOLATED FROM CALVES IN STUDY

O26 (20.1%)

O103 (6.0%)

O^- (34.0%)

O80 (10.0%)

O84 (2.0%)

O156 (2.0%)

O98 (2.3%)

O10 (0.4%)

O145 (2.0%)

TABLE 12

ANTIMICROBIAL RESISTANCE (PERCENTAGE) AMONG AEEC AT EACH COLLECTION

Antimicrobial Agent	T1	T2	T3	T4
Ampicillin	47.0	31.3	33.2	21.2
Penicillin	93.0	89.0	88.3	92.2
Enrofloxacin	0	0	0	0
Tetracycline	57.3	74.2	65.0	58.0
Clindamycin	97.4	100.0	96.0	96.0
Trimethoprim/Sulfamethoxazole	8.0	8.0	10.0	2.0
Florfenicol	0	0	0.1	0
Cephalothin	41.4	29.0	21.0	13.0
Cefoxitin	3.0	0.9	2.0	0.35
Ceftiofur	1.3	0.9	0	0
Gentamicin	4.0	4.0	13.4	19.4
Neomycin	53.5	70.0	48.2	51.0
Spectinomycin	87.1	74.0	53.0	43.3

Incomplete Sampling

Nine calves used in the study died prior to last collection. One calf from farm A died at 10 days of age, however, no necropsy was performed and a cause of death was undetermined. One calf from farm B died a 7 days of age. ETEC was isolated from this calf 6 days previously and enteritis was determined as the cause of death. Six calves from farm D died before the end of the collection period. Two calves, both 8 days of age, died from enteritis with C. parvum and AEEC isolated from fecal samples. One calf, 9 days of age, died of abomasitis, however, no necropsy was performed on this calf. Another calf, 19 days old, died from septicemia and pneumonia, with AEEC, rotavirus and Mycoplasma isolated form necropsy samples. The last calf, 22 days of age, died from enteritis and pneumonia and had AEEC and Mycoplasma isolated form necropsy samples.

DISCUSSION

Results of this study indicated that diarrhea was not associated with the isolation of a potential intestinal pathogens. However, the presence of *Mycoplasma spp.* and *P. multocida* coincided with clinical signs of respiratory disease. Vitamin A deficient calves had a significant increase in the presence of *C. parvum*, rotavirus, AEEC, *Mycoplasma spp.* and *P. multocida* at sampling times T3 and T4.

All calves, with the exception of those from farm E, received vitamin A injections at birth. A study performed in rats showed that vitamin A deficiency impairs the intestinal immune system and results in an increased frequency and severity of enteric diseases caused by microbial agents (Sirisinha, *et al*, 1980). Another study performed on mice looked at the interaction of rotavirus and vitamin A deficiency (Ahmed *et al*, 1990) and concluded that rotavirus and vitamin A deficiency cause few changes alone, but when acting together, provide significant destruction of the mucosa of the small intestines. Intestinal tissue samples were not obtained from vitamin A deficient calves, and therefore histopathology was not performed. However, other studies have demonstrated that vitamin A deficient calves have an impaired intestinal immune system, which could result in an increased frequency and severity of enteric disease caused by microbial agents (Sirisinha *et al*, 1980).

Based on previous studies, relationships have been established between various management factors and their association with the presence of diarrhea and respiratory diseases (Oxender *et al*, 1973; Speicher and Hepp, 1973; Martin *et al*, 1975). Factors such as seasonality, housing, and farm size were not assessed in this study since others

have shown that these aforementioned factors are important in the development of disease (Waltner-Toews *et al*, 1986; Yorke *et al*, 1979).

Growth rate of calves remained consistent on all 5 farms. Feeding practices were similar on all farms and not considered an important factor in determining growth rate of calves. Several studies have shown that diarrhea and pneumonia both can decrease weight gain in preweaned calves (Schmoldt *et al,* 1979; Kleiner *et al*, 1983). Based on a study performed at Pennsylvania State University, heart girth was determined to be the most accurate body measurement used in predicting body weights (Wilson, 1997). Standard body weights for Holstein heifers was determined by Heinrichs and Hargrove, (1987) and stated the average body weight at 1 month of age to be 62 kg, and 97.7 kg by 3 months of age. Virtala *et al* (1996) indicated that a longer duration of pneumonia may reduce a calf's body weight, however, the effects of disease on weight gain were not of practical importance. Their study also concluded that failure of passive transfer reduces the average daily weight gain during the first month of life. Based on the mean weights of all calves in our study, 8.3% were below the average weight at 1 month of age (T3) and were also failure of passive transfer.

Twenty three percent of the calves exhibited clinical signs of diarrhea and 21.4% were concurrently infected with a potential intestinal pathogen. All calves exhibiting clinical signs of diarrhea were positive for an infectious agent at subsequent and multiple collection times. Seventeen (11%) of the calves used in this study showed clinical signs of pneumonia and/or otitis media and 11 calves were simultaneously positive with a respiratory pathogen. Two calves exhibited diarrhea and pneumonia and had intestinal and respiratory pathogens isolated concurrently.

Calves shedding rotavirus antigen did not concurrently show clinical illness when the antigen was isolated. Rotavirus antigen was detected using the Pathfinder (Kallestad) direct antigen detection system. This test assay has a sensitivity of 97.1% and a specificity of 97.9%. Several studies on the excretion of rotavirus antigen by healthy cattle and calves has been reported (Bridger and Woode, 1975; McNulty *et al*, 1976). In our study, all calves received protection against rotavirus infection by passive transfer. Rotavirus was shed by 73.8% of the calves; and diarrhea was not observed among these calves. The continual shedding of rotavirus antigen may have been associated with an avirulent strain or assay specificity. Variations in rotavirus virulence has been identified with some having the ability to replicate without causing clinical signs of disease in non-immune calves (Bridger *et al*, 1992). The variations in virulence has been linked to the fourth gene of the bovine rotavirus which cleaves trypsin and results in enhancement of viral infectivity *in vitro* (Espejo *et al*, 1981; Estes *et al*, 1981).

C. parvum was detected in 46% of the calves evaluated in this study, and was identified in calf feces collected predominately at T2 collection. Of the 2 large farms sampled in our study, 60% of those calves were positive for *C. parvum*. On the smaller herds, 52% of those calves were positive with *C. parvum* oocysts. A heavy pathogen load has been shown to be associated with a larger herd size and animal density (Lance *et al*, 1992). Smaller herds may also have a heavy pathogen load, however this has been associated with more animals in a confined area (Garber *et al*, 1994). Large herds and summer months were significant factors in the shedding of *C. parvum*. By contrast, calves in small herds and during the winter months are less likely to shed *C. parvum*.

Mycoplasma spp. were cultured from calves on all 5 farms, however, most isolations were not associated with clinical signs of disease. Many of the various types of Mycoplasma have been found in the respiratory tract of healthy calves, though rate of isolation varies depending on health status and age of animal (Thomas and Smith, 1972; Bennett and Jasper, 1977; Tanskanen, 1987). Experimental data has also stated change in environmental temperature and humidity to be associated with a change in bacterial flora and could lead to pneumonia (Jones and Webster, 1984). Woldehiwet, Mamache, and Rowan (1990) sampled 24 calves from birth to 3 months of age and determined that the number and type of *Mycoplasma spp.* which colonize the nose and trachea, were influenced by the age of calf and not by environmental temperature and humidity. Their study stated that *Mycoplasma spp* colonizes the upper respiratory tract as soon as a calf is born, then there is a gradual reduction in organism numbers. *Mycoplasma spp.* were most often isolated from the nose of calves at 2-6 weeks of age. Likewise, by weaning age, *M. bovirhinis*, *M. arginini* and *Acholeplasma laidlawii* were the predominate species isolated from the nasal passage and *M. dispar* and *M. bovirhinis* dominated in the trachea. Based on this, the presence of *Mycoplasma spp.* in the respiratory tract of clinically healthy calves in our study was more than likely a normal colonization. However, stress or environmental changes – such as temperature and humidity – could have contributed to some overgrowth of *Mycoplasma spp.* and the presence of *P. multocida.*

Most of our mycoplasma cultures were not speciated. Among those speciated, *M. bovis* and *M. bovirhinis* were the two common isolates. Often clinical signs associated with otitis media were observed concurrently with *Mycoplasma* isolation. Walz *et al,* (1997) described clinical and pathological findings associated with otitis media in

preweaned calves and also demonstrated its presence from the bulk milk tank. Cows with subclinical mastitis serve as a possible source of infection and may be another likely source for the increase isolation of mycoplasma and concurrent otitis media from calves in our study.

This study demonstrated a relationship between the presence of intestinal and respiratory pathogens in calves. Waltner-Toews, *et al* (1986) found that 116 calves used in their study succumbed to both scours and pneumonia, 63.8% were treated for scours before pneumonia and 22.4% simultaneously were treated for scours and pneumonia. In our study, only 5 (3.4%) calves succumbed to both diarrhea and pneumonia. However, calves which had *P. multocida* isolated initially, were more likely to have rotavirus and *Mycoplasma spp.* pathogens and just as likely to shed *E. coli* possessing the *eae/stx* genes at subsequent collection times. Twenty calves had *P. multocida* isolated from their respiratory tract prior to T4 collection period. While *P. multocida* is the bacteria most consistently isolated from young calves with pneumonia, in our study, only 5 calves (25%) showed clinical signs of a respiratory infection and had *P. multocida* isolated at the same time. Subclinical enzootic pneumonia may be a possible explanation for this occurrence and this form of pneumonia may go unrecognized by many producers, with fever and an increase in respiration the only clinical signs seen. If undetected, the pneumonia has the ability to increase in severity and possibly develop into a respiratory disease.

Few studies have shown the effect of having concurrent BVDV and respiratory infections (Broderson and Kelling, 1998; Potgieter *et al*, 1984). Because of the immunosuppressive effects of BVDV, the virus contributes to the presence of

polymicrobial infections and disease. BVDV was isolated from mononuclear cells from 4 calves. Two of the 4 calves were persistently infected as they were positive for BVDV at the time of second culture. All 4 calves had infectious agents isolated from the intestinal and/or respiratory tracts following T1 collections. None of the BVDV positive calves exhibited clinical signs of infection, which is typically seen in calves immunotolerant and persistently infected with BVDV infection.

Enterotoxigenic *E. coli* was detected in 6 of the 145 calves (4.1%) and was associated with diarrhea in a 2-day-old calf. One calf, at one day of age, subsequently died as a result of failure of passive transfer and ETEC infection. The other 4 calves shedding ETEC had adequate serum immunoglobulin concentration and showed no signs of clinical illness. In each of the 6 calves, vaccination of dams for ETEC had been recorded. Based on our results, it appears that ETEC can be isolated from calves that do not show clinical signs of disease.

The second objective of this study was to assess shedding patterns of *E. coli* carrying the *eae* and *stx* genes, determine their predominant serogroups and antimicrobial susceptibility. Calves shedding E. *coli* attaching and effacing genes (*eae*$^+$ and *eae/stx*$^+$) in their feces did not correlate with the presence of diarrhea at that same collection time. Predominant *E. coli* strains varied between farms however, serogroups O26, O118, O80 and O- accounted for 49.5% of all typeable strains. Of these strains, serogroup O26 has been commonly isolated from infants and calves with diarrhea (Karmali, 1987; Robin-Browne *et al*, 1987). The presence of diarrhea was not associated with the isolation of this serogroup and only 4 calves had diarrhea present and concurrent isolation of serogroup O26. Serogroup O157 was not detected among any of the calves in this study.

Several serogroups (**TABLE 11**) isolated from calves have been previously associated with human disease (Willshaw *et al,* 1993; Caprioli *et al*, 1994). Diarrhea was seen in a calf infected with one of these serogroups, however, previous episodes of diarrhea were observed in other calves. Several studies have described the various serotypes associated with *E. coli stx* strains and cattle have been regarded as important reservoirs for humans (Karmali, 1989; Wells *et al*, 1991; Saridakis, 1994). However, Saridakis *et al* (1997) looked at virulence properties of *E. coli* strains in calves and showed that of the 204 calves used in his study, none of the bovine *E. coli* strains produced the *stx* gene. His results supported previous data of human strains (Silva *et al.*, 1983; Guth *et al.*, 1993). In our study, the *E. coli stx* gene does not appear to be a significant contributor to disease in calves, however the presence of the *E. coli stx* gene in the feces of apparently healthy calves is of concern. Calves should therefore be considered a major source of this organism and a possible source of transmission to humans. The occurrence of the various *E. coli* strains isolated from the calves used in this study may have been due to the differences in management practices (Hinton, Hedges and Linton, 1985) and environmental conditions.

The predominant *E. coli* strains isolated from calves were colonies possessing the *eae* gene- only (63.1%) and only 2.5% of the eae^{+} isolates were detected from calves with diarrhea. Isolation of the *E. coli stx* gene - only was less frequently isolated from calves used in this study (12.6%) and was detected in only 3 calves with diarrhea. *E. coli* strains carrying the *stx* gene - only have been detected in several O serogroups, with many of them associated with human disease.

Several studies have shown that enterohemolysin is synthesized by, and may compliment calf strains of *E. coli* which produce Shiga toxins (Beutin *et al*, 1986; Beutin *et al*, 1989; Nataro and Kaper, 1998). Of the 106 randomly selected samples, 35.9% and 14.2% of the *eae* and *eae/stx* strains from calves, respectively, had enterohemolysin activity. It has been proposed that the presence of enterohemolysin correlates with the presence of Shiga toxin producing *E. coli* (Beutin *et al*, 1989; Wieler *et al*, 1995). For this study, enterohemolysin activity was not associated with the presence of diarrhea or Shiga toxin production.

Only 24.2% of calves with diarrhea shedded the *E. coli* attaching and effacing genes which showed multiple antimicrobial resistance. However, not all calves with diarrhea were treated with antimicrobials. Antimicrobial resistant strains were seen extensively in calves not expressing diarrhea and also in calves treated for a respiratory or intestinal infection. One possible explanation for this would be development of resistant strains occurring previously and now becoming persistent in the environment. Linton (1977) and Hinton *et al* (1985), confirmed from their studies that drug resistance is common in *E. coli* strains isolated from calves and uncommon in those from adult cattle. Although source of the resistant strains was not determined in our study, a contaminated environment and feed were probable sources.

Antimicrobial susceptibility profiles of isolated *E. coli* colonies carrying the *eae, stx or eae/stx* genes varied among calves between farms. More than 70% of the *E. coli* strains from calves were resistant to tetracycline and penicillin. Bacterial resistance to various antimicrobial agents has been described by several as being caused by a plasmid-mediated resistance, chromosomal resistance, or mutant strains which have defects or do

not utilize various bacterial pathways (Davies and O'Connor, 1978; Grieco, 1981; Murray, 1986; Chaslus-Dancla *et al,* 1987; Rowe, Ward and Threlfall, 1997). Ampicillin, which has increased activity against many strains of *E. coli*, showed resistance to isolated strains from calves on farm D (68%) but less resistance among calves on the other farms. We recorded one calf from farm D treated with ampicillin during T2 and T3 collections. The *E. coli* strains isolated from this calf and the ampicillin susceptibility varied at each collection time. At T2 collection, this calf had been treated with ampicillin and we were able to isolate *E. coli,* serogroup O80, from the feces which was sensitive to ampicillin. At T3 collection, ampicillin was still being given, however, the *E. coli* isolates were of serogroup O118 and were resistant to ampicillin. By T4 collection no ampicillin had been given in two weeks, and the calf was shedding an untypeable *E. coli* serogroup sensitive to ampicillin. Since it has been shown that the intestinal flora of preweaned calves is continually changing (Hinton, Linton and Hedges, 1985a), it can be concluded that the ampicillin resistant isolates were cleared from this calf and a possible reason why variations in strains occurred. It is unknown as to whether ampicillin was a common antimicrobial agent used on this farm in previous years, but if so, may play an important role in the flow of resistance in *E. coli* strains isolated in the calves on this farm in which we sampled.

Calves having *E. coli* isolates resistant to gentamicin were seen (13.0%) on all farms except calves on farm A. Calves on farm E had the highest number of resistant isolates (83.5%). No calves on this farm were treated with gentamicin, however, neomycin, another aminoglycoside, was supplemented in their milk replacer. Antimicrobial resistance to gentamicin was seen at all 4 collection times. Mechanism of

bacterial resistance to aminoglycoside antimicrobials involves the inactivation of these drugs by plasmid - mediated activation of phosphotransferases, nucleotidytransferases and acetyltransferases (Davies and O'Connor, 1978; Courvalin and Carlier, 1981). In an 18 month study looking at the sensitivity patterns of *E. coli* following oral prophylactic chlortetracycline therapy in beef calves (Brophy, Caffrey and Collins, 1977), results showed an increase in *E. coli* isolates resistant to chlortetracycline and streptomycin while calves were housed in confined conditions. However, as soon as calves were turned out to pasture (between 2-5 months following therapy), several of the *E. coli* isolates became sensitive to chlortetracycline and streptomycin. Based on their results, it seems possible that calves shedding *E. coli* isolates resistant to gentamicin (seen particularly at T3 and T4) could carry isolates sensitive to gentamicin once they are placed in a more open, less confined environment. However, in order to verify this, isolates would need to be obtained and assessed over a more prolonged period of time.

Twenty-six *E.coli* isolates from calves (16%) on farm C show resistance to trimethoprim/sulfamethazole (SXT) even though this was not an antimicrobial used on this farm. All calves on this farm did, however, receive a prophylactic treatment of penicillin G for 5 days. Farm D, who used SXT in many of its sick calves, showed 19 calves (10%) having isolates resistant to this drug, however, only 2 calves treated with this drug carried resistant strains. Development of resistance has been shown to occur in animals receiving medicated feed supplemented with SXT (Guise *et al*, 1986) and by the liberal use of this antimicrobial in humans and animals (Godstein *et al*, 1986). Hariharan *et al* (1989) looked at *E. coli* isolated form pigs and calves with diarrhea and their resistance to SXT. In addition to SXT resistance, the bovine *E. coli* strains also show

resistance to tetracycline (100%), neomycin (92%) and ampicillin (82%). Of the 65 *E. coli* isolates resistant to SXT, 12 were also resistant to tetracycline and 42 of the isolates were also resistant to tetracycline and ampicillin. All farms had some calves with this resistance pattern, however, calves from farm D showed the most strains resistant to all three antimicrobials and calves from farm C had varied resistance patterns. Further studies on the frequency of this resistance pattern are needed. Based on these results, therapeutic use of SXT for treatment of an intestinal infection could possibly lead to resistance of this antimicrobial and possible further resistance to antimicrobials used to treat respiratory disease.

The second and third generation cephalosporins, cefoxitin and ceftiofur, respectively, had a total of 19 resistant *E. coli* strains isolated from farms B and D. Most isolates were from farm D, where 12 calves showed resistance (55 of the isolates were in the intermediate range). The variation in resistance patterns between calves and between farms may be due to exposure to resistant isolates rather than any antibiotics given. Only one calf from farm B was treated with ceftiofur and antimicrobial resistance to cephalosporins was not seen in this calf. Eight calves from farm D were treated with ceftiofur and only one calf showed resistance to cefoxitin in 2 of the 8 *E. coli* isolates tested. Three of the four calves showing *E. coli* resistance to the cephalosporins also showed resistance to SXT. Resistance to enrofloxacin, a fluoroquinolone, was not seen. Second generation cephalosporins and fluoroquinolones are currently not licensed for use in calves.

Multiple antimicobial resistance varied among farms. The calves on farms D and E had more *E. coli* isolates resistant to two or more antimicrobials than any other

farms. Many of the isolates (44.6%) were resistant to ampicillin, penicillin, and tetracycline, however, tetracycline was not a common antibiotic used on any of the farms. Isolates were also resistant to gentamicin and cefoxitin, which are antibiotics not approved for food animal use. Several studies pertaining to *E. coli* isolates showing resistance to antimicrobials, have numerous isolates and serogroups isolated, with many of the isolates resistant to antimicrobials not commonly used on their farms (Howe, Linton and Osborne, 1976; de Lopez *et al*, 1982; Wray *et al*, 1986). Bacterial resistance to multiple antibiotics is a leading cause of treatment failure and mortality. The mar locus (multiple antibiotic resistance) has been identified in *E. coli* bacteria which regulates susceptibility to multiple antibiotics. This multi antibiotic resistant system was discovered by selection of *E. coli* resistant to low levels of tetracycline and chloramphenicol (George and Levy, 1983). Many of the resistances specified are those found on plasmids or chromosomes (Jacoby and Archer, 1991).

CONCLUSIONS

This study focused on shedding patterns of common respiratory and intestinal pathogens in preweaned dairy calves. Data from this study suggest that there is a relationship between isolation of potential respiratory pathogens and subsequent isolation of a potential intestinal pathogen. When *P. multocida* and *Mycoplasma spp.* were isolated from calves initially, these calves were statistically more likely to have rotavirus antigen detected in their feces at later collection times. Likewise, if *P. multocida* or *Mycoplasma spp.* were isolated from calves initially, calves were just as likely to have *Mycoplasma spp.* and *P. multocida*, respectively, isolated at a later collection time. The presence of select potential intestinal pathogens was not associated with isolation of select potential respiratory pathogens. Our data supported previous studies as they related to season, housing, feed, therapy for disease, and occurrence of clinical disease.

Variations in herd size and farm management practices were described but not assessed as they relate to the presence of potentially infectious pathogens. The small number of herds involved in this study was adequate for individual calf data collection, however, a larger sample size would statistically significant when analyzing results between farms. Diversity in management practices among farms has been described previously as they relate to calf mortality and was therefore not evaluated in this study.

The second objective of this study was to assess predominant *E. coli* isolates in calf feces and their antimicrobial susceptibility profiles. *E. coli* possessing the *eae* and *stx* genes was detected in the feces of apparently healthy calves. The predominant serogroups and animicrobial susceptibility did vary among farms and stresses the variations in farm management. Public health concerns and the rising prevalence of this

infectious bacteria in the livestock industry have consumers eager as to prevention and control. In addition, there is concern in regards to emerging antimicrobial resistance to many of the common antimicrobials used to treat an *E. coli* infection. Factors such as practicing a closed herd policy, strict sanitation, and isolation of sick or new animals may have play a role in having no animals becoming infected this organism.

APPENDIX A

MILK REPLACERS AND FEEDS USED IN CALVES ON 5 FARMS

FARM A	SELECT AMPLIFIER FROM LAND O LAKES (CONTAINS A COCCIDIOSTAT) CARGUILD STARTER
FARM B	MICHIGAN PREMIUM (LASALOCID) PURINA NURSE CHOW (OXYTETRACYCLINE AND NEOMYCIN)
FARM C	DECOQUINATE IN MILK REPLACER
FARM D	WHOLE MILK; PURINA CHOW (OXYTETRACYCLINE AND NEOMYCIN)
FARM E	HUBBARD'S MEDICATED MILK (OXYTETRACYCLINE AND NEOMYCIN) CALF 2000

APPENDIX B

COW VACCINATIONS ON 5 FARMS

FARM A	SCOUR GUARD 3(K)[1], CLOSTRIDIAL 8 WAY[2], CATTLEMASTER 4[1], LEPTO 5[2], VIRASHIELD 2®[3]
FARM B	CATTLEMASTER 4[1], CLOSTRIDIAL 8 WAY[2], SCOUR GUARD[1]
FARM C	SCOUR GUARD 3[1], E. COLI -GUARD® COW-SOW[4], HORIZON® 9[4]
FARM D	CLOSTRIDIAL 8 WAY[2], SCOURGUARD[1], CATTLEMASTER 4[1]
FARM E	RESPROMUNE™ 4 IBP-BRSV[2], CLOSTRIDIAL 7 WAY BACTERIN[2], CLOSTRIDIAL C, D ANTITOXIN[5], SCOURGUARD 3[1]

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[1] MANUFACTURED BY SMITHKLINE BEECHAM, [2] MANUFACTURED BY AGRILAB, [3] MANUFACTURED BY GRAND LABORATORIES, [4] MANUFACTURED BY MILES LABORATORIES, [5] MANURACTURED BY COLORADO SERUM

APPENDIX C

CONVERSION TABLE FOR HEART GIRTHS/WEIGHT IN CALVES

Heart Girth in inches	Heart Girth in centimeters	Weight in kilograms
30	76.2	40.7
31	78.7	43.4
32	81.3	47.3
33	83.8	51.2
34	86.4	56.4
35	88.9	61.4
36	91.4	66.9
37	94.0	74.1
38	96.5	80.9
39	99.1	87.3
40	101.6	95.5

APPENDIX D

ZONE DIAMETER INTERPRETIVE STANDARDS (ZONE DIAMETERS IN MM)

ANTIMICROBIAL AGENTS	RESISTANT < MM	INTERMEDIATE MM RANGE	SUSCEPTIBLE >MM
Ampicillin (AM 10)	13	14-16	17
Cefoxitin (FOX)	14	15-17	18
Enrofloxacin (ENO)	12	13-14	15
Gentamicin (GM)	12	13-14	15
Penicillin (P)	14	--	15
Tetracycline (TE)	14	15-18	19
Trimethoprim/Sulfamethoxazole(SXT)	10	11-15	16
Ceftiofur (XNL)	14	15-17	18

APPENDIX E

EXAMPLE FOR DETERMINATION OF TOTAL WEEKS AT RISK AND RELATIVE RISK

COLLECTION TIMES T1	T2	T3	T4	B^+ following an A^+ (NUMPOS)	WAR following A^+ (DENPOS)	B^+ following an A (NUMNEG)	WAR following A^- (DENNEG)
1. A				0	6	0	0
2. A			B	1	6	0	0
3. A	B			1	2	0	0
4.		A		0	2	0	4
5.			A B	0	0	1	6
6. B		A		0	0	0	0
7.		A B	B	1	2	1	4
SUMS				3	12	2	14

A – primary agent isolated
B – agent isolated at later collections
NUMPOS - # cases of given isolate (B) following presence of isolate (A)
NUMNEG - # cases of given isolate (B) following no isolate (A) present
DENPOS –Weeks at risk (WAR) for isolate (B) following presence of primary isolate (A)
DENNEG - Weeks at risk (WAR) for isolate (B) following no primary isolate (A) present
Rate N - Occurrence of isolate (B) in the absence of the primary isolate, (A) in all cases per 10 weeks at risk (NUMNEG/DENNEG)
Rate P - Occurrence of isolate (B) in the presence of the primary isolate, (A) in all cases per 10 weeks at risk (NUMPOS/DENPOS)
RELATIVE RISK - RATE P/RATE N

APPENDIX F

MINIMUM INHIBITORY CONCENTRATION DETERMINATIONS FOR VARIOUS ANTIMICROBIAL AGENTS AGAINST BOVINE *MYCOPLASMA SPP.* ISOLATED FROM 14 CASES OF OTITIS MEDIA

Minimum inhibitory concentrations (ug/ml)

Antimicrobial agent	MIC_{50}	MIC_{90}	Range
Erythromycin	>64.0	>64.0	64.0->64.0
Spectinomycin	64.0	64.0	32.0->64.0
Lincomycin	16.0	64.0	≤8.0-64.0
Premafloxacin	≤0.03	≤0.03	NR[1]
Tilmicosin	≤0.03	≤0.03	NR[1]
Tetracycline	1.0	8.0	0.25-8.0
Lincomycin/Spectinomycin 1:4	16.0	>64.0	4.0->64.0

[1]No Range, all isolates yielded the same value

APPENDIX G

NORMAL VITAMIN A, E, AND SELENIUM VALUES IN BOVINE

VITAMIN A (SERUM)

AGE (DAYS)	NORMAL RANGE	DEFICIENT RANGE
1 - 9	50 - 150 NG/ML	<20 NG/ML
10 - 29	100 - 150 NG/ML	<75 NG/ML
30 - 300	125 - 250 NG/ML	<120 NG/ML

VITAMIN E:CHOLESTEROL RATIO (SERUM)

AGE (DAYS)	NORMAL RANGE	DEFICIENT RANGE
1 - 9	0.7 - 2.1	<0.2
10 - 29	0.7 - 2.0	<0.5
30 - 300	1.5 - 2.5	<.75

SELENIUM (WHOLE BLOOD)

AGE (DAYS)	NORMAL RANGE	DEFICIENT RANGE
1 - 9	100 - 150 NG/ML	<50 NG/ML
10 - 29	60 - 150 NG/ML	<40 NG/ML
30 – 300	60 - 150 NG/ML	<40 NG/ML

APPENDIX H

E. COLI STX ISOLATES TESTING POSITIVE BY PCR AND PREMIER EHEC

FARM	*E. COLI* GENE(S) PRESENT	PCR +	PREMIER TEST +
FARM A	EAEA/STX	13/19	12/19
	STX	6/19	5/19
FARM B	EAEA/STX	8/16	7/16
	STX	8/16	1/16
FARM C	EAEA/STX	9/27	8/27
	STX	17/27	10/27
FARM D	EAEA/STX	51/59	48/59
	STX	8/59	6/59
FARM E	EAEA/STX	0/8	-
	STX	7/8	1/8

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