

BOVINE REPRODUCTIVE INFECTIOUS DISEASE IN GHANA: PREVALENCE AND  
PATHOGENESIS OF EARLY INFECTION WITH AN EMPHASIS ON TRICHOMONIASIS.

by

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## **ABSTRACT**

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Reproductive inefficiency is one of the most costly and production limiting problems facing the livestock development in Africa. Reproductive performance is influenced by a number of important factors, among which are infectious diseases, which result in lost production. Research in these diseases is critical in order to overcome these shortfalls.

The objectives of the following studies were to provide a more in-depth epidemiological analysis of infectious reproductive diseases in Sub-Saharan Africa and further investigate the cytokine environment and molecular factors involved in the innate immune response of the bovine reproductive tract to infectious agents which can play such an important role in livestock production in Africa, in particular *Tritrichomonas foetus*.

The prevalence of important infectious reproductive diseases in a single herd using serology was investigated. Significantly, high seroprevalence of infectious bovine rhinotracheitis and trichomoniasis was demonstrated. The presence of coxiellosis, neosporosis, bovine viral diarrhea virus, and bovine herpesvirus-4, was demonstrated but not serological evidence of brucellosis. Significant associations were found between the seroprevalence of the different viral pathogens but no such association was noted between the other pathogens. The long calving interval in this herd could be due to the presence of these pathogens. Among the diseases we identified, we further studied trichomoniasis (*T. foetus*) and the host immune response in a

mouse model. Serum and reproductive tissues for Th1 and Th2 cytokines (specifically INF- $\gamma$ , TNF- $\alpha$ , IL-2, IL-4, and IL-5) via flow cytometry after 5 days of *T. foetus* infection were analyzed. Mice of varying ages which had different susceptibility to *T. foetus* infection were used to assess whether a Th1 or Th2 cytokine environment was significantly associated with successful infection. Both Th1 and Th2 cytokines (TNF- $\alpha$ , IL-4 and IL-2) were shown to be significantly altered in infected animals. Lastly, by using PCR and IHC techniques, the presence of the novel dual oxidase molecules (DUOX 1 and 2) in the reproductive tract of cows and in bovine endometrial and trophoblast cell cultures was documented. However, there was no significant difference in DUOX 1(P=0.296) or DUOX2 (P=0.480) expression in *T. foetus* infected cells versus their respective controls.

In conclusion, several important infectious diseases in Ghanaian cattle which may affect reproduction, not the least of which is trichomoniasis, were detected. Further, it was demonstrated that the early infection has both Th1 and Th2 cytokine involvement and that while present in the reproductive tract, there is no evidence that the DUOX molecules play a role in early resistance, but they may be important at other time points.

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## Chapter 1

### Introduction

Domestic food animals are susceptible to an array of viral, bacterial, protozoan, and metazoan pathogens which may decrease production efficiency or cause overt disease.

Prevention of diseases is often approached through identification of the causative agent and appropriate immunologic interventions such as vaccination or immunostimulation. To achieve this goal, a thorough understanding of the pathogens and the host resistance mechanisms particularly at the mucosal surfaces such as the gut, respiratory and genital tracts are required.

Sub-Saharan Africa is an excellent example of the economic losses that are possible due to infectious diseases; it is also an excellent example of the opportunities for intervention if the disease ecology, the pathogen, and the host are thoroughly understood. In particular, reproductive losses due to infertility limit the economic advancement of this part of the world, making the reproductive tract an important focal point for future studies. It is with this in mind, that the studies described in the following pages, were conducted (Figure 1).

First, the prevalence of important reproductive diseases in Ghanaian cattle was investigated. It was surprising that only a very few studies have been conducted in this region and only on a very few diseases. I investigated the presence of important reproductive diseases in a single herd of cattle, located at Apollonia near Accra in Ghana.

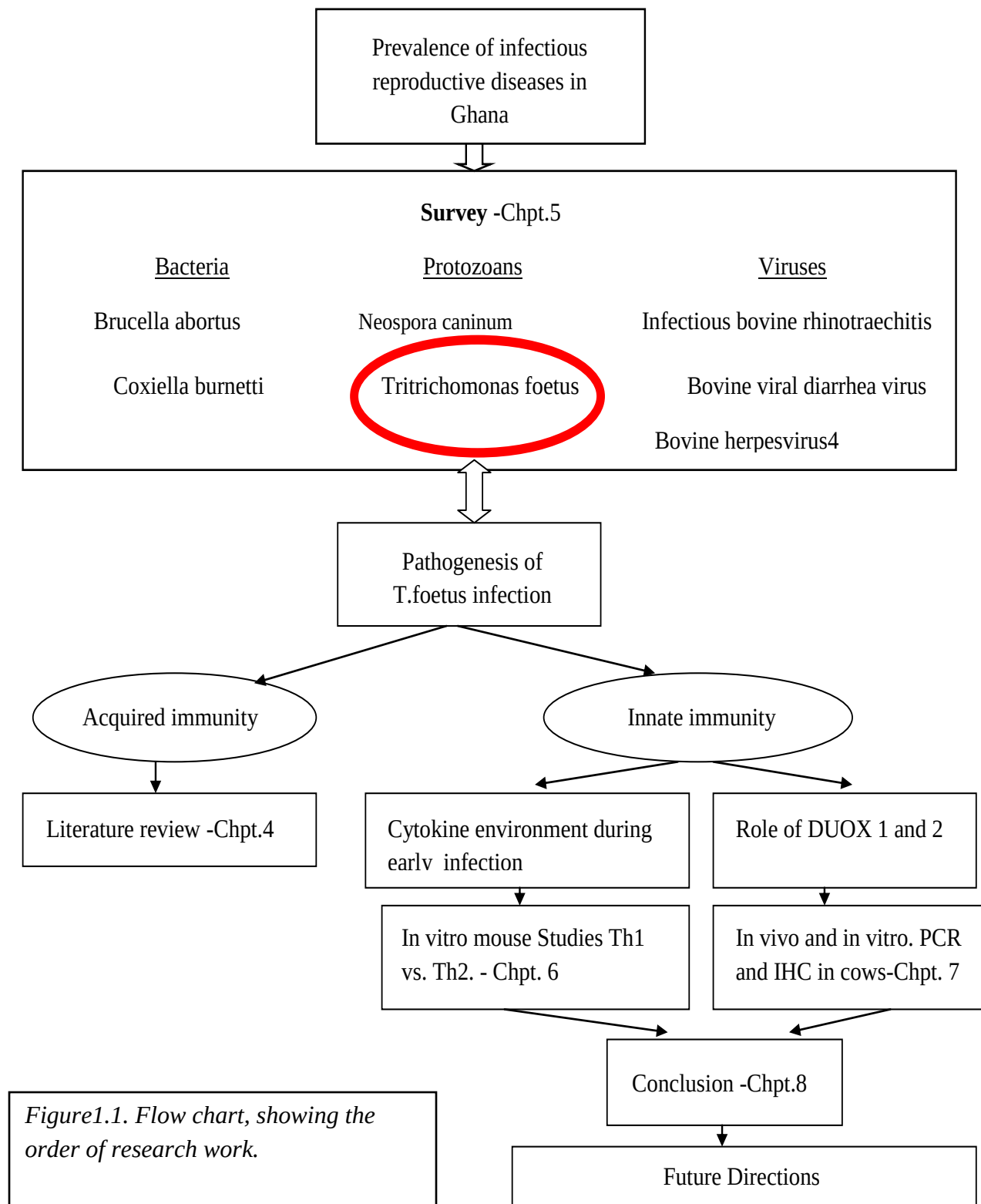
Among the diseases identified in Ghanaian cattle, trichomoniasis, caused by the protozoan *Tritrichomonas foetus* (*T. foetus*) was important. So, secondly using *T. foetus* as a model, the host immune response was studied, emphasizing resistance mechanisms which cooperate to establish protective responses at the mucosal surfaces of the reproductive tract *in vivo* at the earliest point of infection, with the goal to stop the initial colonization via the innate

immune system responses. Due to the challenges of using cattle as a live animal model, an established mouse model was used that showed differential infection rates in mice depending on their ages and an *in vitro* model of infection using bovine endometrial cell lines was developed.

The cytokine environment in infected mice during the early phases of infection was investigated, by assaying for Th1 and Th2 cytokines using flow cytometry in 4 and 8 week old mice, both infected and uninfected. If the cytokine environment can be characterized as to which is most resistant to initial infection, vaccines can be designed towards this goal.

A novel molecule identified in other mucosal organs as important in innate immunity, the dual oxidases (DUOX) 1 and 2 was also investigated. First, the presence of this molecule was documented in the mucosal surface of the reproductive tract in the bovine through PCR and immunohistochemistry. Their presence was demonstrated and an endometrial cell culture model of *T. fetus* infection was developed to look for differences in DUOX1 and DUOX2 expression between the *T. fetus* infected endometrial cells and the uninfected cells.

From these studies, I hoped to demonstrate the importance of both field epidemiological and bench investigations in understanding, and ultimately controlling, infectious reproductive diseases, with the goal of improving the efficiency of livestock production in Africa and around the world.



*Figure1.1. Flow chart, showing the order of research work.*

*For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.*

## Chapter 2

### Literature Review- Ghana

#### **Brief introduction to Ghana Agriculture**

Ghana is located in West Africa. It is bounded in the east by Togo, west by Cote d'Ivoire and the south by the Gulf of Guinea. It lies within 1 to 11 degrees north of the Equator and is bisected by the Greenwich meridian.<sup>6</sup> The climate is tropical, but temperatures vary with season and elevation. Except in the north, two rainy seasons occur, from April to July and from September to November. In the north, the rainy season starts from April to September (<http://www.ghanaweb.com/GhanaHomePage/geography/climate.php>; accessed 28 Jun 07). There are three principal types of vegetation from south to north: coastal savannah, forest zone, and northern savannah (<http://www.africanconservation.org/ghanaprofile.html>; accessed 30 Jun 07). The temperature varies between 22C to 33C annual mean, with an equatorial seasonal variation.<sup>6</sup> Many rivers drain the land including the Volta, which has been dammed to form the largest man-made lake.

Ghana is the first state in the Sub-Saharan Africa to gain political independence from the British rule in 1957, under the leadership of the late Dr. Kwame Nkrumah, the first president of the country, who was later overthrown by a military government. Ghana has since changed to a democratic rule and is considered as one of the leading democratic countries in Africa. The system of government is a constitutional democracy, which is made up of three arms: the executive (President, vice President, and 32 elected ministers), the legislature (parliament and its members), and the judicial (Supreme Court and the chief justice).



The total surface area of Ghana is about 239,460 sq. Km. with a population of 20 million, dominated by the Christian religion (<http://www.crawfurd.dk/africa/ghana.htm> access 30<sup>th</sup> June). It has different ethnic groups, with the following being the major groups and the languages spoken: the Akan- twi; the Ewe- ewe; the Mole Dagbane- molegabane; the Ga-Adangbe- GaAdangbe and the Guan-kyereponi and guan. English is the official language. The Akans are the major ethnic group occupying mainly from the south to the middle part of the country. From the southeast to a zone slightly to the north are the Ewes. Between the southeast and the west of the Accra coastlands are the Ga-Adangbes. The Mole Dangbane occupy the northern and upper part of the country. The Guans are small groups found in pockets in almost all the other communities. They are believed to have migrated from other countries to Ghana in search of jobs in the farming areas.<sup>4</sup> Though Ghana has different ethnics groups based on culture and geographical location, no part in Ghana is ethnically homogenous except perhaps in the villages. It is worth mentioning that, there is always a high degree of tolerance and understanding between the various ethnic groups as well as their dependence on God, which may explain the longstanding peace in Ghana. The country has 10 political administrative regions, and 110 administrative districts. All regions and districts co-ordinate their activities with the seat of the government in the capital, Accra.

## **2.1Agriculture in Ghana**

Agriculture plays a central role of the social and economic development of Ghana. The agricultural sector consists of 5 sub-sectors, which are; crops other than cocoa (63% of agricultural GDP), cocoa (14%), livestock (7%), fisheries (5%) and forestry (11%). (<http://www.ghanaweb.com>: Feature article of Saturday, 4 Feb. 2006 access 30th June 2007). Presently, the sector employs about 65% of the labor force, accounts for over 40% of the gross

domestic product (GDP), contributes about 57% of foreign exchange earnings and provides over 90% of the food needs of Ghana on sustainable basis. (<http://www.ghanaweb.com>: Feature article of Saturday, 4 Feb. 2006 access 30th June 2009). This sector also provides raw materials for the local industries such as soap, food and beverage industries and others.

(<http://www.ghanaweb.com>: Feature article of Saturday, 4 Feb. 2006 access 30th June 2009)

Agriculture practice in most part of Ghana is not scientifically based. Farmers, the majority of whom are women, depend on the rudimentary technology for production that always fails to maximize production.<sup>1</sup>

It is envisaged that, with the current rate of population growth in Ghana, it is expected to exceed 36 million by 2020. This means that, there will be pressure for more land acquisition there by reducing the agriculture land availability per capita from 0.80 hectares (1995) to 0.38 hectares (2020). The immediate solution to this is the government institution of a long term and a broad based national development policy, Vision 2020. This policy is to rapidly transform agriculture practices with the aim of meeting the needs of the anticipated population growth as well as to bring about overall economic growth and its effect on poverty.

(<http://www.ghanaweb.com>: Feature article of Saturday, 4 Feb. 2009 access 30th June 2010).

This policy more or less will depend on the international community for its achievement.

The peaceful political atmosphere and the stability in Ghana, make it a safe investment destination in Africa, and therefore it continues to attract more investors. The recent aid to support its agriculture policy is the amount of \$547 million Millennium Challenge Compact from the US government. Out of this, \$240 million shall be used to support the agricultural program, which is to benefit all types of farmers. This will involve seasonal credit to farmers, irrigation improvement, and the provision of storage and processing structures. To enhance easy

and inexpensive cost of transportation of agricultural products to the consumer market, \$143 million shall be used to improve major feeder roads in the farming communities and then \$100 million will go into expansion of community services and building of local government capacity (<http://www.state.gov/secretary/rm/2006/c18495.htm> access 30<sup>th</sup> June 2007).

The current Accelerated Agricultural Growth Development Strategy (AAGDS), under the livestock services program, has the goal to promote economic growth and social equity from the activities of small-scale producers'. Under the new Livestock Development Project, there is to be an investment more than \$40 million over the period 2004-2010 in a number of districts located in seven regions, focusing mainly on breed development and other husbandry support, animal health improvement, provision of credit and strengthening of farmers groups and general capacity building.<sup>2</sup> In line with this, Animal Production Department (APD), Veterinary Services Department (VSD), and the Livestock Planning and Information Unit (LPIU) have been mandated with specific duties such as: (i) breed improvement, (ii) Forage and pasture development, (iii) Stock water development, (iv) Disease control, (v) Immunization program, (vi) vaccine production (e.g., Anthrax spore vaccine), and (vii) continuous education for farmers.

The animal agricultural development program is supported by a number of research institutes in Ghana among which are the Animal Research Institute of the Council for Scientific and Industrial Research (CSIR), and the Animal Science Departments of the three Universities in Ghana. (Legon, KNUST and, Cape Coast).

Owing to the Accelerated Agricultural Growth Strategy, the government has instituted a decentralization policy of the Ministry of Food and Agriculture. In this regard, the VSD role is to assist in the formulation and implementation of projects and program to bring major economic

livestock diseases under control as well as encouraging the private sector to play active part in animal health delivery. This policy has empowered District Veterinary Officers to take appropriate decisions on disease control and surveillance at the district level and in turn report all cases to the national Technical Directorate of Veterinary Services. The government decentralization process and the Accelerated Growth Development Strategy are supposed to ensure sustainability of livestock program in Ghana.

## **2.2 Livestock in Ghana**

In the previous years, the livestock industry has received less than required attention. Comparing it to crop sector, there is a lack of sufficient information needed for economic analysis.<sup>9</sup>

Table 1 compares the changes in human and cattle population, and number of cattle per 100 people. From the table, there is a consistent declination of number of cattle per 100 persons, which shows that the growth in human population exceeds that of cattle resource base. The meaning of this is that there won't be enough meat for human consumption, and therefore there will be a need to import meat to supplement. With the current emphasis on animal agricultural development in Ghana, there is encouragement to revive and increase production. Among the important categories of animals targeted are: cattle, poultry, pigs and small ruminants (sheep and goats). A new area of livestock being encouraged is grasscutter (cane rat) and ostrich farming which is being sponsored by German Technical Cooperation Assistance (GTZ). Most of these, except for poultry, are reared with rudimentary technology. Approximate populations of livestock are listed in Table 2.2

### **2.2.1 Sheep and Goats**

Every farming household keeps them to supplement the family income. To supplement the inadequate local supply, there is significant importation from neighboring countries. About 80% of the small ruminant farmers practice small-scale farming, where they employ a semi intensive system.<sup>8</sup>

### **2.2.2 Pigs**

In the rural areas, the farmers keep mainly the local breed called Ashanti Black Forest Pig (ABFP), which are smaller in size and less docile. The commercial farms, which are mainly located closer to urban areas, keep imported breeds like the Large White, Landrace, Hampshire etc. Production in the north is limited due to the large Muslim population.

### **2.2.3 Poultry**

Eggs and poultry meat are important components of Ghanaian diets as a source of animal protein. The poultry sector provides employment for significant number of people in Ghana. Poultry farms are found in any part of the country. Small, medium, and large-scale producers form about 60%, 30% and 10% respectively of poultry farmers in the country. Rural poultry constitute about 80% of Ghana's poultry population. Poultry production, especially the broiler industry, is declining as the cost of production in Ghana cannot compete with chicken products imported from Europe, Brazil, and the United States.<sup>8</sup>

### **2.2.4 Fisheries**

Fish trading is more popular in the south, where the ocean serves as a major resource. In the inland sector, fish sources are the rivers and lakes. Per capita consumption is about 20kg representing 60% of all animal protein. Traditionally, smoking and salting is the mode for preservation for about 80% of fish caught. Freezing and canning are used mainly for those to be

exported. The fish industry supports up to 1.8 million people about 10% of the Ghanaian population with employment.<sup>8</sup>

### 2.2.5 Cattle

The majority of Ghanaians depend on beef for their source of protein. The current production of beef contributes less than 30% of the beef demanded nationwide.<sup>8</sup> Ghana thus depends on imports to make up its insufficiency. Therefore increasing domestic production is a goal for making it available at an affordable price for the average Ghanaian.

There are two main breeds of cattle in Ghana known as; (a) *Bos taurus* and (b) *Bos indicus*. The *Bos taurus* includes West Africa Short Horn (WASH), the most popular breed constituting about 60% of the total cattle population.<sup>10</sup> The name WASH is a general descriptive term covering all the variation of small non-humped indigenous cattle. They are generally black and white in color but sometimes fawn and white. Constitutionally, they are thick set with fine boned limbs (eg. Ndama, Muturu, etc). The other breed *Bos indicus* comprise of white Fulani and Zebu which were originally imported from Northern Nigeria to cross the WASH for improvement in size and level of milk production. The resulting genotype between the two is known as Sanga, which has features intermediate between the WASH and the White Fulani. Cattle production in Ghana is mostly sedentary and agro pastoral, rather than nomadic. Cattle distribution in Ghana varies from the South to the North. In the North savannah, cattle herd sizes are generally small, usually between 10 and 50 and may be owned by an individual, a family, or a village. In the South, herd size are quite large most often 50-1000. These animals are usually owned by an absentee owner and are cared by hired Fulani herdsman. In most cases these owners are businessmen, civil servants, or farmers. The herdsman are paid monthly or for

their remuneration, they are allowed to milk the herd and sell products. In some cases, the Fulani may also be given the third calf from any cow.<sup>5</sup>

The major constraint facing the cattle industry in Ghana is disease. Some of the diseases of economic importance are: contagious bovine pleuropneumonia, trypanosomiasis, babesiosis, anaplasmosis, dermatophilosis, and foot and mouth disease.<sup>9</sup> Rinderpest had been a significant disease, but was officially eradicated worldwide in 2010. The role of infectious reproductive diseases in Ghana, however, has not been well characterized, but as in developed countries, these likely play a major role.<sup>3, 7</sup>

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Table 2.1. Human population, Cattle population and number of Cattle per 100 persons in Ghana (1984-1996)

| Year | Humans (x 10) | Cattle head | Cattle per 100<br>persons |
|------|---------------|-------------|---------------------------|
| 1984 | 12.3          | 1.08        | 8.8                       |
| 1986 | 13.04         | 1.13        | 8.7                       |
| 1988 | 13.74         | 1.14        | 8.3                       |
| 1990 | 14.47         | 1.14        | 7.9                       |
| 1992 | 15.24         | 1.16        | 7.6                       |
| 1994 | 16.02         | 1.22        | 7.6                       |
| 1996 | 18.0          | 1.25        | 6.9                       |

Source: Otchere E.O and Okanta S.A "Smallholder dairy production and marketing".

The estimated livestock population in 2000 is indicated in Table 2:

Table 2.2: Livestock Population in Ghana

| Animal Species | Total Population |
|----------------|------------------|
| Cattle         | 1.5 million      |
| Sheep          | 2.5 million      |
| Goats          | 3.0 million      |
| Donkeys        | 14200            |
| Poultry        | 18 million       |
| Horses         | 3000             |

Source: Ghana veterinary services/ministry of agriculture (1996)" Livestock census" 1996

## Chapter 3

### Literature Review- Reproductive Infectious Diseases

#### **Reproductive infectious diseases of cattle in Ghana**

Despite the fact that, Ghana has been successful in controlling many serious epidemic infectious diseases, losses to productivity from other cattle diseases still remain high. These are some of the most costly and production-limiting problems facing the cattle industry in Ghana, which contributes to insufficient animal protein present in the average Ghanaian diet. Presently, only 5% of the average Ghanaian diet is composed of protein of animal origin; the rest is of carbohydrate sources.<sup>13</sup> While there are likely many inter-related factors involved in the lower than desired performance of the Ghanaian cattle industry, reproductive diseases are likely to be significant. Unfortunately, there is little information available on reproductive infectious diseases of cattle in Ghana.

In other countries where infectious reproductive diseases are well studied, they are known to create heavy economic losses mainly due to their insidious nature.<sup>5, 8, 23</sup> The most studied reproductive infectious disease pathogen in Ghana is *Brucella abortus*, and to a lesser degree, *Leptospira spp.*<sup>22</sup>

#### **3.1 Brucellosis**

Brucellosis in Ghana has been extensively studied, partly because of its effect on the extensive economic losses due to abortion and extended calving intervals and also due to its zoonotic nature. The disease in cattle is usually caused by *Brucella abortus*, and occasionally by other species, like *B. melitensis*, *B. suis*, *B. ovis*, and *B. canis*. Brucellosis is prevalent in many countries especially in the tropics. The prevalence of brucellosis in Africa has been variably

reported from zero to 100%<sup>4</sup>, where the wide range of variation was attributed to sample collection and diagnostic techniques. A survey of cattle in Ghana on the Accra plains indicated prevalence of 20%-30%, with high rates of abortion and stillbirth.<sup>22</sup> The disease can be transmitted through copulation. In the host, the bacteria are conveyed to the regional lymph nodes, leading to lymphadenitis. The organism is able to multiply in phagocytic cells like macrophages<sup>3</sup> and disseminate through systemic circulation to the other organs (spleen, lymph nodes, uterus, and mammary gland). During pregnancy, the bacteria localize in the uterus and cause ulceration of the endometrium. Both the membrane and the fetus respond to the infection by increasing the production of erythritol, a carbohydrate compound, which favors the multiplication of the bacteria. The bacterial multiplication spreads the lesions to the placental cotyledons and destroys the villi. Placental insufficiency eventually brings about abortion (typically during the last trimester), stillbirth, premature birth, or weak calves. Infections are life-long in the dam.<sup>6</sup>

### **3.2 Leptospirosis**

Leptospirosis is a bacterial disease characterized by reproductive failure such as abortion, stillbirth, reduced milk production, and delivery of weak offspring. It too, is an important zoonotic disease. The causative organism is *Leptospira sp.*, a spirochaete organism which can be found in many serotypes. Those serotypes found to be associated with bovine abortion are *Leptospira pomona*, *L. canicola*, *L. icterohaemorrhagic*, *L. grippotyphosa*, *L. hardjo*, and *L. borgpetersenii*.<sup>12, 19</sup> Infection is usually through the digestive tract as well as the reproductive tract, eyes, or skin. Following infection and bacteremia, the organism localizes in the kidneys and is shed in the urine, for weeks to years<sup>2</sup>. Direct or indirect contact with the urine of the infected animals is major route of infection, though there can be transmucosal transmission via the

reproductive, digestive, and respiratory tract. An important factor for occurrence of clinical signs is the immune status of the herd. In a previously uninfected herd, clinical signs may be seen in all ages.<sup>2</sup>

Leptospirosis has been little studied in Africa, except in a few places such as Ethiopia where Moch<sup>16</sup> described the prevalence of various domestic animals as: 91.2% in horses, 70.7% in cows, 57.1% in pigs, 47.3% in goats, 43.4% in sheep, 15.4% in camels, and 8.3% in dogs. In Kenya and Uganda, Ball<sup>1</sup> reported prevalence of 34% in cattle and 9.3% in goat samples. Twenty *Leptospira* serovars have been documented from previous studies; 11 of these belong to the species *Leptospira kircheri*, eight of which were found in Democratic Republic of Congo (Zaire); 2 in Kenya and one in Ghana.<sup>7</sup> Studies conducted in Tanzania also confirmed the high prevalence in that country.<sup>14</sup> The climatic and socio-economic environment of the African continent are likely important for the high incidence of the disease; Ghana is not exceptional in the importance of these conditions.

### **3.3 Campylobacteriosis**

Campylobacteriosis (vibriosis) is a venereal disease of cattle. The species most commonly involved in the bovine genital tract infection are *Campylobacter fetus* subspecies *venerealis* serotype A, *C. fetus* subspecies *fetus*. Serotype A and *C. fetus* subspecies *fetus* serotype B. Among these, infertility in cattle is usually associated with *C. f. venerealis* A (90%) and *C.f.fetus* B (10%) and only rarely *C.f. fetus* A and B<sup>27</sup> The disease prevalence in the tropics varies: in Malawi, for example, the incidence as reported by Klastrup and Halliwell<sup>18</sup> was 11.5% in 294 zebu and 11.1% of exotic bulls tested serologically. When the vaginal mucus agglutination test was performed on the cows, it showed that 53.8% of the herds and 13.4% of the samples were infected. When the same test was run in Zimbabwe, 33% of the cows sampled were found

to be positive<sup>10</sup>. Chronic infections are common, but acute infections can also be clinically relevant. Acute infections are associated with infertility and abortion. The chronic phase is usually associated only with abortion.<sup>9</sup> In Ghana, the prevalence of campylobacteriosis in cattle is not known; however, this does not rule out its presence since management techniques are similar across these countries, such as natural breeding. Under these conditions, bulls transmit the infection from one female to another. Spread of the organism to the male is primarily by way of copulation with an infected female. Bulls can remain carriers for up to 18 weeks after infection. Older bulls tend to retain the infection permanently in comparison to younger bulls possibly due to the increase in number and size of the crypts in epithelium of penis.

### **3.4 Infectious bovine rhinotracheitis**

Infectious bovine rhinotracheitis (IBR) is caused by a herpesvirus in the subfamily Alphaherpesvirinae. The infection is characterized by five clinical forms; respiratory, ocular, abortion, infectious pustular vulvovaginitis, and encephalitis depending on predilection site of the individual strain.<sup>25</sup> The disease can be transmitted sexually or by droplet inhalation and the incubation period is between 4 to 6 days. In venereal form it has been associated with infertility. In the respiratory form, there is hyperthermia with red nose (inflammation of the muzzle and the nostril), anorexia and depression. Mortality is rare unless it is complicated with secondary infections. Infectious vulvovaginitis is typically characterized by a fever and whitish discharge from the vulva. On examination of the vulva lips, there will be red spots and discrete pustules.<sup>25</sup> Inflammation of the prepuce and penis with pustular lesions and preputial exudates are seen in the males.<sup>25</sup> The abortion form is seen when infection is extended directly into the cervix or the uterus. This is often seen when cows are artificially inseminated with IBR contaminated semen. The infection of the uterus often affects the cow's estrous cycle and reduces fertility. The uterus

can also be infected from systemic spread from a respiratory infection. The disease incidence has not been reported in Africa; however, the study findings of International Livestock Center of Africa (ILCA) on Ethiopian indigenous zebu cattle (Tekelye Bekele et al, 1989b) showed more than 50% prevalence. The fact that Ghana shares similar climatic and socio-economic environmental factors with Ethiopia suggests that this disease may be significant in Ghana as well.

### **3.5 Bovine viral diarrhea**

Bovine Viral Disease (BVD) is caused by a virus, belonging to the Pestivirus genus within the family Flaviviridae.<sup>21</sup> Bovine viral diarrhea (BVD) is one of the most economically *important* diseases of cattle that has worldwide distribution and tend to be endemic in most populations.<sup>11</sup> The clinical signs of BVD are often subtle, but the clinical disease syndromes can be grouped into three categories: acute BVD, *in utero* infections, and diseases in persistently infected (PI) animals.<sup>26</sup> Acute BVD can vary greatly in presentation from fever, depression, and runny nose and eyes, to diarrhea and respiratory disease. *In utero* infections with BVDV can result in abortion, persistently infected animals, congenital defects, or normal, immuned calves.<sup>20, 24</sup> Persistently infected animals can result from *in utero* infection as described above, or by birth from a PI dam. The spread of BVDV infection through a herd is mainly by PI animals; such animals harbor the virus for life and shed it in high concentration without showing any immune response. In Africa, a few studies conducted on seroprevalence of the disease have indicated that the prevalence was varied from 70–83%.<sup>17</sup> In Ghana, there is no research or published information available on this disease.



### 3.6 Trichomoniasis

Trichomoniasis is an insidious, economically damaging venereal disease of cattle with a worldwide impact. The disease is caused by *Tritrichomonas foetus*. A report looking at samples from most south African countries indicated that, out of the 3,458 samples that were tested for *T. foetus*, 142 (approximately 4.1%) were positive.<sup>15</sup> Trichomoniasis has been found to be related to the form of management practices. No study on tritrichomoniasis in Ghana has been conducted, though the management practices typical of Ghanaian farmers would likely favor the spread of this disease. This is the reason for the following study.

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## Chapter 4

### Literature Review-Bovine Trichomoniasis

#### **Trichomoniasis as a significant disease of cattle in Ghana**

Trichomoniasis is an insidious, economically damaging venereal disease of cattle with a worldwide impact. The disease is caused by *Tritrichomonas foetus*. In culture, *T. foetus* is spindle to teardrop-shaped with a body dimension of 13µm in length and 4 µm in width. *Tritrichomonas foetus* has three anterior flagella originating from the basal body and one recurrent flagellum forming the undulating membrane along the surface of the cell and exiting through the posterior end.<sup>6, 25</sup> Protruding out of the posterior end is the axostyle, a single ribbon of highly ordered microtubules spanning the entire length of the parasite. with an undulating membrane. *Tritrichomonas foetus* is grouped into the phylum *Parabasalia*, the order *Trichomonadidia*, and into the family *Trichomonadidae*.

The economic effect of the disease is attributed to the increased days open, reduced calf crop, reduced average weaning weight because of prolonged conception, culling of infected animals, and the cost of control and preventive procedures.<sup>28, 41, 46</sup> The disease may lead to metritis, early embryonic death, abortion, pyometra and infertility.<sup>8, 20</sup> Due to the insidious nature of the disease once the chronic state is achieved, the owners and herdsmen are often unaware of the presence of the disease in their herds.<sup>4</sup> Based on the pathology of the disease and virulence mechanisms, *Trichomonas vaginalis*, the causative agent of human trichomoniasis, is closely related family member to *T. foetus*. Due to their similarity, *T. foetus* infection in cattle has been proposed for use as an animal model to study the human pathogen and disease.<sup>21</sup> Trichomoniasis may directly cause the death of the embryo or may do so through uterine endometritis and marked leucocytic diapedesis into the endometrium.<sup>60</sup> While trichomoniasis of

cattle has been actively studied in the Americas and to some extent in South Africa, there has been limited work in sub-Saharan Africa, in particular Ghana.

No studies have been done on it partly due to the complex and sometimes time consuming nature of diagnosis. Also there is a perception that this disease is not present, or if present, it has no serious economic consequence and therefore no urgency to study it. This perception may allow trichomoniasis to spread and exert its economic effect on the cattle industry. Diseases such as brucellosis, vibriosis, and leptospirosis have been well documented and may over shadow *T. foetus* infection. In fact, the possibility of dual infection may be under appreciated in many clinical cases. However, in Argentina, dual infection rate in ranch bulls was found to be as high as 11.6% in those areas with extensive cattle raising and natural breeding.<sup>16</sup> Similar to Argentina, Asia, and Africa, including Ghana, may also have a high prevalence of trichomoniasis combine with campylobacteriosis.<sup>16 40</sup>

*Trichomonas foetus* infection has been found to be related to the form of breeding management practiced. Prevalence of the disease is higher in those areas, where natural breeding is practiced.<sup>19, 31, 61, 7</sup> The percentage of infection from a study survey is reported to be from 7 to 15.8%.<sup>10, 57</sup> Cattle management in Ghana is similar to that of other areas where trichomoniasis is endemic; i.e., (i) natural mode of breeding, (artificial insemination is rarely used); (ii) breeding bulls are maintained longer (over 6years); (iii) communal bulls are common; (iv) year round breeding; and (v) *T. foetus* infection is not routinely investigated.

The lack of accurate data on the level of the infection in Ghana presents a fundamental challenge to establishing a control program. As a consequence of this, the disease may continue to multiply, insidiously and negatively impacting the livestock industry in Ghana.

The need to survey trichomoniasis and institute effective management and control measures has potential human health implication. Human trichomoniasis has attracted more attention of late, because it is known to predispose to human immunodeficiency (HIV) infection and cause an abnormal outcome of pregnancy.<sup>27, 35</sup> Until recently, trichomonads were thought to exhibit host specificity, and that *T.foetus* is associated in nature only with bovine disease such as vaginitis. However, it has been confirmed that this parasite is causing feline diarrhea.<sup>39</sup> In humans, there have been only two cases of *T. foetus* like infection. In the first case, this was manifested in the form of meningoencephalitis in a recipient of allogeneic peripheral blood stem cell transplantation.<sup>42</sup> The second case was a patient with AIDS, in association with *Pneumocystis pneumonia*.<sup>22</sup> The implication is that *T. foetus* can cross infect human host which is a threat to veterinarians, farm attendants, abattoir, and laboratory workers from direct contact with infected animals or animal tissues and potentially to the general public if the mode of infection is through some other unknown mechanism.

#### **4.1 Mechanism of immune response in tritrichomoniasis**

Vaginal and uterine mucosae are portals for sexually transmitted diseases (STD) including tritrichomoniasis in both humans and animals, the success of which is influenced by number of factors among which are: extensive layer of mucus, nutrient deficiency, antibody response, constant flow of vaginal fluid and normal flora as being significant during the infection.<sup>17</sup> Macrophage and polymorphonuclear cells (PMN) respond to the site of infection as the first line of defense in a host immune response, which usually results in inflammatory processes including leukorrhea.<sup>48</sup>

Histopathologic studies conducted to examine the changes during early experimental infections in virgin heifers showed inflammation as predominately occurring after 60 days of

infection,<sup>44</sup> with neutrophils and macrophages as the major cellular infiltrates in the endometrium, stratum compactum, glandular epithelium and the uterine lumen. Following the recognition of the parasite, immune cells release pro-inflammatory molecules including tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukins (IL-1, IL-6, IL-8, IL-12) and nitric oxide.<sup>5</sup> These molecules aid the recruitment and activation of more immune cells and stimulate hepatic secretion of acute phase proteins leading to an intense antibody response, mast cell degranulation, and eventually clearance of the parasite. Leukocyte emigration to the site of inflammation is aimed at killing the parasites by the action of released enzymes.

In a study using *T. vaginalis*, a ratio of 10 PMNs to 1 *T. vaginalis* organism was shown to kill the parasite by degrading it into pieces and phagocytized it.<sup>48</sup> Neutrophil importance in *T. vaginalis* infection has been determined to be dependent on the parasite's ability to inactivate the complement involved, through alternate pathway other than that which involves antibody mechanism for killing the parasite. This was demonstrated in a study during which *T. vaginalis* was killed in the absence of parasite-specific antibodies or agglutination; complement from non-immune sera source was included to serve as an indicator.<sup>48</sup> In contrast, for *T. foetus* to be killed by neutrophilic action, parasite-specific antibody and complement are essential.<sup>3</sup>

Studies so far have concentrated largely on the acquired immune response to *T. foetus* and *T. vaginalis*. However, the initiation of the innate immune response and the role of early inflammation in *T. foetus* infection remain less well understood. Studies conducted so far suggest that the inflammatory process during early infection with *T. vaginalis* is stimulated by Toll like receptors-4 (TLR-4) with an unknown ligand, (possibly lipophosphoglycan (LPG)). The TLR-4 is sensitized only by the presence of *T. vaginalis*.<sup>62</sup> In order to understand and delineate the whole mechanism involving the innate response to *T. foetus* and *T. vaginalis*, more research



needs to be done. First a brief overview of what is known about the acquired immune response to *T. foetus* and other trichomonads will be followed by a more in depth review of the innate immune response in general and then more specifically *about* the innate immune response against *T. foetus*.

## **4.2 Acquired immunity**

Specific acquired immunity towards antigens of *T. foetus* has been well studied. In particular, the antibody response towards antigens of *T. foetus* has been reported to act via complement lysis as well as agglutination and opsonization of the parasites.<sup>13, 29</sup> When animals are infected naturally, protection and clearance are based on strong vaginal and uterine IgA and IgG1 production.<sup>58</sup> Vaccination studies have focused on local IgA and IgG1 responses; a vaccination trial indicated enhanced local IgA and IgG1 responses in addition to high serum levels of IgG1 and IgG2 when a surface antigen, TF1.17 was used.<sup>9</sup> In another study, it was noted that, following *T. foetus* vaccination using a whole cell antigen preparation, there was a strong uterine IgA antibody response which correlated to faster clearance of *T. foetus* and less histological evidence of endometritis.<sup>2</sup> Skirrow and BonDurant<sup>58</sup> reported the clearance of vaginal infection to be more linked with the increase of the vaginal antibodies (local) than that of systemic response. The important role of *T. foetus* membrane antigen in providing protection as well as curing infected animals through systemic immunization has also been reported.<sup>18</sup>

Lymphocyte subsets like CD4+/CD8+ T cells have been found to mediate the acquired immune response in microbial infections. CD4+ T cells respond by up-regulating expression of interferon (IFN- $\gamma$ ). Jovanka M. et al (2001) proved that CD4+ T cells which were induced by a purified and functionally characterized antigen of *T. foetus* (so-called Tf190) increased macrophage activation and consequently suppressed parasite growth. This suggests that Tf190

complex (also associated with *T. foetus* surface lipophosphoglycan (LPG)) contains T cell epitopes, a similar finding to LPG complex of *Leishmania spp.*<sup>53</sup>

In one experiment, peripheral blood mononuclear cells (PBMC) previously exposed to purified Tf190, had a phenotypic switch to more CD4<sup>+</sup> cells after re-exposure to Tf190. This was contrary to the case with PBMC exposed to whole *T. foetus* preparations under the similar conditions, implying that immunization with Tf190 induces CD4<sup>+</sup> T cells which increase during re-infection of the parasite. Also, it shows that immunizing cattle with Tf190 can prime a specific subset of CD4<sup>+</sup> T cells, including inflammatory T cells and T cells responsible for facilitating antibody.<sup>1, 11</sup>

### **4.3 General overview of innate immunity**

Innate immune response is universal and evolutionarily conserved mechanism of host defense against infection. Development of the innate response predates the development of the acquired immune response and it is found in all multi-cellular organisms. It is composed of receptors and effectors with ancient lineage. Defects in innate immunity are very rare and almost always lethal. The innate immune system is an evolutionary relic whose major function in higher vertebrates is to contain the infection until the adaptive immune response sets in. It has the following components:

### **4.4 Anatomical barriers**

#### **4.4.1 Mechanical factors:**

These are mechanical factors like squamous epithelial surfaces which form a physical barrier that is impermeable to most infectious agents. The skin for example acts as first line of defense against invading organisms. The desquamation of skin epithelium also helps remove bacteria and other infectious agents that have adhered to the epithelial surfaces. Movement due

to cilia or peristalsis helps to keep air passages and the gastrointestinal tract free from microorganisms. The flushing action of tears and saliva helps prevent infection of the eyes and mouth. The trapping effect of mucus that lines the respiratory and gastrointestinal tract helps protect the lungs and digestive systems from infection. All these same factors are seen in the female reproductive tract. For example, tight epithelial junctions, mucosal secretions, and myometrial contractions are important factors in preventing microbial access to tissues in cow.

#### 4.4.2 Chemical factors:

Fatty acids in sweat inhibit the growth of bacteria. Lysozyme and phospholipase found in tears, saliva and nasal secretions can breakdown the cell wall of bacteria and destabilize bacterial membranes. The low pH of sweat and gastric secretions prevents growth of bacteria. Defensins (low molecular weight proteins) found in the lung and gastrointestinal tract have antimicrobial activity. Surfactants in the lung can act as opsonins (substances that promote phagocytosis of particles by phagocytic cells). In the reproductive tract of cow, vaginal commensals such as *Lactobacillus* spp. produce lactic acid and hydrogen peroxide to create a low (< 5) pH environment. This helps to protect the lower reproductive tract from pathogenic micro-organisms and thereby reduces the possibility of infection reaching the upper reproductive tract.<sup>45</sup> In addition to this, there are defensins which have antimicrobial properties.

#### 4.4. 3 Biological factors:

The normal flora of the skin and the gastrointestinal tract can prevent the colonization of pathogenic bacteria by secreting toxic substances or by competing with pathogenic bacteria for nutrients or attachment to cell surfaces. Normal flora has also been shown to be critical in the protection of the reproductive tract, in particular, the vagina.<sup>45</sup>

## **4.5 Humoral barriers**

### 4.5.1 Acute inflammation:

Humoral factors, here defined as blood-borne factors, including but not limited to antibodies, which play an important role in inflammation, are involved early in edema formation and the recruitment of phagocytic cells. These humoral factors are found in serum or they are formed at the site of infection. Only later are antibodies produced.

### 4.5.2 Complement system:

This forms a major humoral defense mechanism which in the early immune response is non-specific. Complement activation early in the immune response can lead to increased vascular permeability, recruitment of phagocytic cells, and lysis and opsonization of bacteria (considered the “alternate” complement system). Later, during or after the acquired immune response takes hold, complement interacts with antibodies to provide very specific killing functions. Complement has been shown within the vaginal lumen and likely plays an important role in the control of pathogens, particularly *T. foetus*.<sup>32</sup>

### 4.5.3 Coagulation system:

Some products of the coagulation system can contribute to the non-specific defense of a mucosal barrier due to their ability to increase vascular permeability and act as chemotactic agents for phagocytic cells. Also some of the products of the coagulation system can have non-specific antimicrobial functions. For example, beta-lysine protein produced by platelets during coagulation can lyse many Gram-positive bacteria by acting as a cationic detergent.

### 4.5.4 Iron:

Iron serves as an essential nutrient for bacteria. By binding to and sequestering iron, lactoferrin and transferrin limit bacterial growth. The interaction at the mucosal level with iron,

however, can be complicated, as *T. vaginalis* has been shown to penetrate mucin layers more effectively in an iron deficient environment.<sup>37</sup>

## **4.6 Cellular barriers**

### 4.6.1 Inflammation:

One early role of the inflammatory response is recruitment of neutrophils, eosinophils, and macrophages to sites of infection as the main line of defense in the non-specific innate immune system.

### 4.6.2 Neutrophils and eosinophils:

Neutrophils are recruited to the site of infection where they phagocytose invading organisms and kill them intracellularly. In addition, neutrophils can lead to collateral tissue damage to other pathogens and adjacent healthy tissue, sometimes compounding the problem. Eosinophils, more commonly associated with allergic diseases, hypersensitivity, or parasite infections have proteins in granules that are also effective in killing certain parasites.

### 4.6.3 Macrophages:

Tissue macrophages and newly recruited monocytes, which differentiate into macrophages, also function in phagocytosis and intracellular killing of microorganisms. In addition, macrophages are capable of extracellular killing of infected or altered self target cells. Macrophages also can contribute to tissue repair and act as antigen-presenting cells, required for the induction of specific immune responses.

### 4.6.4 Natural killer (NK) and lymphokine activated killer cells:

These cells can nonspecifically kill virus infected and tumor cells. They are not part of the inflammatory response but they are important in nonspecific immunity to viral infections and

tumor surveillance. There is some debate as to whether these cells exist at all in cows, but granular lymphocytes may play a similar role.<sup>36</sup>

#### **4.7 Cytokines in innate immunity**

Cytokines are the hormonal messengers responsible for most of the biological effects in the immune system, such as cell mediated immunity and allergic type responses. The effect of cytokines depends on the balance of Th1 and Th2 pathways based on the “T-helper” cells involved in the process. In mice, Th1 cytokines is typically IFN- $\gamma$ , while Th2 cytokines include IL-2, IL-4, IL-5, and IL-8. Some cytokines, like TNF- $\alpha$  can induce inflammation in either milieu.

The Th1/Th2 pathway paradigm in bovine system is not as well defined as it is in other species. Sometimes this response seems to be heterogeneous, inducing both Th1- and Th2-like responses, whereas in other examples predominant Th1 or Th2 responses occur.<sup>12</sup> For example, T-helper responses induced in Th clones against the rhoptry-associated protein-1 of *Babesia bigemina* are biased toward Th1, as characterized by a strong IFN- $\gamma$  response and a weak IL-4 response.<sup>51</sup> In contrast, clonal T helper responses to *Fasciola hepatica*-infected cattle were characterized by strong IL-4 responses and weak IFN- $\gamma$ .<sup>12</sup>

#### **4.8 Phagocyte response to infection**

##### **4.8.1 Circulating neutrophils and monocytes:**

These cells respond to antigen signals generated at the site of an infection. Such signals include N-formyl-methionine containing peptides released by bacteria, clotting system peptides, complement products and cytokines released from tissue macrophages that have encountered bacteria in tissue. Most of these cells stimulate endothelial cells near the site of the infection to express cell adhesion molecules such as ICAM-1 and selectins which bind to components on the

surface of phagocytic cells and cause the phagocytes to adhere to the endothelium. Vasodilators produced at the site of infection cause the junctions between endothelial cells to loosen and the phagocytes then cross the endothelial barrier. Once in the tissue spaces some of the signals attract phagocytes to the infection site by chemotaxis. The signals also activate the phagocytes, which results in increased phagocytosis and intracellular killing of the invading organisms.

#### 4.8.2 Phagocytosis:

Phagocytic cells have a variety of receptors on their cell membranes to which infectious agents bind to these cells. These include Fc receptors, complement receptors, scavenger receptors, and toll-like receptors. After attachment to a bacterium, the phagocyte engulfs the bacteria and the bacterium is enclosed in a phagosome. During the process, the granules or lysosomes of the phagocyte fuse with the phagosome and empty their contents. The result is a bacterium engulfed in a phagolysosome which contains the contents of the granules or lysosomes.

#### 4.8.3 Respiratory burst:

The process of phagocytosis is accompanied by an increase in glucose and oxygen consumption, referred to as the “respiratory burst.” The respiratory burst results in the production of number of oxygen-containing compounds which kill the organism being phagocytosed. The respiratory burst can take on different forms:

*Oxygen-dependent, myeloperoxidase (MPO)-independent intracellular killing:* During this process, glucose is metabolized via the pentose monophosphate shunt and NADPH is formed. Cytochrome B, which was part of the granule, combines with the plasma membrane NADPH oxidase and activates it. The activated NADPH oxidase uses oxygen to oxidize the NADPH. The result is the production of superoxide anion. Some of the

superoxide anion is converted to  $H_2O_2$  and singlet oxygen by superoxide dismutase.<sup>23, 47,</sup>

<sup>52</sup> In addition, superoxide anion can react with  $H_2O_2$  resulting in the formation of hydroxyl radical and more singlet oxygen. The result of all of these reactions is the production of the toxic oxygen compounds superoxide anion ( $O_2^-$ ),  $H_2O_2$ , singlet oxygen ( $^1O_2$ ) and hydroxyl radical ( $OH\bullet$ ).

Oxygen-dependent, MPO-dependent intracellular killing: In this type, the azurophilic granules fuse with the phagosome and myeloperoxidase is released into the phagolysosome. MPO utilizes  $H_2O_2$  and halide ions (usually  $Cl^-$ ) to produce hypochlorite, a highly toxic substance.<sup>24,30,33,34</sup> Some of the hypochlorite can spontaneously break down to yield singlet oxygen. The result of these reactions is the production of toxic hypochlorite ( $OCl^-$ ) and singlet oxygen ( $^1O_2$ ).

Detoxification reactions: neutrophils and macrophages have means to protect themselves from the toxic oxygen intermediates. These reactions involve the dismutation of superoxide anion to hydrogen peroxide by superoxide dismutase and the conversion of hydrogen peroxide to water by catalase.<sup>30</sup>

Oxygen-independent intracellular killing: In addition to the oxygen-dependent mechanisms of killing there are also oxygen-independent killing mechanisms in phagocytes.<sup>23, 52</sup> Cationic proteins (cathepsin) released into the phagolysosome can damage bacterial membranes; lysozyme breaks down bacterial cell walls; lactoferrin chelates iron, which deprives bacteria of this required nutrient; hydrolytic enzymes break down bacterial proteins. Thus, even patients who have defects in the oxygen-dependent killing pathways are able to kill bacteria. However, since the oxygen-dependent



mechanisms are much more efficient in killing, patients with defects in these pathways are more susceptible and get more serious infections.

#### 4.8.4 Nitric oxide-dependent:

Binding of bacteria to macrophages, particularly binding via Toll-like receptors (see below), results in the production of TNF-alpha, which acts in an autocrine manner to induce the expression of the inducible nitric oxide synthetase gene (i-nos) resulting in the production of nitric oxide (NO). If the cell is also exposed to interferon gamma (IFN-gamma) additional nitric oxide will be produced. Nitric oxide released by the cell is toxic and can kill microorganisms in the vicinity of the macrophage.<sup>26</sup>

#### **4.9 Non-specific killer cells**

They include several cells like NK cells, activated macrophages, eosinophils, and mast cells. These cells are capable of killing foreign and altered self target cells in a non-specific manner.

In the innate response to virus infection and altered self (transformed cells) NK cells utilize two kinds of receptors on their surface: NK receptor and inhibitory receptor. When the NK receptor encounters its ligand on a target cell, the NK cell is signaled to kill. However, if the inhibitory receptor also binds its ligand (MHC class I) then the killing signal is repressed. Normal cells constitutively express MHC class I on their surface; however, virus infected and transformed cells down regulate expression of MHC class I. Thus, NK cells selectively kill virus-infected and transformed cells while sparing normal cells. Innate responses to extracellular microorganisms (parasites) also include eosinophils, a specialized group of cells with the ability to engage and damage large extracellular parasites such as schistosomes. Activated eosinophils release their granule components including major basic protein, eosinophil peroxidase (a cationic

hemoprotein), and eosinophil cationic protein (a ribonuclease that is an eosinophil-specific toxin that is very potent at killing many parasites).

#### **4.10 Pathogen determinants recognized by the innate immune response**

These are determinants recognized by components of the innate (non-specific) immune system which differ from those recognized by the adaptive (specific) immune system. Antibodies and B and T cell receptors recognize discrete determinants and demonstrate a high degree of specificity, enabling the adaptive immune system to recognize and react to a particular pathogen. In contrast, components of the innate immune system recognize broad molecular patterns found in pathogens but not in the host. Thus, they lack the high degree of specificity seen in the adaptive immune system. The broad molecular patterns recognized by the innate immune system have been called PAMPS (pathogen-associated molecular patterns) and the receptors for PAMPS are called PRRs (pattern recognition receptors). A particular PRR can recognize a molecular pattern that may be present on a number of different pathogens enabling the receptor to recognize a variety of different pathogens.

#### **4.11 *Tritrichomonas foetus* innate immunity**

Vaginal and uterine mucosa is the portal entry for sexually transmitted diseases (STD) like trichomoniasis. *Tritrichomonas foetus* is an extracellular organism and infection in cows is generally self-limiting; because of this, animal health researchers have focused on the role of acquired humoral immune responses on resistance rather than the acquired immunity responsible for protection against *T. foetus*. Host and parasite-mediated factors likely influence the initial colonization, development of disease, and the ultimate outcome of infection. For successful colonization in the female reproductive tract, *T. foetus* requires secreted proteases<sup>14, 38, 59</sup> and adhesion to the surface of the host cell.<sup>38</sup> Glycoconjugates on the surface of *T. foetus*, such as

lipophosphoglycan (TF-LPG), facilitate parasite adhesion to bovine vaginal epithelial cells,<sup>56</sup> with subsequent contact-dependent parasite-mediated cytotoxicity.<sup>15</sup>

In a study examining histopathologic changes during early infection in virgin heifers, inflammation was said to occur usually after 60 days of infection.<sup>44</sup> In this study, predominant cellular infiltrates were found to be neutrophils and macrophages within the endometrium, stratum compactum, glandular epithelium, and the uterine lumen. In related studies that examined aborted fetuses from infected cattle, it was noted that macrophages and neutrophils played an important role during infection.<sup>49, 50</sup> In this study, tissues where parasites were found, were usually associated with heavy neutrophil and macrophage infiltration. Also, phagocytized trichomonads were seen within macrophages and giant cells. In a study of neutrophil killing mechanism of *T. foetus* and *T. vaginalis*, it was noted that neutrophils kills *T. vaginalis* without parasite-specific antibodies or agglutination, however, complement derived from non-immune sera was required.<sup>48</sup> On the other hand, neutrophil killing of *T. foetus* was noted to be most effective when parasite-specific antibody and complement were present.<sup>3</sup> In other studies, nitric oxide was shown to be an effector of macrophage-mediated cytotoxicity towards the human pathogen *T. vaginalis*.<sup>43</sup> This study was the first to demonstrate reactive nitrogen species (RNS) as were able to kill the extracellular venereal parasites; though, this did not implicate RNS as being critical in the prevention of the establishment of trichomonad infection within the reproductive tract.

Mechanisms that initiate innate responses and inflammation during *T. foetus* infection are currently unknown. Studies using the human pathogen *T. vaginalis*, indicated that inflammation during infection is caused by stimulation of Toll-like receptor 4 (TLR-4) with unknown substances present only during infection with *T. vaginalis*.<sup>62</sup> This study did not directly implicate

*T. vaginalis*/TLR-4 engagement; however, it pointed to a mechanism whereby inflammation is induced. Related studies with *T. vaginalis* have demonstrated that the parasite has the ability to induce IL-8 production from both human monocytes<sup>55</sup> and human neutrophils.<sup>54</sup> Studies with *T. vaginalis* indicate there are most likely similar mechanisms whereby *T. foetus* is also able to initiate inflammation within the bovine host. Thus, factors such as proteases and TF-LPG, which facilitate parasite colonization, are directly affected by the host immune response.

Since most studies have been focusing on acquired immune responses in trichomoniasis, information such as how the innate response affects early colonization and establishment of infection *in vivo* is not available. Understanding the mechanism of effective early immune response could curtail *T. foetus* infection and other similar resultant pathogens.

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## Chapter 5

### Field Survey of Some Selected Infectious Reproductive Diseases in Ghanaian Cattle

#### **Abstract**

Infectious reproductive diseases of cattle can severely impact the economic viability of the African rural sector. Despite this importance, relatively little is known about the prevalence of reproductive pathogens in African cattle. To emphasize the importance of these diseases, we carried out a cross-sectional study on a cattle herd with decreased fertility (calving interval of 2.5 years; instead of 1.15 years under improved conditions for evidence of seven infectious reproductive diseases: bovine viral diarrhoea virus (BVDV), infectious bovine rhinotracheitis (IBR), bovine herpesvirus-4 (BHV-4), *Tritrichomonas foetus* (Tf), *Coxiella burnetii* (Cb), *Neospora caninum* (Nc), and *Brucella abortus* (Ba). The herd was composed of over 700 cattle from which 18 bulls and 148 cows (2 to 15 years old) were tested. Serum antibodies (ELISA) indicated exposure to IBR (69.0%), BVDV (13.0%), BHV-4 (20.6%), Cb (6.1%), Nc (17.7%), and Ba (0%), and a hemolytic antibody assay detected Tf in 48% of these 166 animals. A significant association was found between all the viral pathogens but not between the other pathogens. This study, the first in Ghana looking at a range of infectious agents, underscores the importance of reproductive infections as potentially limiting factors in livestock production in Africa.

## Introduction

Conditions that interfere with reproduction in cattle are of great economic importance to the rural sector of developing countries, particularly those in Africa. The reproductive rate, as measured by the calving interval, is very often lower in African cattle than in those of more developed countries.<sup>16, 23</sup> This represents a major economic loss for the farmer and the country as a whole. Although infectious conditions, including viruses, bacteria and protozoa, are likely to be major contributors to poor calving intervals in Africa, relatively few studies have been carried out across this vast and rurally oriented continent. To emphasize the need for more information on the prevalence and role of infectious conditions of the reproductive tract in Africa, we have examined the profile of a typical West Africa cattle herd in Ghana. This is the first simultaneous study of multiple infectious agents in Ghanaian cattle.

A range of infectious agents can reduce the calving rate of cattle by causing early embryonic loss, abortion, multiple returns to estrus, and general infertility. Associated with these reproductive losses are decreases in milk production, untimely weaning and poor weight gain in calves, all of which can result in considerable economic loss through reductions in the overall productivity of livestock. In countries where infectious reproductive diseases have been more thoroughly studied, they are known to create heavy economic losses, in part due to their insidious nature.<sup>7, 12, 27, 29</sup>

Viral reproductive diseases are likely to be of importance in Ghana. Infectious bovine rhinotraechnitis (IBR), a herpes virus infection, can affect a wide range of tissues including the reproductive tract where it can cause vulvo-vaginitis, retained placenta, and abortion.<sup>22</sup> Infection of the reproductive tract occurs from either systemic spread of a respiratory infection or from direct inoculation of the cervix or the uterus during artificially insemination with contaminated

semen or during breeding with an infected bull. Bovine herpesvirus-4 (BVH-4) is known to induce a postpartum metritis and pelvic inflammatory disease and is likely to contribute to long-term reproductive tract problems.<sup>15</sup> BVDV affects domestic and wild ruminant species worldwide with a serological prevalence often more than 60%.<sup>14 18 32</sup> Bovine viral diarrhea virus can lead to abortion, stillbirths, congenital defects, and neonatal death, as well as the more widely recognized immunocompromising and diarrheal diseases of older cattle.<sup>14</sup>

Bacteria are also significant causes of reproductive tract infection. *Coxiella burnetii*, an intracellular pathogen causing Q fever, is an important reproductive infectious agent distributed worldwide with the exception of New Zealand.<sup>2, 28, 34</sup> The disease, typically spread via the products of abortion, affects all domesticated ruminants, primarily goats and sheep, causing reproductive failures such as abortions, stillbirth, infertility, and the weakening of offspring. Animals may show no symptoms and can remain chronically infected.<sup>3 28</sup> Brucellosis is prevalent in many countries, and in Africa, the incidence was reported to vary from nearly zero to 100%,<sup>6, 24</sup> with the wide variation attributed to differences in sample collection and diagnostic techniques used. This disease in cattle is usually caused by *Brucella abortus* and occasionally by other species, *B. melitensis*, *B. suis*, *B. ovis*, and *B. canis*. Leptospirosis and campylobacteriosis also can contribute to reduced reproductive tract function in cattle.

*Neospora caninum* is a protozoan that can affect cattle throughout the world causing significant economic loss through abortion especially in countries with intensive dairy production.<sup>9 35</sup> Although this disease can cause abortion at any stage of pregnancy, infected cows can also give birth to either healthy or diseased, sub-clinically infected calves.<sup>9</sup> Another protozoan important to bovine reproductive diseases is *Trichostrongylus axei* which causes irregular breeding cycles, repeat breeding and longer calving intervals as well as early pregnancy

loss and occasionally pyometra. Trichomoniasis has a worldwide distribution and is a major cause of infertility in naturally bred cattle in many countries.<sup>1, 12, 13, 26</sup> Toxoplasmosis also is considered an infection of the reproductive tract of cattle but is thought not to be of great significance.<sup>8</sup>

The rural sector in Ghana is typical of many African countries where infectious diseases represent one of the most costly and production-limiting problems facing the cattle industry. Losses in productivity from disease in cattle are a problem contributing to malnutrition and the excessive reliance on livestock importation. The current cattle production in Ghana satisfies less than 30% of the national beef demand and imports are needed to make up for this insufficiency.<sup>11</sup> The most studied reproductive infectious disease in Ghana is brucellosis and most, if not all, of the other potentially economically important reproductive infections listed above have not yet been studied in Ghana. A survey of cattle in Ghana on the Accra plains in the 1960's indicated an incidence of brucellosis at 20%-30%, with high rates of associated abortion and stillbirth.<sup>25</sup> The lack of data on the other infectious reproductive diseases of cattle in Ghana presents a fundamental challenge to establishing and offering effective veterinary management programs to increase cattle production. A range of infectious diseases is likely to be present because the cattle management practices in Ghana, such as natural breeding, the use of aged bulls (over 4 years) in communal settings, year round breeding, and the lack of routine veterinary care, are known in other developing countries to be associated with a high prevalence of these reproductive tract diseases<sup>1, 13, 26</sup>

This present report examines a typical cattle herd, with reduced fertility, to determine the range of reproductive tract infections present by using a cross-sectional study design to detect the presence of seven important reproductive tract infectious agents (Chapter 1; figure 1),

addressing the hypothesis that multiple reproductive pathogens are present in Ghanaian cattle. This cattle herd is typical of those seen in West Africa and managed by Fulani cattlemen, and using breeding practices that are commonly to such mobile herds in Africa.

## **5.1 Materials and methods**

### **5.1.1 Study area and herd description**

The study was conducted in Apolonia, a village close to the capital city, Accra, located in the coastal region of Ghana in West Africa (Chapter 5; Figure 5.1). The climate is tropical, but temperatures vary with season and elevation. Two rainy seasons occur, from April to July and from September to November. The daily mean temperature varies between 22 C and 33 C, with an equatorial seasonal variation. The selected herd, sampled between July and August 2009, was composed of approximately 700 adult mixed breed African Sanga cattle owned by multiple owners and managed by Fulani herdsman. Herdsmen were queried for the current age and calving history of each cow as well as the routine husbandry procedures practiced. The study subjects have not been previously vaccinated against any of the diseases investigated. At the time of surveying, only 2.4% of the cows were found to be pregnant. At night cattle were kept in small paddocks/kraals near the herdsman's homesteads. Milking was done, twice a day, at about 06:00 am and 03:00 pm each day with calves being allowed to suckle for a few minutes before and after milking. Animals were let out for grazing twice a day after each milking.

### **5.1.2 Sampling.**

Blood was collected by jugular venipuncture from 166 animals (148 of approximately 670 cows and 18 of approximately 30 bulls taken proportionately from each kraal). The minimum sample size was calculated with a power of 0.8 and using the lowest assumed prevalence of any of the diseases of interest (approximately 10% as predicted from the literature); i.e.,

approximately 116 animals (Dean AG, Sullivan KM, Soe MM. OpenEpi: Open Source Epidemiologic Statistics for Health, Version 2.3.1. [www.OpenEpi.com](http://www.OpenEpi.com), updated 2011/23/06, accessed 2012/04/12). Cattle were selected by stratified random procedure, proportional to the size from each kraal. At the end of each day, the blood samples were centrifuged and about 1.8mls of serum were collected into cryovials and stored at 4°C until they were used within 1 week. Commercially available serum ELISA tests was used to determine exposure to the following pathogens: infectious bovine rhinotracheitis virus (IBR), bovine viral diarrhea virus (BVDV), bovine herpes virus 4 (BHV-4), *Neospora caninum*, *Coxiella burnetii*, and *Brucella abortus* (see below for assay details). A hemolytic assay was used to detect antibodies to *T. foetus*.<sup>4</sup> Vaginal and preputial fluid washes were inoculated into a commercial test kit for isolation of *T. foetus*.<sup>a</sup> Each serum sample in the ELISA and hemolytic assays was tested in replicate and the value reported was an average of the two.

#### 5.1.3 Infectious bovine rhinotracheitis (IBR), bovine viral diarrhea virus (BVDV), bovine herpesvirus 4 (BHV-4) ELISA

Antibodies to IBR, BVDV, and BHV-4 in sera were detected using an indirect commercial ELISA kit,<sup>b</sup> according to the manufacturer's instructions. Briefly, all reagents were brought to room temperature at least 30 minutes before starting the test. Ten microliters of the serum samples and positive controls were diluted 1:100, shaken briefly, and 100-µl aliquots of the dilute samples (1:100) were added to the test wells. The microplates were incubated at 37°C for one hour, with gentle agitation after 30 min. Wells were rinsed with washing solution and each plate was emptied by flipping it over sharply above a sink followed by tapping the plate upside down against a piece of clean absorbent paper to remove all the liquid. This was repeated three times. The conjugate was diluted 1:50 in the buffer and 100 µl of the dilute conjugate solution



was added to each well. The plates were incubated for 1 hour at 37°C, and washed as previously described. Chromogen diluted in substrate solution was applied to the plate immediately in volumes of 100 µl per microwell. The plates were incubated for 10 minutes at room temperature and 50 µl of stop solution per microwell was added. Optical densities in the microwells were read using a plate reader and a 450 nm filter immediately after stopping.

The results of the test were then expressed as percentage with the following formula:

$$S/P\% = ((OD_{\text{sample}} - OD_{\text{negative control}}) / (OD_{\text{positive control}} - OD_{\text{negative control}})) \times 100.$$

To overcome the fact that only one serum sample was examined from each cow (not paired sera), the commercial assay was validated against serum samples with a known virus neutralization (VN) titer and a conservative threshold determined. Nineteen serum samples with VN titers varying from < 4 to 512 for IBR and < 4 to 2048 for BVDV were assayed as described above. The S/P% results varied from 2.5% to 120% for IBR and 0.3 to 88% for BVDV (specific data not shown). The correlations between the tests for IBR and BVD were found to be  $r=0.4776$  ( $p=0.0386$ ), 95% CI (0.029, 0.765) and  $r=0.7558$  ( $p=0.0002$ ), 95% CI (0.459, 0.900) respectively. Based on these correlations, a cutoff value for a positive IBR was set at  $S/P\% \geq 75\%$  and that for a positive BVD was set at  $S/P\% \geq 50\%$ . No VN assay for additional validation of the BHV-4 was available, so positive results were conservatively interpreted as S/P% values corresponding to values equal or greater than the positive control.

#### 5.1.4 *Brucella abortus* and Q fever ELISA

*Brucella abortus* and Q fever (*C. burnetii*) antibodies were detected using indirect commercial ELISA kits according to manufacturer's protocol.<sup>c, d</sup> For *B. abortus*, serum samples were diluted at 1:10 and for Q fever, serum was diluted 1:400. The absorbance (OD) was measured at 450 nm by spectrophotometer. The results of the test were then expressed as

percentage using the following formula:  $S/P\% = ((OD_{\text{sample}} - OD_{\text{negative control}}) / (OD_{\text{positive control}} - OD_{\text{negative control}})) \times 100$ . Serum samples for *B. abortus* with the  $S/P\% < 80\%$  was considered as a serum from an animal which has not had exposure to *B. abortus*, while serum sample with  $S/P\% \geq 80$  was classified as positive. Serum samples for Q fever with  $SP\% < 30\%$  were classified as negative, samples with  $SP\% \geq 30\%$  but  $< 40\%$  was classified as suspect and samples with  $S/P\% \geq 40\%$  were classified as positive per the manufacturer's instructions.

#### 5.1.5 *Neospora caninum* ELISA

*Neospora caninum* antibodies were detected using indirect ELISA according to manufacturer's protocol.<sup>e</sup> For *N. caninum* serum samples were diluted at 1:100. The absorbance (OD) was measured at 650 nm by spectrophotometer. The results of the test were then expressed as a ratio using the following formula:  $S/P\% = ((OD_{\text{sample}} - OD_{\text{mean negative control}}) / (OD_{\text{mean positive control}} - OD_{\text{mean negative control}})) \times 100$ . The test is considered valid if the difference between the mean OD of the positive control and that of the negative control is greater than or equal to 0.150. In addition, the OD of the negative control mean must be less than or equal to 0.20. Serum samples for *N. caninum* with  $S/P\%$  of less than 50% are classified as negative and the sample with  $S/P$  ratio greater or equal to 50% are classified as positive per manufacturer's instructions.

#### 5.1.6 *Tritrichomonas foetus* hemolytic assay.

The test was performed as described previously.<sup>4</sup> Briefly, 25µl of 1% fetal calf serum in PBS was added to each well of a 96 well plate, then 25 µl of test serum at two fold dilution was added to each well. Then 25µl 1% *T. foetus* antigen coated RBC addition to each well and the plate was carefully shaken and incubated for 1hr. Later 50 µl reconstituted lyophilized guinea pig

complement<sup>f</sup> at a concentration of 1:10 was added to each well and was incubated for 1 hr at room temperature and finally, the plate was shaken again, incubated for 90 min, and the results were read visually. The extent of hemolysis was categorized subjectively as follows; 0-no hemolysis; 1 - scant (25%); 2 - mild hemolysis (50%); 3 - moderate hemolysis (75%); 4 - complete hemolysis (95-100%). Titers were reported as the highest serum dilution that gave at least 75% hemolysis, i.e. a score of 3 or more.

#### 5.1.7 *Tritrichomonas foetus* isolation

Vaginal and preputial secretions were collected from the vaginal fornix and the prepuce using a dry, sterile Cassou artificial insemination pipette. The external surfaces of the vulva or prepuce were cleaned of extraneous matter and the pipette aseptically inserted and used to scrape the epithelial surfaces of the vagina or the prepuce to obtain sheets of epithelial cells and an ample amount of secretion (approx. 3-6 ml.). The collected materials were inoculated into *T. foetus* specific media<sup>a</sup> and incubated at 35°C in a 5% CO<sub>2</sub>. The cultures were examined daily for 7 days post-inoculation for any motile organisms and compared with positive controls incubated simultaneously.

#### 5.1.8 Statistical analysis

Association between the presence of antibodies to the different infectious diseases agents were examined by the Chi-squared test on all pairs of the examined diseases. Correlations between age, sex, and parity, and the ELISA and hemolytic assay results were examined by Spearman's correlation statistic.

## 5.2 Results

### 5.2.1 Herd information

The mean age of the females was 8.5 years (S.D. +/- 1.8), and that of the males was 4.2 years, (S.D. +/- 1.7). The mean calving interval of 2.5 yr was calculated based on the first calving at 5 years and the number of calves produced as reported by the herdsman.

### 5.2.2 ELISA serology results

The IBR, BVDV, and BHV-4 ELISA results were positive in 69.0%, 13.0%, and 20.6% of the total cattle (Table 5.1). Antibody against *N. caninum* was found in 6.1% of the total number of the animals. Antibody against *C. burnetii* was confirmed in 17.7% of the animals, while 7.3% were classified as suspect with an intermediate OD value. No antibodies against *B. abortus* were detected in any of the animals tested (Table 5.2).

### 5.2.3 Hemolytic assay and culture results for *T. foetus*

The results of the hemolytic assay indicated that 47.6% of the animal had positive sero-reactivity with 47.4% of the bulls being positive and 47.6% of the cows being positive; however, no *T. foetus* organisms were isolated via culture from any animals in the herd (Table 5. 3).

### 5.2.4 Disease Associations

Potential associations between the investigated pathogens were examined and a significant association was observed among the viral pathogens. A positive association was noted between IBR and BVDV (chi-square with 1df = 6.810,  $p = 0.009$ ), between IBR and BHV-4 (chi-square with 1df = 6.028,  $p = 0.014$ ), and between BVDV and BHV-4 (chi-square with 1df = 10.021,  $p = 0.002$ ). No association was found between the bacteria and the protozoan agents with any viral agent or each other.

Age of the cow was negatively correlated with BVD ELISA results ( $p = 0.0261$ ). No bulls were positive for BVD (different from cows,  $p < 0.001$ ). There were no other correlations between age, parity, or sex and any of the investigated pathogens. Only three cows were pregnant at the time of testing; however, there was no consistent pattern of serological results noted among them.

### **5.3 Discussion**

This study examined a typical African herd and found evidence that these animals have been exposed to, or were presently infected with, a wide range of important reproductive tract infectious agents, and thus provides an instructive reminder that there is a need to focus on the control of such infections in order to improve the contribution cattle make to the developing economy of the rural sector.

This herd had an average calving interval of 2.5 years and as the time of our survey, only 2.4% of the cows were pregnant, a common profile for cattle in this geographic area. Herdsmen did not report abortions, but cows with reproductive infectious diseases often lose their pregnancy early when it is difficult to observe, typically require more services per pregnancy than their seronegative herd mates, and are more likely to be non-pregnant at any time point.

This, the first serological analysis in Ghana, examines the prevalence of multiple agents simultaneously, showed strong serological evidence that multiple important reproductive infectious diseases are present in this herd; this is likely to be the case in many cattle herds in this area. These infections are, in all likelihood, impacting livestock production in many parts of Ghana and West Africa.

High seroprevalences of IBR, BHV-4 and *T. foetus* were detected with lower seroprevalences of BVDV, Q fever, and *N. caninum*. Interestingly, there was no serological evidence of brucellosis in this herd. This latter finding is contrary to the earlier findings from a study conducted in a different region of Ghana carried out before significant nationwide efforts had taken place to reduce the impact of this important disease.<sup>25</sup> Literature reports on brucellosis prevalence at individual and herd levels vary considerably across Africa: with estimates of individual animal seroprevalence of 20.2% in Sudan,<sup>18</sup> between 0.3% and 8.2% in Eritrea,<sup>24</sup> 12.3% to 14.1% in Tanzania,<sup>33</sup> 6.6% in Chad,<sup>30</sup> 3.3% in Central African Republic,<sup>21</sup> 14.1% to 28.1% in Zambia,<sup>20</sup> and very recently, as low as 3.1% in Ethiopia.<sup>17</sup> At the herd level, estimates range from 2.4% and 46.1% under different husbandry system in Eritrea,<sup>24</sup> up to 80% in Tanzania,<sup>33</sup> and in Zambia, from 46.2% to 74%. The failure to detect antibodies to *B. abortus* in this herd could be due to improved management and control measures that has been adopted since it was last measured in 1966.<sup>25</sup>

The analysis indicated almost half of the cows had a positive hemolytic titer to *T. foetus*; though no *T. foetus* organism was cultured. This may be due to the low level of sensitivity of culturing cows.<sup>5</sup> Furthermore, it is generally believed that most infected females clear the organism within 2-5 months after infection.<sup>7</sup> Since calving intervals are so prolonged, the actual time cows harbor the organism may be relatively short. It is well known that bulls are an important component of any study addressing transmission and prevalence of reproductive diseases in cattle. Age-related factors have been reported to be significant in the prevalence of trichomoniasis in bulls,<sup>5</sup> and thus it is important to note that only a few bulls, and in fact, only younger bulls were sampled in this study; these may not have been the main source of *T. foetus* in this herd.

A significant association between the viral pathogens was detected, suggesting that similar factors, such as animal density and compromised immunity, may be of importance in the spread of this particular group of diseases. Further investigation of the interaction of these viruses may be a fruitful area of investigation for improving Ghanaian and other regions livestock production.

This study focuses on a single herd in Ghana and is a first approach to provide the data on the current status of reproductive infectious diseases necessary to improve the status of bovine reproduction in this rural region of the world. It is necessary to extend these studies to greater numbers of animals throughout Ghana and in other African countries. Infectious agents not included in this study - such as leptospirosis, toxoplasmosis, and campylobacteriosis - very likely could be present in African cattle herds and need also to be included in future studies, as should other potential causes of poor fertility, such as nutrition. Comprehensive and nation-wide survey and definitive isolation of these pathogens rather than serosurveillance are the logical next steps to confirm the significance of these diseases. In addition to these microbiological approaches, parallel studies to quantify the economic impacts of the pathogens on animal health and production are crucial. The data from this study will be important in the design and pursuit of additional studies and the rational development of local, national, and regional control strategies for farmers, veterinarians, and policy-makers.

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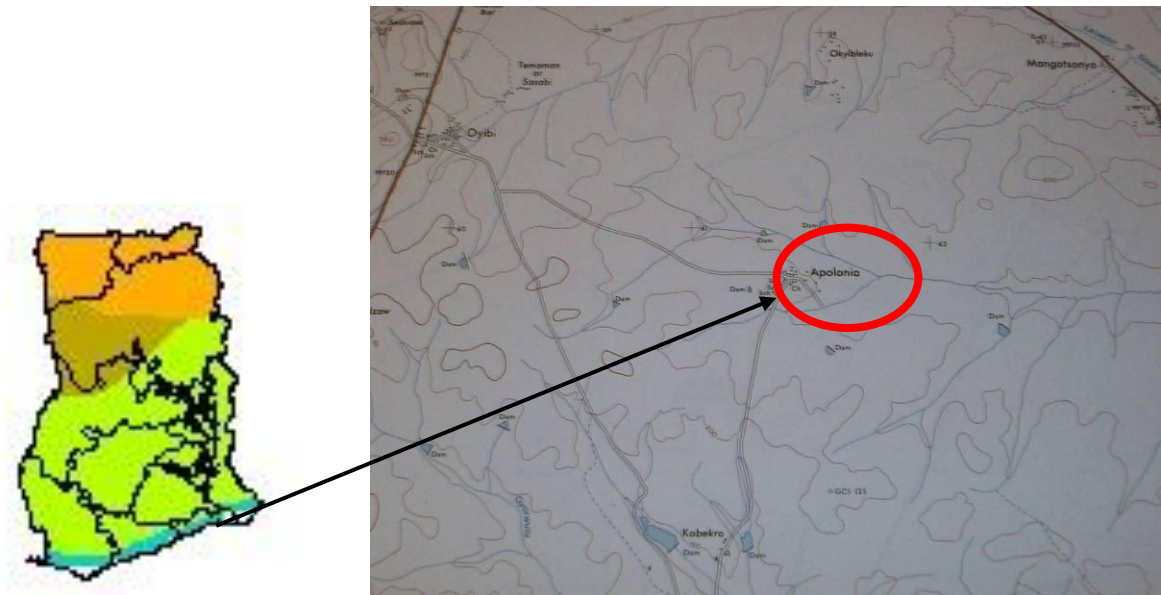


Figure 5.1: Apolonia the study site, a village close to Accra, located in the coastal region of Ghana

Table 5.1. ELISA serology results for infectious bovine rhinotracheitis (IBR), bovine virus diarrhea (BVD), and bovine herpesvirus 4 (BHV-4) from 166 cattle from a herd in Apolonia, in coastal Ghana.

| Test Result | ELISA Seropositivity (% of Animals) |        |       |      |        |       |       |        |       |
|-------------|-------------------------------------|--------|-------|------|--------|-------|-------|--------|-------|
|             | IBR                                 |        |       | BVD  |        |       | BHV-4 |        |       |
|             | Male                                | Female | Total | Male | Female | Total | Male  | Female | Total |
| Negative    | 37.0                                | 30.1   | 31.0  | 100  | 84.9   | 87.0  | 89.5  | 78.1   | 79.4  |
| Positive    | 63.0                                | 69.9   | 69.0  | 0.0  | 15.1   | 13.0  | 10.5  | 21.9   | 20.6  |

Table 5.2. ELISA serology results for *Q fever* (*Coxiella burnetii*), brucellosis (*Brucella abortus*), and neosporosis (*Neospora caninum*) from 166 cattle from a herd in Apolonia, coastal Ghana.

| Test Result          | ELISA Seropositivity (% of Animals) |        |       |            |        |       |            |        |       |
|----------------------|-------------------------------------|--------|-------|------------|--------|-------|------------|--------|-------|
|                      | C. burnetii                         |        |       | B. abortus |        |       | N. caninum |        |       |
|                      | Male                                | Female | Total | Male       | Female | Total | Male       | Female | Total |
| Negative             | 83.4                                | 74.0   | 75.0  | 100        | 100    | 100   | 88.9       | 94.6   | 93.9  |
| Positive             | 5.6                                 | 19.2   | 17.7  | 0.0        | 0.0    | 0.0   | 11.1       | 5.4    | 6.1   |
| Suspect <sup>a</sup> | 11.0                                | 6.8    | 7.3   | 0.0        | 0.0    | 0.0   | 0.0        | 0.0    | 0.0   |

<sup>a</sup> “Suspect” refers to samples that were intermediate in reactivity and could not be accurately interpreted.

Table 5.3. Hemolytic assay results for serum antibody against *Tritrichomonas foetus* in 166 cattle from a herd in Apolonia, coastal Ghana.

| Test Result | Hemolytic Assay for Seropositivity to<br><i>Tritrichomonas foetus</i> (% of Animals) |        |       |
|-------------|--------------------------------------------------------------------------------------|--------|-------|
|             | Male                                                                                 | Female | Total |
| Negative    | 52.6                                                                                 | 52.4   | 52.4  |
| Positive    | 47.4                                                                                 | 47.6   | 47.6  |

## Chapter 6

### Cytokine responses to *Tritrichomonas foetus* infection in a mouse model

#### **Abstract**

Host immune factors are hypothesized to be important in determining the outcome of *Tritrichomonas foetus* infection in cattle. Previous rodent model studies have shown younger mice are more susceptible to *T.foetus* infection and that the success of infection is determined in the first two days post-inoculation. This study was undertaken to determine what factors underlie the different host responses that vary by age in this early period. We hypothesize cytokines which are present in the early innate response vary with the age of the host and are responsible for the variation in susceptibility to infection. In this study, 4- and 8-wk-old BALB/cAnN mice inoculated intra-vaginally with  $10^6$  *T.foetus* organisms (D1 strain; *T. foetus*) were evaluated after 5 days of infection. Susceptibility to infection; serum, vaginal, and uterine tissue cytokine profiles; and histological appearance of the reproductive tract were determined and compared. Successful *T. foetus* infection was determined by culture of vaginal and uterine flushes. All 4-wk-old mice became infected following inoculation, but only 60% of the 8-wk-old mice became infected. Vaginal levels of TNF were significantly higher in the inoculated 8-wk-old mice as compared to both inoculated and control 4-wk-old mice. In serum, by contrast, TNF values were significantly higher in the infected 4-wk-old mice as compared to control 4-wk-old mice, but similar to both infected and control older mice. IL-2 and IL-4 levels in the 4wk infected were significantly lower when compared to their controls. Histopathologically, there were no significant differences between the treatment groups



and controls. In summary, younger mice appear more susceptible to intravaginal infection by *T.foetus* and the cytokine TNF may be involved in resistance to infection.

## **Introduction**

Trichomoniasis is sexually transmitted disease of cattle caused by the protozoa, *Trichomonas foetus* (*T.foetus*).<sup>16</sup> Bovine trichomoniasis is an insidious, economically damaging venereal disease of cattle with a worldwide impact especially in places where natural breeding is practiced.<sup>15</sup> Trichomoniasis in the female host is characterized by mild endometritis and occasionally by pyometra and abortion, while in the bulls, infections cause only mild inflammation and usually ends in a perpetual carrier state.<sup>19</sup> The incidence of disease depends to a large extent on management practices, such as using natural breeding and having older bulls in the herd.

The economic effect of the disease is expressed in infertility; repeat breeding, delayed return to oestrus after mating, early embryonic death and sometimes abortion. Increased prevalence of *T.foetus* in a herd is associated with a significant reduction in calf crop revenue. Currently, there is no treatment for trichomoniasis in cattle which is legal in the U.S.,<sup>15</sup> and infected animals are culled to control spread of the disease.<sup>12</sup>

Studies aimed at understanding the host response in order to control and manage the disease have focused on the acquired immune response. Results from these studies indicates that, cows often recover from infection relatively quickly, which leads to partial but transient immunity to reinfection.<sup>25</sup> In order to elucidate the mechanism of host immune response, different animal models have been studied and the laboratory mouse has been considered a useful model.<sup>2, 7, 26</sup> Very few of these studies have focused on the host immediate response and how it may impact the ability of *T. foetus* to establish an infection.

The innate immune response is key to understanding an antimicrobial challenge to a mucosal organ. Generally, an innate immune response is initiated by binding of microbial pathogen-associated molecular patterns (PAMPs) to Toll-like receptors (TLR) located on phagocytic and epithelial cells.<sup>3,27</sup> As a result of PAMP-TLR binding, chemokines that attract phagocytic cells to the affected site and secretion of type 1 interferons that attract and activate natural killer (NK) cells occur. Epithelial cells in the female reproductive tract express TLR-4 and CD14 as well as TLR1, 2, 3, 5 and 6 which contribute protection.<sup>10</sup>

CD4<sup>+</sup> is a subset of T-cells known to contain both inflammatory T-cells and T-cells responsible for helping antibody responses.<sup>1,6</sup> The protection against *T. foetus* infection through T-cell-dependent responses mechanism has not been well elucidated, although effective immunity against most microbial pathogens, including many parasitic protozoans, is typically T-cell dependent. An example is effective immunity against *Plasmodium* sp. which requires CD4<sup>+</sup> T-cell-regulated immune responses,<sup>20,22,28</sup> including T-cells that produce IFN- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$ .<sup>13</sup> Effective immunity can sometimes be determined by which CD4<sup>+</sup> T-cell pathway response predominates. A Th1 response against *Leishmania* spp., that involves IFN- $\gamma$  and TNF- $\alpha$ , leads to a favorable outcome, whereas a predominately Th2 response exacerbates disease.<sup>14,17,18,23</sup> In contrast, Th2 responses characterized by IL-4, IL-5, and production lead to more favorable outcomes with *Giardia* spp. infections.<sup>17, 23, 29</sup>

The Th1/Th2 pathway paradigm is not well defined in bovine protozoal infections as immune and inflammatory responses are sometimes heterogeneous, inducing both Th1- and Th2-like responses, whereas in other examples predominantly Th1 or Th2 responses occur.<sup>6</sup> For example, T-helper responses induced in Th clones against the rhoptry-associated protein-1 of *Babesia bigemina* are biased toward Th1, and are characterized by a strong IFN- $\gamma$  response and a

weak IL-4 response.<sup>21</sup> In contrast, clonal T helper responses in *Fasciola hepatica*-infected cattle are characterized by strong IL-4 responses and weak IFN- $\gamma$  activity.<sup>6</sup>

The study described here was undertaken to characterize the reproductive tract's immune response to protozoal infectious agents using a mouse model of trichomoniasis (Chapter 1; figure 1), addressing the hypothesis that the cytokine response to infection and success of infection vary with age. The specific objective is to establish the cytokine environments during early *T.foetus* infection in two different age groups of mouse and relate these to the success of intravaginal infection.

## **6.1 Materials and methods**

### **6.1.1 Mice**

Forty BALB/c AnN female mice (National Cancer Research Institute), 4 and 8wks old, were housed at the Vivarium of College of Veterinary Medicine in Michigan State University according to the rules and regulations of IUCAC. Subjects were divided into groups of 10 composing of (1) 4-wk old control, (2) 4-wk old infected, (3) 8-wk old infected and, (4) 8-wk old control. Animals were provided with controlled temperature quality. Light was provided on 12 hour light/dark cycle and food and water offered *ad libitum*.

### **6.1.2 Determination of *T.foetus* infection**

Vaginal washes were obtained initially and at necropsy by aspiration with a micropipette using 50  $\mu$ l of sterile Phosphate Buffer Saline (PBS). To determine *T.foetus* infection, culture aspirates were inoculated into Trypticase Yeast Maltose (TYM), incubated at 37°C and examined daily microscopically daily for 7 days for the presence of *T.foetus*

### 6.1.3 Cytokine profile

Blood was collected from the saphenous vein, placing 0.3-0.5ml into 1.5ml cryovials tube. The cryovials were centrifuged at 3000rpm for 5min, and serum was collected and stored at -20 C until it was used.<sup>4</sup>

### 6.1.4 Estrous cycle determination

All animals were examined to determine the stages of estrous at the time of death. Samples of vaginal mucus were obtained by washing with 50µl of sterile PBS and collecting the sample by aspiration with a micropipette; samples were examined microscopically at 10x magnification as previously described.<sup>5</sup>

### 6.1.5 Parasites

*Tritrichomonas. foetus* strain D1 was originally obtained from a cow with severe post-coital pyometra.<sup>24</sup> This strain, at a intravaginal dose of  $1 \times 10^6$  *T. foetus* organisms, was used in several studies successfully intravaginally infecting 100% of experimental heifers.<sup>5,8</sup> Parasites were maintained in culture by using TYM medium that contained 10% fetal calf serum until inoculation.<sup>9</sup>

A mouse-adapted strain of *T. foetus* (MuTF 52100V) was developed by first intravaginally infecting BALB/ cAnNCr mice with strain D1. Then cultures from vaginal lavages of these infected mice were pooled, grown in culture using TYM media, transferred to fresh media every 2 to 3 days, and subsequently inoculated into susceptible mice. A second-generation mouse-adapted strain (MuTF 10N) was developed by selecting positive cultures from mice infected with strain MuTF 52100V. For inoculation, this material was centrifuged at 3000rpm for 10 min and the sediment/pellet washed 2X with PBS. These were then resuspended into PBS and their concentration was standardized.

#### 6.1.6 Experimental Design

After ear-notching and initial blood sampling, female mice were allowed to acclimate for 2 days before the experiment began. Females in each group were intravaginally inoculated with  $10^4$  motile *T. foetus* strain MuTF10N using organisms suspended in 10 ml trypticase-yeast extraction serum (TYI) media by using an Eppendorf pipette; control animals were sham inoculated with sterile TYI media.<sup>2</sup> A second inoculation of *T. foetus* or TYI at the same dose and volume was given the following day. Animals were killed 5 days after inoculation. At necropsy, they were weighed and blood was collected by cardiac puncture. Before removal of the reproductive tract, the vagina was flushed with 80  $\mu$ l of sterile PBS from which 20  $\mu$ l was cultured in TYM and the remainder was frozen at -80 °C. After removal of the whole reproductive tract, the uterus was separated from the vagina at the cervico-vaginal border, and was bisected. One uterine horn was flushed with 80  $\mu$ l of sterile PBS and cultured in TYM. This uterine horn and its ovary were frozen at -80 °C and the contralateral horn and ovary were fixed in 4% buffered paraformaldehyde. Paraformaldehyde fixed tissues were transferred to PBS after 24hrs, then processed through gradients of progressively more concentrated ethanol, until the tissues were stored long term in 70% ethanol. Cultures were examined microscopically and successful infection was determined by the presence of motile parasites.

#### 6.1.7 Histopathology and Immunohistochemistry

Paraformaldehyde-fixed tissues were trimmed, embedded in paraffin, sectioned at 5-7  $\mu$ m and routinely processed for immunohistochemistry. An enhanced V Red (Alkaline Phosphatase Red) Detection System, (Ventana Medical Systems, Inc., Tucson, AZ, USA) as well as bulk buffers specifically designed for use on the BenchMark Automated Staining System, (Ventana Medical Systems, Inc., Tucson, AZ, USA) were used for immuno-labeling and visualization.

Slides were baked in a drying oven at 60°C for 30 minutes. The slides were then barcode labeled, and placed in the BenchMark for deparaffinization and heat-induced epitope retrieval. Antigen retrieval was performed using retrieval solution CC1 (Medical Systems, Inc., Tucson, AZ, USA) with an 8-minute heating and an 8-minute cooling cycle. A rabbit -polyclonal anti-*T. foetus* antibody at a concentration of 1:500 for 32 minutes was used as a primary antibody. A secondary antibody, goat anti-rabbit IgG, was selected to avoid cross reactivity between irrelevant mouse antibodies. The slides were counterstained using Ventana hematoxylin (Ventana Medical Systems, Inc., Tucson, AZ, USA), and bluing for 2 minutes each, then dehydrated, cleared and mounted. Formalin-fixed, paraffin-embedded sections of uterus from a *T. foetus* infected cow that had previously been tested for trichomoniasis were used as positive controls. Use of an irrelevant primary antibody was used as a negative control.

#### 6.1.8 Flow cytometry

Vaginal and uterine washes were assayed for IL-2, IL-4, IL-5, IFN- $\gamma$  and, TNF- $\alpha$  simultaneously using the mouse Th1/ Th2 – cytokine cytometric bead array (CBA) kit (BD Biosciences). Briefly 50  $\mu$ l of each sample was mixed with 50  $\mu$ l of mixed capture beads and 50  $\mu$ l of the mouse Th1/Th2 PE detection reagent made up of PE-conjugated anti-mouse IL-2, IL-4, IL-5, IFN- $\gamma$  and, THF-  $\alpha$  . The samples were incubated at room temperature for 2 hours in the dark. Following incubation, 1 ml of buffer was added to samples and centrifuged at 200x g-for 5 minutes. The remaining wash buffer was aspirated and discarded from each assay tube and the pellet resuspended in 300  $\mu$ l of wash buffer. Samples were run using BD FACS Calibur Flow Cytometer and data analyzed using the CBA Analysis software (BD Bioscience).

#### 6.1.9 Preparation of Vaginal and Uterine homogenates

Vaginal and uterine homogenates were analyzed using the same procedure as described above, except that tissues were homogenized and their protein content quantified first, using a spectrophotometric assay. Briefly, frozen tissues were ground to a fine powder by hand in a mortar 1/3 filled with liquid nitrogen; this was then resuspended in a homogenate buffer (PBS + 0.05% NP-40 non-ionic detergent + anti-protease tablet). The protein quantity was measured using with a spectrophotometer at a wavelength of 750λ. After flow cytometric assay, cytokine values were adjusted for the respective protein content.

#### 6.1.10 Statistical analysis

The frequencies of successful intravaginal infections in 4 wk and 8 wk old infected mice were expressed as percentages. Statistical analysis of differences in the means between cytokine levels in infected young and adult mice, and between infected and uninfected age –matched controls was performed using the Mann Whitney test (one tailed) based on the hypothesis that cytokine levels would increase in response to infection. In each experiment, a p value  $\leq 0.05$  between infected young and adult mouse was considered significant.

### **6.2 Results**

#### 6.2.1 Stage of estrus

The stages of estrus at the time the mice were killed ranged from proestrus to metestrus. Most of the subjects from both treatment and control groups of both ages were in proestrus, followed by estrus, and then metestrus. No animal was found in diestrus (Table 6.1).

#### 6.2.2 Percentage of vaginal and uterine Infection of *T. foetus* in young and adult mice

The overall frequency of infection in the younger mice was 100% with vaginal infections being more common than uterine; while the frequency of infection in the older mice was 60%, with the uterus more commonly infected (Figure 6.1)

### 6.2.3 Analysis of cytokines levels

An initial comparison of the levels of all measured cytokines, from all infected mice regardless of age, with the control group regardless of age, showed that there was no significant difference between the two groups (data not shown). However, significant differences in some cytokine levels were noted between the different age groups.

The TNF-alpha concentration from vaginal homogenates was significantly higher in the 8-wk old infected mice as compared to the 4 wk old infected mice ( $p=0.032$ ) (Table 6.2; Figure 6.2). IL-2 and IL-4 concentrations from vaginal homogenates were significantly higher in the 4 wk old control mice as compared to the 4 wk old infected ( $p=0.0477$ ) and IL-4 ( $P=0.0412$ ), respectively (Table 6. 2; Figure 6. 3 and 6.4). The uterine homogenates were also significantly higher for IL-2 and IL-4 in the 4wk old control as compared to the 4 wk old infected mice with  $p$  values of 0.0428 and 0.0063, respectively (Table 6. 3; Figure 6. 5 and 6.6). The TNF-alpha concentration in the serum sample was significantly higher in the 4 wk old treated mice compared to the 4 wk old control group ( $p=0.0158$ ) (Table 6.4 ; Figure 6.7); however, there was no significant difference between the 8 wk old infected and its respective control.

### 6.2.4 Histopathology and Immunohistochemistry

Positive labeling of organisms with the specific anti-*T.foetus* antibodies was seen in the lumen of the mouse's vagina and occasionally mixed with the mucinous secretion (Figure 6. 8).



No positive labeling was seen deeper in the tissues. Histopathologically, there was no significant lesion other than presence of some *T.foetus* organisms in the vaginal lumen.

### 6.3 Discussion

The results of this study confirm that younger mice are more susceptible to *T. foetus* infection than older mice and the cytokine environment within the reproductive tract in acute *T.foetus* infection also varies with the age of the host.

In our studies, the 4 wk old mice had a higher frequency of infection, although following successful infection extension to the uterus was more common in the 8 wk old mice. However, in all groups, cellular inflammation and histopathology was generally absent at this early stage of infection. This study also shows that vaginal TNF- $\alpha$  is elevated more in the 8 wk old mice compared to uninfected 8 wk mice and all the younger mice (both infected and uninfected). We also showed that there was a decrease or down regulation of IL-2 and IL-4 respectively in the 4wk old infected mice compared to the uninfected mice and all the adult mice (both infected and uninfected).

Interestingly, the serum levels of TNF-  $\alpha$  are initially low in the uninfected 4 wk mice, but increase in response to infection in these animals to levels similar to those seen in both infected and control 8 wk mice. This suggests that serum TNF-  $\alpha$  levels may be constitutively higher in older mice providing some protection during *T. foetus* infection. Similarly, the data suggests that TNF-  $\alpha$  in vaginal homogenates tends to increase with infection in younger animals and is higher in older animals both uninfected and significantly in infected mice.

Our study demonstrates a phenotype in mice in which TNF-  $\alpha$  is higher and IL-2 and IL-4 resists degradation or is downregulated in older animals inducing an increase in the resistance to infection. TNF- $\alpha$  has been confirmed to play a protective role in host resistance against

microbes. It is possible that *T. foetus* infection of a mucosal surface activates Toll-like Receptors (TLRs) which in turn activate transcription factors (including NF- $\kappa$ B, Egr-1 and AP-1), which bind to the TNF promoter.<sup>4</sup> Following activation of the promoter and subsequent production, TNF- $\alpha$  protects by activating neutrophils via IL-8. TNF- $\alpha$  also activates macrophages, and generates both a respiratory burst and nitric oxide (NO) dependent killing pathways. TNF- $\alpha$  may also induce natural killer (NK) cells to produce interferon-gamma, a significant early host defense mechanism.<sup>4, 11</sup>

These findings suggest that TNF-  $\alpha$  and Th2 cytokines are important in early resistance. Additional studies are needed to identify the specific pathways and effector molecules that these cytokines govern and to apply these findings to preventative and therapeutic strategies.

#### **6.4 Acknowledgements**

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Table 6. 1 Stages of estrus cycle of mice on the 5<sup>th</sup> day of post infection  
with *T.foetus* as determined by vaginal cytology and histopathology post-mortem

|                         | Number of mice in each group |         |           |         |
|-------------------------|------------------------------|---------|-----------|---------|
|                         | 4week old                    |         | 8week old |         |
| Stages of estrous cycle | Treated                      | Control | Treated   | Control |
| Proestrus               | 6                            | 5       | 4         | 7       |
| Estrus                  | 2                            | 3       | 4         | 2       |
| Metestrus               | 2                            | 2       | 2         | 0       |
| Diestrus                | 0                            | 0       | 0         | 0       |

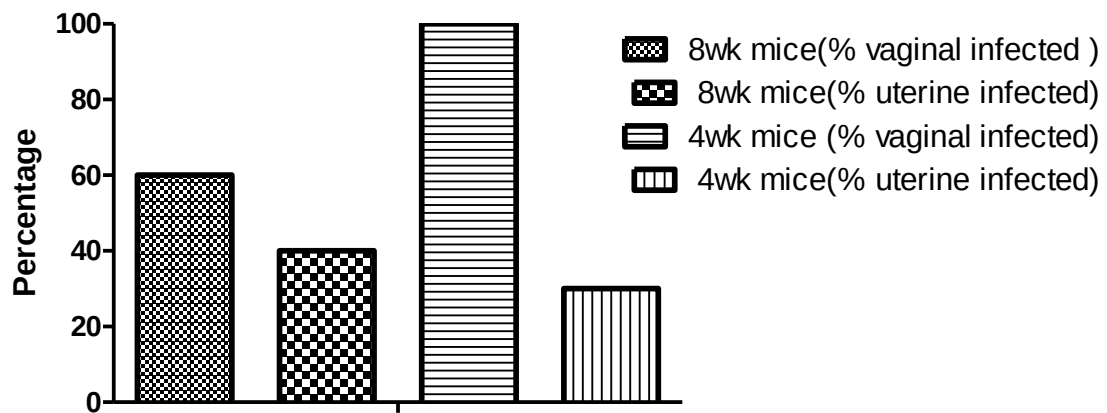
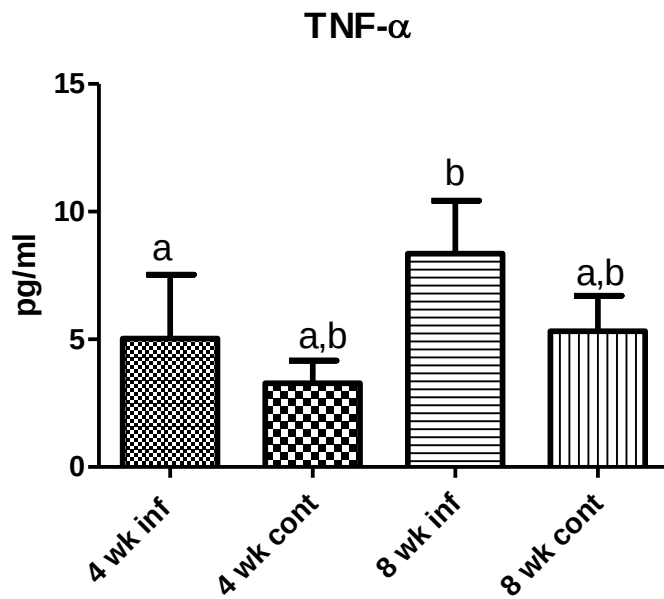


Figure 6.1. The percentage of *T. foetus* infected mice that were successfully infected in either the uterus or the vagina. Note that all 4-wk-old mice had vaginal infections and only three uterine after inoculation. In comparison only 60% (6) of the 8-wk-old mice had vaginal infections and four uterine.



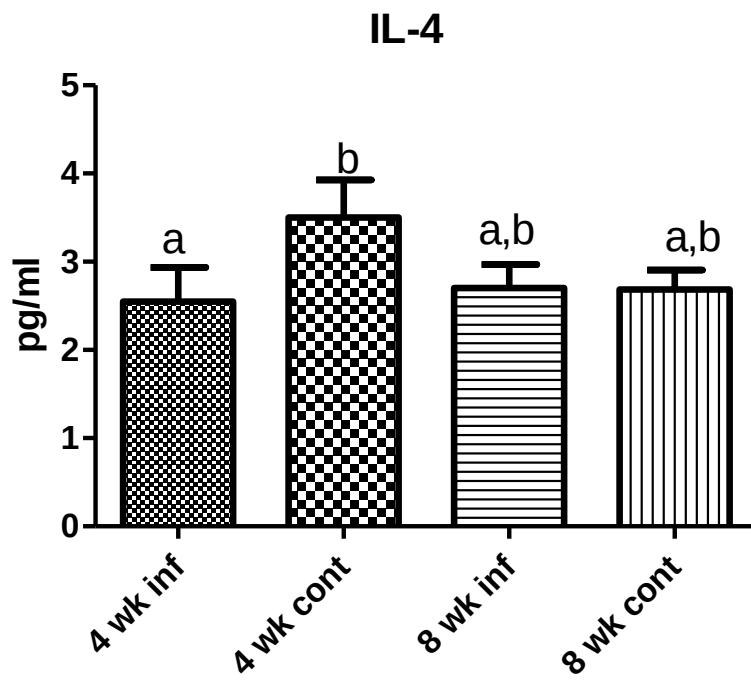
Table 6.2. Vaginal homogenate cytokine concentration in *T.foetus*-infected and control mice (4 wk and 8wk old) 5 days post-inoculation (mean and standard error of the mean (SEM) values)

|           | IL-2               | IL-4              | IL-5              | IFN- $\gamma$     | TNF- $\alpha$     |
|-----------|--------------------|-------------------|-------------------|-------------------|-------------------|
| 4 wk inf. | 0.4300<br>(0.3077) | 2.550<br>(0.3839) | 1.730<br>(0.4169) | 1.020<br>(0.2555) | 5.030<br>(2.495)  |
| 4 wk cont | 1.263<br>(0.3909)  | 3.500<br>(0.4285) | 2.413<br>(0.6043) | 1.438<br>(0.2456) | 3.288<br>(0.8794) |
| 8 wk inf  | 0.9667<br>(0.3308) | 2.700<br>(0.2687) | 2.600<br>(0.5819) | 1.544<br>(0.1260) | 8.356<br>(2.074)  |
| 8 wk cont | 0.8286<br>(0.4004) | 2.686<br>(0.2198) | 1.657<br>(0.5200) | 1.314<br>(0.1100) | 5.314<br>(1.392)  |



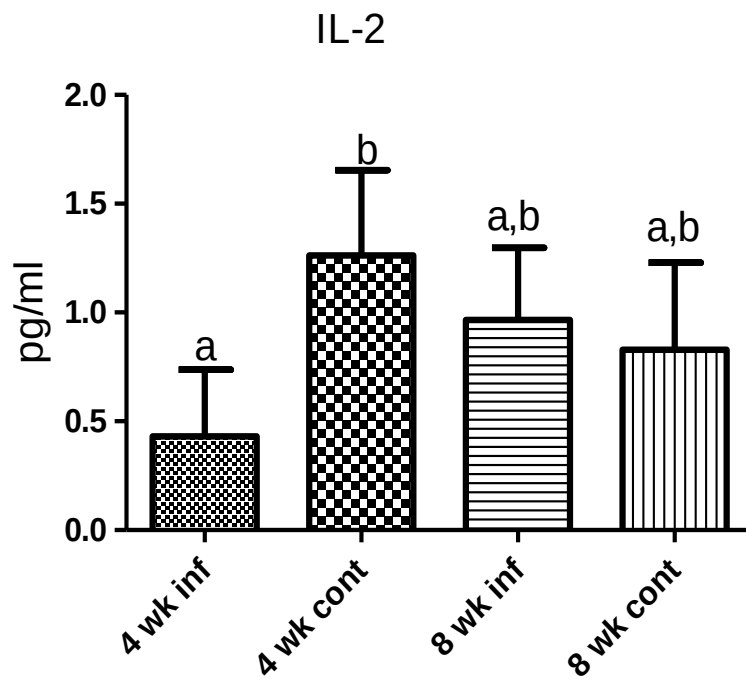
a, b-Different superscript indicate significantly different TNF- $\alpha$  levels in tissue

Figure 6. 2. The levels of the cytokine TNF- $\alpha$  in vaginal homogenates at 5 days post-infection with *T. foetus*. A significant difference is seen between the 8 wk old infected and the 4 wk old infected. Bar represents SEM. (n=10 in each group), p=0.032.



a, b-Different superscript indicate significantly different IL-4 levels in tissue

Figure 6.3. The levels of the cytokine IL-4 in vaginal homogenates at 5 days post-infection with *T. foetus*. A significant difference is seen between the 4 wk old infected and the 4 wk old sham control ( $p=0.0477$ ). Bar represents SEM. (n=10 in each group).

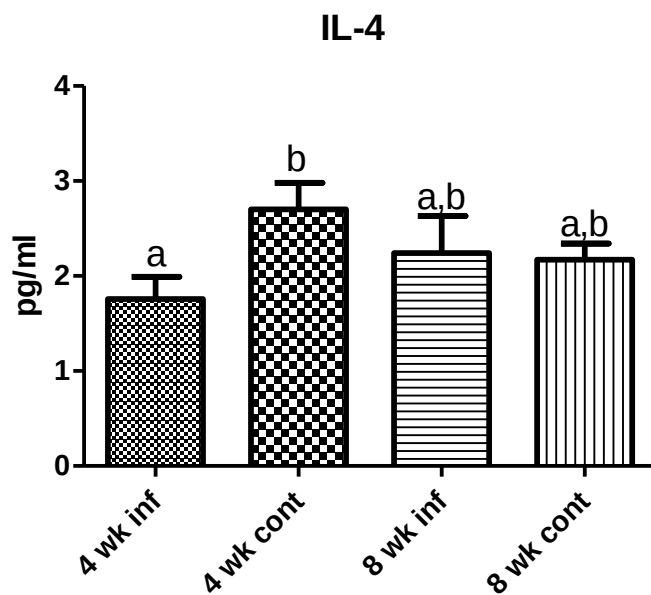


a, b-Different superscript indicate significantly different IL-2 levels in tissue

Figure 6.4. The levels of the cytokine IL-2 in vaginal homogenates at 5 days post-infection with *T. foetus*. A significant difference is seen between the 4 wk old infected and the 4 wk old sham control ( $p=0.412$ ). Bar represents SEM. ( $n=10$  in each group).

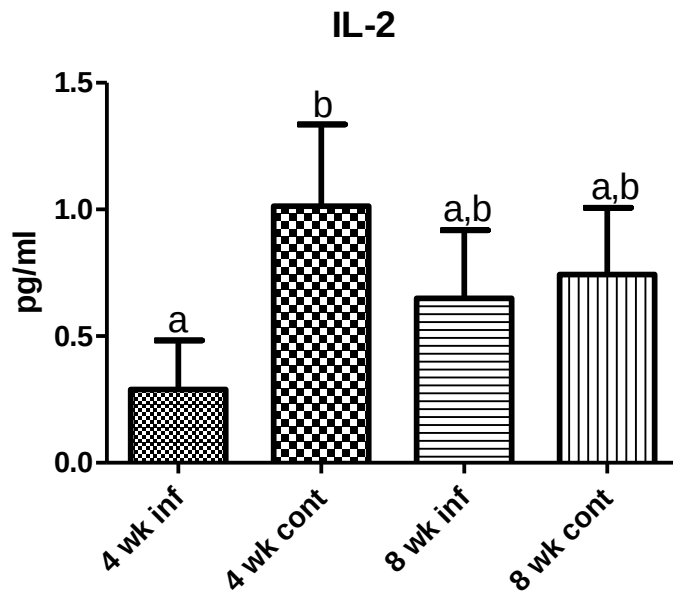
Table 6.3. Uterine homogenate cytokine concentrations in *T.foetus*-infected and control mice (4 wk and 8wk old) 5 days post-inoculation (mean and standard error of the mean (SEM) values)

|           | IL-2               | IL-4              | IL-5               | IFN- $\gamma$      | TNF- $\alpha$      |
|-----------|--------------------|-------------------|--------------------|--------------------|--------------------|
| 4 wk inf. | 0.2900<br>(0.1935) | 1.760<br>(0.2310) | 0.8900<br>(0.3096) | 0.7800<br>(0.2154) | 0.7400<br>(0.3103) |
| 4 wk cont | 1.013<br>(0.3232)  | 2.700<br>(0.2809) | 1.750<br>(0.5751)  | 1.175<br>(0.3239)  | 1.188<br>(0.3796)  |
| 8 wk inf  | 0.6500<br>(0.2684) | 2.240<br>(0.3925) | 1.250<br>(0.3478)  | 1.130<br>(0.2155)  | 1.750<br>(1.834)   |
| 8 wk cont | 0.7429<br>(0.2644) | 2.171<br>(0.1700) | 1.500<br>(0.3970)  | 1.257<br>(0.06494) | 2.571<br>(1.222)   |



a, b-Different superscript indicate significantly different IL-4 levels in tissue

Figure 6.5. The levels of the cytokine IL-4 in uterine homogenates at 5 days post-infection with *T. foetus*. A significant difference is seen between the 4 wk old infected and the 4 wk old sham control ( $p=0.0428$ ). Bar represents SEM. (n=10 in each group).



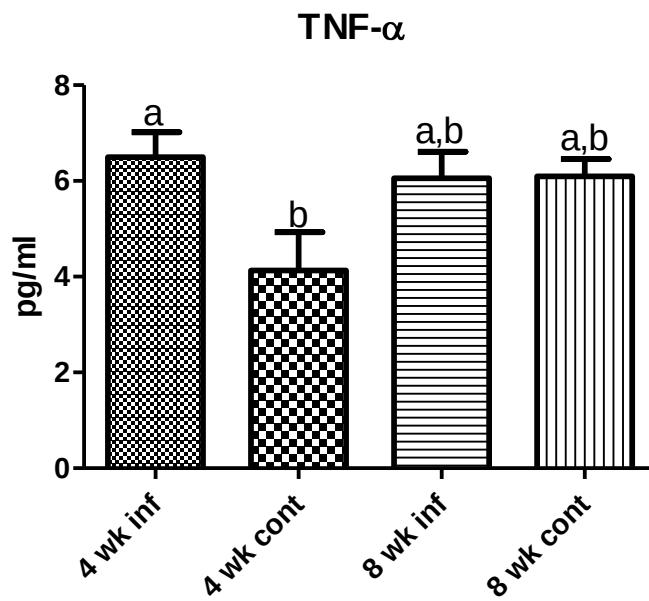
a, b-Different superscript indicate significantly different IL-2 levels in tissue

Figure 6.6. The levels of the cytokine IL-2 in uterine homogenates at 5 days post-infection with *T. foetus*. A significant difference is seen between the 4 wk old infected and the 4 wk old sham control ( $p= 0.00063$ ). Bar represents SEM. (n=10 in each group).

Table 6.4. Serum cytokine concentrations in *T.foetus*-infected and control mice (4 wk and 8wk old) at 5 days post-inoculation (mean and standard error of the mean - SEM)

|           | IL-2               | IL-4               | IL-5              | IFN- $\gamma$       | TNF- $\alpha$     |
|-----------|--------------------|--------------------|-------------------|---------------------|-------------------|
| 4 wk inf. | 1.190<br>(0.2105)  | 1.380<br>(0.4809)  | 3.530<br>(0.3467) | 1.060<br>(0.2459)   | 6.500<br>(0.5160) |
| 4 wk cont | 0.8857<br>(0.3240) | 0.5857<br>(0.3795) | 2.614<br>(0.5059) | 0.4143<br>(-0.2450) | 4.129<br>(0.7981) |
| 8 wk inf  | 1.250<br>(0.1586)  | 1.280<br>(0.3620)  | 3.120<br>(0.2210) | 1.130<br>(0.2044)   | 6.060<br>(0.5476) |
| 8 wk cont | 1.563<br>(0.1463)  | 2.013<br>(0.5191)  | 3.475<br>(0.3793) | 1.163<br>(0.2890)   | 6.100<br>(0.3571) |





a, b-Different superscript indicate significantly different TNF- $\alpha$  levels in tissue

Figure. 6.7 Levels of the cytokine TNF- $\alpha$  in serum 5 days after infection with *T. foetus*. There was significant difference between the 4 wk old infected and the 4 wk old sham control ( $p=0.0158$ ). Bar represents SEM. ( $n=10$  in each group)

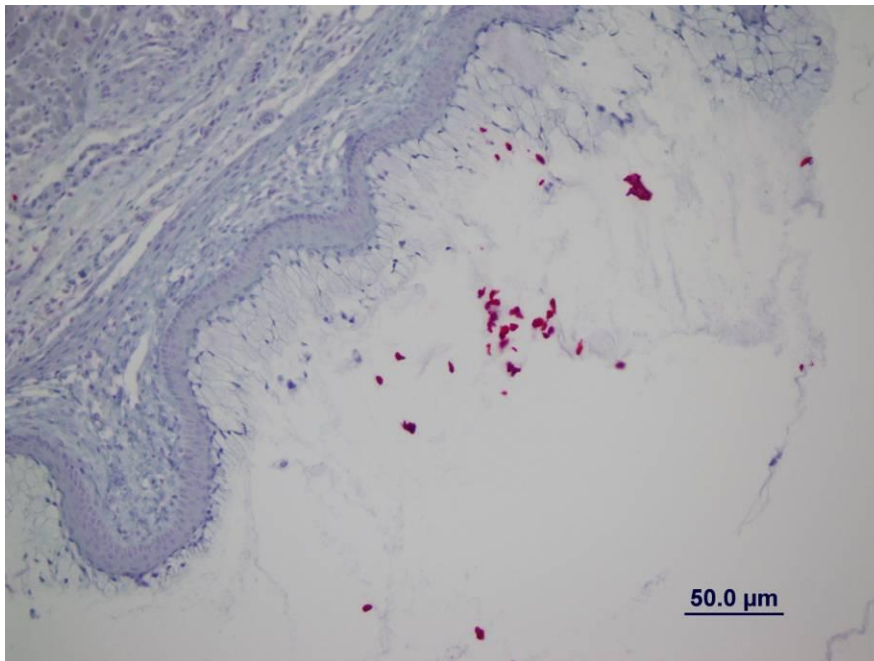


Figure 6.8. *Trichomonas* organisms in the lumen of the mouse's vagina, and occasionally mixed with the mucinous secretion, as detected by immunolabelling with anti-*T.foetus* antibody.

Alkaline phosphatase red with hematoxylin counterstain

## Chapter 7

### Potentially protective dual oxidase enzymes (DUOX1 and DUOX2) in the bovine reproductive tract

#### **Abstract**

Vaginal and uterine mucosa is the portal for sexually transmitted viral, bacterial, and parasitic diseases (STD) in animals and man. The sequelae of STD lead to major economic costs in animal production and human medical care. Understanding the innate immunity response at the mucosal surface of infection is essential to enhancing protection and controlling STD. The recent discovery of the dual oxidases (DUOX1 /DUOX2) in different mucosal membranes, and recognizing the phenomenon of H<sub>2</sub>O<sub>2</sub> associated killing of microbes, suggests that these enzymes may be important in the innate defenses of the reproductive tract. DUOX1 and 2 have been recently identified in the reproductive tract of mice; however, their role in the bovine reproductive tract has not been documented. The aim of the current study is to: 1) investigate the presence of DUOX1 and 2 in the reproductive tract of cows using immunohistochemistry (IHC) and rtPCR, and 2) examine the production of DUOX1 and 2 *in vitro* during acute infection of cultured endometrial cells with *T.foetus* by IHC and PCR, testing the hypotheses that the DUOX molecules exist in the cow and increase in response to infection. Ovaries, oviduct, uterine mucosa, vagina, and intestine were collected in RNAlater from 6 female cattle for analysis of DUOX1 and 2 gene expression normalized to the endogenous control 18S gene. For IHC, samples were labeled with DUOX1 and 2 monoclonal antibodies and examined for specific binding. A comparison of mean critical threshold values (delta Ct) showed the highest DUOX1 and 2

gene expression in the vagina and the lowest in the ovary. DUOX1 and 2 expression was also detected in both bovine trophoblast and endometrial cell lines. Bovine endometrial cells were infected with *T. foetus* for 6 hours, however there was no significant change in DUOX1 and 2 levels, suggesting that if these molecules play a role in innate immunity, it may be later than 6 hrs post infection.

## **Introduction**

The vaginal and uterine mucosa is the portal of entry for sexually transmitted diseases (STD), such as trichomoniasis in cattle caused by *Tritrichomonas foetus*. *Tritrichomonas foetus* is an extracellular organism and the infection in cows is generally self-limiting, though the economically damaging effects of early embryonic death, infertility, and pyometra can be devastating. Historically, research has focused on the role of acquired humoral immune responses. However, few studies investigated how the innate reproductive immune response contributes to protection against *T. foetus* and how these responses might influence initial colonization. One study evaluated the effects of nitric oxide, a component of the early innate immune response, in which it was shown to be an effector of macrophage-mediated cytotoxicity towards the related human pathogen, *Trichomonas vaginalis*.<sup>26</sup> This study was the first to demonstrate that reactive nitrogen species (RNS) were able to kill the extracellular venereal parasites; though, this did not implicate RNS as being a critical molecule in the establishment of trichomonad infection within the reproductive tract.

Mechanisms that initiate immune responses and inflammation during *T. foetus* infection have been rarely studied. In studies of *T. vaginalis* stimulation of Toll-like receptor 4 (TLR-4) has been demonstrated, pointing to a mechanism whereby early inflammation is induced.<sup>34</sup> Related studies with *T. vaginalis* have demonstrated that, the parasite has the ability to induce

IL-8 production from both human monocytes and human neutrophils.<sup>27, 29</sup> Similar processes can be postulated to occur in the early response to *T. foetus*.

Recent studies have shown that dual oxidase molecules (specifically DUOX1 and DUOX2), members of the NADPH oxidase family, which are expressed on the mucosal surfaces of multiple mucosal membranes provide a well-defined, controllable, and dependable source of reactive oxygen species (ROS) (e.g., HOCl, peroxynitrate, hydrogen peroxide), which function in the early innate host response by catalyzing lactoperoxidase to generate antimicrobial hypothiocynate. Reactive oxygen species produced by these DUOX enzymes and their by-products can directly oxidize biomolecules in invading microbes in a nonspecific manner, resulting ultimately in molecular damage and microbial cell death.<sup>17</sup>

Studies have also demonstrated that DUOX1 is associated with specific cytokine pathways. In fact, DUOX1 has been associated primarily with a Th2 cytokine response characterized by IL-4, IL-5, and IL-8; whereas DUOX2 has been more closely associated with a Th1 response, characterized by increases in IFN- $\gamma$ . DUOX1 mediates the production of epithelial mucins, like MUC1 and MUC5AC, in response to inflammatory mediators such as neutrophil elastase or tumor necrosis factor (TNF)- $\alpha$ .<sup>16,30</sup> In other studies, DUOX1 was found as a mediator in epithelial production of the neutrophil chemokine IL-8 in response to stimulation by bacterial lipopolysaccharide (LPS).<sup>25</sup> Induction of DUOX enzymes has also been described. DUOX1 production is induced in response to IL-4 and IL-13 in respiratory tract epithelium. DUOX2 expression is induced in response to IFN- $\gamma$  in respiratory tract epithelium,<sup>13</sup> as well as in response to insulin in thyroid cell lines.<sup>22</sup> In another study, when DUOX enzyme was silenced, it led to increased mortality and infection by gut microbes. This effect was

reversed by reintroduction of the DUOX enzyme, causing infection levels and mortality to fall, demonstrating the key role of DUOX in mucosal immunity.<sup>12</sup>

The role of DUOX1 and DUOX2 in host immune responses has been well studied in the respiratory and gastrointestinal mucosa. However, there is paucity of such information regarding the situation in the reproductive tract mucosa. Recently, the presence of DUOX1 and DUOX2 has been demonstrated in the mouse reproductive tract, including the vagina, uterus, oviduct, and ovary (Agnew et al, manuscript in preparation). However, these molecules have not been demonstrated in a livestock species, nor has the response of these molecules to a venereal pathogen been investigated.

*Tritrichomonas foetus*, the causative agent of bovine trichomoniasis, is an extracellular organism that typically produces Th2 response. Because, it has been demonstrated that Th2 cytokines regulate DUOX1 expression, it is expected that during *T. foetus* infection, DUOX1 and Th2 cytokines production will be important for host protection. We hypothesize that DUOX1 and DUOX2 are present in the bovine reproductive tract and that DUOX1 plays a key role in early resistance to venereal infection by *T. foetus*.

The aim of the current study is to investigate the presence and production of DUOX1 and DUOX2 in the bovine reproductive tract and in bovine endometrial cell cultures (Chapter 1; figure1). This study also hypothesizes that in the presence of *T. foetus* DUOX1 increases in bovine endometrial cell cultures using immunohistochemistry (IHC) and RT-PCR. The specific objectives are;

1. To demonstrate the presence of DUOX1 and DUOX2 expression in the bovine reproductive tract (*in vivo*).

2. To confirm the presence of DUOX1 AND DUOX2 expression in bovine endometrial and trophoblast cell lines (*in vitro*)
3. To show an increase in DUOX1 expression in *T. foetus*-infected bovine endometrial cells (*in vitro*).

## **7.1 Materials and methods**

### **Subject material**

#### **7.1.2 Cow reproductive tract samples**

Reproductive tract tissues (ovary, oviduct, uterus, and ovary) and ileum from six adult cows were collected immediately after slaughter for immunohistochemistry (IHC) and real-time polymerase chain reaction (RT-PCR) investigation. For RT-PCR, approximately 2-4gm each of ovary, oviduct, uterine mucosa, vagina, and intestine were collected and stored separately in a centrifuge tube filled with 2mls of RNAlater (Qiagen, Valencia, CA) and processed for RNA extraction. For IHC, 4mm thick portions of the above listed tissues were collected into 15mls of 4% Paraformaldehyde.

#### **7.1.2 Endometrial and trophoblast cell lines**

The endometrial and trophoblast cell lines were kind donation from Dr. Linda Munson at the University of California Davis.<sup>23,24</sup> Cultures younger than 15 passages were seeded at  $5 \times 10^4$  cells/ml/ $5\text{cm}^2$  density and maintained in DMEM-F12 (Gibco, Life Technologies, 3175 Staley Road NY) medium, 5  $\mu\text{g/ml}$  bovine iron-saturated transferrin (ITS; Sigma-Aldrich), 10 ng/ml murine epidermal growth factor (Becton-Dickenson, San Jose, CA), and 10% fetal bovine serum (Invitrogen) at 37°C in 5% CO<sub>2</sub>. Upon 80-90% confluency the cells were harvested with 0.25% trypsin with gentle rocking to dislodge the cells. For long term storage for later analysis cells

were suspended at the range from  $2.9 \times 10^7$  to  $5.2 \times 10^7$  cells/ml in 1:1000 25-mercaptanol RLT buffer (QIAGEN, Valencia, CA 91355) and then stored at  $-80^\circ\text{C}$ .

#### 7.1.3 Infection of endometrial cell lines with *T. foetus*

The *T. foetus* isolate was originally obtained from a cow with severe post-coital pyometra, and was kind donation from Dr. R. BonDurant (UC Davis) and maintained in culture using trypticase-yeast extract (TYM) medium containing 10% fetal serum.<sup>8</sup> Endometrial cells and trophoblast were grown to 75-90% confluence in  $75\text{ cm}^2$  plates. One milliliter of *T. foetus* inoculum was added to the media at the concentration of  $1 \times 10^6$  cells/ml (multiplicity of infection = 0.1 (MOI)), and the cultures were incubated as previously described for an additional 6 hours. The cells were trypsinized, counted, mixed with 25-mercaptanol/ RLT buffer, and frozen as described above. Each plate infection was run in duplicate, and each experiment was repeated three times.

#### 7.1.4 RNA preparation

RNA was extracted using RNEasy Mini Kits and QIAshredder columns according to the manufacturer's instructions (Qiagen, Valencia, CA). The concentration of the obtained total RNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). RNA purity was determined by the A260/280 value, with ideal numbers ranging between 1.8 and 2.1. The A260/230 ratio (which detects ethanol and salt contaminants from RNA isolation) was provided as an additional measure of sample purity. A value of 1.0 or greater is acceptable, with optimal values being 1.8 or greater. All samples were between 1.97 and 2.09 for the A260/280 value, and all A260/230 values were greater than 1.8 except 3 specimens which had values of 0.35, 1.36, and 1.77.



#### 7.1.5 Primer and probe development

Since Applied Biosystems does not offer off-the-shelf gene expression assays for bovine DUOX1 or bovine DUOX2, custom assays were designed from predicted *Bos taurus* mRNA sequences available in GenBank (XM 587550 and XM 001253634, respectively). DUOX1 and DUOX2 cDNA sequences were analyzed with the NCBI BLAST program to identify regions of the sequence that are similar between the 2 target genes. The gene expression assays were designed in regions that did not show any similarities. Two assays (primers & probe) were designed for each gene by Applied Biosystems and are proprietary.

PCR was run for DUOX1 and DUOX2 using a wide range of cDNA templates (0.32ng to 80ng) synthesized from 1ug of RNA. cDNA samples from ten tissues (Intestine 6 and 7; Ovary 10 and 11; Oviduct 7 and 10; Uterine Mucosa 10 and 12; Vagina 7 and 10) were synthesized for serial dilution plates. This was carried out in order to determine the optimal amount of cDNA template for efficient PCR amplification. Optimal PCR amplification was achieved with 20ng of cDNA template.

#### 7.1.6 cDNA Synthesis

cDNA was synthesized by RT-PCR of 2µg each total RNA using High Capacity cDNA Reverse Transcription Kit according to manufacturer's instructions (Applied Biosystems) and primers. To eliminate amplification of any contaminating chromosomal DNA, the isolated RNA was treated with RNase free DNase (Qiagen, Valencia, CA) according to the manufacturer's instructions. Each cDNA sample was then amplified with Taq polymerase.

#### 7.1.7 q PCR Processing

PCR data quality was assessed using a combination of factors, including visual analysis of the shape of the raw PCR curve and the Ct value. Ct values less than 30 indicated high quality

data and correlate with samples that have high expression levels; Ct values from 30-35 corresponded to lower expression levels and Ct values over 35 were consistent with very low expression levels. Ct values were normalized to the endogenous control (Ct target – Ct endogenous control) to generate a delta Ct (DCT) value for each sample. DCT values were then compared among the different tissue groups to identify statistically significant gene expression differences. The endogenous control was 18S, based on extensive analysis for the most consistent endogenous control from the various tissues (data not shown).

#### 7.1.8 Immunohistochemistry for DUOX1 and DUOX2

Sections of ovaries, oviduct, uterine mucosa, vagina, and intestine were initially fixed in 4% paraformaldehyde, then after 24 hrs, rinsed in PBS. After another 24 hrs, the tissues were placed in 30% ethanol, then 50% ethanol 1 day later, and then finally stored in 70% ethanol 1 day later. Samples stored in 70% ethanol were embedded in paraffin, and sectioned on a rotary microtome at 4 $\mu$ m. Sections were placed on slides coated with 3-aminopropyltriethoxysilane and dried at 56 C overnight. The slides were subsequently deparaffinized in xylene and hydrated through descending grades of ethyl alcohol to distilled water. Slides were placed in Tris buffered saline (TBS) pH 7.5 for 5 minutes for pH adjustment. Endogenous peroxidases were blocked utilizing 3% hydrogen peroxide/ methanol bath for 20 minutes followed by a distilled water rinse. Slides were then placed in TBS + Tween 20 (Thermo Fisher Scientific, Rockford, IL 61105) and stained with standard Avidin –biotin complex. Staining steps were performed at room temperature on DAKO Autostainer. After blocking non-specific protein with normal Goat serum and bovine serum (Vector Labs-Burlingame, CA) for DUOX1 and DUOX2 respectively, for 30 minutes, sections were incubated with Avidin /Biotin blocking system for 15 minutes (Vector Labs-Burlingame, CA). Following subsequent rinsing in TBS + Tween 20, for DUOX1,

slides were incubated for 30min with Polyclonal rabbit antibody DUOX2 ( Santa Cruz-Santa Cruz , CA), diluted 1:200 with Normal Antibody Diluent (NAD) (Scyteck-Logan, UT). Slides were then rinsed in two changes of TBS +Tween 20. After rinsing slides were incubated in Biotinylated Goat and anti Rabbit 1gG H+ L (Vector) in NAD diluted 11ug/ml for 30min. In the case of DUOX2, slides were incubated for 30 min polyclonal goat antibody DUOX2 (Santa Cruz-Santa Cruz, CA), diluted 1:25 with Normal Antibody Diluent (NAD) (Scyteck-Logan, UT). Slides were then rinsed in two changes of TBS +Tween 20. After rinsing, slides were incubated in biotinylated bovine anti-goat IgGH +L (Vector) in NAD diluted 11ug/ml for 30 minutes. After this, slides were rinsed in TBS + Tween 20 followed by the application of R.T.U. Vectastain Elite ABC Reagent (Vector) for 30min. The slides were rinsed with TBS + Tween 20 and developed using Nova Red (Vector) for 15 min. At the completion of Nova Red, slides were rinsed in distilled water, counterstained using Grill 2 Hematoxylin (Thermo Electron – Pittsburgh, PA) for 30 sec, differentiated in 1% aqueous glacial acetic acid, rinsed in running tap water. Slides were then dehydrated through ascending grades of ethyl alcohol; cleared through several changes of xylene and cover slipped using Flotex permanent mounting media (Thermo Scientific –Pittsburgh, PA).

#### 7.1.9 Data Analysis

Data analysis was carried out using Applied Biosystems RQ Manager software v2.1) and StatMiner software (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA). Data analysis was carried out by firstly comparing the mean DCT values for each of the tissue types (for each gene). Ranked and arranged from highest to lowest expression level, and secondly gene expression was measured using the relative quantitation (RQ) method according to Applied Biosystem's recommendations. In this

study, the gene expression level of each tissue was compared to the expression level present in the intestine (control tissue).

#### 7.1.10 Statistical analysis

Three experiments were conducted and each experiment was duplicated. Paired and unpaired t-tests were used to compare control samples with test samples (cultured with *T. foetus*). To determine changes in RNA concentration between each experiment, we compared each duplicate test (cultured with *T. foetus*) with their respective controls for any significant difference. To determine whether total amount of RNA changed was significantly different between the *T. foetus* infected cells and the non- infected cell, each experiment in duplicate, was reported as an average of the three, and unpaired t-test was used to compare any significant difference.

## **7.2 Results**

### 7.2.1 PCR Analysis: Ct Values Generated for Each Gene and Tissue

All three genes (DUOX1, DUOX2 and 18S) were present in each of the samples. The 18S was shown to be highly expressed, indicated by a ct value less than 30. DUOX1 expression was more abundant than that of DUOX2 in the bovine reproductive tract, especially in the vagina (Figures 7.1, 7. 2, 7.3).

### 7.2.2 Cellular Localization of DUOX Protein in Bovine reproductive tract

Positive labeling by the anti-DUOX1 and DUOX2 antibodies was seen in the cytoplasm of the reproductive epithelium (Figure 7.4 A, B, C, D). The majority of the staining was observed both in the apical plasma membrane and to a greater extent, in the apical portion of epithelial cells.

### 7.2.3 In vitro cell results

Preliminary studies showed the presence of DUOX1 and DUOX2 by either qPCR or IHC in the endometrial and trophoblast cells. ( figure 7.5A, B and C). Neither DUOX1 nor DUOX2 was found in the pure culture of *T.foetus* or in the cell culture medium.

### 7.2.4 Cellular Localization of DUOX Protein in Bovine endometrial and trophoblast cell lines

Moderate positive labeling by the anti-DUOX antibodies was seen within the cytoplasm of the endometrial cells and weak staining in the trophoblast cells. ( figures 7. 6A and B).

### 7.2.5 *Trichostrongylus axei* infected bovine cell lines

All three genes (DUOX1, DUOX2 and 18S) were present in each of the cell culture samples. The 18S was shown to be highly expressed indicated by lower ct value less than 30. DUOX1 and DUOX2 were shown to be expressed; however; there was no statistically significant difference in the expression of DUOX1 or DUOX2. (Figures 7.7 and 7. 8)

## **7.3 Discussion**

Our investigation has demonstrated for the first time, using PCR and immunohistochemistry, the presence of DUOX molecules in the bovine reproductive tract, and in bovine trophoblast and endometrial cell lines. Based on our assays, DUOX1 was found to be more abundant than DUOX2 in the reproductive tract. We were not able to show, however, a significant change in either DUOX1 or DUOX2 expression after cells were treated with live *T. foetus* organisms after 6 hr of co-incubation. This suggests that, if DUOX molecules play a role in the immediate immune response to *T. foetus*, this occurs sooner or later than 6 hrs after inoculation. The 6 hrs time course was selected based on the results of our preliminary studies, in which 6hrs *T. foetus* infected endometrial cells showed significant DUOX1 expression comparing to that of 12hrs and 24 hrs. Also, this is supported by the previous studies in

demonstrating the host defense role of DUOX in *Drosophila* by Ha EM et al (2005) in which The host defense role of DUOX was demonstrated in *Drosophila* when the DUOX homologue, dDUOX, was silenced followed by microbes infection in the gut. After, 6 hours post infection, there was massive proliferation of microbes in the insect with the defected gene, and this was significantly different compared to that of the wild type.<sup>12</sup>

This data shows that bovine reproductive epithelial cells make DUOX1 and 2 mRNA and produce DUOX1 and 2 molecules, supporting reports of ROS generation by the endometrial epithelium in rats linked to NADPH oxidase.<sup>14</sup> In addition, other studies in humans indicate that ROS are important in the menstrual cycle<sup>32</sup>. Previous analysis of total mRNA from human uterus, demonstrated expression of NOX1<sup>1, 33</sup> and NOX5<sup>2, 3</sup> which are in the same NADPH oxidase family as DUOX. Other DUOX homologues like NOX2, NOX4, and NOX5 has been reported to be expressed in ovaries<sup>3</sup>.

The members of the NOX oxidase family, including DUOX, produce ROS, which participate in the immune response in a variety of ways such as stimulating the secretion of cytokines or the activation of other killing mechanisms, such as signaling transcription factors like NF- $\kappa$ B, proteins such as kinases and phosphatases, and ion and/or proton channels. DUOX molecules are distinct from other members of this group as they contain not only an NADPH-oxidase domain, but also a domain that is homologous to heme-containing peroxidase such as myeloperoxidase and lactoperoxidase (hence, the “dual” oxidase name). They consist of basic NOX5-like structure fused at the N-terminus with an additional transmembrane  $\alpha$ -helix that is linked at its N-terminus to the peroxidase-like domain.<sup>6,9,18</sup>

DUOX function in the host innate mucosal response has been studied in the respiratory epithelium and the gastrointestinal epithelium. Indeed, preliminary data from our laboratory

supports the importance of DUOX, by showing the expulsion of GI nematodes in rats correlated with a 30 fold increase in DUOX expression (Mackenzie et al, manuscript in preparation)

DUOX's role as a host innate response in the respiratory epithelium is vital for mucosal defense against bacterial infection. Their action against bacteria is similar to that of NOX 2 acting within the phagosome, which is based on NADPH oxidase generation of  $O_2^-$  followed by dismutation to  $H_2O_2$  (by phagosomal superoxide dismutase) and conversion to bactericidal HOCl by myeloperoxidase (MPO).<sup>7,31</sup> Other studies have shown that in the respiratory tract, DUOX isoforms have been confirmed to be the predominant enzymes responsible for the  $H_2O_2$  generation in the presence of lactoperoxidase (LPO) that is needed to catalyze a reaction between  $H_2O_2$  and thiocyanate to produce the bactericidal product hypothiocyanate.<sup>11</sup> Cytokine treatments that increase DUOX mRNA and DUOX protein levels, induce a parallel increase in  $H_2O_2$  production.<sup>13</sup>

Our study results indicate that DUOX1 is more abundant in the bovine reproductive tract than DUOX2. The role of DUOX1 and DUOX2 in both innate and adaptive host defenses of other organs has been demonstrated by their association with either Th1 or Th2 cytokines or by their induction through Toll-like receptors and bacterial stimuli.<sup>13,15</sup> Th2 cytokines have been shown to regulated DUOX1, supporting the speculation that DUOX1 is involved in the Th2 associated clearance of *T. fetus* from the female reproductive tract.<sup>4</sup> Immunohistochemistry showed an apical localization of DUOX in the reproductive tract surface epithelia supporting the findings from previous studies of thyroid gland and isolated cultured airway epithelial cells.<sup>5, 28</sup>

This study also indicated the presence of DUOX1 and DUOX2 in bovine trophoblast cells lines, supporting previous work by others where ROS production in human trophoblasts was linked with an NADPH oxidase characterized as being constitutively active and distinct from the

phagocyte NADPH oxidase.<sup>20, 21</sup> These NADPH oxidases were later confirmed to be NOX2, NOX4, and NOX5, homologues of DUOX enzymes.<sup>3,19</sup> The physiological role of ROS generation in the placental trophoblast has been suggested to include host defense and degradation of noxious substances or signaling and oxygen sensing.<sup>21</sup>

The demonstration here of DUOX1 and DUOX2 presence in the bovine reproductive tract and the endometrial cells support the hypothesis that, DUOX is expressed in the luminal epithelium. Reactive oxygen species produced by these enzymes, play important roles in the immune function. However, DUOX1 and DUOX2 expression in *T. foetus*-infected endometrial cells were not significantly different. It is possible that in this infection model DUOX responses either occur much faster or take longer than expected to be involved. Additional time course studies are necessary to further investigate this. In comparison to the current study, differential expression of DUOX1 and DUOX2 was noted when human tracheobronchial epithelial cells were treated with inflammatory cytokines: DUOX1 increased in response to the Th 2 cytokines IL-4 and IL-13 and, DUOX2 mRNA expression increased significantly in response to Th1 cytokine IFN- $\gamma$  and IL-1 $\alpha$ .<sup>13</sup> DUOX1 and DUOX2 production from the *T. foetus* infected bovine endometrial cell culture is consistent with the above report. However, the DUOX1 and DUOX2 response by protozoan stimuli may be different from that of direct *in vitro* stimulation by either Th1 or Th2 cytokines.

## **7.4 Conclusions**

In this study, we investigated the presence of DUOX1 and DUOX2 in the bovine reproductive tract by using PCR and immunohistochemistry. Results indicated the presence of both types of DUOX: however, DUOX1 was shown to be more abundant than DUOX2 in the reproductive tract, implying that Th2 cytokines, associated with DUOX1, are crucial in the host



immune response. For future studies, we will examine more closely the DUOX response in relation to infections in the reproductive tract *in vivo*. While we have here demonstrated that neither DUOX1 nor 2 significantly change after 6 hours of *T. foetus* infection, and it may be that the response occurs much faster or takes longer than expected.

The presence of DUOX molecules in our cultured endometrial and trophoblast cells demonstrated the potential of this model to further investigate the role of these enzymes and are particularly valuable for examining the potential role of DUOX molecules *in vitro*. In addition, the presence of DUOX molecules in cows suggests that this might be a valuable species for studying the role of innate immunity in the defense against venereal infections that cross taxonomic lines. Our future work will utilize these valuable resources to better characterize the DUOX response to pathogens such as *T. foetus* and develop therapeutic and preventative measures that might exploit this information.

## **7.5 Acknowledgements**

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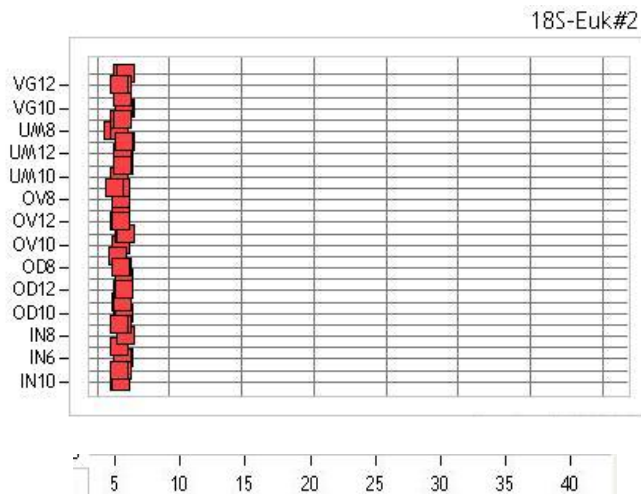
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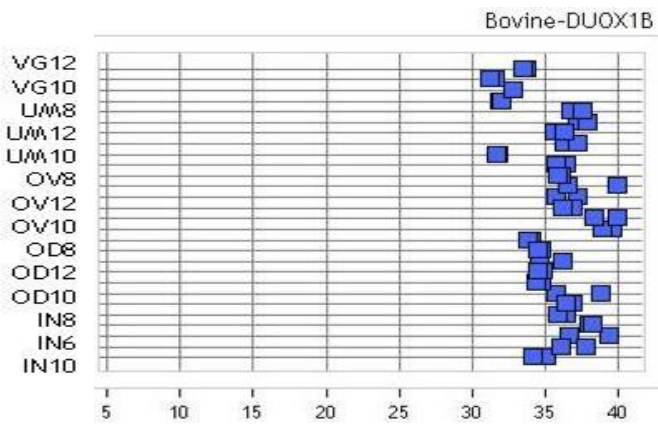
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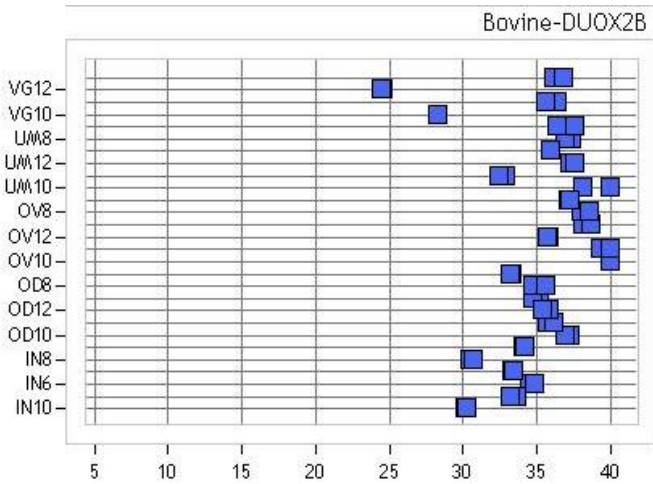


A



B

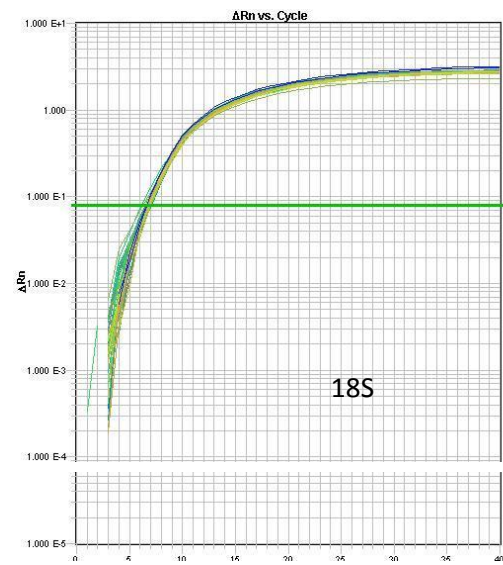
Figure 7. 1. Graph showing Raw CT values for the three genes; (A).18S-EUk; (B). Bovine Duox -1B and, (C) Bovine Duox 2B. Note. The abundance of DUOX 2 in the vagina tissue as compare to DUOX 1.



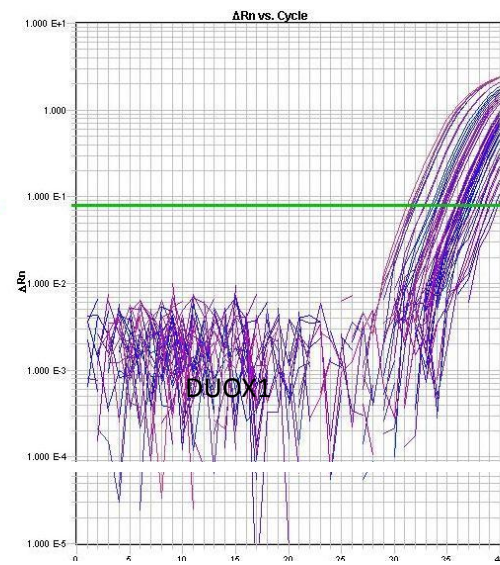
C

Figure 7.1 (cont'd) Graph showing Raw CT values for the three genes; (A).18S-EUk; (B). Bovine Duox -1B and, (C) Bovine Duox 2B. Note. The abundance of DUOX 2 in the vagina tissue as compare to DUOX 1.

A



B



C

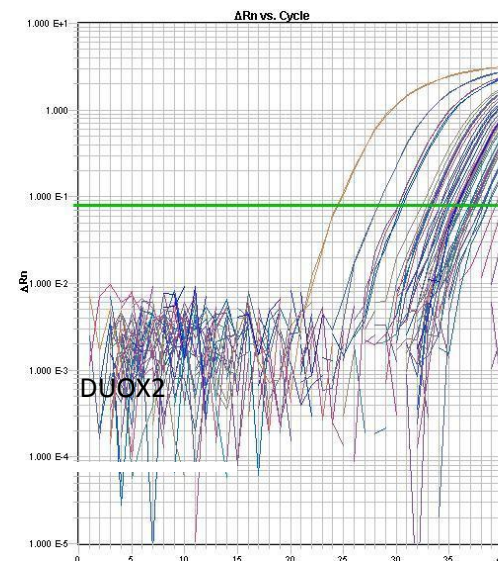


Figure 7. 2. Amplification of the three genes in bovine reproductive tissues. (A).18S (B) . Bovine Duox -1 and, (C) Bovine Duox 2.



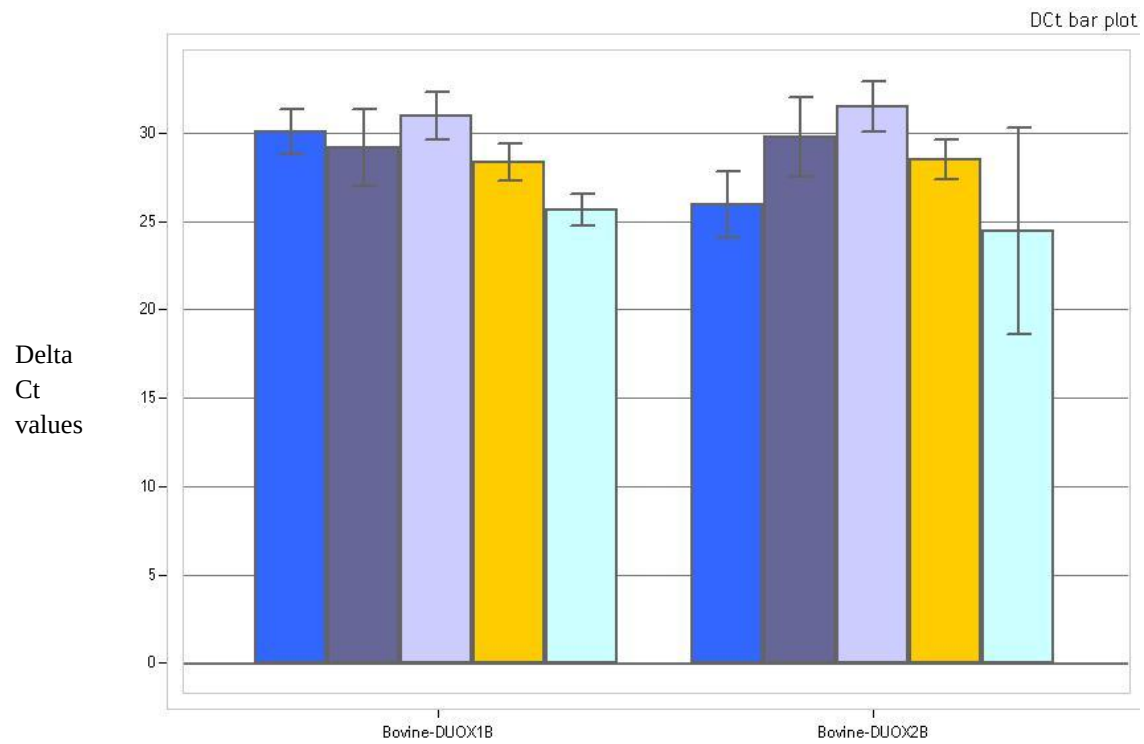
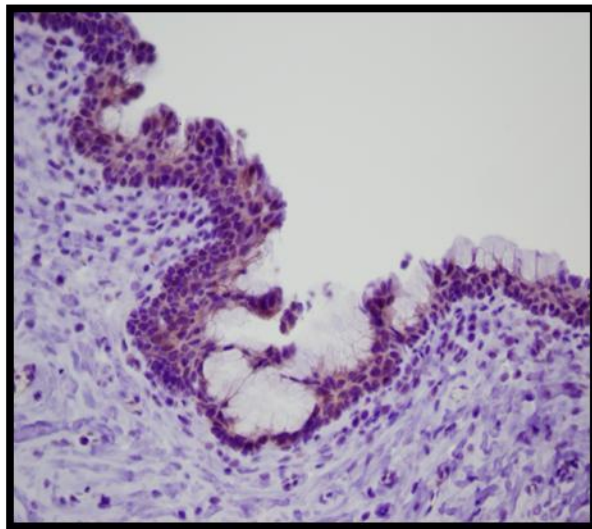
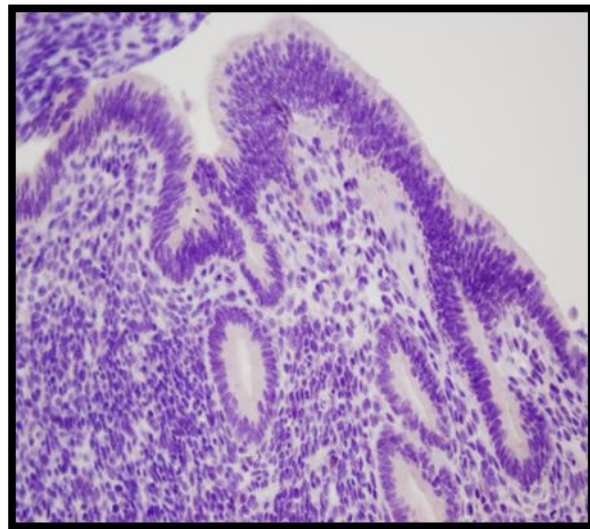


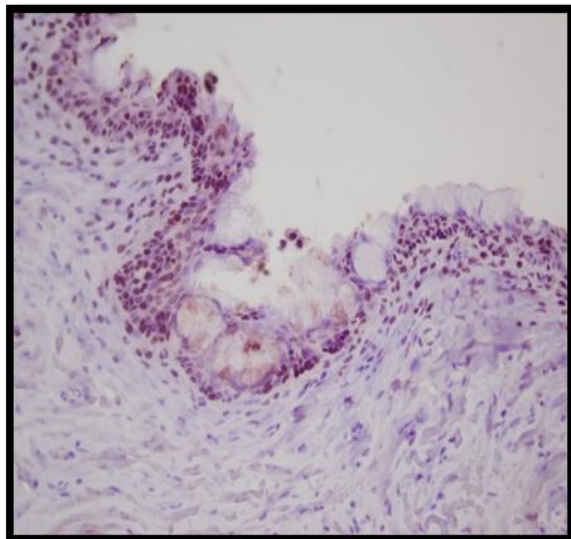
Figure 7.3. Graph showing the Delta Ct values of DUOX1 and DUOX2 in reproductive organs (bars represent the mean CT value from 6 animals with standard deviations). Note. Lower DCT values indicate higher expression levels.



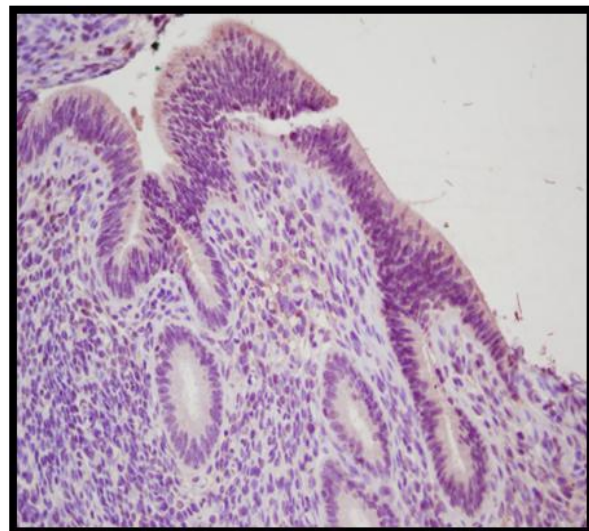
A



B



C



D

Figure 7.4. Localization of DUOX proteins in bovine vagina and uterus by immunohistochemistry A. Immunostaining for DUOX1 in bovine vagina. Note the heavy DUOX1 immunostaining in the cytoplasm of the epithelium. B. Immunostaining for DUOX 1 in bovine uterus with light DUOX immunostaining in the cytoplasm of the epithelium. C. Immunostaining for DUOX2 in bovine vagina. Note the heavy DUOX2 immunostaining in the

Figure 7.4 (cont'd) cytoplasm of the epithelium. D. Immunostaining for DUOX2 in bovine uterus. There is less DUOX2 immunostaining in the cytoplasm of the epithelia relative to the other sections. Immunohistochemistry for DUOX1 and DUOX2, ABC method; counterstained with Gill 2 hematoxylin. 40x.

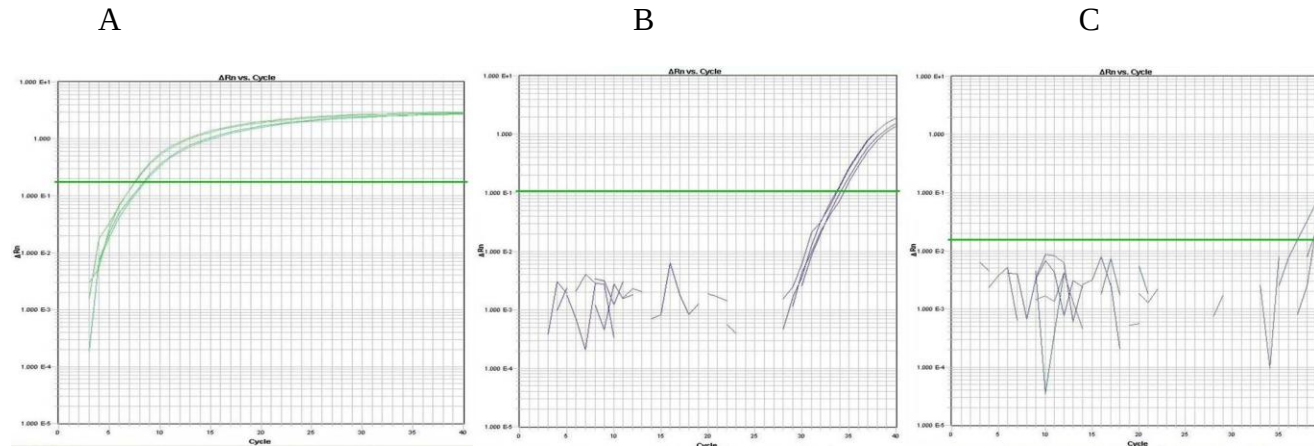
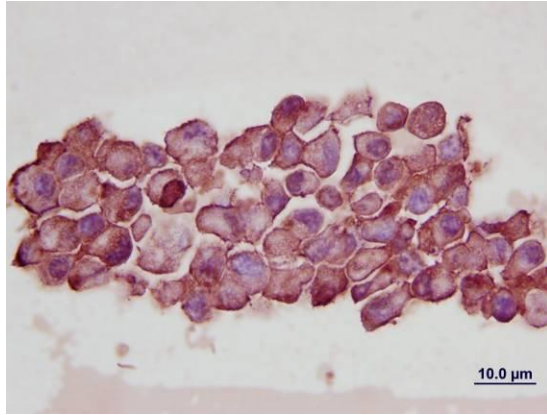
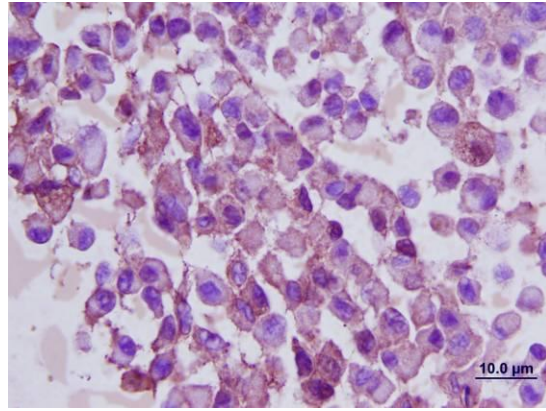


Figure 7.5. PCR results of endometrial and trophoblast cells lines showing expression levels of 18S, DUOX1, and DUOX2. (A) The varied expression levels of 18S for each of the cell lines. (B) Note. The expression of DUOX 1, with amplification of CT values in the mid 30's, indicating lower gene expression levels. (C) Note. No significant expression of either cell.



A. Bovine endometrial cell x40



B. Bovine trophoblast x40

Figure 7. 6. Localization of DUOX1 protein in bovine endometrial and trophoblast cultured cells by immunohistochemistry. A. Immunostaining for DUOX 1 in bovine endometrial cell. Note the heavy DUOX immunostaining in the cytoplasm of the epithelium. B. Immunostaining for DUOX1 in bovine trophoblast cells. Note the weaker DUOX1 immunostaining in the cytoplasm of the trophoblast cells. Immunohistochemistry for DUOX1 with ABC method; Grill 2 hematoxylin counterstain. 40x.

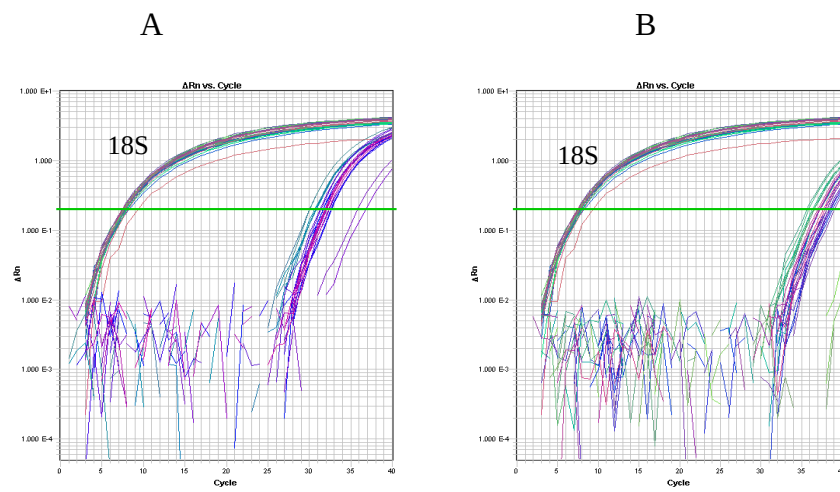
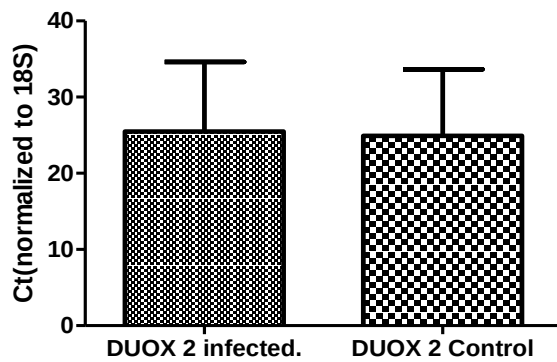
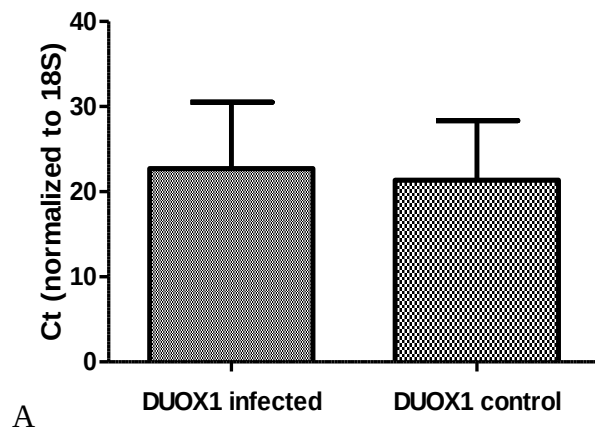
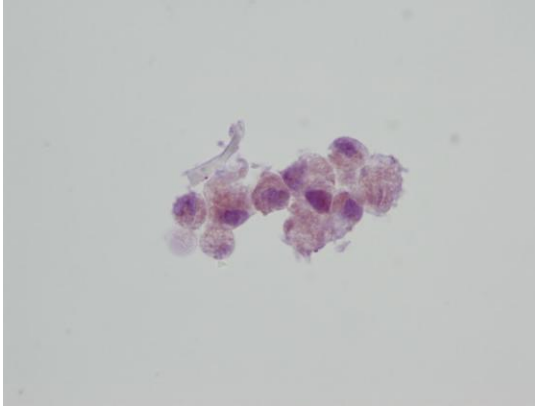


Figure 7.7. qPCR amplification of, 18S, DUOX1 (A), and DUOX2 (B) in *T. foetus*-infected and uninfected bovine endometrial cells.

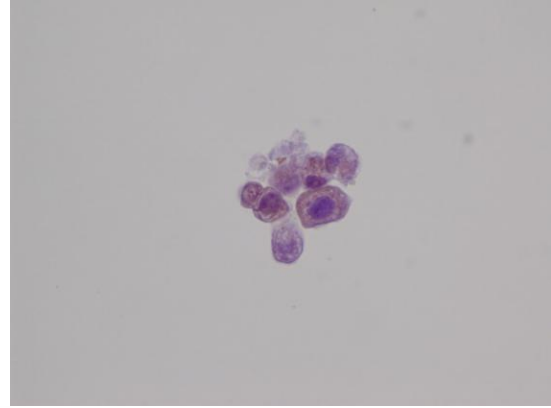


**B**

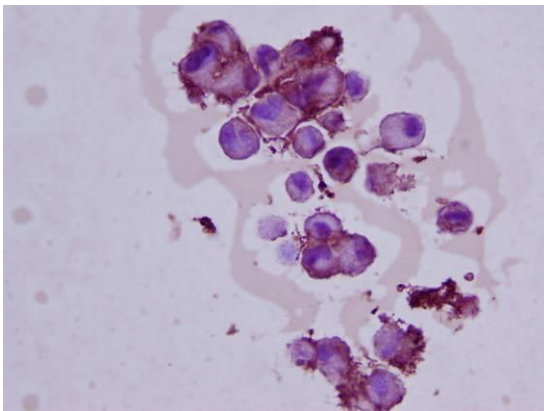
Figure 7.8. DUOX1 (A) and DUOX2 (B) gene expression in cultured bovine endometrial cells. (Bars indicate standard deviation; data is not statistically significant).



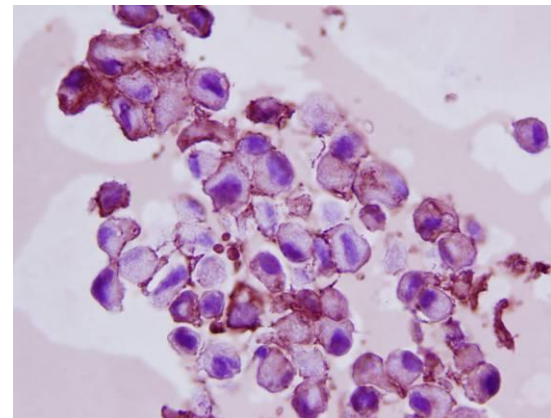
A.



B.



C.



D.

Figure 7.9. Localization of Duox protein in bovine reproductive tissues by immunohistochemistry. A. Immunostaining for DUOX1 in cultured *T. foetus*-infected bovine endometrial cells. Heavy DUOX1 labeling is present in the cytoplasm of the cells. B. Immunostaining for DUOX1 with lighter labeling in the cytoplasm of the uninfected endometrial cells. C. Immunostaining for DUOX2 in infected bovine endometrial cells. Heavy DUOX2 labeling is present in the cytoplasm of the endometrial epithelium. D. Immunostaining for DUOX2 with lighter labeling in the cytoplasm of bovine endometrial cells. Immunohistochemistry for DUOX1 (1:200) and DUOX2 (1:25) with ABC method; Grill 2 hematoxylin counterstain. 40x.



## Chapter 8

### Conclusion

Livestock reproductive diseases can have a significant effect on developing economies, yet they are only rarely studied. Studies conducted in some affected areas in sub-Saharan Africa showed that causative agents of such diseases are significant to livestock development and that successful eradication of such diseases depends on epidemiological studies with the aim of establishing mode of spread and factors that influence transmission. Additional knowledge on the mechanisms of host immune response to these reproductive pathogens at the molecular level including detailed information on the physical and biochemical factors involved can be exploited for possible vaccine development.

The results of the survey using cross-sectional analysis to study some selected infectious reproductive diseases in a Ghanaian herd of cattle indicated that *T. fetus*, IBR, and BHV-4 may be possible causes for a significantly prolonged calving interval and the resulting economic losses. Since, the report of this study is based on a single herd of cattle from a small part of Ghana, these findings do not necessarily represent the total situation in Ghana. Better information about these diseases is needed in order to institute appropriate treatments, as well as prevention and control measures. To achieve this, there will be the need for a country-wide epidemiological studies and definitive isolation of these pathogens. This study, the first to be reported, was undertaken to provide baseline data capable of directing future studies in Ghana with emphasis on *T. fetus*. The data from this study will be invaluable to Veterinary Services Department and animal production sector in Ghana and serve as vital information for better management of cattle.

Experience and many clinical studies indicate that a good understanding of the mechanism of the host innate immune response can be a key in prevention of trichomoniasis, though investigations of the initial colonization phase have been more sparse. Studies on innate immune response during acute stage of *T. foetus* infection were intended to address age variation and susceptibility to infection in a mouse model. This comparative study was done to tease out the difference in resistance between the two age groups based on their cytokine profile. The results showed that 4wk old mice had higher frequency of infection; however, pathological lesions between the two showed no significant difference. There was no significance in the uterine infection, though the 8wk groups showed a higher frequency. The 8wk old mice were shown to be more resistant which correlated with elevation in TNF- $\alpha$  in both infected and the control, implying TNF- $\alpha$  is constitutively secreted in the adult mice and may play a resistance role to *T. foetus* infection. The Th2 cytokines (IL-2 and IL-4) in the vagina and the uterine homogenates of the 4 wk old infected were down-regulated which were found to affect their susceptibility compared to the rest of the group. The mechanism leading to this down-regulation is not understood and needs to be further investigated. Contrary to the earlier reports from various studies that *T. foetus* infection is primarily handled by Th2 pathway cytokines, the results from our study indicated that both Th1 and the Th2 pathways seem to be involved in the response to *T. foetus* infection. However, TNF- $\alpha$  has been variably associated with Th1, Th2, or neither; instead it appears to be proinflammatory, participating in either Th milieu.

DUOX1 and DUOX2, members of the NADPH oxidase family, function in the mucosal surface innate host response. When human tracheobronchial epithelial cells were treated with inflammatory cytokines, DUOX1 increased in response to the Th 2 cytokines (IL-4) and DUOX2

mRNA expression increased significantly in response to Th1 cytokine interferon gamma (IFN- $\gamma$ ), implying differential expression.

The goal of Chapter 7 study was to confirm such differential expression in bovine endometrial cells infected 6hrs with an extracellular protozoan (*T. foetus*), known to respond to Th2 response. These were done in three steps; first, we documented the presence of DUOX in the reproductive tract of bovine (*in vivo*). Second, we confirmed DUOX1 and DUOX2 were present in the bovine endometrial and trophoblast cell lines, but not in *T. foetus* or in the media. Finally, we infected endometrial cell lines with *T. foetus* for 6hrs to test whether DUOX1 or DUOX2 would significantly increase in response to the acute stage of *T. foetus* infection. The results demonstrate that neither DUOX1 nor 2 significantly change after 6 hours of *T. foetus* infection. This implies that if there is a response, it must either occur faster or take longer than expected. In summary, the study did not confirm nor deny the differential expression of DUOX1 and DUOX2 in the reproductive tract mucosal surface; however, it demonstrated that like airway and thyroid epithelial cells, reproductive tract mucosal surface also expressed DUOX1 and DUOX2.

Based on these results, our future studies will focus on additional time course studies from 0-48hrs at 2 hr intervals determining DUOX expression during infection. Also, different models for both *in vivo* and *in vitro* studies will be considered to maximize the chance of DUOX expression. In addition, the mechanism of DUOX innate immune protection in the reproductive tract will be further elucidated. The presence of DUOX molecules in our cultured endometrial cells makes this a particularly valuable model for examining the potential role of DUOX molecules *in vitro*.

Our future work will utilize this valuable model to better characterize the DUOX response to pathogens such as *T. foetus* and develop therapeutic and preventative measures that might exploit this information.