Sveriges lantbruksuniversitet
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A study to evaluate the prevalence of *salmonella* spp and importance of the disease

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Uppsala
2014

Degree Project 30 credits within the Veterinary Medicine Programme

ISSN 1652-8697
Examensarbete 2014:21

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Salmonellos i Peruansk marsvinsproduktion

En studie för att utvärdera prevalens av salmonella och sjukdomens betydelse

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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 2014:21*
***ISSN**: 1652-8697*
***Online publication**: http://stud.epsilon.slu.se*

***Key words**: Salmonella, Guinea pig, Prevalence*
***Nyckelord**: Salmonella, Marsvin, Prevalens*

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SUMMARY

In Peru guinea pigs are raised for meat production and salmonellosis is considered as a major problem. Studies have detected salmonella in animals with enteritis and pneumonia. Salmonellosis exists both in an acute and a chronic form. In the rural areas the diagnosis is made according to clinical signs and macro pathological findings at autopsy.

The major systems applied for the guinea pig production are often classified as family, family-commercial and commercial. These systems differ in number of animals and how industrialised they are. During the last years the demand of meat and subsequently the production of guinea pigs have increased. Improvements of programs for breeding and education have increased the size of the animals and their welfare conditions.

Salmonella spp, the etiological agent of salmonellosis, is a gram-negative bacterium that can cause disease in most animal species including guinea pigs, cattle, pigs and humans. The signs vary between species but enteritis is common. In guinea pigs signs such as diarrhoea, ascites, abortions, paralysis of hind limbs and increased mortality are seen. But the bacteria can also exist in asymptomatic carriers. The diagnosis of salmonellosis is made mainly by bacteriological culture methods but also molecular methods are available today.

This study was performed to investigate the prevalence of salmonella in guinea pig in the Mantaro valley in Peru as a way to evaluate the importance of the disease. Composite faecal samples as well as rectal swabs were taken from a total of 224 randomly selected guinea pigs from farms of different size The samples taken from animals of different gender and age , were cultivated according to standard bacteriological methods using buffered peptone water (BPW) and selective medias (Rappaport-Vassiliadis-Salmonella enrichment broth (RVS) and Xylose Lysine Deoxycholate agar (XLD)). Suspected colonies were biochemically tested using API10S.

In contrast to previously reported information no salmonella was found in either the composite samples or the rectal swab samples. The reason for this discrepancy is questioned due to unforeseen methodological challenges. However, the study also indicates that salmonellosis may be over diagnosed as a problem among guinea pigs in Mantaro valley.

SAMMANFATTNING

I Peru föds marsvin upp för köttproduktion och salmonellos ses som ett stort problem. Studier har detekterat *Salmonella* spp hos djur med bland annat enterit och pneumoni. Salmonellos förekommer både som akut och kronisk form. På landsbygden ställs diagnosen utifrån symtom och makropatologisk bild vid obduktion.

Systemen för marsvinsuppfödning delas upp i tre olika typer som kallas familjära, familje-kommersiella och kommersiella beroende på besättningsstorlek samt hur industrialiserade gårdarna är. Under de senaste åren har efterfrågan på kött ökat och därmed även produktionen av marsvinskött. Avels- och utbildningsprogram har skapat större marsvin och lett till bättre förhållanden för djuren.

Salmonella, det agens som orsakar salmonellos, är en gramnegativ bakterie som kan orsaka sjukdom hos många olika djurslag, däribland marsvin, nötkreatur, gris och även hos människa. Symtomen varierar men enterit och ökad mortalitet är vanligt. Hos marsvin med salmonellos ses symtom som diarré, ascites, aborter, paralys av bakben och/eller plötslig död. Bakterien

kan även finnas hos asymtomatiska bärare. Diagnos ställs främst genom bakteriologisk odling av faeces eller vävnad men idag finns även molekylära tekniker tillgängliga.

Den här studien genomfördes för att undersöka prevalensen av *Salmonella* spp bland marsvin i Mantarodalen i Peru. Faecesprover togs i form av rektalsvabbar samt samlingsprover från totalt 224 slumpvis utvalda marsvin från farmer av olika storlekar. Djuren var av olika kön och ålder. Faecesproverna odlades med hjälp av buffrat peptonvatten (BPV) samt selektiva medium (Rappaport-Vassiliadis-Salmonella enrichment broth (RVS) och Xylose Lysine Dexycholate agar (XLD) och misstänkta kolonier testades med API10S. Till skillnad från tidigare rapporter påvisades ingen salmonella från något av de undersökta djuren . Orsaken till denna skillnad diskuteras och kan vara föranledd av oförutsedda metodologiska utmaningar eller möjligen att salmonella är överdiagnostiserat bland marsvin i Mantarodalen.

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INTRODUCTION

Guinea pigs are mammals that originate from the Andes in Bolivia, Colombia, Ecuador and Peru. Today they are raised in Latin America mainly for their meat. Peru is the country in the Andes that produce the most guinea pigs, or *cuyes* as they are called there. There are around 22 million guinea pigs in Peru and the annual production of guinea pig meat is 16500 tons (Morales, 1994, Lilia, 1997). The total production of meat in Peru was 2002 ca 900 000 tons. And the total consumption of meat per capita has increased over the last 30 year (FAO, 2005). Recent years increasing demand of meat has contributed to a greater and more intensive production of guinea pigs (Layme et al., 2011). The guinea pigs in Peru are produced in three different kind of systems called family, family-commercial and commercial (Lilia, 1997)

Salmonella is a gram-negative bacterium. It is divided into two species; *Salmonella bongori* and *Salmonella enterica.* The specie *S. enterica* is divided into six subspecies (spp); *enterica, houtenae, diarizonae, arizone, indic* and *salamae.* In some of these subspecies there are different serovars. One example of a serovar is *Salmonella enterica* subspecie *enterica* serovar Typhimurium (written *S.* Typhimurium) (Marcela and Verdugo, 2005). Some *Salmonella enterica* (for example *S.* Typhimurium) can infect many different animal species while other are more adapted to their hosts and mostly induce disease only in one (e.g. *S. Dublin* in cattle *and S. gallinarum* in poultry) (Shivaprasad et al., 2013). *Salmonella bongori* is most often isolated from cold blooded animals, where it can be a part of the intestinal flora, but has also occasionally been isolated from humans, birds and water (Foti et al., 2009).

When infected with salmonella, different animal species show different signs but enteritis is very common (Santos et al., 2003). In guinea pig signs such as diarrhoea, abortions, ascites, paralysis of the hind limbs and increased mortality are seen (Iijima et al., 1987, Matsuura S et al., 2010). Acute salmonellosis can cause outbreaks with high morbidity and high mortality. Outbreaks of salmonellosis have been related to chronic, endemic salmonellosis in guinea pigs (Pivnick et al., 1966, Iijima et al., 1987).

Salmonellosis is thought to be a major problem in Peruvian guinea pig production, much because of sub optional management and environment in the production systems applied. In one study *S. enterica* was the most common microorganism isolated in sick guinea pigs (Matsuura S et al., 2010). However the information on the prevalence of salmonella in guinea farms is limited.

Against this background the aim of this study was to investigate the prevalence of *Salmonella* spp in guinea pig farms in Mantaro Valley in Peru and to find out if there was any difference between different types of farms or among animals of different ages. The aim was also to see if salmonella existed endemic in the farms and if asymptomatic shedders could be found. The hypothesis was that if *Salmonella spp* was recovered from faeces from seemingly healthy guinea pigs these asymptomatic carriers could be a source for outbreaks of clinical salmonellosis in a herd. More information about the epidemics of *Salmonella spp* in the region could help the producers reduce their problems and economical losses.

LITERATURE OVERVIEW

Salmonella

Pathogenesis

The pathogenesis of salmonella infection depends on the species and subspecies, the infection dose, virulence factors, the immunological status of the host and interactions of medications. Important virulence factors are capsules (in for example *S.* Typhi, *S.* Paratypi and *S.* Dublin), flagella and fimbriae, which all are important for the adherence to the host cells. Most salmonellas are motile (Marcela and Verdugo, 2005).

The most common infection route is oral, by ingestion of contaminated water or feed (Marcela and Verdugo, 2005). However salmonella can also infect via contact between animals or via conjunctiva (Iijima et al., 1987). When the bacteria reach the intestines it first attaches to the apical surface of the epithelial cells (M-cells and enterocytes). By using effector proteins, mainly two called Sip A and Sip C, salmonella affects the host cell so that it's surface changes and becomes ruffled (Marcela and Verdugo, 2005, Santos et al., 2003). After this the bacteria infect the epithelial cell. The epithelial cell starts to produce chemotactic factors (pathogen-elicited epithelial chemoattractant (PEEC) and interleukin 8 (IL-8)) because of other effector proteins produced by salmonellae, for example SPI-1. The chemotactic factors are released both to the apical and basal side of the cell and attract neutrophils. After approximately one hour the bacteria have passed through the epithelial cell to the lamina propria and can there be phagocytosed by a macrophage or a neutrophil. Once inside the macrophages Salmonellae can induce cell death, apoptosis, through the protein SipB that activates capsase-1. Parallel with this the inflammation progress and more neutrophils are attracted to the inflammation site and start to migrate thorough the epithelial cells into the intestinal lumen. The end result is destruction of the basal membrane and necrosis of the superficial mucosa, which results in fluid secretion into the lumen and diarrhoea. Induced secretion of chloride is also involved in the production of diarrhoea (Santos et al., 2003, Marcela and Verdugo, 2005). In some species, for example mice, salmonellae does not cause diarrhoea and the cell infiltrate in their intestine are mostly mononuclear (Santos et al., 2003). Salmonella can induce septicaemia and even if the exact mechanism for this is not known it is believed that enterotoxins play an important role (Marcela and Verdugo, 2005).

Clinical signs

Other farm animals like pigs and poultry can be asymptomatic carriers of salmonella (Kusar et al., 2010). Funk et al (2000) found the prevalence at two pig farms in North Carolina to be 35, 1% and that different serotypes can exist in the same farm. One other study showed that salmonellae can exist for long periods in a swine herd with only a few chronic carriers (Gray et al., 1996). Cummings et al. (2010) investigated the prevalence of salmonella in cattle in New York and found that it varied between 0 and 53 % and on average was 9, 1 %. In herds with suspected clinical cases the prevalence was highest and larger farms had also a higher prevalence of salmonella (Cummings et al., 2010).

As said earlier *Salmonella spp* can induce different signs in different species. Young animals (neonatal calves and foals and pigs up to four month old) can develop septicaemia with depression, fever, weakness and sometimes neurological signs such as incoordination and paralysis. The mortality among these animals is high (Radostits et al., 2007).

In cattle, in addition to asymptomatic carriers, salmonellae can also lead to acute or chronic enteritis in adult animals. Colic, haemorrhagic diarrhoea and agalactia are common signs in the acute form while inappetence, decreased weight gain and agalactia are typical signs in the chronic form. Gangrene of peripheral body parts (such as ears or tail tip) also is a possible effect of chronic salmonellosis. Abortions in pregnant cows and polyarthritis in calves can be seen both in acute and chronic salmonellosis. Salmonellosis in sheep and goats often results in acute enteritis and sometimes septicaemia. Salmonellosis in swine can cause many different degrees in severity of clinical signs, in addition to asymptomatic carriers. They can suffer from an acute form with pneumonia, enteritis (with diarrhoea and depression), septicaemia and secondary encephalitis. Pigs with septicaemia often have miscoloured (dark red or purple) areas in the skin. In adult horses acute diarrhoea is a typical sign of Salmonellosis. Other signs seen are colic, fever and depression but asymptomatic shedders also exist (Radostits et al., 2007, Gray et al., 1996). In cats and dogs *S.* Typhimurium is the subspecies that causes disease. Signs are acute or chronic diarrhoea. Young animals can die suddenly or after a short period with pneumonia and/or diarrhoea because of septicaemia (Nelson et al., 2009).

Changes in the haematology include most often increase in white blood cell counts (WBC) and this increase is dose dependent. The WBC consists mainly of neutrophils but also the levels of monocytes, lymphocytes and basophiles increases. The changes are seen from day five in mice. Havelaar et al. (2001) tried to isolate salmonella from blood in mice but did not succeed (Havelaar et al., 2001).

Salmonella can also cause delayed-type hypersensitive reactions, which in mice has been shown resulting in thickness of the ears after oral infection with salmonella (Havelaar et al., 2001).

Infection dose

Rats have been used as a model to determine infection doses. A higher infection dose results in increased severity of illness. Doses from 10^8 cfu (colony-forming unit) per animal can result in signs such as weakness, cold, changed fur, nasal discharge and weight loss (but not diarrhoea) already after 2 days. Lower doses may induce gastroenteritis, affecting mostly ileum, caecum and colon, even when the animals seem healthy (Havelaar et al., 2001).

After infection with *S.* Enteritidis in rats the amount of bacteria in faeces first increases, then after one to three days, it decreases before it may increase again. If the challenge dose is lower than 10^4 cfu salmonella might not be detectible in faeces. However, in one study a challenge of 10^3 cfu bacteria caused infection of the spleen and a challenge of 10^5 of the microbes always resulted in an infection of the mesenteric lymph nodes. Lesions such as pneumonia and gastro intestinal changes (including enlarged mesenteric lymph nodes and Peyer´s'

patches and oedema and ulcers in the large intestine) were seen in rats at high doses in the same study. These changes represent a systemic infection (Havelaar et al., 2001).

Diagnosis

Diagnosis of salmonellosis is most often based on bacteriological culture methods. Isolation of the bacteria is required to verify the diagnosis. Salmonellae can be isolated from tissues such as spleen, liver, intestine and mesenteric lymph nodes and from faeces (Kusar et al., 2010). Recovery of salmonella from faeces is dependent on the selectivity of the isolation medium and problems with this may result in false negative results (Havelaar et al., 2001). Faeces are often first pre enriched by incubating in buffered peptone water (BPW) for better results (Funk et al., 2000, Kusar et al., 2010). For the subsequent selective enrichment for example Rappaport-Vassiliadis with Soya (RVS) broth, Muller Kaufman TetraThionate-novobiocin (MKTTn) broth or Modified Semi-solid Rappaport Vassiliadis (MSRV) medium can be used. The next step is cultivating on selective agar plates, for example Xylose-Tergitol 4 (XLT4) agar, Xylose-Lysine-Deoxycholate (XLD) agar, phenol red/Brilliant Green Agar (BGA) or *Salmonella-Shigella* agar, for 24 hours. Biochemical testing is used to confirm and serotype the samples. To obtain isolated colonies to test biochemically a non-selective agar can be used (Kusar et al., 2010, Funk et al., 2000). Kusar et al. (2010) showed that the use of more than one enrichment and isolation media resulted in better detection of salmonella. They also showed that it was often harder to detect salmonella in poultry faeces samples using RVS as enrichment then when using MSRV or MKTTn. However use of RVS might favour detection of serovar Typhimurium rather than serovar Enteritis.

Funk et al. (2000) compared different faecal sample weights (swab, 1 gram, 10 gram and 25 gram) when sampling pigs and found it possible to detect asymptomatic carriers using rectal swabs but that the number of positive samples and the sensitivity of detection was higher using larger amounts of faeces. The proportion positive samples using swabs were 1,6 % while the proportion positive samples from the same pigs using 1- and 10 gram were 4 and 9,5 % respectively. If the prevalence in the population is low the difference in predicted prevalence between swabs and more faeces is lower than if the prevalence is high. A greater sample size is required to be “95 % confident of detecting at least one positive animal” in a group of x animals if rectal swabs are used instead of larger amount of faeces (Funk et al., 2000). Maciel et al. (2011) used 50 mg faeces from asymptomatic carrier mice intragastrically inoculated with *Salmonella* enteritis and could detect bacteria in 22,2 % of the samples. And they got the same proportion of positive samples culturing samples from tissues such as liver, spleen and caecum. Singh at al. (2005) detected salmonellae in experimental inoculated guinea pigs (chronic carriers) using rectal swabs and Singh et al. (2007) also discovered carriers among guinea pigs using swabs (Singh et al., 2005, Singh et al., 2007). Other workers (Salehi et al. 2010) have also shown that it is possible to detect different *Salmonella* serovars, using sampling with rectal swabs, in guinea pigs, rabbits, hamsters and squirrels. They isolated salmonella from 2, 4% of the sampled rabbits and rodents and concluded that these species can also be carriers of salmonella.

The sensitivity of culture methods varies between different methods, different serovars of *Salmonella* and the amount of bacteria in the sample. High sensitivity is important because it is often desirable to detect salmonella in herds with low prevalence and animals secreting low levels of bacteria. The sensitivity is higher when pooled samples are being used. Arnold et al. (2009) found the sensitivity in detecting salmonella in faecal samples using BPW and MSRV to be 86% for individual samples and around 90 % in pooled samples (2-5 animals per sample). Jensen et al. (2012) determined the sensitivity to be 100% in contaminated samples with high levels of cfu/g and 78% for samples with lower levels. They used BPW, MRSV and XLD or BGA. Rostagno et al. (2005) obtained results that varied between 78 and 95 % depending on method used. (Arnold et al., 2009, Jensen et al., Rostagno et al., 2005) The specificity is considered to be 100 % (Rostagno et al).

In biochemical testing salmonella ferment glucose, maltose, mannitol and d-inositol but not lactose, salicin, sucrose and dulcitol. For serological testing agglutination titers with "O"- and "H"-serum and "C"-specific antisera can be used (Pivnick et al., 1966, Onyekaba, 1983). There is a scheme called Kaufman-white that classify salmonella according to their reactions to somatic lipopolysacharide ("O") and the antigenges flagell ("H") and capsule ("Vi")(Shivaprasad et al., 2013).

In later years molecular methods, such as PCR and real-time PCR, have been tested to detect salmonella, using both faeces and tissue samples. Real-time PCR has been shown to be both sensitive and specific and useful manly in detecting small amounts of bacteria, as in for example asymptomatic carriers. Other advantages according to Maciel et al (2011) are for example the speed of the analysis and the ability to get reproducible results. Kausar et al (2010) suggested that molecular methods might be used as a complement, especially for early detection of shedders. But bacteriological culturing is still more useful and required when it comes to isolate a strain, which is necessary for example when identifying the serovar in an infected animal, test sensitivity to different antibiotics or evaluate salmonella-status in herds. By using molecular methods it's not possible to determine if the detected microorganism is viable or not (Kusar et al., 2010, Maciel et al., 2011).

Guinea pigs in Peru

There are many advantages with the production of guinea pigs. The guinea pigs are for example herbivores that can survive on forage that would not be used otherwise, their reproduction cycle is short and they have a great ability to adapt to different environments. Guinea pigs can be raised in the rain forest, in the mountains and in the desserts areas. In addition, the guinea pig meat is rich in protein and low in fat (Lilia, 1997, Morales, 1994).

Besides the production of meat guinea pigs are important in traditional medicine and religious rituals. The production and consumption of guinea pigs also promotes connections between the countryside and the cities, people with different background and cultures (Lilia, 1997, Morales, 1994).

The guinea pig

Females can become fertile when they are three months old. They manage litters of five to six pups, but average is three to four. The interval between partum is on average 77 days, improved breeding has shortened the interval from 86 days 2000 to 77 year 2006. The interval depends on the environment, management and diseases but shorter interval has also been correlated to larger litters and larger numbers of earlier litters (Oshiro et al., 2006). Although guinea pigs can live up to nine years it is common to slaughter the females after their third litter because they grow larger and require more feed, which is less cost effective. Additionally the mortality among them also rises (Morales, 1994).

There are different kinds of guinea pigs. They are divided into groups according to either body conformation (type A and B) or coat (type 1, 2, 3 and 4). Type A are the typical type used for meat production as they develop more musculature and are calmer then type B. Type 1 have straight and short coat and are common as meat producers in Peru. Type 2 also have short hair but they have rosettes in their coat. They are usually not used for meat production. The third type have a long and straight coat, some have rosettes and some have not. This type is most common as pets. The last type (type 4) is born with curly hair and as they grow older the coat gets more straight and "spiky". The average weight of an adult guinea pig, used as meat producer, is one kilo at the time of slaughter. The males used for reproduction can weigh up to three kilos. Common colours are white, brown and grey but they have often two or more of these colours (Lilia, 1997, Morales, 1994). Recent years breeding programs conducted by the universities (for example La Universidad Nacional Mayor de San Marcos, Lima) has resulted in increased slaughter weight and rate of growth.

A newborn guinea pig weighs around 120 grams and at the age of 13 weeks the weight can be between 400 and 740 grams depending mainly on the genetics. Larger guinea pigs can be sold earlier and this is especially important for the breeders with poor finances (Oshiro et al., 2006). The breeding programs has resulted in six different kind of genotypes in Peru called "Peru", "Andino", "Inti", "type 2", "type 4" and "Criollo". They differ in size (length and weight) and percentage muscles. "Peru" and "Andino" are the largest ones and "Criollo" the smallest ones (Oshiro et al., 2006). Programs funded by the government to educate the people in hygiene routines and management practices have also improved the health of the guinea pig. The improved Peruvian bred guinea pigs are being exported to Honduras and the Dominican Republic (Lilia, 1997, Morales, 1994).

Breeding systems

The family (small) system is according to Food and Agricultural Organization of the United Nations (FAO, 1997) the most common in the Andes. Between 77-95% of the guinea pigs were produced this way in the regions Junín and Cajamarca 1997. In the region around Lima the situation is different as only about 20 % of the guinea pigs are produced in family systems. The animals are managed by the members of the family, most often by the women or children. Almost half of the meat that is produced is consumed within the family and it serves as an important source of protein. The guinea pigs can also be sold when the family needs money. These guinea pigs are mainly fed weed and plant and vegetable leftovers from the kitchen.

However alfalfa is also one of the most important feedstuff, especially around Lima (Oshiro et al., 2006). The guinea pigs are housed in either the kitchen or in a small building adjacent to the house, depending on the family's possibilities. The conditions for the animals are often poor with bad hygiene, inbreeding and high mortality. Microbial and parasitic infections are quite common. Dependent on the family's economy and the amount of forage they can afford the number of guinea pigs varies, but 1990 the median was between 20 and 26 animals/farm. The guinea pigs are often small but robust, which is needed because of the conditions and the climate (Lilia, 1997).

Family-commercial (also called middle sized and semi-industrial) farms have many similarities to the family system but they are more organised and they have more guinea pigs. The farmers cultivate forage and cereals for feed and the farms are often located close to cities where they can sell their products. Also in this type of system the amount of feed that can be produced for the guinea pigs decides how many animals that can be kept. Normal is between 100 and 500 animals, with a maximum of 150 reproducing females. The animals are held in a specially constructed separate building and grouped according to age and sex (Lilia, 1997).

The commercial (large, industrial) farms are located in the valleys of the Andeans. They use selected breeding lines for genetic improvement of growth and the amount of meat produced/guinea pig. This type of guinea pig farming is developing and makes it possible to offer guinea pig meat in urban areas and the cities. Commercial farms can have from 1000 up to 5000 animals/farm (Lilia, 1997, Morales, 1994).

Salmonella in guinea pigs

Clinical signs of salmonellosis in guinea pigs include anorexia, changed coat, swollen abdomen, diarrhoea, abortions, infertility, paralysis of the hind limbs and cachexia. Salmonellosis can also be acute and cause sudden death without any signs or death 24-48 hours after the first signs. *Salmonella* spp can cause outbreaks within a colony and cause increased mortality. Outbreaks of salmonella have been associated with chronic, enzootic salmonellosis in herds. Presence of rats in the stables has also been associated with outbreaks (Matsuura S et al., 2010, Iijima et al., 1987, Pivnick et al., 1966).

Guinea pigs can also become infected without showing any signs of illness, so called chronic salmonellosis. Chronic salmonellosis can be enzootic in a colony for many years and for example in the case of *S.* Typhimurium, impair the animal's health and cause increased mortality (Pivnick et al., 1966). Guinea pigs with chronic salmonellosis can also suffer from alopecia. *S.* Abortusequi has been correlated to hair loss starting around one month after infection. The degree and onset of the alopecia varies between different strains (Singh et al., 2005). Chronic salmonellosis can also be a cause of infertility. Sing et al (2007) inoculated guinea pigs with strains of *S.* Abortusequi and found that the guinea pigs showed no clinical sign of illness, and no guinea pig died, but the conception rate was lowered. The exact mechanisms and pathogenesis are not fully understood (Singh et al., 2007).

The bacteria can be isolated from the liver, spleen, intestines, lungs and uterus in guinea pigs (Matsuura S et al., 2010). Pathological findings are most common in the same organs, the liver followed by the intestines being the most affected. Inflammation (mostly catarrhal but also haemorrhagic and necrotic) is the most common type of lesion but circulatory disorders such as petechia, oedema (found in the abdominal cavity and the pericardial sac) and emphysema (in the lungs) are also common. These changes are characteristic of septicaemia. Infected guinea pigs have in average five affected organs (Layme et al., 2011, Iijima et al., 1987, Pivnick et al., 1966). According to Iijima et al. (1987) a common lesion in guinea pigs with salmonellosis is enlarged cervical lymph nodes, occasionally containing pus. They also report that conjunctivitis was common in infected guinea pigs and isolated salmonella from the conjunctiva in 64 % of infected guinea pigs (Iijima et al., 1987).

Salmonella can, but not always, cause rise in antibody titers within one to two weeks after infection. After this rise the bacteria might not be isolated from the guinea pig, even if lesions in for example liver and lymph nodes remain (Iijima et al., 1987).

The most common serovars in domestic guinea pigs are *S.* Typhimurium and *S.* Enteritidis (Richardson, 2000). *S.* Typhimurium can cause chronic salmonellosis in guinea pigs (Pivnick et al., 1966).

Salmonella in guinea pigs in Peru

Matsuura et al (2010) found salmonellae in 61, 5% of tissue samples taken from guinea pigs in Peru with clinical signs of salmonellosis. Most positive samples were found in females close to or close after partum, followed by other adults. Females were more affected then males (Matsuura S et al., 2010). Layme et al. (2011) detected salmonellae in 64, 8% of their samples, consisting of tissues with pathological lesions typical for salmonellosis. However more positive results were obtained from the males. Onyekaba (1983) found that the mortality in an outbreak was highest among weaners and new-borns.

Antibiotics such as enrofloxacin, tetracycline and sulfa-trimethoprim are being used to treat salmonellosis in guinea pigs in Peru. A study performed in the province of Carhuaz showed that salmonella was sensitive to enrofloxacin and sulfa-trimethoprim but also to amoxicillin and streptomycin. However, amoxicillin is not being used because penicillin, along with macrolides, is thought to be toxic to guinea pigs. The same study found salmonella that were intermediate or resistant to furazolidone and tetracyclines (Matsuura S et al., 2010). Onyekaba (1983) treated guinea pigs with tetracycline in an outbreak of salmonellosis and while some animals survived their illness, other developed chronic salmonellosis and died. In his study the salmonella was sensitive to tetracycline, ampicillin, streptomycin and chloramphenicol (Onyekaba, 1983). Administration of ampicillin in doses >6mg/kg causes mortality in guinea pigs and has been associated with detection of *Clostridium difficile* in the caecum. It is thought that the antibiotics disturb the intestinal flora and that overgrowth of *C. difficile* causes enterotoxemia (Young et al., 1987).

MATERIAL AND METHODS

A literature study was performed and articles found by using the database "Web of Knowledge" and "PubMed" searching for words as "guinea pig", "salmonella", "Peru", "pathogenesis", combinations of these words etcetera. References from original articles and reviews were also used.

The animals and the sampling

All samples were collected in the Mantaro Valley, Junín region of Peru, during September and October 2013. The guinea pigs were selected using randomised samples with strata. The strata were; large farms (with a total of 2000 – 5000 animals), medium sized farms (300-1200 animals) and small/family farm (10-200 animals).

Picture 1. Map of Peru, ring indicate the area of Mantaro Valley (CIA World Fact Book, 2004)

The larger farms were very organised and the animals were grouped so that animals of different ages and sexes were separated. The breeding was thereby controlled and the feeding adapted to meet the different animal's needs. For the time of mating one adult male was placed in a box together with four to seven females. The rest of the time the adult males were kept in single boxes. Pregnant females and/or females with neonates were held either in small groups (three to six adults) or in single boxes. When the neonates were around 15 days they were separated from the mother and sorted according to sex. These young animals (so called "recria") were often held in large groups (10-20 animals) until they were around 45 days old. The small farms were less organised, sometimes the animals in the farms visited were kept loose in the kitchen or in big boxes in the backyard. Nonetheless, most of the small farms tried to divide animals of different sexes and ages into different boxes or cages.

According to the farmers treatment with antibiotics was manly used in animals with signs of disease. However in some farms antibiotics were given regularly to the whole herd on prophylactic basis. The extent of these prophylactic treatments is not exactly known.

Guinea pig housing in a large and a small farm.

In the first part of the study a total of 80 individual samples were collected from two large farms (40 samples per farm). These samples were faecal samples collected using rectal swabs. First the total numbers of boxes on each farm (N) were counted and then one animal from every k^{th} box (k being the sampling interval) were tested so that the desired number of animals (n) were sampled.

$$k = \frac{N}{n}$$

The sampled animals in each box were randomly chosen. And from each sampled animal the following information were recorded; age of the animal (neonatal (0-15 days) young (15-45 days) or adult (>45 days)), gender (male or female), clinical signs (if any) and if the female was pregnant. The total numbers of animals on each farm sampled was also recorded. The first farm had approximately 3500 animals and the other one 5000 animals. The distribution of samples in different groups of age is shown in Table 1.

Table 1. Distribution of individual swab samples, groups of different age

Age of animal	***No. of samples***
Young	21
Adults (pregnant)	55 (19)
Neonates	4
	80

In the second part of the study a total of 144 composite samples were taken. This was made to obtain a larger amount of material to analyse. Presumed fresh faeces were collected from the floor of the boxes making up pooled samples. The number of animals in each pooled sample

varied because the number of animals in each box varied. In the large farms the boxes were randomly selected by picking the boxes with every k^{th} animal in, based on the total number of animals on the farm (N) and the desired number of samples (n). This method was not possible in the smaller farms however as the owners often did not know exactly how many animals they had. On these farms the boxes were selected in the same way as in the first part of the study. Information about the number of animals in the box, their age (neonatal, young or adult) and if there were any dead animals or whether any guinea pigs in the box showed obvious clinical signs were recorded. The composite samples were distributed among the different age-groups, types of farms and animals per box as showed in Table 2, 3 and 4. The mean value of animals per box was 10 and the median number 8.

Table 2. Distribution of composite samples, groups of different age

Age of animals in the box	***No. of samples***
Young	48
Adults	59
Neonates and adults	35
Mixed ages	2
	144

Table 3. Distribution of composite samples, different types of farms

Size of farm	***No. of farms***	***No. samples***
Large farms	2	50
Medium sized farms	6	55
Small farms	10	39
	18	**144**

Table 4. Distribution of composite samples, number of animals per box

No. of animals in the box	***No. of boxes***
1-5	39
6-10	42
11-20	41
>20	9

The laboratory work

In the laboratory of Department of Biomedical Sciences and Veterinary Public Health (BVF) at the Swedish University of Agricultural Science (SLU) the laboratory methods was worked out and practical training in the procedures, including preparation of medias, undertaken and tested before leaving for Peru.

All laboratory work in Peru was done in the microbiology laboratory in the research station Instituto Veterinario de Investigaciones Tropicales y de Altura (IVITA) situated in Mantaro Valley. It is a part of La Universidad Nacional Mayor de San Marcos (UNMSM) in Lima.

The laboratory in IVITA, to the right the autoclave.

The method of culturing salmonella was performed according to the guidelines of "Nordic committee on food analysis" (No. 71.5 1999) with some adjustments to local techniques and available equipment in the laboratory in IVITA. The media for cultivating salmonella were made in the laboratory at the research station according to the manufactures instructions.

The rectal swabs were collected and transported in transport medium (Deltalabb) to the laboratory. The samples were first pre enriched in 10 or 5 ml buffered peptone water (BPW, Merck) at 37º C for 24 hours. At one occasion, the samples were stored in fridge for 20 hours before cultivated. After the pre-enrichment 0,1 ml of the solution were transferred to a sterile test tube with Rappaport-Vassiliadis-Salmonella enrichment broth (RVS, Merck) and incubated at 41,5 º C for 24 hours for selective enrichment. The samples were then plated out on selective agar plates, Xylose Lysine Deoxycholate agar (XLD, Merck), and incubated at 37º C for 24 hours. Suspected colonies (black colour) were subcultured on the non-selective agar Tryptone Soy Agar (TSA, Britanialab) for 24 hours at 37 º C. XLD-plates with no growth after 24 hours were incubated for 24 additional hours. Biochemical testing was performed using oxidase test and API10S-system (Biomérieux). In this study samples were considered positive for *Salmonella* spp if confirmed and sereotyped with the API10S-system. The numerical profiles yield in the API0S was interpreted according o the manufacturers list of numerical profiles. Codes that were not found in that list were checked in the "API web" by the manufacturer.

The composite samples were pre enriched, 1g faeces in 9 ml in buffered peptone water, at 37º C for 24 hours. The following steps of the analysis were the same as with the swabs.

The temperature in the incubator was controlled one to three times per day using both the digital thermometer of the incubator and a thermometer placed inside the incubator. The temperatures mentioned above are the one aimed for, but the temperatures sometimes varied ± 2 degrees C.

Four samples (tissue from liver and contents from the intestine) were taken from one guinea pig with suspected acute salmonellosis and one with suspected chronic salmonellosis. The diagnosis of the animals was made according to clinical signs and macroscopic pathology by a pathologist at IVITA.

Testing methods

Due to the lack of isolation of salmonella (see below) a test consisting of two parts; "A" and "B", was performed to test the impact of temperature in autoclave when preparing media and in the incubator when incubating RVS on the final recovery of *Salmonella* spp. These tests were performed in the laboratory at BVF in Uppsala after the field study in Peru.

In "A" RVS was prepared according to the instructions of the manufacturer (i.e. autoclaved at 115 ºC for 15 minutes) and the RVS inoculated with strains of *Salmonella* was incubated at 43 ºC. In "B" the RVS was autoclaved at 123 ºC in 15 minutes and then incubated at 41, 5 ºC. In both "A" and "B" the pH was measured in the RVS when it had returned to room temperature after the autoclave sterilization. Strains from Swedish National food Agency (SLV) were used to inoculate a total of 30 samples, 10 of each serovar. These were *S.* Dublin (number 248), *S.* Enteritidis (number 397) and *S.* Typhimurium (number 242). The strains were first plated on blood agar and incubated at 37 ºC, then one colony from each plate were plated on to a new blood agar plate and incubated at 37 ºC for 24 hours. One separate colony (from the second round blood agar) was mixed with 10 ml 0, 1 % peptone saline diluent and diluted to $1{:}10^4$. 100 µl of this suspension was put in10 ml RVS broth. A suspension (2008:2 from SLV) of mixed aerobes (6,7-7,4 cfu of coliform bacteria, Enterobacteriaceae, E.coli, Enterocci, Bacillus cereus and yeast) was mixed with 104 ml 0,1 % peptone saline diluent and 100µl of this suspension was also added to the sample before the RVS was incubated for 24 hours. After the incubation the samples were plated out on XLD-plates and incubated at 37 Cº before read. Each strain was tested in five samples, making the total number of samples 15 in both "A" and "B".

RESULTS

In the first 80 samples, which were taken with rectal swabs, no salmonella were detected. About 70% the XLD-plates showed no growth at all after a total of 48 hours incubation. One plate hade black areas on the agar surface but most of the other plates with growth had turned yellow and had white, sometimes lightly swarming colonies (Table 5). These were not further analysed but might have been *Escherichia, Enterobacter, Klebsiella, Proteus* or *Serratia* because according to the manufacturer of the media all these bacteria grow with yellow opaque colonies on XLD. According to the API10S the black colonies turned out to be "*Pantoea* spp, *Klebsiella oxytoca, Citrobacter koseri* or *Serratia odorifera"*.

Table 5. Results rectal swab samples

No of animals on the farm	***No. of swab samples taken***	***No. of XLD with black colonies***	***No. samples confirmed as Salmonella* spp *with API***
3500	40	0	0
5000	40	1	0
	80	**1**[1)]	**0**

1) The bacteria turned out to be either *Pantoea* spp, *Klebsiella oxytoca, Citrobacter koseri* or *Serratia odorifera*

Among the 144 composite samples a total of 18 XLD plates showed black colonies (Table 6) and these colonies were therefore tested with the API10S. No *Salmonella* spp was confirmed and the results of the API10S-test included "*Pantoea* spp/*Klebsiella oxytoca/Citrobacter* spp", "*Citrobacter spp/Salmonella spp*", "*Yersinia enterocolitica/pseudotuberculari*s" and "*Shigella* spp/*E.coli*". The numerical profiles that was not included in the list of numerical profiles provided in the API10S-kit gave the result "doubtful result" when the manufacturer used their web based numercial list to control them.

Table 6. Results composite samples

Type of farm	***No. of composite samples taken***	***No. of XLD-plates with black growth***	**No. of samples confirmed as *Salmonella* spp with API**
Large farms	50	10	0
Middle sized farms	55	6	0
Small farms	39	2	0
	144	**18**[1)]	**0**

1) Confirmation by API10S included the following species: "Pantoea spp/Klebsiella oxytoca/Citrobacter spp", "Citrobacter spp/Salmonella spp", "Yersinia enterocolitica/pseudotuberculari*s*" in addition to strains giving "doubtful result"

In the first part of the study four guinea pigs had signs of diarrhoea, one had ascites and three had focal alopecia. The rest of the animals were seemingly healthy. In the second part of the study dead animals were found only in two boxes and signs seen were skin lesions such as alopecia and wounds.

The tissue samples taken from the two sick guinea pigs were also negative for *Salmonella*.

When testing the laboratory methods at the laboratory at BVF in Uppsala it was found that *S.* Enteritidis and *S.* Typhimurium were able to survive incubation of RVS in 43 Cº (test "A"). In all samples with these strains black colonies in pure culture were found on the XLD-plates. *S.* Dublin did not survive and no growth on the XLD-plates was observed. When cultivated in RVS autoclaved in 123 ºC and incubated in 41,5 ºC (test "B") all three strains survived. Growths of black colonies in pure culture were observed on all 15 XLD-plates (se Table 7). The ph after autoclaving was 5,22 in "A" and 5,29 in "B". This is within normal variation (pH 5,2± 0,2) according to the manufacturer.

Table 7. Results of the test at BVF

Serovar	***No positive samples in "A [1)]" (%)***	***No positive sample in "B[2)]" (%)***
S. Dublin	0 (0)	5 (100)
S. Typhimurium	5 (100)	5 (100)
S. Enteritidis	5 (100)	5 (100)
	10 (66,7)	**15 (100)**

1) RVS autoclaved at 115 ºC and RVS incubated at 43ºC
2) RVS autoclaved at 123 ºC and RVS incubated at 41,5 ºC

DISCUSSION

No *Salmonella* was isolated from any of the animals examined, neither from the rectal swab samples in the first part of the study nor in the composite samples in the second part of the study. Animals of different age, sex and from different types of housing were sampled but in none of these groups *Salmonella* was detected in faeces. These results contrast to the general opinion that salmonellosis is a common problem in the Peruvian guinea production and also the result of some other studies as described above. In case of regional differences it is noted that no papers have been found on the prevalence of *Salmonella spp* in guinea pigs from the geographical area of this study. However, in an un published study performed by the National Institute for Agricultural Innovation in another region, Huancayo, 192 animals were sampled and salmonella was not isolated from the faeces from any of the animals (Arce et al., 2013).

Before an assessment of the result of this study can be done the focus on first hand had to be directed to possible failure or limitations of the used methodology. Because no salmonella were isolated it would have been of basic importance if a positive control had verified that salmonella could be isolated by the bacteriological methods onsite in IVITA. In spite of repeated attempts it was however not possible to obtain a type strain of *Salmonella* for that purpose from the laboratory of UNMSM in Lima. An assessment of the reliability of the culture methods is focused on the media used and the temperature during media preparation and incubation. The autoclave as well as the incubator used were of older models and their regulation of the internal temperature was not optimal which might have influenced the results.

In two studies on *Salmonella spp* in guinea pigs in Peru the specie *Salmonella enterica* was found but the serovar was not determined (Layme et al., 2011, Matsuura S et al., 2010). However, the most common serovar among guinea pigs are *S.* Typhimurium and *S.* Enteritidis (Richardson, 2000). Because RVS has been found to be a good alternative in detecting *S.* Typhimurium RVS was a suitable medium for enrichment in this study, even if according to some studies other selective enrichment medias such as MSRV or MKTTn are preferred according some studies (Kusar et al., 2010).

The autoclave used was of an old model and the regulation of the temperature inside was not optimal. It had no thermometer for controlling the temperature while running. Therefore it is a possibility that when the autoclave was used to prepare the BPW and RVS the temperature was a bit too high, compared with the instructions from the manufactures. Problems with the selective medium might result in false negative results (Havelaar et al., 2001). But the tests at BVF again show that RVS autoclaved at a higher temperature (123 ºC) then the manufacturers recommendations can be used to cultivate at least some strains of *Salmonella.* Although the exact temperature in the autoclave used in IVITA is not known the risk of having a too high proportion of false negative results compared to controlled conditions can be considered as low.

To select for *Salmonella* spp, in addition to using RVS, a temperature of 41, 5 ºC was used. Problems with the incubator led to temperatures fluctuating one to two degrees above and below the set temperature. This might have affected the cultivating, and in the end making it harder to detect *Salmonella* (Peterz et al., 1989, Vanschothorst et al., 1977). However, although not optimal it is posible to cultivate *Salmonella* spp at higher temperatures. Salmonella can grow between 37º C and 44 ºC, but the multiplication is lower at the higher temperatures (Van schothorst et al., 1977). In one study isolation of *Salmonella* spp after incubation in different incubation temperatures (37 or 43 ºC), times (24 or 48 hours) and three different mediums was compared. The conclusion was that the best results were obtained by incubation for 24 hours at 43 ºC (Kafel and Bryan, 1977). Peterz et al. showed that a higher incubation temperature (43 ºC versus 40) in Rappaport-Vassiliadis (RV) broth suppressed the growth of salmonella, especially *S.* Dublin. They also showed that there are differences between different types of RV, that a higher concentration of magnesium chloride might explain a poor result in detecting salmonella (Peterz et al., 1989).

Even if the number of samples tested at BVF was limited the results shows that *S.* Typhimurium and *S.* Enteritidis are able to growth in RVS at 43 Cº. This is in accordance with the studies mentioned above. Due to the fact that these strains are the most common associated with salmonellosis in guinea pigs (Richardson, 2000) it is likely that any salmonella in the fecal samples would have been able to survive and grow even if the temperature in the incubator at IVITA was sometimes higher than 41, 5 Cº.

The growth of most *Enterobacteriaceae* increases in BPW but decreases in selective enrichment at high temperatures. Beckers et al (1987) found *Salmonella* spp in the final plating in samples were salmonella had grown both in BPW and the selective enrichment. In the samples were salmonella had not increased, or even decreased, during the selective

enrichment no salmonella were found in the final plating. They also concluded that the number and type of competition flora determinate if *Salmonella* spp can be recovered. *Enterobacter, E.coli, Citrobacter, Proteus* was found to play an important role (Beckers et al., 1987). Isenberg el al (1989) have shown that less *Salmonella* spp is found on XLD-plates if the percentage of *Salmonella* spp compared to other bacteria in the sample is low (Isenberg et al., 1969). In this study colonies of other *Enterobacteriaceae* were frequently found on the XLD-plates indicating the presence of competitive flora in the sample. It is possible that this fact might have resulted in some false negative results.

Because of the randomised sampling it is possible that none of the sampled animals were shedding salmonella in contrast to the non-sampled animals. Havelaar et al (2001) showed that guinea pigs that are infected with salmonella shed large amounts of bacteria in their faeces the first days and that the amounts decline and may rise again later. Because the shedding seems to be intermittent it is theoretically possible to get negative results because the animals were sampled on a day with low amount of bacteria in the faeces. However, all animals on a farm are unlikely to be infected at the same time and therefore when considering the amount of samples taken it is unlikely that randomized sampling or sampling on the "wrong" days are the explanation for the negative results. Nonetheless, a very low prevalence of salmonella infected animals in a herd may result in diagnosis failure due to overly low sensitivity of the testing methods, even on a herd level.

Both Pivnick et al (1966) and Singh et al (2007) have shown that chronic, asymptomatic salmonellosis exist in guinea pigs. However, when only low amounts of salmonellae ($< 10^4$ cfu/ gram faeces) are shedded , as for example in chronic carriers, Havelaar et al (2001) could not detect the bacteria in faecal samples. Because mainly seemingly healthy animals were sampled in this study again it is possible that low shedding animals were not detected. However, if salmonella is a clinical problem in a herd some animals are likely to shed the microbe in such high counts that would be picked up by testing procedure.

Funk et al. (2000) showed that the size of faecal samples affect the possibility of detecting salmonella. Smaller amount of faeces yield higher number of false negative samples. This might have been a problem, especially in the first part of the study, because it was hard to get material from some of the smaller animals. The difference is not as apparent if the prevalence in the area is low. If the prevalence is low the number of samples needed to be 95 % sure to find at least 1 positive sample needs to be larger, and the same is true if lower faecal weight is used (Funk et al., 200). There are also studies (for example Singh et al.,2005 and Singh et al. 2007) were *Salmonella* spp has been found in subclinical guinea pigs using rectal swabs. This suggests that the method used could have detected salmonella in the faeces of the guinea pigs if there were any.

Some methods were changed during the field work because of new knowledge or following negative results. To lower the risk of not getting enough faeces composite samples were taken instead of rectal swabs in the second part of the study. Also the method for selection of the animals sampled was changed. Because young animals were held in large groups while for example adult males were held alone in one box there is a risk of selection bias in the first

part of the study. The adult animals had a greater chance to get sampled than the younger ones. Because of this the method of selection was changed in the second part of the study so that al animals had the same chance to get sampled. The first 40 rectal swab samples were enriched in 10 ml BPW while the other 40 was enriched in 5 ml of BPW. This was made to get a higher concentration of bacteria in the BPW to transfer to the RVS. No published literature to support this method has been found.

It is possible that some clinical signs of disease were missed in the second part of the study because when there were more than five to ten animals per box it was hard to examine them all. However, at least no animals were so severely ill that they did not run around when people were approaching the box, that severely sick animals are unlikely to have been missed. From a clinical point of view the use of antibiotics can also be considered. Although no indication of this aspect were present in this study, it is known that administration of antibiotics may decrease the faecal shedding of salmonellae (Williams et al., 1978). As well, antibiotics have been used to mask infection with salmonella in poultry (Martin Wierup, 2014, pers. comm.).

The samples taken from two dead animals with suspected salmonellosis in this study were also negative. This again might indicate that something was wrong with the methods used. However another possible explanation is that the guinea pigs did not die from salmonellosis. The diagnosis was not confirmed using either histology or microbiology methods. There are other bacterial infections that can cause similar signs as salmonellosis. For example, *Yersinia pseudotuberculosis* exists both as chronic and acute disease in guinea pigs. Septicaemia and death within 48 hours is seen in the acute form and diarrhoea and weight loss in the chronic form. Its pathological picture also resembles that of salmonellosis with for example focal necrosis in spleen and liver. *Clostridium spp* and *Escherichia coli* (*E.coli*) can also cause enteritis sudden death in guinea pigs (Richardson, 2000). Both *Yersinia* and *E.coli* were found in faeces in this study demonstrating that these agents exist in guinea pigs in Mantaro. Examples of other causes of abortion (in addition to salmonellosis) is high temperature and infections with *Bordertella bronchiseptica*, *Streptococcus pneumoniae* and *Toxoplasma gondii* (Richardson, 2000).

In summary it is reasonable to conclude that the total lack of isolation of *Salmonella* spp reflects the real situation and is not only the result of limitations of the methods applied. Salmonellosis might be over diagnosed in the area and not as widespread as currently believed. Maatsura et al (2010) analyzed tissue samples from animals with suspected salmonellosis according to clinical signs and found salmonella in 61,5% of the samples. Similar results were reported by Layme et al. (2011) who isolated *S.* Enterica in 64,8% of necropsies from guinea pigs with suspected salmonellosis (Layme et al., 2011). But then again, in the non-published study performed by the National Institute for Agricultural Innovation in Huancayo (2013) no salmonella was found in guinea pig faeces (Arce et al., 2013). This might indicate that around one third of the diagnoses made according to clinical signs and macroscopic pathology may not be correct. Because cultivating is not regularly used to confirm the diagnoses there is also a risk that other infectious agents are being overlooked. A study in cattle has shown that clinical cases of salmonellosis are related to a higher

prevalence of *Salmonella* spp in the herd (Cummings et al., 2010). No such studies have been found in guinea pig but outbreaks of salmonellosis have been related to presence of asymptomatic carriers same in guinea pig colonies (Iijima et al., 1987). One explanation might be that salmonellosis occurs in larger outbreaks when salmonella are introduced in the herd and that the animals then get rid of the pathogen and that it does not survive on the farm. However that type of epidemiology is not supported by for example Lijima et al (1987), Pivnick et al (1966) or Singh et al (2007) and is not known to occur in other farm animal species such as pigs and poultry (Kusar et al., 2010).

If *Salmonella* spp is responsible for most problems in the guinea pig production in Mantaro Valley it is therefore likely that the prevalence would be high and salmonella would have been isolated in this study regardless of the initial and formal doubts with the methodology. Problems with the temperatures at incubation and autoclaving might be an explanation for some of the negative results but it is unlikely that all of the samples were false negative. There is thus need for more research on the epidemiology of *Salmonella* spp in guinea pigs. Such studies should also focus on other pathogens in the guinea pigs in the Mantaro Valley. Based on the experiences from this study, future studies should ensure that type strains of *Salmonella* spp are available to be used as a positive control of the reliability of bacteriological methods applied for cultivation.

Acknowledgments

Great thanks to supervisor John Pringle (Department of Clinical sciences), assistant supervisor Martin Wierup (Department of Biomedical Sciences and Veterinary Public Health) and Cesar Gavidia (National University of San Marcos, Lima). Also a great thanks to Lise-Lotte Fernström (Department of Biomedical Sciences and Veterinary Public Health), Miguel Siever Morales (National University of San Marcos, Lima), Ronald Jimenez and Juan Raúl Lucas (IVITA) for help and support.

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