Journal of Animal and Veterinary Advances 8 (12): 2447-2452, 2009
ISSN: 1680-5593

Presence of Zoonotic Interest Bacteria in Slaughter Pigs

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Abstract: The objective of this study was to recognize, the presence of *Salmonella, Brucella*, *Leptospira* and *Yersinia* in slaughter swine that are apparently healthy. Samples were taken from blood, tonsils, ileum, kidney and spleen of 100 pigs that came from the States of Jalisco, Guanajuato and Sonora. For brucellosis diagnosis, card test and bacteriology from spleen were performed. For leptospirosis a micro agglutination technique was used and bacteriology from kidney. Bacteriology study was performed for *Salmonella* diagnosis using samples from ileum and tonsil samples were used for diagnosis of *Yersinia enterocolitica. Salmonella* study results were negative for all animals. Five reactors were found for *Brucella* by serology (5%), but no isolation was achieved. Twenty-five percent positive reactors to two serovars of *Leptospira* were found: Bratislava 19 animals (19%) and Panama 6 (6%) with no isolation of the bacteria. Twenty-two percent isolations of *Yersinia enterocolitica* biotype 1 serotypes 0:3 and 0:9 were obtained. Positive animals came from the States of Jalisco and Guanajuato. It is concluded that of the 4 diseases that were studied, the one that had more relevance was *Y. enterocolitica* with 22% of isolations in slaughter swine that are apparently healthy.

Key words: *Brucella*, pigs, *Leptospira*, *Salmonella*, *Yersinia*, bacteriology

INTRODUCTION

Animal slaughter is one of the professional activities with a higher degree of risk, with a very significant role in the transmission of zoonotic diseases. Slaughterhouse operators that are in direct contact with carcasses or viscera are the ones that are more at risk (Boqvist *et al*., 2003; Godfroid *et al*., 2005; Campagnolo *et al*., 2000).

Among the main bacterial zoonosis in swine are salmonellosis, brucellosis, yersiniosis and leptospirosis. Swine brucellosis is one of the infectious diseases that affect the reproductive organs and it is generally caused by *Brucella suis* and according to the contact possibilities with other animal species by *Brucella abortus* and *Brucella melitensis*. It is a chronic disease that occasionally may pass undetected and it affects sows as well as boars. It is characterized by the production of abortion, infertility, birth of weak animals and pathologically, it can produce epididymitis, orchitis, endometritis, laminitis and spondylitis.

Brucella suis has five biovars. Biovar 2 is found in Europe and its natural host is the hare, in swine it causes lesions in the uterus. In Mexico biovar 1 (Luna and Mejia, 2002) is the most common, while worldwide the most common one is biovar 3.

Biovars 1 and 3 of *Brucella suis* are very pathogenic for man, since there are numerous reports in the United States of human brucellosis caused by this species. In some Latin American countries *B. suis* biovar 1 is the major cause of human brucellosis (Corbel, 1997; Godfroid *et al*., 2005).

Recently, *Yersinia enterocolitica* has been recognized as an emerging pathogen, of which the main reservoir is the pig. Most of the *Yersinia* isolations in humans correspond to biotypes 1, 0:8; 2, 0:5, 0:7 and 0:9, 4, 0:3, that have been isolated from swine, mainly serotype 0:3 from tonsils and tongue (Bissett *et al*., 1990; Kwaga and Iversen, 1992).

Even though, it is still not precisely determined how the infection is established between humans and domestic animals, there are strong evidences that transmission may be related with the consumption of pork or direct contact with these animals, mostly by slaughterhouse, butchery and farm workers (Hurvell *et al*., 1982). Nevertheless,

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association of human disease has been indicated, by contact with animal waste and non-chlorinated water contaminated with *Y. enterocolitica* together also, with improper refrigeration conditions in food processing plants during their preservation and storage, where the bacteria is capable of survival and increasing in number (Weagant, 1991).

In the United States and European countries, studies have been carried out in slaughter swine, mainly in butcher shops and slaughterhouses and the serotypes that have been isolated from throat, tongue and tonsils have been serotypes 0:3 and 0:5 (Hanna *et al*., 1980; Saumya *et al*., 2005) with a strong association between serotypes isolated in man and animals. Predominant serotypes in Europe have been 0:3 and 0:9 (Asplund *et al*., 1990).

Leptospirosis is caused by the spirochete *Leptospira interrogans* of which, there are >230 serovars. Its importance lies in the fact that it affects domestic mammals as well as humans being one of the most important zoonosis (Levett, 2001).

In pigs, it is frequent that infection is not clinically seen. Animals remain as asymptomatic carriers during long periods of time and in this manner they disseminate the disease by eliminating the bacteria by urine. Characteristic signs are infertility, mummifications, abortions and weak piglet births (Faine *et al*., 1999).

After the leptospiremic phase, bacteria shows great affinity for kidney tissue. It has been identified in the intra-tubular space and proximal tubule lumen of kidneys, where it can remain during many months. It has been possible to isolate several serovars of *L. interrogans* from the genital tract of non-gestating sows and from boars and this demonstrates that it can also remain viable in these tissues (Ellis and Thiermann, 1986).

On the other hand, humans are considered to be accidental hosts, although potentially susceptible to any serovariety of *L. interrogans*. In open populations, it is accepted that pets are the most important source of contagion, nevertheless, the highest risk groups are those, whose activity is related with mammals, such as the swine farm workers. Handling of contaminated viscera and fluids in slaughterhouses are factors that predispose to leptospirosis contagion (Boqvist *et al*., 2003; Campagnolo *et al*., 2000).

Human symptoms are not very specific and may comprise a wide range of symptoms, among which initially are fever, headaches, abdominal pain, myalgia and anorexia. After that there can be febriculas, jaundice, renal damage, lung alterations (Hill and Sanders, 1997; Levett, 2001).

Serology studies performed in swine farms in Mexico found that the most frequent ones were: Bratislava, Icterohaemorrhagiae Palo Alto strain and Portland vere strain Sinaloa ACR, these two strains were recently isolated in Mexico (Cisneros *et al*., 2002b).

Swine salmonellosis manifests itself by septicemia or enterocolitis. The septicemic form of *S. choleraesuis* is found in pigs from 5 weeks of age, in finishing pigs and in breeding sows it may cause sudden death or abortion. The first clinical sign is yellowish watery diarrhea without mucus or blood. A characteristic of the disease is the rapid dissemination getting to most of the animals in a pen. When infected, pigs excrete bacteria in feces and contaminate the facilities establishing a continuous infectious cycle in the herd. Recuperated pigs excrete *Salmonella* during >5 months (Morilla, 2005).

Pigs remain infected during long periods and pork may become a source of these bacteria for humans. The reservoir for *Salmonella* is the intestinal tract of animals. *Salmonella choleraesuis* has been the cause of 60-80% of salmonellosis cases, 8% of the cases are produced by *S. typhimurium* and *S. derbi* (Morilla, 2005).

Serology is useful to recognize an infected herd, but it is not so for individual carrier pigs detection (Morilla, 2005).

Studies that have been carried out in carcasses and tissues (ileum, iliac lymphatic and mandible nodes and tonsils) have demonstrated contamination during slaughter. *Salmonella* has been identified in 16.7% of the pigs and 12.9% in carcasses. The greater amount of isolations has been found in iliac node and ileum indicating fecal origin contamination, during the pig slaughtering process in the slaughterhouse. These results indicate that node analysis may be more sensitive for *Salmonella* detection than other tissues (Vieira-Pinto *et al*., 2005). In other studies, isolation has not been achieved from cervical nodes (prescapular) (Bahnson *et al*., 2006).

The objective of this study was the isolation and identification of brucella, yersinia, leptospira and salmonella in slaughter swine in a cold-storage slaughter plant of the State of Mexico.

MATERIALS AND METHODS

Since, the frequency of these organisms in swine in Mexico is not know, a pilot study with 20 samples was done to estimate infection frequency and based on this estimate the sample size (n) was established with the equation (Cannon and Roe, 1982):

$$n = v^2(p)(1-p)/T^2$$

The minimum sample size was estimated in 62 pigs, nevertheless, it was increased to 100. Once, the sample

size was estimated, sampling was performed systematically, considering a daily slaughter average of 1,100 animals. Also, the interval was estimated, thus sampling was carried out with 16 samples/day/week (Mendez, 1998).

The origin of the animals was the States of Jalisco, Guanajuato, Sonora and Michoacan. From each one of the slaughtered and apparently healthy animals samples were taken from blood, tonsils, ileum, kidney and spleen.

Samples were individually deposited in sterile glass flasks and plastic bags, properly identified and preserved at refrigerator temperature (4°C).

For serological diagnosis of *Brucella, B. abortus* 8% antigen was used in a buffer lactate solution with pH 3.5, stained with Bengal rose. To do the test, 30 μL of serum and 30 μL of antigen, were perfectly mixed, after that a slight rotation movement was applied for 4 min, after which the reading was performed using the agglutinascope indirect light source (Alton *et al*., 1988). To isolate brucella, Farrell's selective medium was used. This is made of an agar with dextrose base to which, bovine fetal serum is added and a series of antibiotics: bacitracin 25,000 UI, polymycin B 5,000 UI, cyclohexamide 100 mg, vancomycin 20 mg, nalidixic acid 5 mg and nistatin 100,000 UI (Alton *et al*., 1988). Spleen samples were placed in sterile bags with 10 mL of saline physiological solution, to then be processed in the Stomacher macerator for 3 min. In this case, the supernatant, as well as part of the macerated product was spread in plates with Farrel culture medium and then incubated at 37°C with 10% CO_2 during 10 days (Marin, 2001).

In the case of *Salmonella*, ileum was used for isolation; it was cut and macerated to be spread in an enrichment thioglycollate broth, incubated during 24 h at 37°C and after that it was inoculated in xylose lysine deoxycollate agar and brilliant green, incubating at 37°C during 48 h.

Finally, for *Yersinia enterocolitica* isolation, identification and serotyping, 10 g of tonsils sample were chopped and mixed in 20 mL of sterile peptone water where, it was allowed to rest during 10-15 min at room temperature (±24°C), then filtered through sterile gauze to eliminate larger fragments. From the macerate, 1 mL of the filtrate was mixed with 20 mL of modified Rappaport medium, added with a CIN supplement (Novobiocin, Irgasan and Cefsulodine) (CIN), in 6 sterile glass tubes and incubated at 22-25°C for 48 h in aerobiosis. After that, it was spread in duplicate in Salmonella-Shigella (SS) agar plates supplemented with sodium desoxycollate and calcium chloride and McConkey agar, incubating at 33°C for 48 h in aerobiosis (Diaz *et al*., 1998).

Isolates identified as *Yersinia* were then placed for biochemical tests and incubated during 48 h in aerobiosis at different temperatures, at 25°C: Oxidation-Fermentation (OF), H_2S, Motility and Indol (SIM), Voges-Proskauer (VP). At 36°C: Triple Sugar Iron (TSI), urea, Iron-Lysine (LIA), nitrates, Motility-Iron-Ornithine (MIO), Methyl Red (RM) and Simons citrate, as well as catalase and oxidase reactions and carbohydrate reactions (Macfaddin *et al*., 2003).

Once, the genus was identified, then corresponding biotype and serotype were identified, each one of the isolates of *Y. enterocolitica* were challenged with reference antisera 0:3, 0:8 and 0:9 using adsorption agglutination techniques and slow agglutination. Together with this, biotyping was carried out by hydrolysis Tween 80 (Wayne method) technique.

Semisolid Korthoff and EMJH culture mediums were used added with 5 fluoruracile in final concentration of 0.1 mg mL^{-1} to reduce bacterial contamination (Cisneros *et al*., 1994). Kidneys were refrigerated and transported to the laboratory. In order to carry out the spreading, a spatula was heated over a burner and applied to the surface of the kidney. After that and in asepsis conditions a portion of the sample was taken, including cortical and medullar zones, then passed through a sterile 3 mL discardable syringe and deposited in a tube with 5 mL phosphate buffer solution at pH 7.2-7.4, it was stirred and allowed to rest during 60 min, with a sterile pipette a drop of supernatant was taken and deposited in each one of the three tubes with each one of the culture mediums, then incubated at 30°C during eight weeks, with microscope revisions every 7 days (Myers, 1985).

Serology: Micro Agglutination Test (MAT) described by OPS (Myers, 1985) was used with an initial dilution of 1:50.

In an assay tube 2.4 mL of Phosphate Buffer Solution (PBS) was deposited and 0.1 mL of problem sera was added to obtain a dilution of 1: 25. In a 96 well serology plate, 50 μL of problem sera was added for each line of 12 wells. To each of the wells 50 μL of the different antigens were added giving a final dilution of 1: 50. A negative control was included for each one of the serovars.

It was incubated 60 min in moist chamber at room temperature and the reading was done in a dark field microscope observing the degree of agglutination in each dilution, comparing this with the negative control. If the test came out positive with >50% of agglutination observed in the dark field observation to the initial test 1: 50 for any of the serovars that were studied, then double dilutions were performed, until a final titer was obtained. Positive sera were those that reacted at 1:100 or

above with 50% agglutination or disappearance of field cells, in the observation of the dark field microscope (Myers, 1985).

Antigens: These were cultured in liquid medium and incubated at 30°C during 5-7 days until good growth was obtained. Serovars of *L. interrogans* that were used were: Icterohaemorrhagiae, Pyrogenes, Grippotyphosa, Canicola, Pomona, Wolffi, Panama, Hardjo strain Hardjoprajitno and Hardjobovis, Tarassovi and Bratislava of international reference; Hardjo strain H-89 genotype Hardjoprajitno of bovine origin; Portland vere strain Sinaloa ACR of porcine origin and Icterohaemorrhagiae strain Palo Alto of canine origin, Mexican strains recently isolated (Cisneros *et al*., 2002b).

RESULTS

By 8% Bengal rose test, 5% of sera were positive to brucella; nevertheless, brucella was not isolated from the spleen of the sampled pigs.

In the case of *Salmonella* there weren't any strain isolates from the ileum samples. Of the total samples 22 (22%) had *Yersinia enterocolitica* biotype 1. 16 of the isolated strains were serotyped, it was not possible to serotype 6 strains, with the antisera that were used, found serotypes were O:3, 36.36% (8/22) and 36.36% of serotype O: 9 (8/22) and it was not possible to identify any of serotype O:8. In one sample, it was possible to identify two serotypes of *Yersinia enterocolitica* 0:9 and 0:3.

Of the 100 analyzed sera, 25.0% were positive to *Leptospira*, of the 13 serovars that were tested in the MAT, there were only sera positive to *L. bratislava* 19% and Panama 6%. The range of titers that were obtained were 1:100-1:200 for both serovars, there was no isolation obtained from the kidney samples.

DISCUSSION

In Mexico swine brucellosis has not been studied very much, although there are evidences that *B. suis* is present since there have been some isolations obtained from cattle (Pacheco and Luna, 1999). Nevertheless, there are no data of swine farms infected with brucella; in this study, the percentage of seropositive animals was very low. *Yersinia enterocolitica* was not isolated from pigs that were positive to brucella and animals with positive isolation of *Y. enterocolitica* were not positive to brucella and therefore there were no crossed reactions in this case.

Presence of *L. bratislava* and *L. panama* in the studied samples, indicates that these serovars are very important in swine in the central region of the country (Diosdado *et al*., 2004).

In the last few years, a notorious increase in the relative frequency of *L. bratislava* has been observed by serodiagnosis performed in Mexico. In a study that lasted 10 years (1975-1984) and included 4,456 sera sent to diagnostic laboratory, only in 3 years (1980-1982) 12 reactors to Bratislava were identified of a total of 1791 sera (Jimenez *et al*., 1986), while in another study that took 10 years (1983-1994), 2,097 sera were studied and then the most important serovars were *L. panama* (16%) and *L. icterohaemorrhagiae* (9%), while *L. bratislava* was of little importance (0.6%) (Rojas *et al*., 1994).

Recently, serological studies in swine carried out in swine areas of Mexico have reported that these two serovars turn out positive reactors (Diosdado *et al*., 2004).

In a farm of breeder gilts located in the State of Sonora, *L. panama* was present in 27% animals by detection of antibodies thus, being the most frequent serovar (Cisneros *et al*., 2002a).

In a study, in the zone of Guanajuato in the center of the country, it was mentioned that 52 sampled farms 71% had serology reactors to *L. bratislava*, 56% to *L. icterohaemorrhagiae* and 46% to *L. panama*, thus being Bratislava the most frequent. In this same study, of 482 third parity sows or more, 32.3% were positive to *L. bratislava* and 25% to *L. panama*, making these the two most frequent serovars (Moles *et al*., 1997). Those frequencies are higher than the ones found in this study with 19 and 6%, respectively for *L. bratislava* and *L. panama*.

Likewise, in another study in the region of the high plateau of Mexico, done with 114 pigs raised in a technically managed farms and 50 raised in backyard premises it was found that both systems had 28% positive reactors to *L. bratislava*, while for *L. panama*, it was 34% of the backyard farms and 28% of the technically managed farms (Moles *et al*., 1998).

Even though, the percentage results were lower, the results indicated that only these two serovars were present. This is important since *L. panama* has always been associated with pigs and it was not identified as an important serovar.

It is probable that pigs play a very important role in the transmission of *L. bratislava* since, they become asymptomatic carriers and serve as reservoirs for the disease and spreading of infection to other animals and even man. Therefore, the relevance of this serovar is highlighted especially in the aspect of zoonosis.

Viscera handling by slaughterhouse workers and even by persons dedicated to the sale of these in markets makes these people a high risk population.

Salmonella serotypes in swine are more fastidious for their culture than other serotypes from other animal

species, probably, because of this no isolation was achieved from ileum. Viera and Pinto found that the greater number of isolations may be performed from the lymph nodes.

Isolation of *Y. enterocolitica* from pigs demonstrated the presence of the microorganism in these slaughter animals and that the agent is located in the digestive tract, specifically in tonsils.

Finding serotypes 0:3 and 0:9 in swine tonsils in Mexico, agrees with similar results published in Europe, Japan and Canada and also in those countries it is indicated that frequently swine are a source of infection for man and that they are common reservoirs of *Y. enterocolitica* (Merilahti *et al*., 1991).

In humans, transmission has been established as caused by contact of persons with infected animals, the transmission from person to person, within an infected family and the consumption of contaminated pork, those involved are mainly workers that have to do with slaughterhouses, farm hands and swine producers. Another form of transmission is related to the use of contaminated water during slaughter, contributing then in some way to the quick dissemination of the microorganism by the carcass. These elements, combined with inappropriate hygiene measures in storage places and the handling of food are risk conditions that should be taken into consideration as contributors to the infection by zoonotic microorganisms.

CONCLUSION

It is concluded that of the four diseases that were studied, the one that shows more relevance is *Y. enterocolitica* since, there were 22% of isolates in apparently healthy slaughter animals.

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