Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Veterinary Medicine
and Animal Science
Department of Clinical Sciences

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Uppsala
2014

Degree Project 30 credits within the Veterinary Medicine Programme

ISSN 1652-8697
Examensarbete 2014:10

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Clostridium difficile-toxin bland köttmarsvin i Perus högländer.

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Degree Project in Veterinary Medicine

***Credits**: 30 hec*
***Level**: Second cycle, A2E*
***Course code**: EX0736*

***Place of publication**: Uppsala*
***Year of publication**: 2014*
***Number of part of series**: Examensarbete 2014:10*
***ISSN**: 1652-8697*
***Online publication**: http://stud.epsilon.slu.se*

Photografer: Sandra Lundgren

***Key words**: guinea pig, Clostridium difficile, toxin, typhlitis, Peru*

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SUMMARY

The guinea pig is an important meat-producing animal in Peru as well as in several other South American countries. They are held in different kinds of systems, from the small backyard breeders with few animals to the large commercial farms with thousands of guinea pigs. One of the major issues at the farms is animals dying of unknown causes. Salmonellosis is often considered as a major cause, but samples for confirmation are rarely taken. It has been known for a long time that *Clostridium difficile* (*C. difficile*) toxins are lethal to guinea pigs. Several studies have also investigated and confirmed that treatment with antibiotics is a significant risk factor for development of *C. difficile* infection (CDI) in laboratory animals. *C. difficile* is a large, anaerobic, gram positive rod with two essential virulence factors, toxin A and toxin B. Until now there appear to be no published studies or reports on the possible occurrence of CDI in meat producing guinea pigs. The aim of this study was to investigate whether *C. difficile* infection exists in meat producing guinea pigs in the highlands of Peru. The study was performed by taking samples from 77 guinea pigs from 15 farms with 120-5000 animals on each farm, in the Mantaro valley in Peru. The samples were taken either by a rectal swab (22.0 %) or directly from the cecum (77.9 %) at necropsy. The clinical and pathologic findings in the selected animals primarily consisted of signs of diarrhea, poor general condition, ascites, typhlitis and enteritis. Some of the animals had been treated with antibiotics during the last week before the samples were taken. An enzyme immunoassay (EIA) was used to demonstrate the presence of *C. difficile* toxin A and/or B in the samples. One (1) animal was found toxin positive in the test. It was a neonate male from a large farm that at necropsy showed lesions consistent with severe typhlitis.

SAMMANFATTNING

I Peru, liksom i flera andra Sydamerikanska länder är marsvinet ett viktigt köttproducerande djur. De hålls i olika system, från små uppfödningar på bakgårdarna med ett fåtal djur till stora kommersiella farmer med tusentals marsvin. Djur som dör av okänd anledning är ett av de stora problemen på farmerna. Salmonella antas ofta vara orsaken, men prover för konfirmering tas sällan. Det är sedan länge känt att toxiner producerade av *Clostridium difficile* (*C. difficile*) är dödliga för marsvin. Flera studier har undersökt och konfirmerat att antibiotikabehandling är en viktig riskfaktor för utvecklandet av *C. difficile*-infektion (CDI) hos försöksdjur. *C. difficile* är en stor, anaerob, grampositiv stav med två huvudsakliga virulensfaktorer, toxin A och toxin B. Inga tidigare rapporter eller studier över förekomst av CDI hos köttproducerande marsvin har påträffats. Syftet med den här studien var att undersöka om *Clostridium difficile*-infektion existerar bland köttproducerande marsvin i de Peruanska högländerna. För att studera detta togs prover från 77 marsvin från 15 olika farmer med 120-5000 djur per farm, i Mantarodalen i Peru. Proverna togs antingen med en rektalsvabb (22.0 %) eller direkt från cecum (77.9 %) i samband med obduktion. De kliniska och patologiska fynden hos de utvalda djuren bestod i huvudsak av diarré, nedsatt allmäntillstånd, ascites, tyflit och enterit. Några av djuren hade behandlats med antibiotika under veckan före provtagning. En ”enzyme immunoassay” (EIA) användes för att påvisa förekomsten av *C. difficile* toxin A och/eller B i proverna. Ett (1) djur var toxinpositivt i testet. Det var en ung hane från en stor farm. Vid obduktionen sågs lesioner överensstämmande med kraftig tyflit.

CONTENTS

INTRODUCTION

Guinea pigs (*Cavia porcellus)* are small herbivorous, monogastric rodents that are kept and bred as a meat producing animal in Peru and several other countries in South America (FAO, 1997). It is a growing industry in Peru with an increasing amount of meat being exported. The guinea pig is an important source of nutrients such as proteins for the Andean people, as well as a significant socioeconomic factor for the poor rural families. Compared to other livestock the guinea pig is cheaper and easier to keep. They are raised according to three principally different systems, the family, the family-commercial and the commercial systems. In the family systems the animals are kept at home, often in the kitchen or in the backyard and are taken care of by the women and children in the family. This type of husbandry is very common. The family-commercial and commercial farms are increasingly well organized and larger in size.

In the larger farms there are often a greater awareness connected with the breeding for traits that affect production (FAO, 1997). The animals are of different types and selected and bred with different goals, such as numbers of offspring, growth rate and feed conversion, to name a few. In the family held systems there is often a negative selection because the biggest/fastest growing animals get eaten or sold and the less productive ones are left to reproduce.

Clostridium difficile (C. difficile), is a gram positive, spore forming, toxin producing anaerobic bacterium. *C. difficile* infection (CDI, formerly known as *C. difficile*-associated disease (CDAD)) may, in humans as well as in several animal species, result in the whole panorama of clinical expression from asymptomatic carrier state to fulminant potentially fatal pseudomembranous colitis (Keel & Songer, 2006). The symptoms vary by animal species including humans, age groups and other characteristics of the population but in general appear as diarrhea following after treatment with antibiotics. The bacteria were first found by Hall and O'Toole in 1935 in feces from healthy newborn human babies. They found the bacteria difficult to cultivate and named it *Bacillus difficilis (B. difficilis)* due to that trait and its morphology. The high carrier rate among asymptomatic newborns made Hall and O'Toole discount the bacteria an important pathogen (Keessen *et al.*, 2011a). Only over the last 30 years *C. difficile* has been recognized as a pathogen, and primarily in humans where it is now one of the most important nosocomial diseases. In veterinary medicine it is also now considered an important emerging pathogen (Songer, 2004). Most of the studies are done in humans and laboratory animals, but the infection has been described in many other species as well.

The purposes with this study were:

- To investigate if CDI (i.e. *C. difficile* toxin production) exists in meat producing guinea pigs in the highlands of Peru.
- To examine what the symptoms are in the farmed guinea pigs with CDI and if that is in concordance with previous findings in laboratory guinea pigs.
- To assess if there is correlation between treatment with antibiotics and the presence of *C. difficile* toxins in feces or cecal contents from guinea pigs.

LITERATURE REVIEW

Pathogenesis of CDI

The predisposing factors for development of CDI have been thoroughly investigated in humans. It has been postulated that the two main factors for appearance of clinical disease are exposure to the pathogen in combination with a disruption of the intestinal flora (Kelly & LaMont, 1998). This is most likely true for animals as well. The most important risk factors for humans are hospitalization,

antibiotic treatment and advanced age (Hurley & Nguyen, 2002; Simor *et al.*, 2002). These risk factors are not fully evaluated in various animal species, but it is known that for some species, like guinea pigs and hamsters, treatment with antibiotics is a significant risk factor for development of CDI (Knoop, 1979; Lowe *et al.*, 1980; Rehg, 1980; Rothman, 1981). Other risk factors that have been suggested but not proven for animals are, for example in horses, stress, fastening, surgical or medical treatment and hospitalization (Båverud *et al.*, 1997; Gustafsson *et al.*, 2004). The results from some studies are in conflict, for example if antibiotic treatment is a risk factor for CDI in horses, where a strong relation between the two has been shown in some studies (Madewell *et al.*, 1995; Båverud *et al.*, 1997) whereas in other investigations antibiotics was not found being a prerequisite for CDI in horses (Weese *et al.*, 2006).

C. difficile has two essential virulence factors, the two large exotoxins toxin A (TcdA) and toxin B (TcdB). Only the toxin producing types among the over 400 different *C. difficile* strains cause CDI (Tonna & Welsby, 2005). The two toxins are both cytotoxic and can cause disease on their own, but often act synergistically (Poutanen & Simor, 2004). The cytotoxic effect of TcdB is 1000 times more potent than that of TcdA (Tonna & Welsby, 2005), but for long TcdB was thought not to be able to cause disease on its own (Lyerly *et al.*, 1985). It was shown that TcdA caused damage to the mucosa of the intestines and TcdB then seemed to affect the epithelial cells under it (Poutanen & Simor, 2004). However, after finding TcdB positive, TcdA negative strains that cause disease in humans (al-Barrak *et al.*, 1999; van den Berg *et al.*, 2004) it was concluded that TcdA is not necessary for developing CDI.

The receptor-binding domains of TcdA and TcdB seem different from each other (von Eichel-Streiber *et al.*, 1992). The receptor for TcdA is primarily located on the brush border of some of the intestinal epithelial cells. A receptor for TcdB has yet to be demonstrated. TcdA is taken up by endocytosis when it binds to the receptor of a cell, and then fuses with a lysosome (Frisch *et al.*, 2003; Pfeifer *et al.*, 2003). Through several steps the toxin becomes activated and released into the cytoplasm. TcdB is activated in endolysosomes as well, but the exact mechanism is not known (Qa'Dan *et al.*, 2000; Pfeifer *et al.*, 2003).

The toxins exert their effect in several ways. They inactivate Rho GTPases, damage the actin cytoskeleton and cause the loss of cell-to-cell contact by disruption of cell adhesion molecules, and eventually induce apoptosis (Pothoulakis & LaMont, 2001; Pfeifer *et al.*, 2003). TcdA and TcdB have several other direct and indirect effects on cell signaling. The toxins initiate an inflammatory cascade involving several proinflammatory interleukins and tumor necrosis factor-alpha. Neutrophils, monocytes, macrophages and mast cells also play an important role in the pathogenesis of CDI. TcdA causes the macrophages to express cyclooxygenase-2 with production of prostaglandin E_2 (Alcantara *et al.*, 2001). All this leads to increased secretion of fluid into the intestinal lumen and tissue damage, with or without pseudomembrane formation. There is a neural component as well in the pathogenesis of CDI (Pothoulakis & LaMont, 2001) wherein TcdA stimulates the release of substance P from neurons. Substance P causes degranulation of mast cells as well as having a direct vascular effect, both contributing to the inflammation. The toxin also exerts its effect directly in the small intestine where it inhibits the release of norepinephrine from sympathetic postganglionic nerve fibers (Xia *et al.*, 2000).

In addition to TcdA and TcdB a few *C. difficile* strains produce a binary toxin (CDT) (Perelle *et al.*, 1997; Gülke *et al.*, 2001; Geric *et al.*, 2006). CDT has been demonstrated to induce fluid accumulation in one study (Geric *et al.*, 2006) and is toxic to eukaryotic cells in culture, but the clinical relevance is still not known.

CDI in humans and different animal species

Most of the epidemiologic data are from humans (Kessen *et al.*, 2011a). Until quite recently *C. difficile* has not been recognized as a pathogen for animals and prevalence studies done often include only a small number of animals and are based on use of different methods for detecting the bacteria and its toxins as there is no reference standard. The consequence is large discrepancies in the results found in the various studies.

Humans

Hall and O'Toole (1935) first found the bacteria and its toxins in healthy newborn babies. Later studies have isolated the bacteria in feces from asymptomatic babies with a prevalence as high as 50-80 % (Bolton *et al.*, 1984; Hurley & Nguyen, 2002). Human neonates seem to be almost completely resistant against the *C. difficile* toxins even when the colonization and level of toxins are as high as found in adult humans with severe disease (Larson *et al.*, 1982; Bolton *et al.*, 1984). The carrier rate among healthy adults in general is usually found low, around 3 % (Hurley & Nguyen, 2002) with the exception of a study where Iizuka *et al*. (2004) used a RT-PCR method where they found toxigenic RNA from *C. difficile* in approximately 50 % of the 30 asymptomatic adults tested. Probably more common is carrier status among asymptomatic patients at hospitals and long-term care facilities where the prevalence is about 4-20 % (Johnson *et al.*, 1990; Simor *et al*., 2002). This is most likely a consequence of a higher burden of spores in the environment. Most of the symptomatic humans are elderly persons of at least 65 years of age that are hospitalized and have received antibiotic treatment (Hurley & Nguyen, 2002; Simor *et al*., 2002).

Guinea pigs

Several early studies regarding *C. difficile* and CDI were done in laboratory guinea pigs, but to the authors knowledge no studies have investigated *C. difficile* in commercial and/or familiar guinea pig farms for food production.

Snyder (1937) found that the toxin of *B. difficilis* (now known as *C. difficile*) was lethal when injected subcutaneously in guinea pigs, but was not taken up from the intestines when given orally. The main findings were a gelatinous hemorrhagic edema at the injection site, and that the guinea pigs died in convulsions that were not seen in the other species tested.

That several types of antibiotics, among them penicillin, ampicillin and clindamycin, cause typhlitis due to *C. difficile* toxins in guinea pigs has been known for a long time (Lowe *et al*., 1980; Rehg, 1980; Rothman, 1981). Rehg (1980) studied a toxin found in the cecum of guinea pigs with clindamycin-associated colitis. Most of the animals died after a single subcutaneous injection with clindamycin and the cecal filtrate from these animals was lethal to the guinea pigs that were injected with it intraperitoneally. The toxin found was neutralized by *C. sordelli* antitoxin. The *C. sordelli* antitoxin is also known for neutralizing *C. difficile* toxin (i.e. the toxin could possibly be from *C. difficile*). Another study also demonstrated that guinea pigs injected with clindamycin die from colitis, and that the cecal filtrates from these animals contain a cytotoxin that is lethal to other guinea pigs (Knoop, 1979).

Rothman (1981) investigated what caused the colitis and death of guinea pigs after administration of a single dose of penicillin. She found a toxin in cecum of the animals that was cytotoxic in cell cultures and also lethal to guinea pigs after injection into ileum or cecum. The animals died within 24 hours after injection. Congestion and hemorrhage were seen in the cecum. The results strongly indicated that the toxin was produced by *C. difficile.*

Boot *et al.* (1989) investigated the presence of *C. difficile* and its toxins in cases of typhlitis in specific pathogen free guinea pigs that had not received antibiotics or any other treatment. They found the *C.*

difficile toxins to be the cause of the spontaneous acute pseudomembranous typhlitis detected in most of these animals. Another common finding was normal feces in the rectum, with fecal impaction of the first part of the colon. External signs of diarrhea were rarely observed.

Other species

Adults

Of the common laboratory species, the hamster is by far the species most likely to develop CDI after treatment with antibiotics because of its high sensitivity to *C. difficile* toxins (Chang *et al.*, 1978; Lyerly *et al.*, 1985). Rats are the least sensitive laboratory animal to *C. difficile* toxin, and mice are between the hamsters and the rats in sensitivity (Lyerly *et al.*, 1985).

There is a significant association between *C. difficile* and enterocolitis in horses (Båverud *et al*., 1997; 2003; Weese *et al*., 2001b). In a study by Båverud *et al.* (2003) 28 % of the horses with antibiotic-associated diarrhea were positive for *C. difficile* toxins. In adult healthy horses the reported prevalence of *C. difficile* is generally low, 0-1 % (Jones *et al*., 1987; al Saif & Brazier, 1996; Weese *et al.*, 2001b; Båverud *et al*., 1997; 2003). In a study of mainly racetrack horses in Canada the estimated prevalence of *C. difficile* was 7 % (Medina-Torres, 2009).

Whether *C. difficile* should be considered a pathogen in dogs is a topic of discussion and further investigation. The prevalence of *C. difficile* found in healthy dogs ranges from 10.5-58 %, but there are also several reports about the isolation of *C. difficile* and its toxins in dogs with severe acute, as well as chronic, diarrhea, with a significant association between detection of the toxins and symptoms of diarrhea (Marks *et al.*, 2002; Borriello *et al.*, 1983; Riley *et al.*, 1991; Lefebvre *et al.*, 2006; Clooten *et al.*, 2008; Weese *et al.*, 2001a; Cave *et al.*, 2002).

The situation for cats seems similar to that as in dogs. *C. difficile* is found in asymptomatic cats (Borriello *et al*., 1983; Riley *et al*., 1991; al Saif & Brazier, 1996; Madewell *et al.*, 1999) but there has also been indications that *C. difficile* can cause diarrhea in cats as well (Weese *et al.*, 2001c).

Neonates

Toxin resistance seen in human neonates is also observed in neonatal rabbits and hamsters that do not seem to be affected by the toxins either (Rolfe & Iaconis, 1983; Eglow *et al.*, 1992; Keel & Songer, 2007). In contrast, neonates of other species, like piglets, hares and foals are very sensitive to the *C. difficile* toxins (Dabard *et al.*, 1979; Jones *et al.*, 1987; Waters *et al.*, 1998; Songer *et al.*, 2007; Yaeger *et al.*, 2007; Debast *et al.*, 2009).

The reason for this difference in susceptibility between different species is not fully understood. It has been implicated that it is dependent on the absence or presence of a significant number of TcdA receptors, though newborn rabbits have very few receptors (Eglow *et al.*, 1992; Borriello & Wilcox, 1998). However, in other studies it was discovered that the binding of toxins in the intestines of neonate hamsters is significant and similar to the one that occurs in adult hamsters and piglets, implicating that there is another still not discovered factor (Keel & Songer, 2007; Rolfe, 1991).

Source of infection

For humans, the most common way of transmission is nosocomial at hospitals and long-term care facilities where the prevalence of *C. difficile* in the environment is high (Simor *et al.*, 2002). The same association is not proved for hospitalized animals like dogs, cats and horses even if some studies done in horses indicate that it occurs but that community-associated infection is probably more common (Madewell *et al., 1995;* Aroyo *et al.*, 2007).

Similar ribotypes of *C. difficile* are found in humans and animals which implicates that transmission between animals and humans is a possibility (Arroyo *et al*., 2005; Rodriguez-Palacios *et al*., 2006; Keel *et al.*, 2007; Goorhuis *et al*., 2008; Hammitt *et al*., 2008; Jhung *et al.*, 2008; Debast *et al.*, 2009; Indra *et al.*, 2009). Another explanation could be the fact that *C. difficile* is a ubiquitous bacteria so the source could be common for both the animal and human isolates.

No exact data were found concerning how fast the toxins are degraded in dead animals but according to Bartlett (2008) they seem very resistant. He tested stool specimens that had "been stored in unspecified conditions for 4 to 5 years" and found all of them still positive for *C. difficile* toxins.

Strains of *C. difficile* with the same ribotypes commonly found in humans have been isolated from food products like meat, vegetables and ready-to-eat salads, but no foodborne outbreaks have been reported (al Saif & Brazier, 1996; Rodriguez-Palacios *et al.*, 2007; 2009; Bakri *et al*., 2009; Songer *et al.*, 2009; Jöbstl *et al*., 2010; Weese *et al.*, 2010). Whether the origin of the organism is from humans or animals has to be evaluated further.

Clinical expression

The symptoms and the pathologic changes seen in humans and different animal species are largely similar. The *C. difficile* toxins cause an inflammation in the intestines. The distribution of the lesions differs among different species and age groups (Rothman, 1981; Jones *et al*., 1987; Perkins *et al.*, 1995; Kelly & LaMont, 1998; Waters *et al.*, 1998). For humans the lesions are found in the distal part of the colon. In guinea pigs, neonatal pigs and adult horses the main location for the lesions are the cecum, and for adult horses with extension to the proximal colon. Two groups differ from the rest, in rabbits and foals the lesions are mainly found in the small intestines.

The clinical signs within a certain species can vary from mild diarrhea to severe pseudomembranous colitis and death (Kelly & LaMont, 1998). In humans CDI is often asymptomatic, or with only milder diarrhea. Some people develop more or less extensive colitis with watery diarrhea, nausea and abdominal pain and in more severe cases pseudomembrane formation is also seen. In the most severe cases the disease progresses with complications such as ileus, perforation or megacolon and could be life-threatening (Rubin *et al.*, 1995; Kelly & LaMont, 1998).

In hamsters and guinea pigs the most common findings are typhlitis with accumulation of fluid in the cecum, enlargement of the mesenteric lymph nodes, more or less extensive hemorrhage and congestion as well as thinning of the epithelium or thickening of the cecal wall (Rothman, 1981; Blankenship-Paris *et al.*, 1995). In hamsters, besides the cecal lesions, involvement of the jejunum and colon is also seen. Occasionally a fully developed pseudomembranous typhlitis is seen and most of the time it leads to the death for the animals (Boot *et al.*, 1989). Rothman (1981) described the moribund guinea pigs exhibit signs like: "ruffling of the fur, unsteady gait and rapid respiration".

In piglets additional symptoms like ascites, hydrothorax and dyspnea may be seen (Waters *et al.*, 1998). Sometimes the animals, guinea pigs as well as piglets, present with obstipation instead of diarrhea even if the content in the cecum is watery, most likely because they develop a paralytic ileus and toxic megacolon (Boot *et al.*, 1989; Waters *et al.*, 1998).

Laboratory diagnosis

There are several ways of diagnosing CDI. All laboratory tests are done on fecal samples and the diagnosis is based on either a method for demonstrating the bacteria, the toxins or a combination of both. The method used for indicating the presence of *C. difficile* is stool culture where selective, cycloserine-cefoxitin-fructose agar is used (O'Connor *et al.*, 2001). The major disadvantage with only

culturing the bacteria is the rather high rate of asymptomatic carriers among some groups (Rolfe & Iaconis, 1983; Bolton *et al.*, 1984; Johnson *et al*., 1990; Kuijper *et al*., 2006). Even if the bacteria are found it is not sufficient evidence to prove that it causes the disease, since some strains of *C. difficile* do not produce toxins (Mathis *et al.*, 1999).

There is no official reference standard for detection of *C. difficile* toxins, even if the cell cytotoxicity assay (CTA) is often considered the optimal method with its high sensitivity and specificity (Chang *et al.*, 1979; Wilkins & Lyerly, 2003; van den Berg *et al.*, 2007). The main disadvantages with that method are that it has a rather long turnaround time, at least 24-48 hours, and it requires a laboratory with a cell line, which makes it more technically demanding and labour intensive than other tests (Chang *et al.*, 1979; Delmée *et al.*, 2005; Ticehurst *et al.*, 2006).

The most common test for routine diagnosis of CDI in humans as well as in animals is one of the many rapid commercial enzyme immunoassays (EIA) available for detecting *C. difficile* toxin A and/or B (Barbut *et al.*, 2003). The advantage of these tests is their quick turnaround time. A result is achieved within a few hours. They are also relatively inexpensive, easy to use and do not require advanced laboratory equipment. The main disadvantages with using these enzyme immunoassays in animals are that they are not fully evaluated for use in animals and that they in some studies, in dogs for example, were found having an unacceptably low sensitivity and for several of the assays poor specificity (Chouicha & Marks, 2006).

The EIAs are developed and validated for use in humans. Several studies have investigated the sensitivity and specificity for different EIAs when tested on human fecal samples. Rüssmann *et al.* (2007) studied three different EIAs, one of them ProSpectT *C. difficile* Toxin A/B Microplate Assay, and found all of them satisfying in terms of sensitivity (88.3-93.3 %) and specificity (100 %). The samples used were human stool specimens from patients with antibiotic-associated diarrhea. Yücesoy *et al.* (2002) tested another EIA and proved that it was as good as the CTA, which means that the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were all 100 %. In other studies where the same EIA has been used the sensitivity ranged from 83.3-96 %, the specificity between 99.3-100 %, the PPV was 100 % and the NPV 90-99.5 % (Lyerly *et al.*, 1998; Aldeen *et al.*, 2000).

The sensitivity and specificity are lower when the EIAs are evaluated in animals like pigs and dogs. The sensitivity, specificity, PPV and NPV for pigs in some studies were, respectively: 54.8-91 %, 31.5-92.6 %, 53.1-86 % and 66.7-86 % (Keessen *et al.*, 2011b; Anderson & Songer, 2008; Post *et al.*, 2002. Chouicha & Marks (2006) performed two studies with five EIAs. In the first study they tested 143 fecal specimens from dogs with and without diarrhea and found a sensitivity that ranged from 7-33 % and a specificity that ranged from 65-100 % compared to the CTA. In the second study they tested the same five EIAs on 29 *C. difficile* isolates cultured from the same 143 fecal samples as used before and found a sensitivity of 93 % and a specificity ranging from 87-100 %.

OBJECTIVES OF THE STUDY

The primary reason for performing this study was that CDI seems to be overlooked as a potential problem in guinea pig production in the Peruvian highlands in spite the fact that it has been proved lethal in laboratory guinea pigs and is also the cause of serious problems in humans as well as in several other animal species (Rehg, 1980; Rothman, 1981; Boot *et al.*, 1989; Rubin *et al.*, 1995; Kelly & LaMont, 1998). A common belief is that the Peruvian guinea pig production has significant problems with salmonellosis. In two studies *Salmonella spp.* was isolated in about 60 % of the animals with clinical symptoms and/or pathological lesions consistent with salmonellosis (Matsuura *et al*., 2010; Layme *et al.*, 2011) but in another recent study where the prevalence of *Salmonella spp.* in the

guinea pig population was investigated the bacterium was not found in any of the animals studied (Ellen Pettersson, data at press: SLU Epsilon 2014). The basis of this study was the theory that all morbidity/mortality might not be salmonellosis and the aim was therefore to study whether CDI, that can be manifested similar at gross necropsy, exists in the guinea pig population in the Peruvian highlands.

MATERIALS AND METHODS

Selection of farms and animals

77 guinea pigs were selected for this study from 15 farms in the Mantaro valley in the Peruvian highlands, at an altitude of about 3300 meters, within a distance of 50 km from Huancayo city. The samples were collected during October 2013. About 40 farms were visited at least once during this period.

All animals found ill on the visited farms were clinically examined and sampled if they fit the inclusion criteria (see below). These samples consisted of a small amount of feces taken with rectal swabs from the individual animals (stored in Amies transport medium without charcoal). Samples from 17 (22.1 % of the animals included in the study) live guinea pigs were collected this way. Animals considered having a poor prognosis were, when possible, sacrificed and underwent gross necropsies together with animals found dead, about 150 guinea pigs in total. Of these animals 60 fit the inclusion criteria and were sampled by collecting a small amount of cecal material. The necropsies were either performed on the farms or at IVITA (Veterinary Institute of Tropical and Highland Research), the research station in the Mantaro valley, which is part of the National San Marcos University, Peru.

Inclusion criteria consisted of: live animals with symptoms of diarrhea or a poor general condition where CDI could not be ruled out (where no other obvious reason for the condition was observed) and necropsied animals that had been dead for less than 24 hours with signs of typhlitis (hemorrhage, congestion, hyperplasia of the Peyer's patches, watery and/or hemorrhagic content in cecum) or with signs of generalized enteritis. Animals that did not show any signs of intestinal disease at necropsy but had received antibiotics the previous week were also included. All animals fitting the criteria were sampled without regard to farm size or the animal's age or sex.

At the smallest farms visited, with about 30-120 guinea pigs, no ill or dead animals were found. The size of the farms where animals fitting the inclusion criteria were found ranged from about 120 animals up to 5000 animals. The animals were divided into different age groups according to a local system with neonates (cría) = 0-14 days old, growing (recria) = 15-45 days old and adult (engorde and reproductor) > 45 days old. In this study there were 11 (14.3 %) neonates, 37 (48.0 %) growing and 29 (37.7 %) adult animals. Of the 77 animals 48 (62.3 %) were female and 29 (37.7 %) were male.

Detection of *C. difficile* toxin

In this study a rapid solid phase enzyme immunoassay (EIA) (ProSpecT^{TM} *C. difficile* Toxin A/B Microplate Assay. Remel, part of Thermo Fisher Scientific, 12076 Santa Fe Drive, Lenexa, KS 66215, USA) was used for detecting the presence of *C. difficile* toxins A and/or B. Specific antibodies, mouse monoclonal anti-Toxin A and rabbit anti-Toxin B antibodies, were used to bind the toxins in the microplate wells. Through a few steps, where first an enzyme conjugate (horseradish peroxidase labelled goat anti-Toxin A and rabbit anti-Toxin B) and later a substrate (3,3'5,5'-tetramethylbenzidine) were added, a coloured (yellow) product developed in the positive samples. The reaction can be read spectrophotometrically at 450/620 to 650 nm (dual wavelength) or visually on a scale from 0 (no color, negative reaction) to 4+ (very distinct yellow) where 1+-4+ are regarded as

positive results (presence of toxin A and/or B). In this study the reactions were read visually against a white background. The wells were washed and incubated at room temperature (20-25 °C) several times during the process. The swabs, when used, were dispensed in the sample diluent in a test tube, and otherwise some of the cecal content was put directly in the tube with the diluent when the sample was taken from the animal. The samples were stored refrigerated (2-8 °C) and tested within 48 hours, or 72 hours if diluted in the diluent. The test was performed according to the manufacturer's instructions.

Clinical data including use of antibiotics

A minor survey was also performed on the farms visited. Questions included were about the farm in general, such as how many animals they had, occurrence of recent increased morbidity or mortality over the last week or month, and if so, possible causes of disease, use of prophylactic antibiotics, as well as questions about the individual animals sampled. The individual questions were, for example, the age and sex of the animal, how long the animal had been ill, what signs they had observed, if it had been administered any treatment and in particular if it had received any antibiotics either as a treatment or prophylactic.

RESULTS

The answers in the survey showed that it was commonly presumed that the animals died from salmonellosis, followed by the owners who had no idea at all. At most farms, except IVITA, a diagnosis was normally never established. At IVITA all animals found dead are taken for a gross necropsy where they are examined visually. Many of the animals are found with suspected salmonellosis during the necropsies with signs of enteritis, often edema (ascites or hydropericardium) and inflammatory (often hemorrhagic or necrotizing) changes in various organs like the liver, heart, lungs and mesenteric lymph nodes. As samples for further diagnostics are rarely taken the diagnosis is seldom substantiated.

Only two of the 15 farms reported increased morbidity and mortality prior to the farm visits. On one of these farms salmonellosis was suspected as the underlying cause, and animals were prophylactically administered a mixture of tetracycline, enrofloxacin, florfenicol and sulfaquinoxalin together with some other ingredients such as electrolytes and clay, on regular basis. They treated all the animals for five consecutive days every fourteenth day. Animals showing signs of illness were given additional enrofloxacin. On the other farm all of the guinea pigs had received antibiotics, (enrofloxacin), for five days with the last treatment two days before sampling, since the owner had observed an increased mortality.

At 6 of the farms (40.0 %) in this study one or more selected animal had received treatment with antibiotics, primarily enrofloxacin, but also tetracycline, florfenicol and sulfaquinoxalin one to several times the last week. Eighteen of the sampled animals (23.4 %) had received antibiotics sometime during that period. Some animals (5 (6.5 %)) that at necropsy did not show any involvement of the gastrointestinal tract, but had received antibiotic treatment as described above, were sampled anyway to assess whether *C. difficile* toxins could be found after antibiotic treatment.

Out of the 77 samples (Table 1 and Attachment 1), one (1) animal (1.3 %) was found positive for *Clostridium difficile* toxin A and/or B. The animal was a neonate (0-14 d.) male found dead at a large farm (around 5000 animals). At gross observation during necropsy it showed signs of severe typhlitis with a hemorrhagic cecum with watery content. The animal had not received any treatment and no signs of illness had been observed prior to its death.

At necropsy twenty-five of the animals in this study (32.5 %) showed signs of typhlitis, including one or more of the following: hemorrhage, congestion, hyperplasia of the Peyer's patches, watery and/or hemorrhagic content. Two of the guinea pigs had a more severe typhlitis than the others, one of which was found toxin positive. Among the 25 guinea pigs with typhlitis 40.0 % (n=10) also had signs of generalized enteritis. In this study 40.0 % (n=30) of the animals showed signs of enteritis without affection of the cecum. In addition 3.9 % (n=3) of the included animals had diarrhea but did not undergo necropsy.

Of major importance to this current work are the findings of a companion study about the prevalence of *Salmonella spp.* that was done simultaneously on the farms in the same area by another student who was not able to detect a single case of that organism by cultivation of fecal samples (Ellen Pettersson, data at press: SLU Epsilon 2014).

Table 1. *Findings at necropsy and at clinical examination in sampled animals*

Signs	Number of animals				
	Total	Age (N/G/A)	Sex (F/M)	Given antibiotics (Y/N)	Test result *C. difficile* toxin (Pos/Neg)
Necropsied animals	60	9/28/23	36/24	15/45	1/60
Typhlitis	15	2/8/5	8/7	7/8	1/15
Typhlitis and enteritis	10	0/7/3	4/6	2/8	0/10
Enteritis	30	7/12/11	19/11	1/29	0/30
Other + antibiotics	5	0/1/4	5/0	5/0	0/5
Live animals	17	2/9/6	12/5	3/14	0/17
Poor general condition	14	2/6/6	10/4	3/11	0/14
Diarrhea	3	0/3/0	2/1	0/3	0/3
Total	77	11/37/29	48/29	18/59	1/76

Explanations: N=neonate, G=growing, A=adult, F=female, M=male, Y=yes, N=no, Pos=positive, Neg=negative.

DISCUSSION

For the first time (to the author's knowledge) a guinea pig on a meat producing farm in Peru was found positive for *C. difficile* toxins A and/or B. If the CDI in this animal was primary or secondary to something else is not known but the necropsy findings were in concordance with the ones described in previous studies in laboratory guinea pigs with CDI so it is possible that the CDI was the cause of this animal's death.

One of the purposes with this study was to examine what the symptoms are in industrial guinea pigs with CDI. Another purpose with the study was to assess if there is a correlation between treatment with antibiotics and the occurrence of *C. difficile* toxins in feces or cecal content from the guinea pigs. As only one animal was found positive no significant conclusions could be made in either of these matters.

Worth noticing is that the positive animal was not previously treated with antibiotics, which in several species is often considered the most important risk factor. Spontaneous cases of CDI have however been described in guinea pigs before (Boot *et al.*, 1989) so it is not unlikely to believe that it exists

among the industrial animals as well. It is not known whether any other guinea pig in the same building as the positive case was being treated with antibiotics at the moment for the toxin positive animal's death and if this could have had any impact of the case (i.e. by shedding and spreading of the bacteria and/or antibiotics in the building).

In this study the results of the survey show a rather high rate of farms treating their animals with antibiotics (40 %). It is somewhat falsely high because farms using antibiotics were selected for sampling more often than farms not using it. Only one or two more farms, except the 6 where samples were taken, out of the 40 visited reported use of antibiotics in the previous week.

The importance of the finding of CDI in one single animal is hard to interpret, but it at least indicates that the disease exists in the population. The manufacturer of the EIA used stress that a negative result in the test does not exclude presence of toxins since the level of toxins in the sample could be below the detection limit so it is possible there were false negative results in this study. The EIA used in this study is not validated for use in guinea pigs, but when evaluated in other species the specificity in most cases is higher than the sensitivity, sometimes approaching 100%. With that in mind it is more likely that there might have been false negative rather than false positive results. On the other hand, the prevalence of CDI in the Peruvian guinea pig population is not known. If the prevalence is low, the positive predictive value (PPV) for the test would be low and therefore a higher risk of false positive results.

Considering the fact that the lesions in the intestines caused by CDI are quite similar at visual examination to the ones found in other enteric infections the positive animal in this study is an important finding as the treatment and precautions to be considered to prevent the diseases are quite different for CDI and various other diseases. Salmonellosis, for example, is thought to be very common and is often treated with antibiotics in the highlands. Considering that the other study done at the same time (Ellen Pettersson, data at press: SLU Epsilon 2014) was unable to detect *Salmonella spp.* in any single sample and also that one of the most important things to do in case of CDI is to withdraw the antibiotics it is highly inappropriate to misinterpret the signs for salmonellosis and start/continue antibiotic therapy.

In the *Salmonella spp.* prevalence study done by the other student (Ellen Pettersson, data at press: SLU Epsilon 2014) both *Yersinia spp.* and *Escherichia coli* were cultivated from fecal samples and confirmed by API10S. This together with the detection of *C. difficile* toxins in one animal indicates that all morbidity/mortality is not only due to salmonellosis and great care should therefore be taken when interpreting clinical and/or pathological findings as all of them, at gross observation, are largely similar.

In this study the main goal was to sample animals with typical cecal lesions, but due to shortage of time because of logistic problems only a few animals with the preferred pathologic findings could be sampled. Considering this being the first investigation of its kind a broader spectrum of symptoms was included in order to see in what type of cases the clostridial toxins could be found. Animals with other pathological lesions in their intestines, apart from typhlitis, as well as animals that had received antibiotics and were found dead or in a poor bodily condition were included.

The EIA used in this study has been evaluated in humans with results showing a very good sensitivity (93.3 %) and specificity (100 %) compared to the results from different combinations of other diagnostic tests (Rüssmann *et al.*, 2007). While these EIA's have not been evaluated in guinea pigs they have in several other animal species. In this study the EIA was chosen for several reasons. First of all they are commonly used in routine diagnostics for humans as well as for animals even if the sensitivity and specificity are found low in some studies when tested on feces from animals (Post *et*

al., 2002; Anderson & Songer, 2008). Secondly the EIA is easy to use even in a modestly equipped laboratory such as the one used in this study. The CTA and stool culture were not an option because they require more advanced laboratories not feasible in the Andean highlands.

Sometimes a low sensitivity and specificity was found when EIA's were evaluated for use in animals (Keessen *et al.*, 2011b). The reason for this is still not known, but inhibitors reducing the binding of the toxins have been suggested as well as increased toxin degradation due to protease activity, or simply toxin levels too low to detect (Chouicha & Marks, 2006; Anderson & Songer, 2008).

The results from this study indicate that CDI was associated with typhlitis in the farmed guinea pigs similar to the one seen in laboratory animals. This study also suggests that not all the cases of typhlitis are caused by *C. difficile* toxins. But still, the positive case was one of the two animals with the most severe lesions in the cecum, most animals only had minor lesions, so CDI should not be forgotten in cases of severe typhlitis.

Whether the *C. difficile* toxin positive case is truly positive cannot be known for sure. The relatively high use of antibiotics at the farms would be expected to result in CDI to a larger extent than found in this study. On the other hand enrofloxacin is not the type of antibiotic most commonly associated with CDI in guinea pigs. The results found in this study could indicate that the prevalence of *C. difficile* is low in the Peruvian guinea pig population which together with a suspected low sensitivity and specificity for the EIA used would make the PPV unreliably low, meaning the risk for false positive results is high. However, as the prevalence is not known, it could be much higher than indicated in this study. For example, if it had been possible to choose the sampled animals with greater care (i.e. only those with severe typhlitis) the number of toxin positive animals could have been higher, which in turn would make the PPV much higher and significantly lower the risk for false positives. With a narrower selection, focusing only on animals with pronounced signs of typhlitis, it is likely that a higher frequency of CDI would be demonstrated.

CONCLUSIONS

One animal was found positive for TcdA and/or TcdB in this study. It was a neonate male with the same pathologic findings as earlier described for laboratory guinea pigs with CDI. The macroscopic findings at necropsy together with the positive result of the EIA indicates that CDI actually exists in meat producing guinea pigs in the Peruvian highlands but one has to bear in mind that there is a possibility that the result could have been false positive. Further studies focusing on guinea pigs with pronounced signs of typhlitis are therefore required and would further elucidate to what extent CDI is a problem in the Peruvian guinea pig production.

ACKNOWLEDGEMENTS

I wish to thank my supervisor John Pringle and assistant supervisor Martin Wierup for all help through this whole process, Cesar Gavidia, my supervisor in Peru, for invaluable advise when planning this study, Ulf Emanuelson for great input on my thesis, Miguel Siever, Lise-Lotte Fernström and Annie Engström for support with laboratory issues, Ronald Jiménez for help with getting out to the farms and necropsies, Juan Lucas for help with collection of the data and in the laboratory, and all the other people at IVITA for making me feel welcome. I would also like to thank my family and friends for always supporting me. Finally I wish to thank the fund of the Faculty of Veterinary Medicine, Gulli Strålfeldt's fund and Swedish SIDA for granting me the scholarships that made this project possible.

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APPENDIX 1

Table 2. *Findings at necropsy and at clinical examination in sampled animals*

Nr	Age	Sex	Necropsy/live	Symptoms	Antibiotics	Test result (EIA)
1	Adult	Female	Necropsy	Enteritis	No	Negative
2	Adult	Male	Necropsy	Enteritis	No	Negative
3	Adult	Female	Necropsy	Enteritis, ascites	No	Negative
4	Growing	Male	Necropsy	Enteritis	No	Negative
5	Adult	Female	Necropsy	Enteritis	No	Negative
6	Adult	Female	Necropsy	Enteritis	No	Negative
7	Growing	Male	Live	Diarrhea, paralysed hindlimbs	No	Negative
8	Growing	Female	Live	Hemorrhagic diarrhea	No	Negative
9	Adult	Male	Live	Poor general condition	No	Negative
10	Growing	Female	Live	Poor general condition	No	Negative
11	Growing	Female	Necropsy	Enteritis	No	Negative
12	Growing	Female	Necropsy	Enteritis	No	Negative
13	Growing	Female	Live	Poor general condition, growth retardation	Yes[1]	Negative
14	Neonate	Male	Live	Poor general condition, artritis	No	Negative
15	Neonate	Female	Live	Poor general condition	No	Negative
16	Adult	Female	Live	Poor general condition, ascites	No	Negative
17	Adult	Female	Live	Poor general condition, ascites	No	Negative
18	Adult	Female	Live	Poor general condition	No	Negative
19	Neonate	Female	Necropsy	Enteritis	No	Negative
20	Adult	Female	Necropsy	Enteritis	Yes[2]	Negative
21	Adult	Female	Live	Poor general condition	No	Negative
22	Adult	Female	Necropsy	Enteritis	No	Negative
23	Neonate	Male	Necropsy	Typhlitis	No	Negative
24	Growing	Male	Necropsy	Enteritis	No	Negative
25	Growing	Female	Necropsy	Enteritis	No	Negative
26	Growing	Female	Necropsy	Enteritis	No	Negative
27	Growing	Female	Live	Poor general condition	No	Negative
28	Growing	Male	Live	Poor general condition	No	Negative
29	Growing	Male	Necropsy	Enteritis	No	Negative
30	Growing	Male	Necropsy	Typhlitis	No	Negative
31	Neonate	Male	Necropsy	Enteritis	No	Negative
32	Neonate	Female	Necropsy	Enteritis	No	Negative
33	Neonate	Female	Necropsy	Enteritis	No	Negative

34	Adult	Female	Necropsy	Enteritis	No	Negative
35	Neonate	Male	Necropsy	Typhlitis	No	Positive, 2+
36	Adult	Female	Necropsy	Typhlitis	No	Negative
37	Growing	Male	Necropsy	Typhlitis	No	Negative
38	Adult	Female	Necropsy	Enteritis, typhlitis	No	Negative
39	Growing	Male	Necropsy	Typhlitis	No	Negative
40	Growing	Male	Live	Poor general condition	No	Negative
41	Growing	Female	Live	Diarrhea, poor general condition	No	Negative
42	Growing	Male	Necropsy	Enteritis, typhlitis	No	Negative
43	Growing	Male	Necropsy	Enteritis	No	Negative
44	Growing	Male	Necropsy	Typhlitis	No	Negative
45	Adult	Female	Necropsy	Enteritis	No	Negative
46	Growing	Male	Necropsy	Enteritis, typhlitis	No	Negative
47	Growing	Male	Necropsy	Enteritis	No	Negative
48	Growing	Male	Necropsy	Enteritis	No	Negative
49	Growing	Female	Necropsy	Enteritis	No	Negative
50	Adult	Female	Necropsy	Enteritis, typhlitis	No	Negative
51	Adult	Female	Necropsy	Enteritis, typhlitis, ruptured intestines	No	Negative
52	Adult	Female	Necropsy	Enteritis, massive ascites	No	Negative
53	Growing	Male	Necropsy	Enteritis, typhlitis	No	Negative
54	Growing	Male	Necropsy	Typhlitis	No	Negative
55	Growing	Male	Necropsy	Enteritis	No	Negative
56	Growing	Female	Necropsy	Typhlitis	Yes[3]	Negative
57	Adult	Female	Necropsy	Severe typhlitis, ascites	Yes[3]	Negative
58	Growing	Male	Necropsy	Enteritis, typhlitis	Yes[3]	Negative
59	Growing	Female	Necropsy	Typhlitis	Yes[3]	Negative
60	Growing	Male	Necropsy	Enteritis, typhlitis	Yes[3]	Negative
61	Growing	Female	Necropsy	Typhlitis	Yes[3]	Negative
62	Adult	Female	Necropsy	Pneumonia	Yes[3]	Negative
63	Growing	Female	Live	Slightly poor general condition	Yes[4]	Negative
64	Growing	Female	Necropsy	No necropsy findings	Yes[4]	Negative
65	Adult	Female	Live	Slightly poor general condition, increased mortality in the box	Yes[3]	Negative
66	Adult	Female	Necropsy	Lethargy, ascites, hepato- and splenomegaly	Yes[5]	Negative
67	Adult	Female	Necropsy	Pneumonia	Yes[6]	Negative
68	Adult	Female	Necropsy	Typhlitis	Yes[7]	Negative
69	Adult	Female	Necropsy	Typhlitis, ascites, peritonitis	Yes[7]	Negative
70	Adult	Female	Necropsy	Typhlitis	Yes[8]	Negative

71	Adult	Female	Necropsy	Gas extended intestines, ileus? Suspected megacolon.	Yes[8]	Negative
72	Growing	Male	Necropsy	Enteritis, typhlitis	No	Negative
73	Adult	Female	Necropsy	Enteritis	No	Negative
74	Growing	Female	Necropsy	Enteritis, typhlitis	No	Negative
75	Neonate	Male	Necropsy	Enteritis	No	Negative
76	Neonate	Female	Necropsy	Enteritis	No	Negative
77	Neonate	Male	Necropsy	Enteritis	No	Negative

Explanations: 1. Enrofloxacin (Ef) prophylactic one or two times per month, last time two days ago. 2. Treated with Ef the last four days. 3. Ef prophylactic every 14th day, last time four days ago. 4. Ef prophylactic every 14th day, last time one day ago. 5. Single dose of Ef the same day. 6. Ef for the last five days, increased mortality at the farm. 7. Ef for five days, last time two days ago, increased mortality at the farm. 8. Ef for six days, last time seven days ago, increased mortality at the farm.