

Isolation of Aerobic Bacteria in Internal Specimens from Domesticated Pigs Used in Biomedical Research and the Association with Bacterial Translocation

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Abstract: Swine are extensively used in biomedical research as animal models. Although, ideally they are procured from specialized breeders of pathogen-free animals, animals from meat production farms may be used. The aim of the present study was to investigate the potential existence of subclinical disease of domestic pigs used for research. In order not to sacrifice additional animals, 46 pigs from two other studies in progress were investigated: group A, n = 24, aseptic minor procedures; group B, n = 22, similar procedures plus induction of ventricular fibrillation leading to Cardiac Arrest (CA). Microbiological cultures of blood, liver and lung tissues were executed. Overall 60% samples contained pathogenic or opportunistic pathogens protocol comparison demonstrated positive blood cultures in one animal of group A (4.2%) and 5 of group B (22.7%, p>0.05). Liver cultures were positive in two animals of group A (8.3%) and 15 of group B (68.2%, p = 0.0041). Lung cultures were positive in 10 animals of group A (41.7%) and 17 of group B (77.3%, p>0.05). *Bordetella bronchiseptica* was isolated in liver (10.8%) and lung cultures (26%) overall. There was a significant difference regarding *Stenotrophomonas maltophilia*, isolated at a greater rate in group B (liver p = 0.00007, lung p = 0.0043). This consisted an unexpected finding, leading to the assumption that group B animals may have sustained Bacterial Translocation (BT) through an intestinal ischemia/reperfusion mechanism due to CA. These results not only showed the impact the use of non pathogen-free animals may have on the results in research but also provided for the first time indication that CA may contribute to systemic infection early after successful resuscitation, by BT.

Key words: Domestic swine, pigs, biomedical research, health status, bacterial translocation, subclinical disease, *Stenotrophomonas maltophilia*, *Bordetella bronchiseptica*

INTRODUCTION

The acquisition, care and use of laboratory animals for experimental and other scientific purposes are regulated by international, national and institutional guidelines with the aim of safeguarding the animals' health and welfare and additionally resulting in valid scientific results (Council of European Communities, 1986; Institute for Laboratory Animal Research, 1996; Klein and Bayne, 2007). It is also scientifically accepted that only healthy animals not harboring infections potentially hazardous to other animals or humans or detrimental to the scientific procedure should be used in research (Voipio *et al.*, 2008). In accordance with such guidelines, it is advised that laboratory animal breeders and suppliers

are evaluated for the quality of their animals (Institute for Laboratory Animal Research, 1996) and that user establishments follow health monitoring programs (Nicklas *et al.*, 2002; Rehbinder *et al.*, 1998).

During the last 25 years, certain member states of the European union adopted national implementing measures of the Directive 86/609/EEC (Council of the European Communities, 1986) that ensured a high level of animal protection with strict regulations while others applied only the minimum requirements laid down in that Directive. The new directive, effective 01.01.13, comes to harmonize discrepancies and set stricter measures (Council of the European Communities, 2010). Although, ideally all laboratory animals for research use should be procured from specialized breeders of pathogen-free

animals (American Association for Laboratory Animal Science Learning Library, 2005), there are some countries where breeders of farm pigs produce small numbers of animals for research establishments in parallel to their meat production purposes. These breeders are not required by national law to screen their animals by a specialized diagnostic laboratory for the list of pathogens traditionally recommended for research purposes (Rehbinder *et al.*, 1998). This however, lack of health screening, may result in the use of animals with subclinical disease having an impact on research results.

The aim of the present study was therefore to investigate the potential existence of subclinical disease of outwardly healthy farm pigs that were delivered to and used in a University research establishment in Greece. The lack of local breeders of specific pathogen-free swine or minipigs and the expenses associated with their importation has resulted in the use of these farm pigs raised mainly for meat production. The investigation of their health status was carried out by microbiological cultures of samples of whole blood, liver and lung tissue removed under aseptic procedures. We considered that potential positive results would consist strong scientific evidence in addition to the literature and persuasive arguments for changes in procurement policies.

MATERIALS AND METHODS

Laboratory animals: Forty-six domestic pigs (*Sus scrofa domestica*) of the cross Landrace x Large White, both male and female, 3-4 months of age with a mean body weight of 19 ± 3 kg, originating from the main local breeder for research use were investigated. The study was conducted after permit (K/7406/2006) from the Veterinary Directorate of the Prefecture of Athens according to the Greek legislation in conformance to the Directive 86/609/EEC of the European Union. All activities directly related to the pigs to promote their welfare such as during transportation, housing, anaesthesia, analgesia, surgery and euthanasia were carried out according to the above legislation and scientific recommendations. The samples were taken from pigs that were being used over a 15 months period by other researchers of two protocols (with license no. K/227/2004, K/3131/2006, K/3312/2006 and K/8025/2006) in order not to sacrifice other animals for the present study. The inclusion criteria of these protocols were that the specific aseptic procedures followed would not influence the results of the study and that they would be maintained under anaesthesia for 1 h until euthanasia.

The animals from the first protocol (group A, n = 24) were subjected to minor aseptic procedures of 1 h duration while the animals from the second protocol

(group B, n = 22) were subjected to similar aseptic procedures with the addition of the induction of Ventricular Fibrillation (VF) leading to Cardiac Arrest (CA).

The anaesthesia protocol was similar in both groups. Initial sedation in each animal was achieved with intramuscular 10 mg kg⁻¹ ketamine hydrochloride (merial), 0.5 mg kg⁻¹ midazolam (Dormicum, Roche) and 0.05 mg kg⁻¹ atropine sulphate (Demo S.A.). The marginal auricular vein was then catheterized (BD Venflon, Luer-Lok, 20GA). Anaesthesia was initiated with an intravenous bolus dose of 2.0 mg kg⁻¹ of propofol (Diprivan, AstraZeneca) via this vein and maintained at 54 mL min⁻¹. While anaesthetized and still breathing spontaneously, pigs were intubated with a 4.5 or 5.0 endotracheal tube (MLTt 4.5 or 5.0 Oral 27 mm Mallinckrodt Medical). Correct placement of the endotracheal tube was ascertained with inflation and auscultation of both lungs. The right jugular vein and the left carotid artery were aseptically surgically exposed and catheterized. The blood samples for culture were withdrawn from the arterial catheter at this time.

VF was induced only in Group B with the use of a 5F pacemaker catheter (Pacelt, St Jude Medical, 100 cm) that was inserted into the right ventricle with aseptic procedures, through the right jugular vein. VF was induced with a 9 V ordinary cadmium battery and was confirmed electrocardiographically. After induction of VF leading to CA, the animals were left untreated for 5 min according to the experimental protocol. Then resuscitation with precordial compression, mechanical ventilation and electrical defibrillation was applied as described elsewhere (Xanthos *et al.*, 2007).

The animals restoring spontaneous circulation were monitored for another 45 min while anesthesia was maintained. The animals that did not restore spontaneous circulation after resuscitation were not tissue sampled or included in the results.

Animals from both groups were euthanized by an intravenous solution of 2 g of sodium thiopental (Pentothal, Hospira) while still under propofol anaesthesia. Using aseptic technique, a 50 cm midline incision was performed parallel to the sternum from the 3rd intercostal space to the navel.

Lung samples were acquired by incision in the caudal portion of the right lung. Using new sterile forceps and scissors, a lung tissue specimen was harvested under sterile conditions. For the liver samples, an incision was made in the right medial hepatic lobe using new sterile instruments and the specimen was also harvested under sterile conditions.

Specimen collection

Blood samples: A quantity of 5 mL of whole blood was withdrawn from the arterial catheter and placed aseptically in a blood culture vial (BacT/ALERT i AST Culture Bottles, bioMerieux). A quantity of 0.1 mL of diluted blood was added into glass tubes with 0.9 mL of Muller-Broth medium and diluted 1:10 into sodium chloride four consecutive times. An aliquot of 0.1 mL of every dilution was plated onto McConkey agar, chocolate agar and blood agar. Numbers of viable colonies were counted after multiplying with the appropriate dilution factor. The lower limit of detection was 50 counting Colony Forming Units (CFU) mL⁻¹.

Lung and liver samples: Every tissue segment was weighed, homogenized and cultured. The lower limit of detection was 10 CFU g⁻¹. Identification of gram-negative isolates was done by lactose fermentation and according to the API20E and API20NE systems (bioMerieux). Identification of enterococci was done by a negative catalase test and according to bile and salt solubility. The number of viable cells in blood and tissues was expressed as its log₁₀ value.

Statistical analysis: Percent prevalence data were subjected to the Fisher's exact test. Bacterial concentrations (quantified by counting CFU) were represented as mean values±SEM of log₁₀ CFU mL⁻¹ (for cultures of blood) or CFU g⁻¹ (for cultures of lung and liver tissues) and were statistically compared by Mann-Whitney U test. Only samples whose bacterial growth was higher than zero were used in the statistical analysis in order to compare bacterial concentrations. A significance level of 5% was set for all analyses.

RESULTS AND DISCUSSION

In total, all cultures were sterile in 18 of the 46 animals (39.1%). In the positive cultures (28/46, 60.9%), the main bacterial species detected in the blood and the tissues of both groups were *Stenotrophomonas maltophilia* and *Bordetella bronchiseptica* (Table 1).

Concerning the detection of *B. bronchiseptica*, all blood samples were negative while 5 out of 46 liver samples were positive (10.8%) and 12 out of 46 lung samples were also positive (26%) as shown in Table 1.

Bacteria were isolated in blood from one animal of group A and 5 animals of group B (p = 0.1928) in liver samples from two animals of group A and 15 animals of group B (p = 0.0041) and in lung samples from 10 animals of group A and 17 animals of group B (p = 0.2341) as shown in Table 2.

Table 1: Absolute numbers of positive cultures in blood, liver and lung samples from total 46 animals

Culture	Blood	Liver	Lung
<i>Stenotrophomonas maltophilia</i>	6	12	13
<i>Bordetella bronchiseptica</i>	0	5	12
<i>Klebsiella pneumoniae</i>	0	0	1
<i>Escherichia coli</i>	0	0	2

Total number of animals = 46; Number of healthy animals (%) = 18 (39.1%)

Table 2: Absolute numbers of positive cultures from animals separated in 2 groups. Absolute numbers of positive cultures in blood, liver and lung samples from the animals separated into 2 groups according to the experimental protocol. Group A: minor surgery, group B: minor surgery plus cardiac arrest p<0.05

Organs	Group A (n = 24)	Group B (n = 22)	p-value
Blood	1	5	0.1928
Liver	2	15	0.0041
Lung	10	17	0.2341

Table 3: Specific bacteria isolated from groups A and B. Type of bacteria isolated from the blood, liver and lung samples from the 2 groups of animals. Group A: minor surgery, group B: minor surgery plus cardiac arrest. Absolute numbers of animals infected and their percentage per group are shown p<0.05

Type of bacteria	Group A (n = 24)	Group B (n = 22)	p-value
Blood number of animals (%)			
<i>Stenotrophomonas maltophilia</i>	1 (4.2)	5 (22.7)	0.1928
Liver number of animals (%)			
<i>Stenotrophomonas maltophilia</i>	0 (0.0)	12 (54.5)	<0.0001
<i>Bordetella bronchiseptica</i>	2 (8.3)	3 (13.6)	0.6680
Lung number of animals (%)			
<i>Stenotrophomonas maltophilia</i>	1 (4.2)	12 (54.5)	0.0043
<i>Bordetella bronchiseptica</i>	7 (29.2)	5 (22.7)	0.7556
<i>Klebsiella pneumoniae</i>	1 (4.2)	0 (0.0)	1.0000
<i>Escherichia coli</i>	1 (4.2)	1 (4.5)	1.0000

There was a significant difference between groups regarding the presence of *S. maltophilia*. It was isolated at a greater rate among animals of group B than among animals of group A. Specifically although, there was no significant difference regarding the blood cultures this was not the case for the liver and lung cultures. No liver sample of group A was positive for *S. maltophilia* while in group B there were 12 positive samples (p<0.0001). Additionally, there was only one positive sample in the lung samples of group A while *S. maltophilia* was detected in 12 samples of group B (p = 0.0043) (Table 3).

We note that the difference in the sums of Table 2 and 3, regarding the total positive lung samples of group B is due to the fact that there were two bacteria isolated (both *Escherichia coli* and *B. bronchiseptica*) in one animal from this group.

Concerning the total numbers of bacterial counts, there was no significant difference between the two studied groups regarding the blood, liver and lung tissue cultures of the positive animals as shown in Fig. 1.

The counts of bacteria isolated from liver samples (log₁₀, CFU g⁻¹) of positive pigs from group A (minor surgery) and group B (minor surgery plus CA) are shown

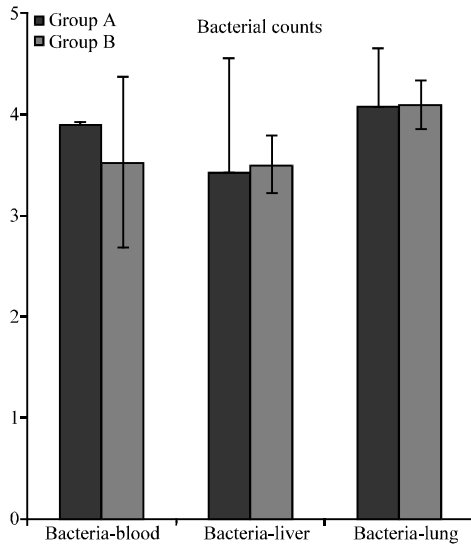


Fig. 1: Total counts of bacteria isolated from blood (\log_{10} CFU mL⁻¹), liver and lung (\log_{10} CFU g⁻¹) of positive pigs from group A (minor surgery) and group B (minor surgery plus cardiac arrest). Values are mean \pm SEM

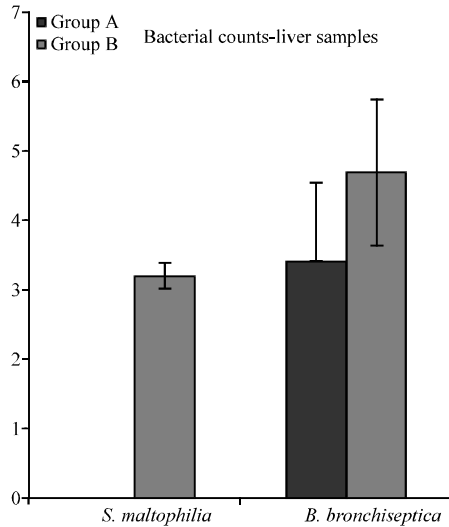


Fig. 2: Counts of bacteria isolated from liver samples (\log_{10} CFU g⁻¹) of positive pigs from group A (minor surgery) and group B (minor surgery plus cardiac arrest). Values are means \pm SEM. There were no *S. maltophilia* isolated from the liver of group A animals. (*Stenotrophomonas maltophilia*, *S. maltophilia*, *Bordetella bronchiseptica* and *B. bronchiseptica*)

in Fig. 2. There was no *S. maltophilia* isolated from the liver of group A animals. The counts of bacteria isolated from lung samples (\log_{10} CFU g⁻¹) of positive pigs from

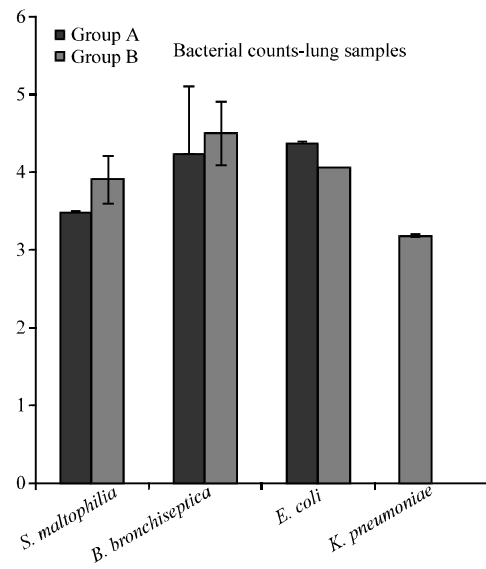


Fig. 3: Counts of bacteria isolated from lung samples (\log_{10} CFU g⁻¹) of positive pigs from group A (minor surgery) and group B (minor surgery plus cardiac arrest). Values are means \pm SEM. There was only 1 animal from group A and no animal from group B with *K. pneumoniae* isolated from the lung; (*Stenotrophomonas maltophilia*, *S. maltophilia*, *Bordetella bronchiseptica*, *B. bronchiseptica*, *Klebsiella pneumoniae*, *K. pneumoniae* and *Escherichia coli*)

group A (minor surgery) and group B (minor surgery plus CA) are shown in Fig. 3. The current study undertook to investigate the microbiological status of domestic swine, raised in meat-production farms which are used in research establishments in Greece.

Blood cultures as well as lung and liver tissue cultures were chosen as parameters to evaluate health status (Choi *et al.*, 2003; Zou *et al.*, 2010). The results demonstrated that the majority of the pigs used had negative blood cultures indicating the initial absence of active disease. That was not the same for lung and liver samples.

The results of the study concerning the detection of *Bordetella bronchiseptica* indicated that it was not isolated from any of the blood samples while 5 out of 46 liver samples (10.8%) and 12 out of 46 lung samples (26%), were detected positive (Table 1). According to a recent study (Zhao *et al.*, 2011), a large population of pigs was examined through a 6 years period in order to identify the occurrence of *B. bronchiseptica* among animals with respiratory disease. Based on the results, *B. bronchiseptica* was found to be the fourth most frequent

isolated pathogenic bacterium (after *Streptococcus suis*, *Haemophilus parasuis* and *Escherichia coli*) with an average isolation rate of 18.6%. This is a finding of great interest not only because the frequency of *B. bronchiseptica* in the lung in the study was much higher than in the previous one but mainly because in the case the animals were initially considered healthy.

Bordetella bronchiseptica is a gram-negative coccobacillus causing respiratory infection in many animal species (Goodnow, 1980) as pigs, dogs, rabbits, rats, mice (Bernis *et al.*, 2003) and rarely in humans, especially in immunocompromised patients (Patel *et al.*, 2011). Particularly in pigs, it is considered as one of the main etiological agents causing atrophic rhinitis and pneumonia (Brockmeier *et al.*, 2002a). It is usually detected from the respiratory system but can be found even in sites such as the brain and heart blood which may lead to the conclusion that it can invade deep organs leading to systemic infection (Zhao *et al.*, 2011). According to previous studies infection mainly occurs with or without symptoms in pigs after the age of 3 weeks, when maternal immunity decreases (De Jong and Borst, 1985). This is in accordance with the animals we examined as they were 3-4 months old. *B. bronchiseptica* may exist in herds causing subclinical infection. As a result, a co-infection with other organisms may lead to respiratory tract disease and further cause an animal to be vulnerable to opportunistic bacteria (Brockmeier *et al.*, 2000, 2002b; Dugal *et al.*, 1992). Although, the role of *B. bronchiseptica* in causing a respiratory disease is very important, it should not be underestimated that its existence, even subclinically may predispose the animals to colonization and infection with other pathogens. Accordingly, it is uncontested that pigs infected by *B. bronchiseptica* may lead an experimental study to invalid results, especially in cases where respiratory system physiology or microbiological data are involved. Concerning the finding of *E. coli* (1 positive lung sample, 2%) and *Klebsiella pneumoniae* (2 positive lung samples, 4.3%), it is considered to be of low interest because of the limited number of the total positive samples.

As it is shown in Table 1, the bacterium *Stenotrophomonas maltophilia* was detected in 6 blood samples, 12 liver samples and 13 lung samples. However, we unexpectedly observed a highly increased percentage of positive liver and lung cultures determined after euthanasia in group B which recovered from CA. These unexpected findings indicate the possibility that the reduction of tissue blood flow because of splanchnic hypoperfusion due to CA in this group followed by reperfusion after successful resuscitation, may induce functional disorders of the intestine and increase the

permeability of the mucosal barrier, resulting in endotoxin release and bacterial translocation. Bacterial Translocation (BT) is defined as the invasion of indigenous intestinal bacteria through the gut mucosa into normal sterile tissue causing disease (Berg and Garlington, 1979; Balzan *et al.*, 2007; Feng *et al.*, 2007). There are two major routes that bacterial compounds might gain access to systemic circulation: through the enteric venous system to the portal vein or following lymphatic enteric drainage (Deitch, 2002). No evidence is available to the knowledge if CA due to VF followed by successful resuscitation and systemic ischemia/reperfusion may be accompanied by translocation of bacteria through the gut barrier. There are several experimental studies showing that BT frequently occurs in acute inflammatory conditions like acute pancreatitis (Van Minnen *et al.*, 2007) liver cirrhosis (Natarajan *et al.*, 2006), trauma (Deitch and Bridges, 1987), thermal injury (Deitch and Berg, 1987; Fazal *et al.*, 2000) intestinal obstruction (Ikuta *et al.*, 2004), hemorrhagic shock (Sun *et al.*, 1997; Schlag *et al.*, 1999), stroke (Tascilar *et al.*, 2010) and obstructive jaundice (Deitch, 1990; Reynolds *et al.*, 1996).

Although, there is substantial data of the occurrence of BT both in animal models and in humans (Tsujiimoto *et al.*, 2009), the mechanism that connects mesenteric ischemia to BT and secondarily to systemic sepsis has not been totally clarified. It has been reported (Paradis *et al.*, 2007) that at the initial stages of ischemia, generation of oxygen radicals and other mediators of tissue injury might occur causing an increase of tissue permeability followed by leakage of endotoxins into the peritoneal cavity. That stimulates BT into Mesenteric Lymph Nodes (MLN) and increases the levels of the circulating endotoxins at the later stages of the ischemic damage (Kibbler, 1989; Ledingham and Ramsay, 1989; Wells *et al.*, 1989), bacteria probably infect the liver via the portal vein. The infection of the MLN, the liver and potentially the peritoneal cavity may lead to systemic sepsis. Other investigations have suggested that the main route through which the bacterial compounds might gain access to the systemic circulation is the lymphatic enteric drainage (Deitch, 2002).

This is the first experimental study providing indication that BT may contribute to a systemic infection early after successful resuscitation from CA. The accidental finding may be explained as a consequence of the ischemia/reperfusion injury due to CA and resuscitation in the splanchnic vascular system which usually increases mucosal permeability and activates inflammatory mediators (Jakob, 2003), further leading to the pathogenesis of systemic infection and inflammation

and to multiple organ dysfunction. Based on the reported findings, we note two observations of potential clinical importance. The former is that *Stenotrophomonas maltophilia* is the main species isolated from blood and tissues of the studied animals. This pathogen in animals has been isolated mainly from porcine semen and rarely from the liver (Althouse and Lu, 2005; Petridou *et al.*, 2010) while in humans it affects immunocompromised and debilitated hosts causing lethal episodes of bacteremia and sepsis (Senol, 2004). The second is that from the current study results, future investigation can be suggested, regarding empirically prescribed antimicrobials for microorganisms of the enteric flora that could potentially be administered in human practice to patients as early as possible after successful resuscitation from CA in view of the observed increase of bacteria in the lung and liver of CA animals.

It is also acknowledged that additional sampling sites (e.g., portal vein, MLN) would have provided more information but this was not included in the initial aim and study design. The present results with these culture differences between the two groups were not expected; this consists a noteworthy significant observation. Additionally, a larger sample number would have been desirable. However, in order not to sacrifice additional pigs for this study and to apply the principle of reduction only those animals that were being used for other licensed protocols were examined (Russell and Burch, 1959).

CONCLUSION

The results of the present study demonstrate that the health status of the pigs that are procured from existing conventional breeding establishments raised for meat production was of an undesirable level for experimental purposes with approximately 60% of them infected. Conventional farm animal breeding facilities are therefore to be expected to produce less than optimal animals, regarding their health status for research purposes. Contrarily, the use of purpose-bred specific pathogen free pigs would ensure valid research results. Additionally, the observation regarding the cultures from the two different protocols used can be further examined in future studies. It could be proposed that the use of antibiotics after a resuscitated CA at least those targeting the gut flora could be of great clinical interest in order to prevent possible BT but this hypothesis remains to be confirmed with future studies.

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