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Veterinary public health aspects of hazards occurring along the pig production line

Coordinatore
Ch. mo Prof.
Maria Luisa Cortesi

Tutor
Ch. mo Prof.
Adriano M. L. Santoro

Co-Tutor
Ch. mo Prof.
Roger Stephan

Candidata
Dott.ssa
Eleonora Sarno

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a Maria, Rosa, Teresa
e Violetta

Long words name little things. All big things have a little name, such as life and death, peace and war, or dawn, day, night, love, home. Learn to use little words in a big way - It is hard to do. But they say what you mean. When you don't know what you mean, use big words: They often fool little people.

-SSC Booknews, July 1981

Table of contents

Veterinary public health aspects of hazards occurring along the pig production line: Aims.....4
References.....12

Research study no. 1: Occurrence of *Erysipelothrix* spp., *Salmonella* spp., and *Listeria* spp. in tonsils of healthy Swiss pigs at slaughter.....15
Materials and Methods.....30
Results and Discussion.....41
References.....46

Research study no. 2: Seroprevalence of anti-HEV and anti-*Salmonella* antibodies in pigs at slaughter in Switzerland.....53
Materials and Methods.....63
Results and Discussion.....66
Conclusion.....69
References.....70

Research study no. 3: High prevalence of antibodies against hepatitis E virus in sows serum samples collected between 1988 and 1991 in Switzerland.....76
Samples and serological analysis.....78
Results and Discussion.....80
References.....82

Research study no. 4: Detection of anti-HEV antobodies in saliva samples using a modified commercial ELISA: preliminary investigation.....84
Materials and Methods.....86
Results and Discussion.....87
References.....88

Research study no. 5: Lack of evidence so far for carbapenemase-producing *Enterobacteriaceae* in food-producing animals in Switzerland.....89
Materials and Methods.....91
Results and Discussion.....94
References.....96

Veterinary public health aspects of hazards occurring along the pig production line: Aims.

Key words: Biohazards, Public Health, Pork Meat

Pork has long been the most consumed meat globally. It is estimated that the annual average consumption of meat in European Union (EU) is approximately 43 Kg per head (2005)[1] and the annual average amount of slaughtered pigs (1000 tonnes) is about 1882.41 (2008)[2]. In recent years swine have been involved in the transmission of several microbial agents to humans acting as primary or intermediary reservoir of infection. Healthy live animals harboring pathogenic microorganisms have the potential to transmit them to human caretakers via direct contact on farm, to butchers via direct contact with carcasses, and to the general community via contaminated foodstuffs.

The management of biological hazards (bacteria and viruses for instance) occurring along the pig production line and transmitted to humans by contact with pigs or by the consumption of pork is therefore of major public health and economic significance.

In this context the purpose of this thesis was to collect information on the prevalence of five biological hazards, which are responsible for zoonotic and food-borne diseases in humans, among the Swiss healthy pig population.

Furthermore prevalence was investigated as (i) prevalence linked with lymphoid tissues (prevalence obtained from tonsil samples collected at slaughter), (ii) serological prevalence (antibody detection from meat juice and serum samples collected at slaughter and from oral fluid (saliva) samples collected on-farm

level), (iii) prevalence linked with fecal shedding (prevalence obtained from fecal (rectal content) samples collected at slaughter).

Among biological hazards of concern to pork safety, *Erysipelothrix* spp. (*Erysipelothrix* (*E*) *rhusiopathiae*), *Salmonella* spp., *Listeria* spp. (*Listeria* (*L*) *monocytogenes*), hepatitis E virus (HEV) and carbapenemase-producing *Enterobacteriaceae* (CPE) were investigated in the following research studies. The point of sampling in the pig production line, the type of sample for each biological hazard and its estimated prevalence is given in Table 1.

Table 1. Prevalence of five biological hazards from different samples collected at-slaughter and on-farm level among the Swiss healthy pig population.

	Pig Production Line		
Biological Hazard	Sampling Level	Sample type	Prevalence (%)
Erysipelothrix spp.	Slaughter line	Tonsils	Nd [a]
Salmonella spp.	Slaughter line	Tonsils/Meat juice	0.8 [b]/6 [c]
Listeria spp.	Slaughter line	Tonsils	5.6 [d]
hepatitis E Virus	Slaughter line/Farm level	Meat juice (Pig)-Serum (Sow)/Saliva	49 [c] - 97.5 [c]/65 [c]
CPE	Slaughter line	Feces	Nd [a]

[a] Nd, not detected; [b] *S.* Bredeney and *S.* Kedougou isolates; [c] Results obtained using a commercial ELISA test kit; [d] Nine *L. monocytogenes* isolates belonging to serotypes 1/2a, 1/2b, 4b, four *L. ivanovii* isolates, and one *L. innocua* isolate.

Erysipelothrix spp. are pathogens or commensals in a wide variety of animals. The disease of greatest economic importance is swine erysipelas, which is caused by *E. rhusiopathiae.* In addition, *E. rhusiopathiae* is an occupational hazard causing zoonotic disease occasionally in those people, whose jobs (farmers, veterinarians, abattoir employees) are closely related with pigs, their products or wastes, and soil. Human infection occurs via skin lesions mainly by contact with infected carriers. The domestic pig is an important reservoir shedding bacteria in

feces, urine, saliva, and nasal secretions, creating continue sources of infection (Wood, 1992).

Since it has been estimated that 30 to 50 % of healthy pigs harbor the organism in their tonsils and lymphoid tissues (Wood, 1999), tonsil samples (*tosilla veli palatini*) were collected from pig carcasses at slaughter as described in research study no. 1. Samples were then analyzed by culture methods in order to investigate the epidemiological status of the Swiss swine herds.

Surprisingly, tonsil samples tested all negative (Table 1). Our findings do not concur with the previous assumption and moreover *E. rhusiopathiae* seems to occur only in low number in the Swiss healthy pig population.

Salmonella spp. are worldwide a major cause of acute bacterial gastroenteritis (EFSA/ECDC, 2011). Pigs are usually asymptomatic carriers of different *Salmonella* serovars and a considerable part of human cases is attributed to the consumption of pork (Berends *et al*., 1998; EFSA, 2010). Isolation of any *Salmonella* serovar in pork is therefore regarded as public health hazard (Boyen *et al*., 2008; EFSA, 2006). Research study no. 1 and 2 outlined *Salmonella* prevalence in the Swiss healthy pig population. For this purpose two different types of samples were collected and then analyzed.

Firstly, pig palatine tonsils (*tosilla veli palatini*) were collected during slaughter and subsequently analyzed in accordance with ISO 6579:09.2006 using a two-step enrichment procedure. Two *Salmonella* isolates were found and serotyped as *S.* Bredeney and *S.* Kedougou (0.8%). Although this serotypes have been identified in the EU survey on *Salmonella* in holdings with breeding pigs, they represent in each case less than 5% of the *Salmonella* positive holdings (EFSA, 2009) and are

only rarely reported as cause of human cases or foodborne outbreaks (EFSA/ECDC, 2011).

Lastly, meat juice samples from diaphragm muscles were collected at slaughter in order to investigate the prevalence of antibodies against *Salmonella* spp. using a commercial ELISA kit. Our findings showed a low prevalence rate of 6% (Table 1) reflecting the constant low prevalence of *Salmonella* spp. in domestic pigs in Switzerland as has also been described previously (Jost *et al*., 2011).

L. monocytogenes is a food borne pathogen causing human illness in a well-defined risk population as pregnant women, neonates, and immunosuppressed. Human infection primarily results from eating contaminated food products including pork. With regard to meat production, *Listeria* spp. have been recovered from slaughterhouse environments, carcasses, tonsils (Autio *et al*., 2000). It has been suggested that *L. monocytogenes* originating from tonsils, even though rejected after inspection, and tongue of healthy pigs may contaminate the slaughtering equipment. Equipment can in turn spread the pathogens to carcasses (Autio *et al*., 2000) and in this way carcasses can introduce the tonsil-originating pathogens into food processing plants.

Pig palatine tonsils (*tosilla veli palatini*) were collected (research study no.1) at slaughter and analyzed using a two-step enrichment procedure (ISO 11290-1:2004). Of the 14 *Listeria* isolates (5.6%), nine, four, and one were identified as *L. monocytogenes*, *L. ivanovii*, and *L. innocua*, respectively. Of the nine *L. monocytogenes* isolates, three belonged to serotype 1/2a, two to serotype 1/2b, and four to serotype 4b (Table 1).

The majority of human cases are associated with *L. monocytogenes* of serotypes 1/2a, 1/2b, and 4b and the proportion associated with isolates of serotype 1/2a has

increased in recent years (Lukinmaa *et al*., 2003; Parihar *et al*., 2008; Allerberger and Wagner, 2010). Our findings suggest that tonsils might be a more accurate predictor of *L. monocytogenes* carrier status in pigs than other samples as feces.

HEV is the causative agent of an acute liver disease in developing countries with poor hygienic standards. In developed countries the infection has been linked to history of travel in endemic regions. In recent years several autochthonous cases with no travel history have been described. Ongoing discoveries on hepatitis E virus raise questions about its zoonotic and meat borne potential (EFSA, 2011a). Several investigations showed that farmed pigs are highly infected with and shed mostly genotype 3 in Europe. Despite the large widespread distribution of HEV and the possible role of domestic pigs, wild boars and other animals as reservoirs (Meng, 2010; Pavio and Mansuy, 2010; Meng, 2011), the virus has become of interest in view of public health including food borne transmission, although rare, only recently.

For the first time HEV prevalence was investigated among the Swiss domestic pig population. Presence of anti-HEV immunoglobulin G (Ig G) was studied using a commercial ELISA kit. For this purpose three kinds of samples were collected during research studies no. 2, 3, and 4.

Meat juice samples were collected during slaughter from diaphragm muscles of six-month old pigs. Analyses gave positive results for 49% of all tested animals suggesting the high prevalence and the need to identify the origin of the infection of pigs to be able to minimize the potential zoonotic transmission to humans.

Serum samples collected between 1988 and 1991 from sows (pigs older than six months) were investigated in a retrospective serological study in order to get a clearer idea of the circulation of the virus throughout the National territory.

Additionally serum samples from current (year 2012) slaughtered sows were evaluated.

Anti-HEV Ig G were found in 97.5 % of all stored serum samples (Table 1) and in 67.5% of current serum samples from healthy sows at slaughter.

Although our data suggest that HEV is highly prevalent among sows, hidden biases related to the aged samples cannot be discarded. Our results also indicate that HEV circulates in Swiss pig herds since at least 1988.

Finally, oral fluid samples from breeding pigs were collected on-farm level, and subsequently analyzed by using a modified ELISA method adapted to the typology of the matrix. Detecting Ig G anti-HEV from oral fluid pen-based samples provided a prevalence rate of 65%. Prevalence found in this preliminary study is in agreement with our previous data set and suggests the chance of using oral fluid samples as potential approach to get information on the herd immunity and history of prior infection with the virus.

Carbapenems are a class of β-lactam antibiotics (penicillin derivatives, cephalosporins, monobactams, and carbapenems) with a broad spectrum of antibacterial activity. Recently, alarm has been raised over the spread of drug resistance to carbapenem antibiotics among *Enterobacteriaceae* (Nordmann *et al*., 2012). There are currently no new antibiotics in the pipeline to combat bacteria resistant to carbapenems, and worldwide spread of the resistance gene is considered a potential nightmare scenario. Recent reports prove that the intestinal flora of pigs (Fisher *et al*., 2012) and cattle (Poirel *et al*., 2012) constitute a possible reservoir of carbapenemase producers. To screen for the occurrence of carbapenemase-producing *Enterobacteriaceae* in food-producing animals at slaughter in Switzerland, fecal samples were collected from fattening pigs and

subsequently analyzed by culture method. Antimicrobial susceptibility and PCR searching for $bla_{carbapenemase}$ genes were evaluated too (research study no. 5). No evidence so far for the occurrence of carbapenemase-producing *Enterobacteriaceae* needs to be postulated.

To sum up, *Erysipelothrix* spp. and carbapenemase-producing *Enterobacteriaceae* were not detected, whereas *Salmonella* prevalence has not changed in recent years and the two detected isolates seem not to be linked with human infection. *L. monocytogenes* isolates, although the low prevalence, belong to the serotypes responsible for the majority of human cases. The role of hepatitis E virus and its high prevalence needs further considerations in view of its food-borne and zoonotic potential.

The present work can be put in the context of the recent interest in modernization of the meat inspection system across the EU, where there has been a considerable concern about the sources and the spread of zoonotic foodborne pathogens along the food production line.

A new "risk-based" meat inspection approach, so-called "from farm to fork", has been issued in the European Union with basic Regulation (EC) No. 178/2002 (Anonymous, 2002) and the "Hygiene Package" Regulations (EC) No. 852/2004, 853/2004, 854/2004 and 882/2004 (Anonymous, 2004a,b,c,d).

In other words, as already stated in the last EFSA scientific opinion on the public health hazards to be covered by inspection of meat, an effective control of the main biological hazards is possible only through a longitudinally quality control system along the entire production line, combining a range of preventive measures and controls applied both on-farm and at-abattoir in an integrated way (EFSA,

2011b). The new resulting food safety approach focuses on the food chain, from primary production to consumption, with process optimization instead of end product inspection.

In contrast to the so far idea of protecting the consumer by just condemning carcasses and organs during the official *post-mortem* inspection at slaughter and preventing products not-fit for consumption from entering the food chain, the new goal is to assure production processes at farm level that results in healthy animals for slaughter, which in turn results in carcasses fit for consumption.

Healthy live food animals, that are symptomless carriers of zoonotic agents, can harbor such microorganisms in their digestive contents (i.e. rectal tract) and in the digestive tissues (i.e. digestive glands, tonsils) and moreover can shed them in feces creating sources of infection both on-farm and at-slaughter (Fosse *et al*., 2009). Consequently their presence on meat, which cannot be detected by the solely macroscopic examination during the *post-mortem* inspection, stems from contamination events that can occur anytime during harvest and processing until meat is served to the end consumer.

Therefore intervention such as identification of pathogen sources, farm management practices that lead to animal exposure, application of good hygiene practices both at abattoir and food-processing stages are required.

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Research study no. 1: Occurrence of *Erysipelothrix* spp., *Salmonella* spp., and *Listeria* spp. in tonsils of healthy Swiss pigs at slaughter.

Key words: Zoonotic and foodborne pathogens, Tonsils, Healthy slaughtered pigs

Zoonotic and foodborne diseases are widespread, thus affecting lives, business, and economies worldwide. They have a major health impact in industrialized countries and remain responsible for high levels of morbidity and mortality in the general population but particularly for at-risk-groups such as infants, young children, pregnant women, elderly, or immunocompromized people. With regard to meat production, healthy food animals including pigs were recognized in recent years as carriers of pathogens causing human illness. Such pathogens harbored by healthy animals may enter the food chain during slaughter (Nørrung and Buncic, 2008; Fosse *et al*., 2009). Thereby, it must be considered that pork is today the most frequently consumed meat in Europe. To estimate the risk involved, baseline data on the animals' probability of carrying such pathogens are required. For this purpose, *Erysipelothrix* spp. (*E. rhusiopathiae*), *Salmonella* spp., and *Listeria* spp. (*L. monocytogenes*) were selected as target organisms in the present study.

Erysipelothrix spp. are zoonotic pathogens or commensals in a wide variety of wild and domestic animals, birds and fish (Conklin and Steele, 1979). Until 1987, the genus *Erysipelothrix* was thought to be comprised of only one species, *Erysipelothrix rhusiopathiae* (Jones 1986). However, on the basis of DNA–DNA hybridization (Takahashi *et al*. 1987b, 1992), multilocus enzyme electrophoresis (Chooromoney *et al*., 1994) and restriction fragment length polymorphism (Ahrne *et al*., 1995), the genus *Erysipelothrix* has been divided into four species, *E.*

rhusiopathiae (comprising serotypes 1a, 1b, 2, 4, 5, 6, 8, 9, 11, 12, 15, 16, 17, 19 and 21 and type N), *Erysipelothrix tonsillarum* (comprising serotypes 3, 7, 10, 14, 20, 22 and 25), *Erysipelothrix* sp. strain 1 (comprising serotype 13) and *Erysipelothrix* sp. strain 2 (comprising serotype 18). Among the four species, *E. rhusiopathiae* is commonly associated with erysipelas in swine (Takahashi *et al*., 1987a,b; Takeshi *et al*., 1999). *Erysipelothrix rhusiopathiae* is facultative anaerobe, Gram-positive rod, catalase negative, non-motile and non-spore forming, that is generally straight, though sometimes curved. The bacteria can readily become decolorized with Gram stain, so that they appear as Gram-negative.

The disease of greatest prevalence and economic importance is swine erysipelas, which is caused by *E. rhusiopathiae* and occurs in three forms: acute, sub-acute and chronic (Grieco and Sheldon, 1970; Conklin and Steele, 1979; Wood, 1992). Acute erysipelas in swine is characterized by sudden death or general signs of septicemia. Virulent organisms cause the infection and bacteremia usually develops within 24 h of exposure, quickly resulting in clinical signs of generalized infection and septicemia. Presence of diffuse areas of erythema and sometimes vesicles, petechiae and necrosis are also characteristic features. Sub-acute erysipelas shows signs that are less severe than the acute form. The animals do not appear as sick. Cutaneous lesions, urticarial, or diamond-skin lesions appear as early as the second or third day after exposure to infection. These lesions may in a few days gradually lose their swelling and coloration, and disappear with no subsequent effect other than a superficial desquamation. In other instances, the lesions overlap and cover large areas of the skin. The intensity of these skin lesions has a direct relation to the prognosis. Light pink to light purplish red

lesions will disappear within several days whereas the deep-purplish red lesions can precede either death or necrosis of the skin.

The chronic form of infection may follow acute or sub-acute disease and is characterized most commonly by signs of local arthritis or proliferative pathological changes in the heart (endocarditis). Chronic arthritis results in joints showing various degrees of stiffness and enlargement. This is the most important clinical manifestation of swine erysipelas from an economic standpoint.

In addition to animal disease, *E. rhusiopathiae* causes various forms of human disease that can originate from an animal or environmental source. Clinical manifestations seen in humans closely resemble those seen in swine. There are three clinical categories of human disease: a localized cutaneous form (erysipeloid), a generalized cutaneous form, and a septicemic form often associated with endocarditis (Brooke and Riley, 1999). Erysipeloid is the most common form of human infection. It is an acute localized cutaneous infection usually occurring on the hand or fingers, described as a local cellulitis.

The lesion consists of a well-defined, slightly elevated, purplish zone, the peripheral edge of which spreads as the center fades. The pain is often severe and may be described as a burning, throbbing, or itching sensation. Systemic symptoms can occur in some cases: fever, joint aches, lymphadenitis and lymphadenopathy. Arthritis of an adjacent joint may be seen. The absence of suppuration, lack of pitting edema, and disproportionate pain helps to distinguish erysipeloid from staphylococcal or streptococcal infection. The disease is self-limiting and usually resolves in 3–4 weeks without therapy (Reboli and Farrar, 1992). The generalized cutaneous form of the disease caused by *E. rhusiopathiae* involves lesions that progress from the initial site to other locations on the body or

appear at remote areas. The lesions are similar to those of the localized form. Systemic symptoms such as fever and joint pains are more frequent than in the localized form. The clinical course is more protracted and recurrences are common (Klauder, 1938).

Septicemia is a more serious manifestation of *E. rhusiopathiae* infection, almost always linked to endocarditis. It rarely develops from localized infection. Fifty cases with systemic infection in 15 years were reported with an extremely high incidence (90%) of endocarditis (Gorby and Peacock, 1988).

Human infections are often occupationally related (farmers, veterinarians, abattoir employees) and occur via skin lesions. It occurs mostly in those people whose jobs are closely related with contaminated animals, their products or wastes, or soil. The people with the highest risk of exposure include butchers, abattoir workers, veterinarians, farmers, fishermen, fish-handlers and housewives (Reboli and Farrar, 1989). The infection has been also associated with a wide variety of occupations, including meat cutters, meat-processing workers, poultry-processing workers, meat inspectors, rendering-plant workers, knackers, animal caretakers, bone button makers, game handlers, furriers, leather workers, soap makers, fertilizer workers, sewer workers, bacteriology laboratory workers and stockyard workers (Wood, 1975). The common names for human infection reflect this occupational mode of acquisition. These include whale finger, seal finger, speck finger, blubber finger, fish poisoning, fish handler's disease, and pork finger. Infection is initiated either by an injury to the skin with infective material or when a previous injury is contaminated. Most cases in humans and other animals may occur via scratches or puncture wounds of the skin (Wood, 1975).

The domestic pig is an important reservoir of *E. rhusiopathiae* and it has been estimated that 30 to 50 % of healthy pigs harbor the organism in their tonsils and lymphoid tissues (Wood, 1999). Carriers can shed *Erysipelothrix* spp. in feces, urine, saliva, and nasal secretions, creating sources of infection. So soil, bedding, food and water can be contaminated by infected pigs, leading to the indirect transmission of the organism (Wood, 1992). Over 30 species of wild birds and at least 50 species of wild mammals (Shuman, 1971; Wood and Shuman, 1981) are known to harbor *E. rhusiopathiae*, providing an extensive reservoir. The organism can survive for long periods in marine environments. It survives and grows on the exterior mucoid slime of fish without causing disease in the host (Wood, 1975). The slime on fish appears to be an important source of infection for man. The organism has been isolated from the environment but this may be secondary in importance to animal reservoirs as a source of *E. rhusiopathiae.*

Although *E. rhusiopathiae* is killed by moist heat at 55 °C for 15 min, it is resistant to many food preservation methods, such as salting, pickling and smoking (Conklin and Steele, 1979). It was long believed that the organism could live in soil indefinitely. However, some studies have not supported this and found that the organism survived for a maximum of only 35 days in soil under various conditions of temperature, pH, moisture content, and organic content (Wood, 1973).

Salmonella spp. are zoonotic pathogens and one of the major causes of food-borne illnesses in humans. In 2009, 10’9884 cases of salmonellosis in humans were reported in the EU (EFSA, 2011).

Salmonella spp. belong to the family of *Enterobacteriacaee* and morphologically are short, ovoid Gram-negative rod-shaped bacteria. Their motility is due to

peritrichous flagella. *Salmonella* nomenclature is complex, and often scientists use different system to refer to and to communicate about this genus. According to the Kauffmann-White scheme, the genus *Salmonella* contains two species, each of which contains multiple serotypes (Table 2).

Table 2. *Salmonella* species, subspecies, serotypes, and their usual habitats, Kauffmann-White scheme [a]

Salmonella species and subspecies	No. of serotypes within subspecies	Usual habitat
S. *enterica* subsp. *enterica* (I)	1,454	Warm-blooded animals
S. *enterica* subsp. *salamae* (II)	489	Cold-blooded animals and the environment[b]
S. *enterica* subsp. *arizonae* (IIIa)	94	Cold-blooded animals and the environment
S. *enterica* subsp. *diarizonae* (III[b])	324	Cold-blooded animals and the environment
S. *enterica* subsp. *houtenae* (IV)	70	Cold-blooded animals and the environment
S. *enterica* subsp. *indica* (VI)	12	Cold-blooded animals and the environment
S. *bongori* (V)	20	Cold-blooded animals and the environment
Total	2,463	

[a] The Kauffmann-White scheme has been described elsewhere (Popoff *et al*., 1997, Popoff and Le Minor,1997).
[b] Isolates of all species and subspecies have occurred in humans.

The two species are *S. enterica*, the type species, and *S. bongori*, which was formerly subspecies V (Popoff and Le Minor, 1997; Reeves *et al*., 1989). *S. enterica* is divided into six subspecies (Brenner and McWhorter-Murlin, 1998; Popoff and Le Minor, 1997), which are referred to by a Roman numeral and a name (I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *indica*). *S. enterica* subspecies are differentiated biochemically (Brenner and McWhorter-Murlin, 1998; Popoff and Le Minor, 1997) and by genomic relatedness (Crosa *et al*., 1973; Popoff and Le Minor, 1997; Reeves *et al*., 1989). Centers for Disease Control and Prevention

(CDC) uses names for serotypes in subspecies I (for example, serotypes Enteritidis, Typhimurium, Typhi, and Choleraesuis) and uses antigenic formulas for unnamed serotypes described after 1966 in subspecies II, IV, and VI and in *S. bongori*. The name usually refers to the geographic location where the serotype was first isolated. For named serotypes, to emphasize that they are not separate species, the serotype name is not italicized and the first letter is capitalized (Table 3).

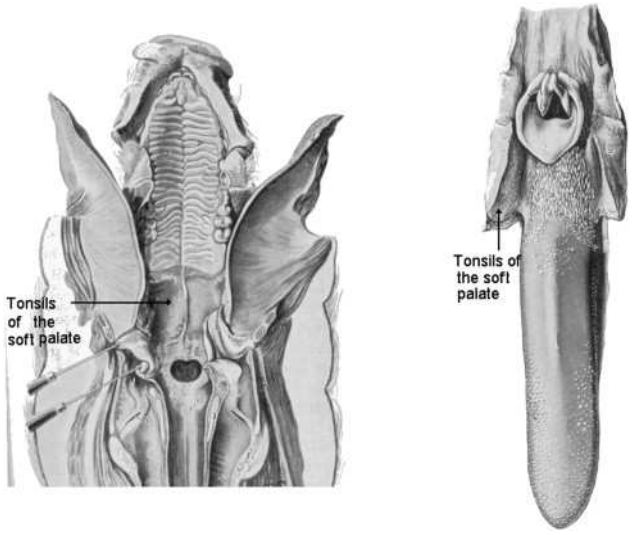
Table 3. *Salmonella* nomenclature in use at Centers for Disease Control and Prevention (CDC), 2000[a]

Taxonomic position	Nomenclature
Genus (italics)	*Salmonella*
Species (italics)	*enterica*, which includes subspecies I, II, IIIa, IIIb, IV, and VI
	bongori (formerly subspecies V)
Serotype (capitalized, not italicized)[b]	The first time a serotype is mentioned in the text; the name should be preceded by the word "serotype" or "ser."
	Serotypes are named in subspecies I and designated by antigenic formulae in subspecies II to IV, and VI and *S. bongori*
	Members of subspecies II, IV, and VI and *S. bongori* retain their names if named before 1966

[a] In 1984 Farmer *et al*. updated the reporting system used at CDC for Salmonella. The major changes that CDC made and that result in a difference from the 1984 reporting system are (i) capitalization of the serotype name, (ii) inclusion of subspecies VI and *S*. bongori, and (iii) adoption of the type species name *S*. enterica.
[b]Examples of serotype designations are *Salmonella* serotype (ser.) Typhimurium, *Salmonella* II 50:b:z6, *Salmonella* IIIb 60:k:z, and *Salmonella* ser. Marina (IV 48:g,z51:2).

Serotype names designated by antigenic formulae include the following: (i) subspecies designation (subspecies I through VI), (ii) O (somatic) antigens followed by a colon, (iii) H (flagellar) antigens (phase 1) followed by a colon, and (iv) H antigens (phase 2, if present) (for example, *Salmonella* serotype IV 45:g,z51:2). For formulae of serotypes in *S. bongori*, V is still used for uniformity (for example, S. V 61:z35:2). The majority (59%) of the 2,463 Salmonella serotypes belong to *S. enterica* subsp. I (*S. enterica* subsp. *enterica*) (Popoff and Le Minor, 1997). Within *S. enterica* subsp. I, the most common O-antigen

Figure 1. Oral cavity and tongue of a pig presenting the tonsils of the soft palate. Adapted from Nickel *et al*., 1979.



serogroups are A, B, C1, C2, D and E. Strains in these serogroups cause approximately 99% of Salmonella infections in humans and warm-blooded animals (Popoff and Le Minor, 1997). Serotypes in *S. enterica* subspecies. II (S. enterica subsp. salamae), IIIa (S. enterica subsp. arizonae), IIIb (*S. enterica* subsp. *diarizonae*), IV (*S. enterica* subsp. *houtenae*), IV (*S. enterica* subsp. *indica*), and *S. bongori* are usually isolated from cold-blooded animals and the environment but rarely from humans (Farmer *et al*., 1984).

Pigs can be infected by several *Salmonella* serotypes and the occurrence of these serotypes is also geographically determined (Fedorka-Cray *et al*., 2000; Loynachan *et al*., 2004). The European Food Safety Authority (EFSA) considers all isolated serotypes from pigs as important hazard for public health (EFSA, 2006). Several studies in literature reported a worldwide distribution of such pathogen among the pig population insomuch as the EU-wide baseline survey (EFSA, 2008) reported that *Salmonella* prevalence in pig breeding holdings and in pig production holdings was 28.7 % and 33.3 % and that *S*. Derby was the most frequently isolated serovar followed by *S*. Thyphimurium detected respectively in

29.6 % - 25.4 % of *Salmonella*-positive breeding holdings and in 28.5 % - 20.1 % in *Salmonella*-positive production holdings.

Pig infection is thought to occur mainly via the fecal-oral route and pathogenesis is characterized by three phases: (1) colonization of intestines, (2) invasion of enterocytes, and (3) bacterial dissemination to lymph nodes and organs.

Briefly, porcine epithelial beta-defensin 1 is expressed in the dorsal tongue at antimicrobial concentrations and may contribute to the antimicrobial barrier properties of the dorsal tongue and oral epithelium (Shi *et al*., 1999). *Salmonellae* that overcome this barrier may colonize the tonsils.

The palatine tonsils (Figure 1) are often heavily infected in pigs and should, therefore, not be underestimated as a source of *Salmonella* contamination during slaughter (Wood *et al*., 1989; Kühnel and Blaha, 2004). During ingestion, *Salmonella* spp. enter the tonsils in the soft palate and persist within the tonsillar crypts (Fedorka-Cray *et al*., 1995; Horter *et al*., 2003). No detailed information has been gathered on how *Salmonella* spp. interact with and persist in the porcine tonsillar tissue, although some observations mention persistence of *Salmonella* spp. on the superficial epithelium of the tonsillar crypts (Horter *et al*., 2003) resulting in asymptomatic carrier animals not detectable by macroscopic examination of carcasses during meat inspection. As long as *Salmonella*-positive animals enter the slaughter line, there will be the possibility of transmission to the consumers, even if the process is carried out according to best hygiene conditions. Nowadays, in the European Community Member States, tonsils must be compulsory removed (Regulation EC No. 854/2004) during the pork slaughter procedure and they cannot be present in the final products. This means that the main risk that tonsils may assume as a source of *Salmonella* to other edible tissues arises indirectly through the operators when they remove tonsils and cut other

tissues without knife sterilization. Moreover the complete extraction of the tonsillar tissues from the carcass is frequently impracticable due to the anatomical collocation contributing in this way to a source of contamination.

The way of colonization of the tonsils may therefore be much different than the mechanism of colonization of the intestine.

Following ingestion *Salmonella* spp. must survive the low pH of the stomach. It has been shown that *Salmonellae* can adapt to and survive in acidic environments up to pH 3 by producing acid shock proteins (Audia *et al*., 2001; Smith, 2003; Berk *et al*., 2005). Bacteria that survive passage through the stomach travel to the small intestine where they encounter other antibacterial factors including bile salts, lysozyme and defensins. Even though *S*. Typhimurium can be highly resistant against the direct antibacterial effects of bile salts (van Velkinburgh and Gunn, 1999), these salts repress the invasion of *Salmonella* in epithelial cells, possibly by decreasing virulence gene expression (Prouty and Gunn, 2000). Since high concentrations of bile salts are present in the upper part of the small intestine, this might explain why *Salmonella* preferentially colonizes the ileum, caecum and colon.

In the distal parts of the intestine, adherence to the intestinal mucosa is generally accepted as the first step in the pathogenesis of *Salmonella* infections in pigs. Following adhesion, *Salmonella* spp. invade the intestinal epithelium. It is generally accepted that *Salmonella* can spread throughout an organism using the blood stream or the lymphatic fluids and infect internal organs, although this has not yet been studied in swine. The colonization of the mesenteric lymph nodes, spleen and liver can result in prominent systemic and local immune responses (Dlabac *et al*., 1997). Macrophages are the cells of interest for host-restricted or–adapted *Salmonella* serotypes to disseminate to internal organs. The bacteria

replicate rapidly intracellularly and cause the systemic phase of the infection, while interfering with the antibacterial mechanisms of the macrophages and inducing cell death (Waterman and Holden, 2003; Hueffer and Galan, 2004).

Human salmonellosis is usually characterized by the acute onset of fever, abdominal pain, nausea, and sometimes vomiting, after an incubation period of 12-36 hours. Symptoms are often mild and most infections are self-limiting, lasting a few days. However, in some patients, the infection may be more serious and the associated dehydration can be life threatening. In these cases, as well as when *Salmonella* causes bloodstream infection, effective antimicrobials are essential for treatment. Salmonellosis has also been associated with long-term and sometimes chronic sequelae e.g. reactive arthritis.

In view of foodborne pathogens, *Salmonella* spp. are worldwide a major cause of acute bacterial gastroenteritis and has long been recognized as an important pathogen of economic significance in humans. In the European Union (EU), a total of 108'614 confirmed human cases of salmonellosis (23.7/100'000) have been reported in 2009 (EFSA/ECDC, 2011). A considerable part of the human cases are attributed to the consumption of pork (Berends *et al*., 1998; EFSA, 2010).

Contamination often occurs when organisms are introduced in food preparation areas and are allowed to multiply in food, e.g. due to inadequate cooking or cross contamination of ready-to eat (RTE) food.

L. monocytogenes has significant public health and economic impacts as a foodborne pathogen. Human infections primarily result from eating contaminated

food and may lead to serious and potentially life-threatening listeriosis (Doganay, 2003). Because of its high case fatality rate, listeriosis ranks among the most frequent causes of death due to foodborne illness. In the EU, a total of 1'645 confirmed human cases of listeriosis (0.4/100'000) have been reported in 2009 (EFSA/ECDC, 2011). In several EU member states, the annual incidence rate has increased over the last few years, especially in the elderly population. *Listeria* spp. are widely distributed in the environment and certain strains may become established and persist in the processing environment (Thévenot *et al*., 2006; Wulff *et al*., 2006; Blatter *et al*., 2010). Other reservoirs include domestic and wild animals, but their significance in view of foodborne diseases and potential transmission routes (during slaughter) remain to be elucidated.

The genus *Listeria* currently comprises eight species: *Listeria (L) monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seeligeri, L. grayi, L. marthii* (Graves *et al*., 2010) *and L. rocourtiae* (Leclercq *et al*., 2010). Only two species of the genus are generally considered to be pathogenic, *L. monocytogenes* is an opportunistic pathogen in human beings, whereas *L. ivanovii* affects mammals mainly causing abortion. However, there have been some reports of *L. seeligeri* and *L. ivanovii* (Rocourt *et al*., 1986, Cummins *et al*., 1994) causing illness in humans.

L. monocytogenes is a Gram-positive, facultative, intracellular bacterial pathogen that causes morbidity and mortality in humans and livestock. It is a significant food-borne pathogen due its widespread distribution in nature, its ability to survive in a wide range of environmental conditions, and its ability to grow at refrigeration temperatures. However, epidemiological studies have revealed that only *L. monocytogenes* and only strains belonging to serotypes 1/2a, 1/2b and 4b were implicated in 90% of outbreaks of listeriosis (Schuchat *et al*., 1991). It is

unclear why only three of the 13 serotypes are implicated in food-borne outbreaks whilst other serotypes are also found as food contaminants.

Domestic animals contribute to amplification and dispersal of *L. monocytogenes* into the farm environment, and the farm ecosystem maintains a high prevalence of bacteria, including subtypes linked to human listeriosis cases and outbreaks. The clinical manifestations varied with the species affected. Spontaneous abortion, subclinical mastitis, encephalitis or septicemia were described. Listeriosis in adult swine is uncommon. The most common form is septicemia in young piglets, with death within 3 to 4 days.

Nevertheless an outbreak of listerial meningoencephalitis was observed in an Indian pig farm (Rahman *et al.,* 1985). Moreover pregnant sows aborted macerated fetuses and *L. monocytogenes* was isolated from the brain of affected animals in India (Dash *et al*., 1998).

However, animals may also commonly be asymptomatic carriers and shed *L. monocytogenes* in significant numbers contaminating the environment. The organism is thought to be harbored in the intestinal tract: the prevalence of *L. monocytogenes* in pig fecal samples ranges from 0% to 47% with a highest prevalence reported in Eastern Europe (Fenlon *et al*. 1996).

Carcasses might be contaminated when the large intestine is ruptured during evisceration (Skovgaard and Norrung 1989). However, Kanuganti *et al*. (2002), detected *L. monocytogenes* in only 4% of pork carcasses sampled but not in the rectal contents of animals prior to slaughter. Authors have suggested not all *L. monocytogenes* detected in carcasses have a fecal origin. Buncic *et al*. (1991) showed that pigs were more likely to harbor *L. monocytogenes* in their tonsils than to excrete the bacteria in their feces. Autio *et al*. (2000) also found that 14% of pig

tongues and 12% of tonsils sampled in slaughterhouses contained *L. monocytogenes*. Reported prevalence of *L. monocytogenes* in tonsils range from 0% to 61%; this range is probably due to differences in sampling techniques and/or farm management methods (Fenlon *et al*. 1996). Autio *et al*. (2000) hypothesized that *L. monocytogenes* spread through contact between the tonsils and tongue and the other viscera and carcass during the evisceration process.

Moreover *L. monocytogenes* may then survive in food industries because it grows at low temperature, adhere to various food contact surfaces and certain strains have adapted to disinfectants (Salvat *et al*. 1995). In fact, Nesbakken *et al*. (1996) found *L. monocytogenes* at every stage of the fresh pork meat industry, with increasing prevalence from the slaughterhouse to the cutting room. It also occurs frequently in raw pork meat (Norrung *et al*. 1999), although the origin of the contamination is unclear. *L. monocytogenes* has been occasionally isolated on farms from the feces and skin of presumably healthy pigs (Skovgaard and Norrung 1989).

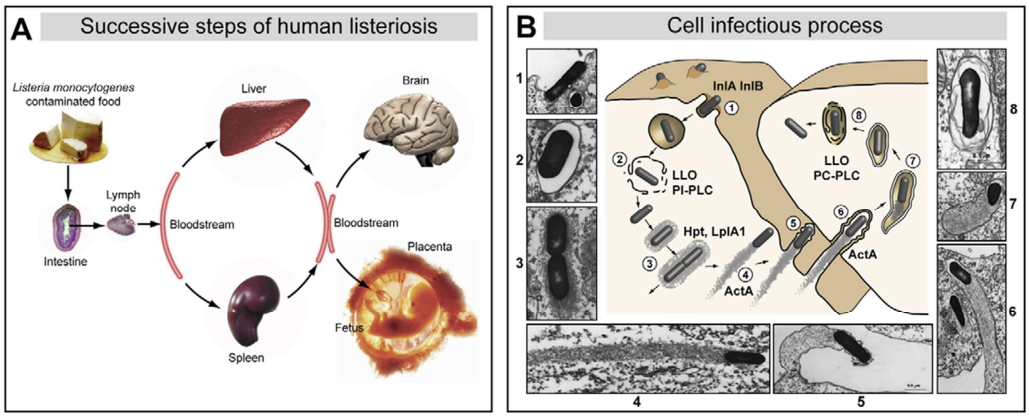
In humans the main route of transmission is believed to be through consumption of contaminated food. However, infection can also be transmitted directly from infected animals to humans as well as between humans.

The clinical signs of *L. monocytogenes* infection are very similar in all susceptible hosts. Two basic forms of presentation can be distinguished: perinatal listeriosis and listeriosis in the adult patient. In both instances, the predominant clinical forms correspond to disseminated infection or to local infection in the central nervous system (CNS). In adults pure meningeal forms are observed in some cases, but infection normally develops as a meningoencephalitis accompanied by severe changes in consciousness, movement disorders, and, in some cases,

paralysis of the cranial nerves. The encephalitic form, in which *Listeria* organisms are isolated with difficulty from the cerebrospinal fluid (CSF), is common in animals but rare in humans.

There are other atypical clinical forms (5 to 10% of cases), such as endocarditis (the third most frequent form), myocarditis, arteritis, pneumonia, pleuritis, hepatitis, colecystitis, peritonitis, localized abscesses (e.g., brain abscess, which accounts for about 10% of CNS infections by *Listeria* spp.), arthritis, osteomyelitis, sinusitis, otitis, conjunctivitis, ophthalmitis, and, in cows, mastitis (Vázquez-Boland *et al*., 2001).

Figure 2. Listeriosis. (A) Successive steps of human listeriosis. (B) Electron-micrographs and schematic representation of the successive steps of the cell infectious process. Major virulence factors are indicated (Source Cossart and Toledo-Arana, 2008).



Only limited data are available in literature for the occurrence of *Erysipelothrix* spp., *Salmonella* spp., and *Listeria* spp. in healthy slaughtered pigs and data were so far lacking in Switzerland. The aim of the present study was therefore to assess the occurrence of these pathogens in tonsils from Swiss pigs at slaughter and to further characterize isolated strains.

Materials and Methods

This study was based on investigations carried out within eight months (January to August 2011) in a Swiss abattoir working 5 days per week and processing 3000 pigs per week with an average of 250 pig carcasses per hour.

The project was performed in two phases:

- The aim of the first phase (pilot-phase, about 1-2 months) was to (i) establish the sampling procedure at the cantonal slaughterhouse in Zurich and (ii) develop the culture methods for isolation of *Erysipelothrix* spp.;
- The aim of the second phase (official phase 2-6 months) was to (iii) assess the occurrence of *Erysipelothrix spp*., *Salmonella* spp. and *Listeria* spp. in healthy pig tonsils and further characterize isolated strains.

i. Establishing sampling procedure at the slaughterhouse

Briefly, the slaughter process consisted in several operations. After lairage pigs were stunned by electrical tongs, immediately exsanguinated and then immersed in a scald tank for about 5 minutes with an average tank water temperature of 60 °C and an average carcass surface temperature of 56.0 °C. Carcasses were dehaired using a rotating drum with scrapers, passed through a polishing and then a singeing step. Carcasses where then wet polished with a series of flails and moved into a separated clean area. Evisceration involved several step as slitting the belly, removal of the gut, and removal of the thoracic viscera, each of which performed by a different operator. Carcasses were then split along the midline from back to front using a splitting saw. Subsequently a specific operator removed kidneys from each half carcass. The heads were still hung up on the half-carcasses thanks to a flap of skin. No contact with other carcasses was observed. After

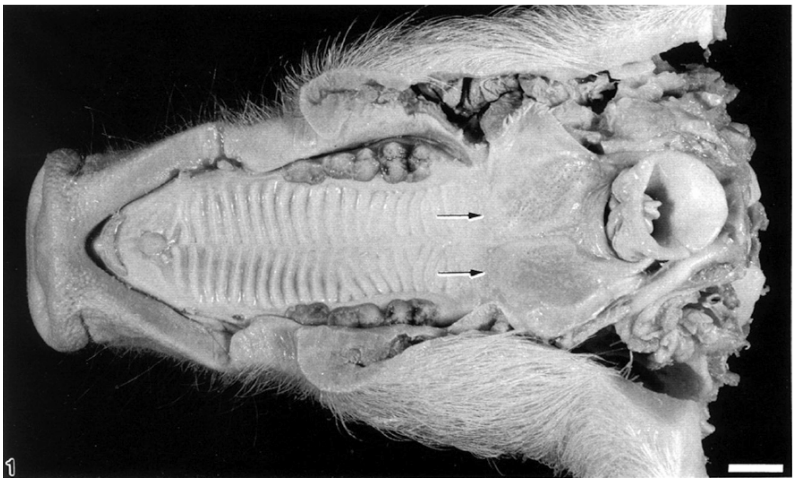
trimming and stamping, carcasses were weighed and then washed with potable water (about 10°C per 10 s). The heads were cutting off only after washing and before the final chilling (Spescha *et al*., 2006).

The point of sampling was determined in accordance with the official veterinarian to avoid slaughtering-impediments.

Samples consisting in tonsils were collected at the end of the line when the head had been cut off. Subsequent the heads were leant to the meat hook, cleaned from the ears, washed and conducted to the cool-room trough the assembly line track. Butchers often separated tongues from the heads making easier samples' collection.

Pig palatine tonsils are paired oval lympho-epithelial organs situated on either side of the median furrow of the soft palate differently from domestic animals and humans, where they are situated in the *tonsillar fossae* of the lateral walls of oropharynx (Figure 3).

Figure 3. Photograph of the mucosal surface of a plastinated pig's head showing the anatomical location of the tonsils of the soft palate (arrows). Bar = 10 mm. From Belz and Heath (1996), p. 103. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. Copyright The Anatomical Record, 1996. Wiley-Liss, Inc.



After leaning the head on its frontal region on a support, the epiglottis was pressed down to close the digestive tube with the help of a finger. In this way the oral cavity was explored to search for the rough surface of the tonsils localized at the beginning of the hard palate. Once identified, using scissors and a mouse-tooth forceps (with teeth at the tip of each blade) previously washed and immersed in 96% alcohol solution for two-thirds of their length, the *velum palatinum* was stretched and cut with two parallel lines on both sides of the median furrow of the soft palate. With a crosscutting tonsils were excised and cleaned from other tissues (muscles and connective tissues). All operations were made trying to avoid cross-contamination.

When butchers cut the tongue collection of tonsils became easier. Once the head had been taken and leant on the support as already described, the oral cavity was free and empty and thus the well visible tonsils were ready to be cut. Tools were washed and dipped into the 96% alcohol solution after each sampling. Collected samples were kept in sterile stomacher bags and stored in a cool-box until laboratory examination. Each bag was labeled with a number corresponding to the slaughtering number of the carcass. Traceability was guaranteed by recording the necessary data for every sampled carcass (place of origin, producer, date of slaughter, etc.) in a sampling protocol.

ii. Laboratory detection methods for *Erysipelothrix spp.*

Isolation for *Erysipelothrix* spp. was performed using one-step enrichment. Basically tonsil samples were enriched in 10 ml of a non-selective broth and then one aliquot was spread on different agar plates. Identification of the colonies centered on phenotypic and biochemical markers. For this purpose cultural morphology on agar plate, hemolytic characteristics and catalase tests were

evaluated in comparison with a strain of *E. rhusiopathiae* used as positive control. Subsequently, Gram's stain followed by microscopic observation was performed. *E. rhusiopathiae* is as Gram positive slight and slender rod easily decolorized. Suspicious colonies were studied for their motility, hydrogen sulfide (H_2S) and indole activities using the Sulfide-Indole-Motility Media test (SIM-test) incubated for 48 h at 37°C. *E. rhusiopathiae* is a non-motile, H_2S producer and indole negative microorganism. Sugar fermentation properties were investigated biochemically using a commercial identification kit.

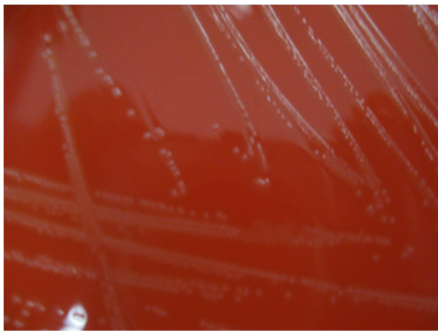
With regard to Gram's stain suspicious colonies were first diluted in a drop of sterilized water and spread on a slide using an inoculation loop. The slide was fix over a gentle flame with circular movements to avoid localized overheating. About 5 drops of crystal violet stain were added for 60 seconds over the fixed culture and then washed under running water. Five drops of the iodine solution were added for 30 seconds covering the smear and then washed under running water. Few drops of decolorizer were added for 5 seconds and five drops of the safranin solution were added for 20 seconds. The red safranin solution was washed off under running water and the final stained slide was observed under a microscope.

Firstly, a total of 64 samples were collected at slaughter in four sampling days. A quantity of them (20 samples) was enriched in 10 ml of non-selective Brain Heart Infusion (BHI, CM1135, Oxoid Ltd., Hampshire, UK) for 24 h at 37°C and subsequent plated onto non-selective blood agar (Dehydrated Columbia Blood Agar Base EH, *Difco*™ Laboratories, Detroit, MI, USA), incubated for 24 h at 41.5 °C. The remaining 44 out of 64 samples were enriched in 10 ml of non-selective BHI for 24 h at 41.5°C and subsequent plated onto blood agar incubated

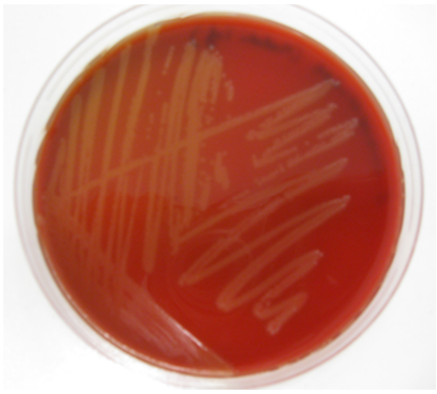
for 24 h at 41.5 °C. *E. rhusiopathiae* grows on blood agar as very small white colony surrounded by a small zone of hemolysis when incubated for 24 h at 41.5 °C (Figures 4 and 5) and surrounded by a wider zone of hemolysis when incubated for 48 h at 41.5 °C (Figures 6 and 7).

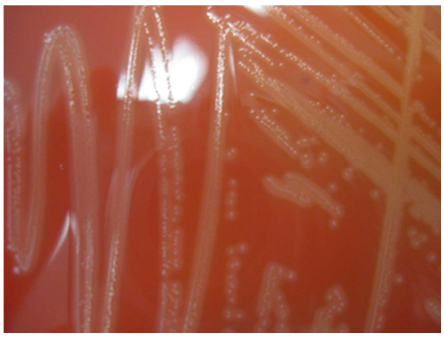
In both cases the 64-blood agar plates were characterized by bacteria overgrown with white, whitish and grayish colonies with or without the zone of hemolysis. Suspicious *E. rhusiopathiae* colonies were further studied under microscopic observation. Stained colonies revealed Gram positive/negative coccal bacteria.

Figures 4-5. On the left, *E. rhusiopathiae* (positive control) haemolysis on blood agar (41.5 °C per 24h). On the right, detail of *E. rhusiopathiae* (positive control) haemolysis on blood agar (41.5 °C per 24h).



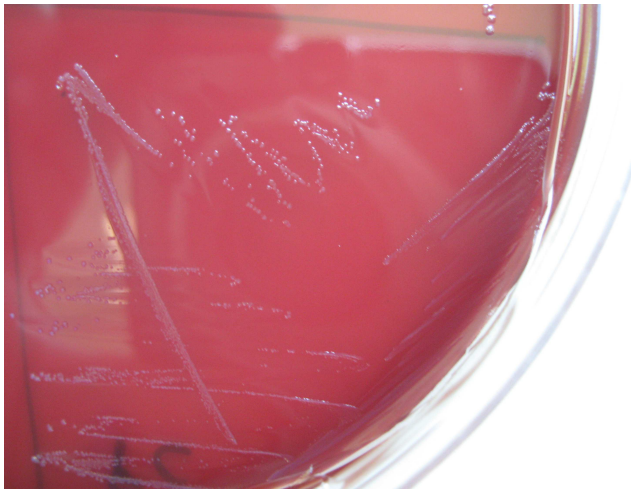
Figures 6-7. On the left, *E. rhusiopathiae* (positive control) haemolysis on blood agar (41.5 °C per 48h). On the right, detail of *E. rhusiopathiae* (positive control) haemolysis on blood agar (41.5 °C per 48h).





Secondly, a total of 70 samples were collected in six sampling days at the same abattoir. Tonsils were enriched in 10 ml of non-selective BHI for 24 h at 41.5°C. Subsequently an aliquot was plated onto a modified selective Packer's medium incubated for 48 h at 42°C (see official phase for medium composition). *E. rhusiopathiae* grows on this medium as small bluish colony surrounded by a small narrow zone of hemolysis (Figure 8).

Figure 8. *E. rhusiopathiae* (positive control) morphology on modified Packer's medium (41.5°C per 48 h). Small bluish colony surrounded by a small zone of haemolysis.



All the 70-selective agar plates showed blue colonies with different size (Figure 9, left). For the sake of simplicity this colonies were labeled as big blue colonies and identified after staining as Gram positive coccal bacteria and small bluish colonies sometimes surrounded by a zone of hemolysis and identified after staining as Gram positive coccal bacteria.

Figure 9. Bacteria growing (left) as big and small blue colonies compared with *E. rhusiopathiae* positive control colonies (right, up and down) on modified Packer's medium (41.5°C per 48 h).

In only one case a suspicious small blue colony was observed microscopically as Gram-positive rod and thus sub-cultured in sheep blood agar and incubated for 24 h at 37°C. Following the established protocol, the bacterial colony was studied for its motility, hydrogen sulfide (H_2S) and indole activities using SIM-test incubated for 48 h at 37°C. The blackening of the stab line gave a positive reaction. Biochemical properties were investigated using Api Coryne System in accordance with the manufacturer's instructions (bioMérieux SA, Marcy l'Etoile, F). *E. rhusiopathiae* was found with a 92.9 % ID. Confirmation was obtained by the use of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). *E. rhusiopathiae* was isolated and identified with 88% ID.

Additionally 48 samples were collected in two sampling days at slaughter. Based on previous *E. rhusiopathiae* finding, samples were enriched in 10 ml of BHI broth supplemented with horse blood (SR048C,Oxoid Ltd.) for 24 °C at 41.5°C in order to increase the chances of detection. An amount (one loopful) was plated

onto Modified Packer's medium incubated for 24 h at 41.5 °C. After incubation plates were characterized by bacteria overgrown with white, whitish and grayish colonies sometimes with a narrow zone of hemolysis. Suspicious colonies were Gram stained and identified as mostly Gram positive and negative coccal bacteria.

In summary, collection of samples at slaughter was based on the specifications and findings of the first pilot phase. With regard to the laboratory analyses, tonsils samples were enriched in BHI for 24°C at 41.5°C and plated onto modified Packer's medium incubated for 48°C at 41.5°C.

In addition to *Erysipelothrix* spp., *Salmonella* spp. and *Listeria* spp. were also investigated.

iii. Occurrence of *Erysipelothrix* spp., *Salmonella* spp. and *Listeria* spp. in healthy pig tonsils at slaughter and further characterization of isolated strains.

To investigate the occurrence of *Erysipelothrix* spp., *Salmonella* spp. and *Listeria* spp., tonsil samples were collected from healthy slaughtered pigs. Palatine tonsils are known as a portal of entry and a site of multiplication and persistence for several microorganisms in animals including pigs (Salles and Middleton, 2000). Sampled pigs were about six month old and the average weight of each carcass was about 80 kg. On each sampling day, 22 to 49 samples were collected and not more than two samples originated from the same batch/producer. The majority of the producers were distributed over the north and central part of Switzerland. Sampling comprised two phases. In the first phase, 250 samples were collected during eight sampling days and examined for *Erysipelothrix* spp. and *Salmonella* spp. These samples were obtained from carcasses originating from 138 different batches and 108 producers. In the second phase, 250 samples were collected

during seven sampling days and examined for *Listeria* spp.. These samples were obtained from carcasses originating from 126 different batches and 108 producers. Tonsil samples (*tonsilla veli palatini*) were obtained at the end of the slaughter process when the head had been cut off. For this purpose, forceps and scissors previously sterilized in 96 % ethanol solution were used. After leaning the head with its frontal region on a support, the *velum palatinum* was stretched and cut with two parallel lines on both sides of the median furrow of the soft palate. Tonsil samples were then excised and placed into sterile stomacher bags. Samples were transported cooled to the laboratory and bacteriological examinations were carried within 3 h after sampling.

Examination for *Erysipelothrix* spp. was done by culture method after an enrichment step. Briefly, half of each tonsil sample was homogenized for 60 s in 10 ml of Brain Heart Infusion Broth (BHI, CM1135, Oxoid Ltd., Hampshire, UK) and incubated for 24 h at 41.5 °C. Subsequently, subsets (one loopful) of the enrichment broth were streaked onto a modified Packer's selective medium (pH=7.58) containing per liter 39 g of blood agar base (Dehydrated Columbia Blood Agar Base EH, *Difco*™ Laboratories, Detroit, MI, USA), 0.01 g of crystal violet (Sigma-Aldrich, St Louis, MO, USA), 0.7 g of sodium azide (Sigma-Aldrich), and 5 % of horse blood (SR048, Oxoid Ltd.). Plates were incubated for 48 h at 41.5 °C. Suspicious colonies (very small size, bluish color, surrounded by a narrow zone of hemolysis) were sub-cultured onto sheep blood agar (*Difco*™ Laboratories; 5 % sheep blood, SB055, Oxoid Ltd.) for 24 h at 37 °C. After Gram staining, Gram-positive rods were tested for catalase reaction and H_2S production. For confirmation and species identification, presumptive *Erysipelothrix*-positive colonies were verified (i) by appraisal of their biochemical properties using the

Api Coryne System in accordance with the manufacturer's instructions (bioMérieux SA, Marcy l'Etoile, F) and (ii) by the use of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS; Mabritec AG, Riehen, CH).

Examination for *Salmonella* spp. was done in accordance with ISO 6579:09.2006 using a two-step enrichment procedure. Briefly, the other half of each tonsil sample was pre-enriched in 10 ml of Buffered Peptone Water (BPW, CM1049, Oxoid Ltd.) for 24 h at 37 °C. From the first enrichment, 1 ml was incubated for 24 h at 37 °C in 10 ml of Kauffmann Tetrathionate-Novobiocin Broth (CM1048, Oxoid Ltd.) supplemented with Novobiocin Sodium Salt (74675, Sigma-Aldrich) in accordance with the manufacturer's instructions and 0.1 ml was incubated for 24 h at 41.5 °C in 10 ml of Rappaport-Vassiliadis Soya Pepton Broth (CM0866, Oxoid Ltd.). After plating onto Mannitol Lysine Crystal Violet Brilliant Green Agar (MLCB, CM0783, Oxoid Ltd.) and Xylose-Lysine-Desoxycholate Agar (XLD, CM0469, Oxoid Ltd.), plates were incubated for 24 h at 37 °C. Suspicious colonies were tested for biochemical properties of *Salmonella* by using the following tests: oxidase reaction, acid production from mannitol, o-nitrophenyl-β-D-galactopyranoside (ONPG) test, H_2S and indole production, and proof of urease and lysine decarboxylase. Isolated *Salmonella* strains were affirmatively identified and serotyped at the Swiss National Reference Centre for Enteropathogenic Bacteria and Listeria (Institute for Food Safety and Hygiene, University of Zurich, CH).

Examination for *Listeria* spp. and in particular *L. monocytogenes* was done in accordance with ISO 11290-1:2004 using a two-step enrichment procedure.

Briefly, each sample was incubated in 10 ml of Fraser Broth (CM0895, Oxoid Ltd.) with Half Fraser Supplement (SR0166, Oxoid Ltd.) for 24 h at 30 °C. From the first enrichment, 0.1 ml were incubated in 10 ml of Fraser Broth (CM0895, Oxoid Ltd.) with Fraser Supplement (SR0156, Oxoid Ltd.) for 24 h at 37 °C. Subsequently, subsets (one loopful) were then plated onto Palcam Agar (Merck Eurolab GmbH, Darmstadt, D) and onto Chromogenic Listeria Agar (CM1084, Oxoid Ltd.) supplemented with Listeria Selective Supplement (SR0226, Oxoid Ltd.) and Listeria Differential Supplement (SR0244, Oxoid Ltd.). Both plates were incubated for 48 h at 37 °C. On the chromogenic agar, colonies of *Listeria* spp. grew with a green-blue colour, whereas colonies of *L. monocytogenes* and *L. ivanovii* grew with a green-blue colour surrounded by an opaque halo. Presumptive *L. monocytogenes* and *L. ivanovii* colonies on the chromogenic agar were streaked onto sheep blood agar for appraisal of hemolysis (CAMP test with *S. aureus* and *Rhodococcus equi*). To identify other *Listeria* species, the API Listeria identification Kit was used (bioMérieux SA). Isolated *L. monocytogenes* strains were affirmatively identified and serotyped at the Swiss National Reference Centre for Enteropathogenic Bacteria and Listeria (Institute for Food Safety and Hygiene, University of Zurich, CH).

Results and Discussion

Using the described method for detection of *Erysipelothrix* spp., all 250 tonsil samples obtained from healthy Swiss pigs at slaughter tested negative (Table 4).

Table 4. Occurrence of *Erysipelothrix* spp., *Salmonella* spp., and *Listeria* spp. in tonsils of healthy pigs at slaughter.

	Erysipelothrix spp.	*Salmonella* spp.	*Listeria* spp.
No. of sampled animals/ batches	250/138	250/138	250/126
No. (%) of positive animals	nd[a]	2 (0.8 %)	14 (5.6 %)
No. (%) of positive batches	nd	2 (1.4 %)	14 (11.1 %)
Isolates (No.)	nd	*S.* Bredeney (1) *S.* Kedougou (1)	*L. monocytogenes* (9)[b] *L. ivanovii* (4) *L. innocua* (1)

[a] nd, not detected;
[b] *L. monocytogenes* isolates belonging to serotypes 1/2a, 1/2b, and 4b.

Based on these results, *Erysipelothrix* spp. seem to occur only in low numbers in the Swiss pig population. Our findings are in contrast to the assumption that 30 to 50 % of healthy pigs might harbor the organism (Wood, 1999). As a limitation of the present study, the use of solely the described cultural detection method must be mentioned. In our study, agar plates were often overgrown with different bacteria so that potentially present *Erysipelothrix* spp. may have been competitively suppressed in their growth or missed. Using a cultural method with addition of selected antibiotics, Takahashi *et al*. (1987a) isolated *E. rhusiopathiae* from the tonsils of 10.5 % of 600 healthy slaughter pigs. Nevertheless, none of the available media is considered ideal (Wang *et al*., 2010). To get a clearer and more accurate picture on the prevalence of *Erysipelothrix* spp. in the pig population, PCR-based methods have been proposed and evaluated (Takeshi *et al*., 1999;

Wang *et al*., 2002; Yamazaki, 2006; Pal *et al*., 2010). In view of pig colonization, such methods are also of interest for differentiation of *Erysipelothrix* species, in particular as the role of *E. tonsillarum*, which is considered non pathogenic for pigs, needs to be further elucidated. In a survey from Australia, which investigated 109 abattoir samples (collected from various parts of pig and sheep carcasses, as well as from different sections of the slaughter line, pen, soil and effluent), genus-specific PCR yielded 32.1 % *Erysipelothrix*-positive samples, whereas culture yielded only 15 (13.8 %) *Erysipelothrix* isolates (Wang *et al*., 2002). However, *Erysipelothrix* spp. were thereby not detected in tonsil samples with either method.

Salmonella spp. were detected in only 0.8 % of the 250 tonsil samples obtained from healthy Swiss pigs at slaughter (Table 4). The two *Salmonella*-positive animals originated from two farms located in the central part of Switzerland. With regard to the batch level, *Salmonella* spp. were detected in 1.4 % of the 138 examined batches. Reported detection rates of *Salmonella* spp. in pig tonsils at slaughter vary between different surveys. From tonsils of Bavarian fattening pigs, no *Salmonella* were isolated (Fredriksson-Ahomaa *et al*., 2009), whereas Bonardi *et al*. (2003) detected *Salmonella* in 5.3 % of 150 tonsil samples from slaughter pigs in northern Italy, and a high *Salmonella* prevalence of 19.6 % has been reported in tonsils of slaughter pigs in the Netherlands (Swanenburg *et al*., 2001). In a European baseline survey investigating slaughter pigs, average *Salmonella* prevalence was 10.3 % in lymph nodes and 8.3 % on carcasses (EFSA, 2008). Prevalence thereby ranged from 0 % in Finland to 29.0 % in Spain for lymph nodes and from 0 % in Slovenia and Sweden to 20.0 % in Ireland for carcasses.

The two *Salmonella* isolates found in the present study belonged to serovars Bredeney and Kedougou. In the study of Bonardi *et al*. (2003), five *Salmonella (S.)* Bredeney strains and three *S.* Derby strains were isolated out of eight positive tonsil samples. *S.* Bredeney and *S.* Kedougou were also identified in the EU survey on *Salmonella* in holdings with breeding pigs, but these serovars represented in each case less than 5.0 % of the *Salmonella*-positive holdings (EFSA, 2009). The most frequently detected serovars were thereby *S.* Derby and *S.* Typhimurium, which were isolated in 20.1 to 29.6 % of the positive holdings. With regard to human illness associated with *Salmonella*, *S.* Bredeney and *S.* Kedougou are only rarely reported as cause of human cases or foodborne outbreaks (EFSA/ECDC, 2011). In the year 2009, only one verified outbreak (three cases) caused by *S.* Bredeney has been observed in the EU. The majority of human *Salmonella* cases were caused by *S.* Enteritidis and *S.* Typhimurium. Of all 324 *Salmonella*-associated verified foodborne outbreaks reported in 2009, *S.* Enteritidis and *S.* Typhimurium accounted for 59.6 % and 15.7 %, respectively (EFSA/ECDC, 2011).

Listeria spp. were detected in 5.6 % of the 250 tonsil samples obtained from healthy Swiss pigs at slaughter (Table 4). The 14 *Listeria*-positive animals originated from 11 farms located in the north-central part of Switzerland. With regard to batch level, *Listeria* spp. were detected in 11.1 % of the 126 examined batches. Of the 14 *Listeria* isolates, nine, four, and one were identified as *L. monocytogenes*, *L. ivanovii,* and *L. innocua*, respectively. Overall, *L. monocytogenes* were therefore detected in 3.6 % of the examined animals and 7.2 % of the batches. Reported detection rates of *L. monocytogenes* in pig tonsils at slaughter vary between different surveys. Amongst Bavarian fattening pigs, *L.*

monocytogenes was isolated from 32.0 % of 50 tonsil samples (Fredriksson-Ahomaa *et al*., 2009). In other European studies examining tonsils of pigs, the prevalence of *L. monocytogenes* ranged from 12.0 to 44.6 % (Buncic, 1991; Autio *et al*., 2000; Autio *et al*., 2004). Autio *et al*. (2004) thereby reported that the prevalence of *L. monocytogenes* in tonsils of fattening pigs (22.0 %) was significantly higher than in sows (6.5 %) and that the prevalence among pigs from five abattoirs varied from 3.3 to 25.0 %. In the U.S., Wesley *et al*. (2008) recently detected *L. monocytogenes* in only 0.6 % of 181 tonsil samples from cull sows, whereas Kanuganti *et al*. (2002) found *L. monocytogenes* in 7.1 % of 252 slaughter pigs. Interestingly, several studies reported that *L. monocytogenes* was isolated more frequently from tonsils than from fecal samples (Buncic, 1991; Wesley *et al*., 2008; Fredriksson-Ahomaa *et al*., 2009). Thus it was hypothesized that tonsils might be a more accurate predictor of *L. monocytogenes* carrier status in pigs than fecal samples. Of the nine *L. monocytogenes* isolates, three belonged to serotype 1/2a, two to serotype 1/2b (three strains), and four to serotype 4b. The majority of human cases are also associated with *L. monocytogenes* of serotypes 1/2a, 1/2b, and 4b and the proportion associated with isolates of serotype 1/2a has increased in recent years (Lukinmaa *et al*., 2003; Parihar *et al*., 2008; Allerberger and Wagner, 2010).

In conclusion, although *Erysipelothrix* spp. were not isolated, this study demonstrates that *Salmonella* spp. and *L. monocytogenes* could be detected in tonsils from healthy Swiss pigs at slaughter. Compared to the data from some other European countries, detected prevalence of *Salmonella* spp. and *L. monocytogenes* was low. However, it must be considered that tonsils colonized with pathogens might play a role in the contamination of pluck sets, carcasses,

and the slaughterhouse environment during slaughter (Fredriksson-Ahomaa *et al*., 2009). To encounter this threat, prevention of contamination during slaughter is of major importance, in particular adherence to good hygiene practices and application of effective cleaning and disinfection procedures to prevent equipment contamination.

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Research study no. 2: Seroprevalence of anti-HEV and anti-*Salmonella* antibodies in pigs at slaughter in Switzerland.

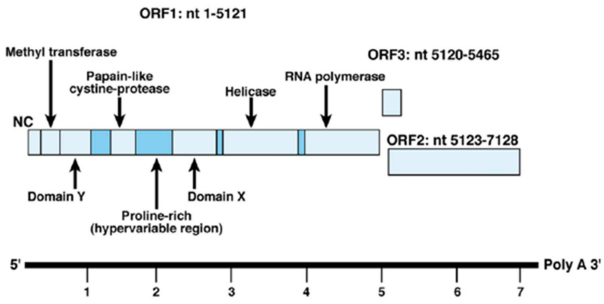
Key words: Hepatitis E virus, *Salmonella* spp., Seroprevalence, Swine

Hepatitis E virus (HEV) is the causative agent of the human hepatitis E, an inflammatory liver disease with important public impact in both developing countries and industrialized countries. It's responsible for an enterically transmitted acute self-limiting non-A, non-B hepatitis in humans and generally does not progress to chronicity (Purcell and Emerson, 2001). HEV is a spherical positive single-stranded non-enveloped RNA virus and it is the only virus within the genus *Hepevirus* and the *Hepeviridae* family (Emerson *et al*., 2004, Meng, 2010).

The HEV genome includes 2 short non-coding regions that surround 3 open reading frames (ORFs) (Figure 10). The RNA genome of HEV is capped at the 50 end, and polyadelylated at the 30 end (Huang *et al*., 1992; Reyes *et al.*, 1990) with approximately 7.2 kb in length. ORF1 encodes the non-structural proteins required for the replication and protein processing, including RNA helicase, an RNA-dependent RNA polymerase, a methyltransferase, and a cysteine protease (Mushahwar, 2008). ORF-1 contains a hypervariable region that does not have a major role for virus infectivity (Pudupakam *et al*., 2009). The icosahedral HEV capsid is 27–34 nm in diameter; a crystal structure of HEV-like particles has recently been characterized (Yamashita *et al.*, 2009). Distinct amino acid mutations in the capsid could attenuate the virus and have implications for vaccine development (Cordoba *et al*., 2011). The small, 114 amino acid protein encoded

by ORF-3 has multiple functions that regulate the cellular environment (Ahmad *et al.*, 2011).

Figure 10. Genomic organization of HEV including the ORFs. The scale shows nucleotides in thousands (Source of the drawing: Wedemeyer *et al.*, 2012).



HEV replicates in the cytoplasm, with a sub genomic RNA producing capsid proteins and full genomic RNA encoding nonstructural proteins and serving as a template for replication.

The HEV strains can be grouped into 4 mammalian genotypes plus an Avian HEV, with different geographical distribution and host range. Genotype 1 is responsible for most endemic and epidemic cases of hepatitis E in Asia and Africa. Genotype 2 is endemic in Mexico and western Africa. Whereas these genotypes have been found exclusively in humans, genotypes 3 and 4 have been also detected in pigs and other animal species (Meng, 2010). Genotype 3 is spread worldwide and genotype 4 is restricted to Southeast Asia. Thus the endemic strains found in Europe are usually of genotype 3. The particularity of HEV is that, among all known major hepatitis viruses (A, B, C and D) HEV (genotype 3 and 4) is the only one with animal reservoirs.

Swine HEV was first isolated and genetically characterized from pigs in the USA in 1997 (Meng *et al.*, 1997).

HEV infection in developing countries is mostly a waterborne disease associated with large epidemics due to the contamination of water and water supplies, and poor sanitation conditions (Purcell and Emerson., 2008). In contrast, in industrialized countries, including many European countries, USA and Japan, acute hepatitis E occurs sporadically via oral-fecal route and the contamination pathways are still not fully understood (Purcell and Emerson., 2008). It is often reported in patients with history of travelling in endemic areas (Péron *et al*., 2006). However, in the last decade an increasing number of sporadic human HEV cases (due to genotypes 3 and 4) without history of travelling have been reported.

Under experimental conditions, genotypes 3 and 4 HEV can infect across species barriers. The ability of HEV genotype 3 to cross species barrier has been reported in non-human primates experimentally infected (Meng *et al*., 1998). The reverse, anthropozoonotic potential of HEV genotype 3 was proven by infecting pigs with HEV derived from a liver transplant recipient with persistent HEV infection (Pischke *et al*., 2010). No efficient cell culture system for swine and wild boar HEV strains has been established, nevertheless it has been reported that A549 cells could support the replication of swine HEV of genotype 4 derived from a fecal specimen (Zhang *et al*., 2011). Takahashi *et al*., 2012 showed that swine and wild boar HEV strains can replicate as efficiently as human HEV strains in human cultured cells, including A549 lung cancer cells and PLC/ PRF/5 hepatocarcinoma cells. Thus, zoonotic transmission represents an important mode of transmission for HEV genotype 3 and should be considered as the main source for autochthonous HEV infection in North America and Europe. It is not clear whether HEV incidence rates are really changing or if more cases are detected trough increased surveillance.

Figure 11. Typical evolution of the serological titer of HEV-specific antibodies and levels of alanine-transferase (ALT) levels during acute self-limited infection (Hoofnagle *et al*. 2012).

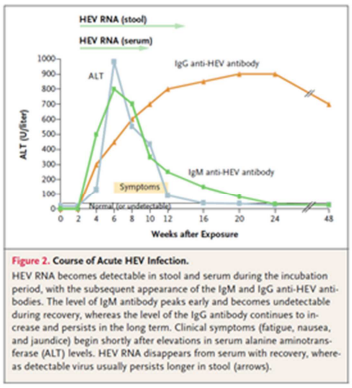


Figure 2. Course of Acute HEV Infection.
HEV RNA becomes detectable in stool and serum during the incubation period, with the subsequent appearance of the IgM and IgG anti-HEV antibodies. The level of IgM antibody peaks early and becomes undetectable during recovery, whereas the level of the IgG antibody continues to increase and persists in the long term. Clinical symptoms (fatigue, nausea, and jaundice) begin shortly after elevations in serum alanine aminotransferase (ALT) levels. HEV RNA disappears from serum with recovery, whereas detectable virus usually persists longer in stool (arrows).

Accumulating evidence indicates that hepatitis E is a zoonotic disease, and swine (and likely other animal species) can act as reservoirs (EFSA, 2011). Swine and human strains of HEV are genetically closely related and, in some cases, indistinguishable (Lu *et al*., 2006).

In humans, incubation time ranges from 2 weeks to 2 months with an average of 40 days (Purcell and Emerson, 2008). Most HEV infections have a clinically silent course and rarely associated with clinical symptoms during childhood (Buti *et al*., 2008). Initial symptoms of acute hepatitis E are typically unspecific and include flulike myalgia, arthralgia, weakness, and vomiting. Some patients have jaundice, itching, uncolored stools, and darkened urine, accompanied by increased levels of liver transaminases, bilirubin, alkaline phosphatase, and γ - glutamyltransferase (Figure 11).

Diagnosis for acute HEV infection is based on detection of anti-HEV Ig M. Increased titers of anti-HEV Ig G can indicate recent HEV infection. HEV RNA can be detected in blood and stool for several weeks during acute HEV infection.

The overall mortality rate associated is generally low between 1% and 5 % (Pavio *et al*., 2010) except for pregnant women, who may exhibit mortality rates up to 28% (Purcell and Emerson, 2001). The severe course in pregnant women might result from hormonal and immunologic features of pregnancy. Reduced expression of the progesterone receptor was associated with fatal outcomes from hepatitis E in pregnant women (Bose *et al*., 2011).

The HEV receptor has not been identified and the detailed mode of entry into hepatocytes is unknown. Similar to other viruses, binding of the HEV capsid involves heparin sulfate proteoglycans.

Several transmission routes (Figure 12) have been reported for HEV:

i. Fecal-oral transmission. Due to contamination of drinking water especially in developing countries (water-borne disease);

ii. Foodborne transmission. In the last years an increase of several case reports has been recorded. In June 2000, an English 58-year-old woman, who worked as a shop assistant, was seen at a rapid access "jaundice hotline" clinic with a 5-day history of myalgia and jaundice. She had not traveled outside the United Kingdom for 10 years and had no contact with farm or domestic animals. She was not a vegetarian and, although she admitted to eating raw sausage and bacon in the past, she claimed not to have done so in the 3 months before her illness. Isolated HEV strain showed a

Figure 12. Source and route of HEV 1-4 infection.

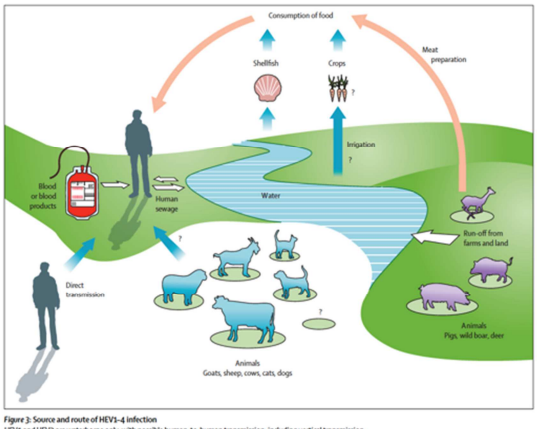


Figure 3: Source and route of HEV1–4 infection
HEV1 and HEV2 are waterborne only, with possible human-to-human transmission, including vertical transmission.

100% amino acid identity with strains of HEV circulating in pig herds in the analogous region. This case supports the theory that autochthonous HEV infection in industrialized countries is a zoonotic disease (Banks *et al*., 2004). In November 2010, a Portuguese 65-year-old male patient was referred to the hospital with a provisional diagnosis of acute hepatitis and a progressive onset of a fever, fatigue, loss of appetite, upper abdominal discomfort and dark-colored urine. The patient had no travelling history in the last six months and contact with animals. However he recalled to have eaten traditional homemade pork sausages made of raw meat about two weeks prior to the development of the clinical manifestations of acute hepatitis. Anti-hepatitis E virus Ig M and Ig G were found with ELISA test in blood samples (Duque *et al.,* 2012). A French and immune-compromised 30-year-old Caucasian female with no travelling history and contact with animals, living in East of France, was hospitalized for acute hepatitis with common clinical features: jaundice and anorexia. Physical examination revealed moderate abdominal pain and dark urines. Hepatitis E infection was diagnosed on both HEV RNA and serological samples. Genotype 4 was identified for the first time in France. The recent characterization of genotype 4 HEV through swine surveillance in Europe and the description of the first human case in France open and confirm interesting questions about the circulation of this genotype: health risks in human population, transmission patterns, and zoonotic reservoir. Two cases of confirmed zoonotic transmission of HEV through the consumption of contaminated animal food products have been reported in Japan. In these two cases, clinical symptoms occurred 40 or 60 days after consumption of Sika deer (sushi) or wild boar (grilled) meat. In both cases, HEV RNA was

successfully amplified in the patients as well as in the leftover frozen animal meat. The HEV viral sequences recovered from the patients and from the leftover frozen meats were either identical or near identical with 99.95% identity, confirming the zoonotic nature of transmission through the consumption of animal food products (Li *et al*., 2005; Tei *et al.*, 2003). Autochthonous HEV case reports have also been described in Germany and Italy (Veitt *et al*., 2011; Zanetti *et al*., 1999). Thus, among the possible contamination pathways of HEV, contaminated food must be seriously considered and also it must be considered that viruses do not multiply in foods, but may persist for extended periods of time as infectious particles in the environment, or in foods and studies on natural persistence of HEV are missing (EFSA, 2011).

iii. HEV exposure through direct contact with animals. Higher anti-HEV antibody prevalence within individuals in close contact with pigs has been reported: slaughterhouse staff, veterinarians and pig breeders (Meng *et al*., 2002). Vulcano *et al*., 2007 investigating HEV prevalence in the general population and among workers at zoonotic risk in Latium Region reported prevalence in the general population of 2.9% against 3.3% of pig breeders. In Italy it is estimated that the prevalence in the general population varies among Italian regions but seems to be on the same level: 3.3% in the North (Romanò *et al*., 2011), 2.9 % in the Centre (Vulcano *et al*., 2007), 3.8% in the South (Cacciola *et al*., 2011) and 4.3 in Sardegna (Masia *et al.*, 2009). Similar value of 4.9% was found in Switzerland in Kaufmann *et al*., 2011 study.

iv. Transmission by transfusion of infected blood products (Matsubayashi *et al*., 2008) and organ transplantation. Recently, a German patient was reported to

have become infected with HEV from a liver transplant. The patient tested positive for HEV RNA 150 days after transplantation and phylogenetic analysis indicated that a similar strain of HEV was isolated from the donor, who was negative for anti-HEV. Therefore, occult HEV infection (asymptomatic presence of HEV RNA) is a potential concern (Schlosser *et al*., 2012);

v. Vertical maternal-fetal transmission (Aggarwal and Naik, 2009);

vi. Direct horizontal transmission of HEV between humans is unusual.

Data are missing on the incidence of hepatitis E in EU Countries. Also, the distinct transmission pathways of HEV and especially the proportion of foodborne cases out of total hepatitis E cases are not known (EFSA, 2011).

Infected animals do not normally show clinical signs of disease. Under natural conditions of infection, the dynamics of HEV infection is similar to what is described for most viral infections in pigs: acquisition of passive immunity through colostrum absorption (60% of the piglets), progressive decline of these passive antibodies at 8–10 weeks of age, then seroconversion between 14 and 17 weeks of age (Figure 13) corresponding to the peak of viremia observed at 15 weeks of age (40% of animals). In a Spanish study, the percentage of viremic pigs increased from 9 weeks to 15 weeks of age and gradually decreased towards slaughter age (de Deus *et al.*, 2008). The Ig M anti-HEV increased from 9 weeks of age, and approximately 100% of the pigs studied (n = 16) were Ig G anti-HEV-positive at 22 weeks of age. This dynamics observed in a Spanish herd is also in accordance with what was observed in Japan where the peak of fecal virus excretion was between 1 and 3 months of age (75 to 100% of the animals) then decreased to 7% of the animals at 5–6 months of age (Nakai *et al*., 2006). The

seroprevalence observed at the end of fattening reveals an effective transmission of the virus between the animals from the same fence.

Figure 13.. Representation of the kinetics of seroconversion: maternal anti-HEV antibodies (dark blue), Ig M anti-HEV (green), Ig G anti-HEV (light blue) and fecal excretion of HEV (red) in pigs infected naturally naturally (From Pavio *et al*., 2010).

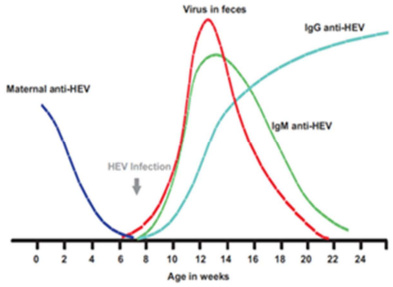


Table 5. Hepatitis E virus (HEV) prevalence studies in Europe (2008-2012) using ELISA (Enzyme-Linked ImmunoSorbent Assay) and RT-PCR (Reverse Transcription-Polymerase Chain Reaction) in pigs and products thereof (from Sarno *et al*., 2012. XXII Convegno Nazionale AIVI)**.**

Reference	Country	Method/Sample type	Prevalence (%)
WACHECK *ET AL*., 2012	SWITZERLAND	ELISA (Meat juice)	49
CASAS *ET AL*., 2011	SPAIN	ELISA (Meat juice)	64
CASAS *ET AL*., 2011	SPAIN	ELISA (Serum)	64
DI BARTOLO *ET AL*., 2011	ITALY	ELISA (Serum)	87
ROSE *ET AL*., 2011	FRANCE	ELISA (Serum)	31
BREUM *ET AL*., 2010	DENMARK	ELISA (Serum)	73.2
BAECHLEIN *ET AL*., 2010	GERMANY	ELISA (Serum)	49.8
MARTINELLI *ET AL*., 2011	ITALY	ELISA (Serum)	50.2
DI BARTOLO *ET AL*., 2011	ITALY	RT-PCR (Bile)	51.1
MASIA *ET AL*., 2009	ITALY	RT-PCR (Bile)	6.3
KOSINOVA *ET AL*., 2012	CZECH REPUBLIC	RT-PCR (Bile)	34.9
KOSINOVA *ET AL*., 2012	CZECH REPUBLIC	RT-PCR (Feces)	22.2
HAKZE-VAN DER HONING *ET AL*., 2011	THE NETHERLAND	RT-PCR (Feces)	15
HAKZE VAN DER HONING *ET AL*., 2011	BELGIUM	RT-PCR (Feces)	7
MCCREARY *ET AL*., 2008	ENGLAND	RT-PCR (Feces)	21.5
STEYER *ET AL*., 2011	SLOVENIA	RT-PCR (Feces)	20.3
WIDÈN *ET AL*., 2011	SWEDEN	RT-PCR (Feces)	29.6
BREUM *ET AL*., 2010	DENMARK	RT-PCR (Feces)	49.5
DI MARTINO *ET AL*., 2010	ITALY	RT-PCR (Feces)	7.3
BERTO *ET AL*., 2012	PORTUGAL	RT-PCR (Feces)	22
DI BARTOLO *ET AL*., 2011	ITALY	RT-PCR (Feces)	33.3
FORGÁCH *ET AL*., 2010	HUNGARY	RT-PCR (Feces)	21
DI BARTOLO *ET AL*., 2011	ITALY	RT-PCR (Liver)	20.8
FORGÁCH *ET AL*., 2010	HUNGARY	RT-PCR (Liver)	31
WENZEL *ET AL*., 2011	GERMANY	RT-PCR (Liver)	4
KABA *ET AL*., 2010	FRANCE	RT-PCR (Liver)	2,5
ROSE *ET AL*., 2011	FRANCE	RT-PCR (Liver)	4

Recent prevalence studies in Europe (2008-2012) using ELISA (Enzyme-Linked ImmunoSorbent Assay) and RT-PCR (Reverse Transcription-Polymerase Chain Reaction) in pigs and products thereof are showed in Table 5. Ig G anti-HEV investigated in meat juice samples using ELISA test range between the Swiss 49% (Wacheck *et al*., 2012a) and the Spanish 64 % (Casas *et al*., 2011). Values comprised between the French 31% (Rose *et al.*, 2011) and the Italian 87% (Di Bartolo *et al.,* 2011) are found in sera samples. Using molecular methods HEV prevalence in bile samples varies between the two Italian 6.3% (Masia *et al*., 2009) and 51.1% (Di Bartolo *et al*., 2011). Considering molecular analyses on fecal samples minimum Belgian value of 7% (Hakze-Van Der Honing *et al*., 2011) and maximum Danish of 49% (Breum *et al.*, 2010) was recorded. Data from liver samples using molecular methods range from the German and Frech 4% (Wenzel *et al*., 2011; Rose *et al.*, 2011) to the Hungarian 31% (Forgách *et al*., 2010).

Salmonella is a zoonotic pathogen and one of the major causes of food-borne illnesses in humans. In 2009, 10’9884 cases of salmonellosis in humans were reported in the EU and 1325 cases in Switzerland (EFSA, 2011). Pigs are usually asymptomatic carriers of different salmonella serovars, and thus, isolation of any salmonella serovar in pork is regarded as public health hazard (Boyen *et al*., 2008; EFSA, 2006). In the EU, regulation (EC) No 2160/2003 on the control of *Salmonella* and other specified food-borne zoonotic agents gives the setting for the reduction of the prevalence of *Salmonella* in pigs (Regulation (EC) No 2160/2003). In Switzerland, no such control or monitoring regulations for the pig production exist.

Materials and Methods

Between September and October 2011 in nine sampling days a total of 400 samples were taken in a Swiss slaughterhouse sited in Zürich with an average processing capacity of 850 slaughtered pigs per week. To investigate hepatitis E virus (HEV) and *Salmonella* spp. seroprevalence in the healthy swine population with unknown exposure, samples consisting in diaphragm muscle (approximately 10 grams per sample) were taken from slaughtered pigs. Sampled pigs were about six months old with an average weight of 80 Kg per half-carcass. According to sampling design on each collecting day from 14 to 42 pairs of samples were collected and then divided in two groups. From each running batch on the slaughter line, three to five different pieces of diaphragm muscle were collected and pooled in a stomacher sterile bag forming the first group and another sample from the same batch was collected and put in a stomacher sterile bag as individual sample forming the second group. Samples originated from 167 different producers located in 136 different villages geographically distributed for a 12.5 % in the Northwest, 61.7 % in the Central and 72.7 % in the Northeast part of Switzerland. Diaphragm muscle samples were collected directly from the hooked thoracic organs immediately after opening the chest cavity from the butcher. For this purpose scissors previously sterilized in 96% ethanol solution were used and dipped in hot water after each sampling. Samples were transported cooled to the laboratory of the Institute for Food Safety and Hygiene, Vetsuisse Faculty of Zürich (Switzerland) and immediately stored at -20 °C until processing.

Between 200 and 1000 µL of meat juice was then obtained from each pooled sample as well as individual sample by thawing - squeezing of the diaphragm tissue and stored at -20°C until tested. For detection of swine HEV and

Salmonella spp. seroprevalence enzyme-linked immunosorbent assays (ELISA) were used. ELISA tests were performed at the Institute of Food Hygiene, Faculty of Veterinary Medicine, Ludwig-Maximilians University, Munich, Germany.

Swine meat juice were tested for HEV by the commercial kit ELISA Priocheck® HEV Ab porcine (Prionics, Schlieren, Switzerland) intended for detection of antibodies directed against hepatitis E virus following a four steps protocol consisting of sample preparation, incubation, conjugate incubation and detection.
In brief, 10 µl of meat juice were diluted with 90 µl dilution buffer and brought onto the microwell plate (sample preparation). After an incubation time of 60 minutes at 37°C (incubation) the plate was washed for four times and the conjugate was added. Conjugate incubation (30 minutes at 37°C) was followed by washing and addition of the substrate. The substrate reaction was stopped after 30 minutes at room temperature (detection) and the reaction was read using Microplate Reader 680 (BioRad, Hercules, USA) at 450 nm within 60 minutes.

The interpretation of the results followed the manufacturer's instructions. Thus, samples with an optical density (OD) above or equal to the cut off value are considered as positive, samples with an OD between the OD of the mean of the cut off control and the cut off value are considered as questionable, and samples with an OD below the mean of the cut off control are negative. The Cut Off value was calculated as mean OD_{450} of the Cut-Off control multiplied with 1.2 following this formula:

$$mean\ OD_{450\ nm}\ of\ Cut\text{-}Off\ Control * 1.2 = cut\ off$$

Following the manufacturer's instructions all meat juice samples were analyzed on the presence of anti-*Salmonella* antibodies (SALMOTYPE® Pig Screen Labor Diagnostik GmbH Leipzig, Leipzig, Germany). Briefly, 10 µl of meat juice were diluted with 90 µl of dilution buffer, brought onto the microwell plate and incubated for 60 minutes at room temperature. After washing the plate for three times the conjugate was added and incubated (30 minutes at room temperature). This was followed by another washing step and addition of the substrate. The substrate reaction was stopped after 10 minutes and the OD was measured using Microplate Reader 680 (BioRad). OD results were interpreted according to the manufacturer's instructions: samples with an OD% value above or equal to 20 are considered positive, samples with an OD% value between 10 and 20 are considered questionable and samples with an OD% value less than 10 are negative. The sample OD% value was calculated according to the following formula:

$$Sample\ OD\%\ Value = (OD_{sample} - MV\ OD_{NC} / MV\ OD_{PC} - MVOD_{NC})\ 72.1\ OD\ \%$$

Results and Discussion

Taking into account the sampling design and the sensitivity and specificity of the serological tests used in this study, 120 out of 200 pooled samples and 97 out of 200 corresponding individual samples collected from healthy Swiss pigs at slaughter tested positive for the presence of antibodies against HEV in their meat juice. Estimated seroprevalence at farm-level (pooled samples) and individual pig-level (individual samples) was therefore 60.0 % and 48.5 %, respectively. Moreover in view of sampling design, pooling strategy showed a higher detection rate compared to the individual one with a 20.0 % versus 7.0 %. Pooling the samples on herd level gave a statistically significant higher (*P<0.0001,* Fisher's test) detection rate (40/200) compared to individual samples (14/200).

In 18 of 200 pooled samples and in 10 of 200 individual samples results were considered questionable (Table 6).

Table 6. Detection rate of anti-HEV and anti-*Salmonella* antibodies in pooled and individual meat juice samples from slaughtering pigs in Switzerland using Priocheck® HEV Ab porcine (Prionics) and SALMOTYPE® Pig Screen (Labor Diagnostik Leipzig).

Sampling method (n=200)	ELISA test kit			
	Priocheck® HEV Ab porcine (%)		SALMOTYPE® Pig Screen (%)	
	positive	questionable	positive	questionable
pooled	120 (60.0)	9 (4.5)	8 (4.0)	0
individual	97(49.0)	6 (3.0)	12 (6.0)	0
Pooled and individual[1]	74 (37.0)		0	

[1]detection rate of antibodies in the paired pooled and individual samples of one producer

No standardized methods are available for detection of HEV in meat and meat products, all of the described tissue culture systems are limited as they are inefficient and relay on high inoculation titres. Therefore molecular methods as conventional PCR or real time PCR are preferred for HEV detection in food. Some of ELISA available kits can detect anti-HEV immunoglobulins independently from the analyzed species thus also enabling testing of pigs and other animal species. In other cases, antigens from human assay have been used in combinations with species-specific secondary antibodies for serological testing of animal species. It also should be considered that RNA presence does not necessary correlate with the infectious titre of viruses.

Several descriptive studies have been reported in literature on HEV prevalence in swine herds in different countries and the different nature of the collected information varies among studies in terms of samples used (sera, livers, meat juice) and methodologies adopted for detection (adapted-ELISA, ELISA, RT-PCR). Nevertheless similar findings have been recently reported in the European Countries. Di Bartolo *et al.* (2011) testing by an adapted-ELISA showed an Italian pig seroprevalence of 87% (40 of 46 samples) in sera and testing by RT-PCR showed a prevalence of 51 % (23 of 45 samples) in bile, 33 % (16 of 48 samples) in feces and 20 % (10 of 48 samples) of liver samples. The French seroprevalence was estimated (using an adapted-ELISA) as 65% on farm-level and 31% on individual pigs-level while HEV-RNA positive livers (by RT-PCR) were found in 4% of pigs and 24% of pig tested farms (Rose *et al.,* 2011). Wacheck *et al*. (2012b) reported a German seroprevalence of 68 % analyzing meat juice and sera samples using the ELISA assay for swine anti-HEV antibodies and Wenzel *et al*. (2011) reported a prevalence of 8% in German pig livers using RT-qPCR method. Commercially sold porcine livers have been found to contain HEV RNA, with

detection rates of 6.5% in The Netherlands, (Rutjes *et al*., 2007) 1.9 % in Japan (Yazaki *et al*., 2003) and 11 % in the USA (Feagins *et al*., 2007). Our findings about the detected Swiss HEV seroprevalence seem to reflect the European prevalence as reported in several studies.

With regard to *Salmonella* results, 8 out of 200 pooled samples and 12 out of 200 corresponding individual samples tested positive for the presence of antibodies against *Salmonella* showing a seroprevalence in Swiss pig population of 4 % on farm-level and 6 % on pig-level (Table 6).

There was no congruency between the pooled and individual samples on herd level. Thus, the seroprevalence in the present study on herd and animal level was 4.0% and 6.0%, respectively. None of the samples gave a questionable result. This finding is in accordance with our previous studies regarding the occurrence of *Salmonella* spp. in the Swiss pig population whereas *Salmonella* spp. have been detected from pig tonsils by cultural method in 5.6 % (14 of 250 tonsil samples) on individual level and 11.1 % (14 of 126 tested batches) on farm level (Sarno *et al*., 2012).

Conclusion

The importance of the hepatitis E Virus is well documented in the last EFSA Scientific Opinion and the high prevalence found in Switzerland as well as on European level need to be elucidated. In our study all the tested pigs were about six months old corresponding to the period (Figure 5) of 22 weeks were Ig G anti-HEV and fecal shedding of the virus are higher. Young animals are more susceptible to the HEV infection, due to the loss of maternal immunity (Meng *et al*., 1997; Kanai *et al*., 2010) or to an incomplete or short-lasting protective immunity permitting continuous reinfection (Fernandez-Barredo *et al*., 2006).

On the other hand if the virus is present in the muscle it is important to understand how much dangerous it could be and what kind of impact it could have on public health considering that the EFSA interest was attracted. Evidence of foodborne infections as well as higher prevalence in at risk workers suggest the potential role of the pig population in the epidemiology of human infections and the similar distribution of similar genotypes in autochthonous human cases suggest a high potential for zoonotic transmission.

With regard to *Salmonella* spp. this study shows that only 12 of 200 tested pigs have antibodies anti-HEV and only 8 batches can be considered positive.

However, it must be considered that contamination with viral as well as bacterial pathogens might play a role in the contamination of the abattoir environment and carcasses, (Fredriksson-Ahomaa *et al*., 2009) and it is a source of infection for at-risk-workers. By the same token, prevention of contamination during slaughter is of major importance, in particular adherence to good hygiene practices and application of effective cleaning and disinfection procedures to prevent equipment contamination.

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Research study no. 3: High prevalence of antibodies against hepatitis E virus in sows serum samples collected between 1988 and 1991 in Switzerland.

Key words: Seroprevalence, Hepatitis E, Swine

Hepatitis E virus (HEV), the causative agent of an acute hepatitis (Pavio and Mansuy, 2010; Bonney *et al*., 2012), is a non-enveloped positive-stranded RNA virus of about 7.2 kb in length containing three open reading frames (ORFs) (Emerson and Purcell, 2003) and belonging to the *Hepeviridae* family (Emerson *et al*., 2004). HEV is classified into at least four major genotypes. Genotypes 1 and 2 are restricted to humans and mainly associated with waterborne outbreaks in developing countries with low hygienic standards. Genotypes 3 and 4 have been described in both humans and animals worldwide.

In recent years, there is growing evidence of zoonotic transmission (Anonymous, 2011), since domestic pigs, wild boars and other animals are considered to be reservoirs for HEV (Meng, 2010; Pavio and Mansuy, 2010; Meng, 2011). With regard to pigs, they are normally infected at the age of 4-8 weeks but usually no clinical signs are evident (Pavio *et al*., 2010). Although rare, food-borne transmission of HEV from animal products to humans is of emerging concern. Human infections were reported after consumption of contaminated Sika deer, wild boar and pig meat in Japan and recently after consumption of pig liver sausage in France (Tei *et al*., 2003; Colson *et al*., 2010; Colson *et al*., 2012).

Swine HEV was first isolated from pigs in 1997 (Meng *et al*., 1997), but retrospective serological studies on swine hepatitis E infection are rare in literature. In order to assess the occurrence of HEV in the Swiss pig population

before the first isolation, serum samples collected between 1988 and 1991 from healthy sows at slaughter were investigated for the presence of HEV antibodies.

Samples and serological analysis

A total of 240 serum samples from sows collected during a four-year period (1988 to 1991) were investigated. For all of these sows, data on geographical origin, abattoir, and date of slaughter were available. For each of the four years, samples from 60 animals originating from 20 cantons (three pigs per canton) were included. The origin of the 240 sampled sows was distributed almost throughout Switzerland and comprised 216 different villages (50-60 different villages for each of the four years). Additionally, serum samples from current slaughtered sows were collected. Thus, a total of 37 samples from 24 different producers were selected (one to two samples per producer). The majority of producers were distributed over the north and central part of Switzerland.

Samples were tested for anti-HEV immunoglobulin G (Ig G) using a commercial ELISA kit, Priocheck® HEV Ab porcine test (Prionics, Schlieren, Switzerland) (sensitivity 91.0%, specificity 94.0%). Briefly, 10 µl of samples were diluted 1:100 (dilution buffer) and brought onto the test plate coated with a recombinant HEV antigen of ORF2 and ORF3 of the genotypes 1 and 3. A positive, negative, and cut-off controls were included in each run. After incubation for 60 min at 37°C, microwell plates were washed four times with 300 µl of washing fluid and the conjugate (a peroxidase (POD) labeled anti-pig antibody, 100 µl) was added. Conjugate incubation (30 min, 37°C) was followed by washing and the addition of a chromogen (TMB) substrate (100 µl). Substrate reaction was stopped after 30 min at room temperature by adding 100 µl of stop solution. Color development was measured at 450 nm within 60 min (Tecan Group Ltd., Männedorf,

Switzerland). Interpretation of results followed manufacturer's instructions. Thus, samples with and optical density (OD_{450}) above or equal to the cut-off value (*mean* $OD_{450\ nm}$ *of Cut-Off Control * 1.2*) were considered positive. Samples with an OD_{450} between the OD_{450} of the mean of the cut-off controls and the cut-off value were considered doubtful and retested as recommended by the manufacturer.

Results and Discussion

Of the 240 serum samples collected between 1988 and 1991, 234 out of 240 (97.5%) tested positive for the presence of antibodies against HEV. Doubtful results were obtained for five samples. After retesting, three remained doubtful, one proved to be positive and one tested negative. Thus, only three samples (1.25%) yielded negative for antibodies against HEV .

Of the 37 samples collected in 2012, 24 out of 37 (64.9 %) tested positive. Doubtful results were obtained for six samples. After retesting, five were confirmed negative and one positive. Thus, 25 out of 37 (67.5 %) serum samples and 21 out of 24 (87.5 %) producers tested positive for the presence of antibodies against HEV (Table 7).

This is the first study reporting the occurrence of anti-HEV antibodies in pigs older than six months in Switzerland (Wacheck *et al*., 2012). Although our data show a high occurrence of Ig G anti-HEV in the sow population, hidden biases related to the aged samples cannot be discarded. This study also indicates that HEV circulates in Swiss pig herds since at least 1988.

Table 7: Prevalence of anti-HEV Ig G in sow serum samples from a four-year period (1988-1991) and 2012 in Switzerland.

Year	No. of analyzed sera	No. (%) of positive sera	No. (%) of negative sera	No. (%) of doubtful sera[a]
1988	60	57 (95%)	1 (1.7%)	2 (3.3%)
1989	60	58 (96.7%)	2 (3.3%)	0
1990	60	60 (100%)	0	0
1991	60	59 (98.3%)	0	1 (1.7%)
Total	**240**	**234 (97.5%)**	**3 (1.25%)**	**3 (1.25%)**
2012	**37**	**25 (67.5%)**	**12 (32.5%)**	**0**

[a] Sera samples remaining positive after retesting

Previous studies on HEV in sows were reported from Spain, Denmark, and the United Kingdom (UK). In retrospective studies from Spain, Seminati *et al*. (2008) found an anti-HEV Ig G prevalence of 60.8% in gilts and sows (1998-2000) and Casas *et al*. (2009) reported an average farm percentage of seropositive sows of 47.8% (1985 to 1997). Even higher seroprevalence rates ranging from 73.2% to 85.5% have been described in sows from Denmark and the UK (Banks *et al*., 2004; Breum *et al*., 2010).

In conclusion, HEV seems to be highly prevalent among sows since at least 1988 in Switzerland. Although HEV has become of interest in view of public health aspects including foodborne transmission only recently, our findings clearly indicate that HEV is circulating in the pig population already for many years.

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Research study no. 4: Detection of anti-HEV antobodies in saliva samples using a modified commercial ELISA: preliminary investigation.

Key words: Hepatitis E virus, Saliva, Prevalence, Swine

Saliva is the oral fluid originating mainly from three pairs of major salivary glands (parotid, mandibular and sublingual glands) and from a large number of minor salivary glands. Its functions consist in helping bolus formation by moistening food, protecting the oral mucosa against mechanical damage, playing a role in the preliminary digestion of food through the presence of α-amylase and other enzymes. The various compounds of saliva are inorganic (ions), organic compounds (non-protein and lipids), hormones and protein/polypeptide. Among these, the immunoglobulins have function of protection; IgA are mainly produced by the B-lymphocytes present near the salivary glands and Ig G and IgM are mainly derived from crevicular fluid or from plasma leakage.

Oral fluid samples have already been used in human medicine for the diagnosis or detection of a variety of infectious agents (Malamud *et al*., 1992), hormones (Lippi *et al*., 2009), and drugs (Danhof *et al*., 1978). Although oral fluid testing has not been widely applied to livestock health and wellness management, veterinary literature on the presence of antibodies, pathogens, and acute phase proteins in oral fluids from animals reflects the findings in human beings (Prickett and Zimmerman, 2010). Increasingly, oral fluid samples have been used for the surveillance of Porcine Reproductive and Respiratory Syndrome virus (PRRSV) infections in commercial swine operations using polymerase chain reaction (PCR)-based assays (Chittick *et al*., 2011; Kittawornrat *et al*., 2010). While PCR-

based assays are useful for detecting the circulation of PRRSV, antibody-based assays are informative regarding herd immunity and history of prior infection.

Diagnostic strategy using oral fluid has already been adopted in swine literature. Kittawornrat *et al*. (2012) suggests that the Ig G oral fluid ELISA can provide efficient, cost-effective PRRSV monitoring in commercial herds and PRRSV surveillance programs.

Thus, the purpose of this preliminary investigation was to evaluate the use of oral fluid for the assessment of health and diagnosis of hepatitis E in pigs. Moreover the diagnostic performance of a modified commercial ELISA was evaluated.

Materials and Methods

A total of 20 oral fluid samples were collected from five Swiss pens of pigs by allowing access to a short length of cotton rope suspended in the pen. After about 30 minutes of pig interaction (biting and chewing) with rope, oral fluid was extracted by squeezing the rope manually to release the liquid in a small sterile tube. Samples were chilled immediately following collection. Upon arrival at the laboratory, sample fluids were clarified by centrifugation (5010 rpm, 15min, 20°C) and stored at -20°C.

Samples were tested for anti-HEV immunoglobulin G (Ig G) using the commercial ELISA kit, Priocheck® HEV Ab porcine test (Prionics, Schlieren, Switzerland) (sensitivity 91.0%, specificity 94.0%) modified to calibrate the reactivity of the assay to the lower concentration of Ig G present in the oral fluid compare to the high concentration of serum and meat juice. Briefly, 10 µl of samples were brought onto the test plate coated with a recombinant HEV antigen of ORF2 and ORF3 of the genotypes 1 and 3 without dilution. The positive, negative and cut-off controls were diluted according to manufacturer's instruction (1:100) and included in the run. After incubation for 60 min at 37°C, microwell plates were washed four times with 300 µl of washing fluid and the conjugate (a peroxidase (POD) labeled anti-pig antibody, 100 µl) was added. Conjugate incubation (30 min, 37°C) was followed by washing and the addition of a chromogen (TMB) substrate (100 µl). Substrate reaction was stopped after 30 min at room temperature by adding 100 µl of stop solution. Color development was measured at 450 nm within 60 min (Tecan Group Ltd., Männedorf, Switzerland). Interpretation of results followed manufacturer's instructions. Thus, samples with and optical density (OD_{450}) above or equal to the cut-off value (*mean* $OD_{450\ nm}$ *of*

*Cut-Off Control * 1.2*) were considered positive. Samples with an OD_{450} between the OD_{450} of the mean of the cut-off controls and the cut-off value were considered doubtful and retested as recommended by the manufacturer.

Results and Discussion

Ig G anti-HEV from oral fluid pen-based samples by using a modified commercial ELISA provided 13 positive (65%) out of 20 samples.

The early work led to the conclusion that oral fluid samples might be a good predictor of the healthy status of swine herds and might provide an efficient, cost-effective approach to help the official veterinarians to evaluate the status of pigs to be slaughtered. In particular could be used as a valid aid for meat official veterinarians as FCI (food chain information are a set of declarations including epidemiological data, herd health data, production data) to classify herds intended for slaughter into food safety risk categories so that slaughter procedures and /or decisions for fitness for consumption can be adapted to the risk category.

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Research study no. 5: Lack of evidence so far for carbapenemase-producing *Enterobacteriaceae* in food-producing animals in Switzerland.

Keywords: Carbapenemase-producing *Enterobacteriaceae*, Swine.

The increasing prevalence of *Enterobacteriaceae* that produce extended-spectrum-β-lactamases (ESBLs) undermines the efficacy of many β-lactam therapies based on penicillins and cephalosporins (Pitout and Laupland, 2008) and render carbapenems (imipenem, meropenem, ertapenem and doripenem) in human medicine crucial drugs of last resort for the treatment of infections due to multi-resistant gram-negative bacteria (Queenan and Bush, 2007). However, carbapenemase-producing *Enterobacteriaceae* have been increasingly reported world-wide (Nordmann *et al*., 2011), a development which is observed with great concern by the scientific and medical community and threatens to become a public health problem of global dimension (Akova *et al*., 2012).

Carbapenemases are a diverse group of β-lactamases belonging to the Ambler classes A, B, and D or Bush groups 2f, 3 and 2d, accordingly (Amber *et al*., 1991; Bush and Jacoby, 2010). Class A carbapenemases (Bush group 2f) include the serine β-lactamases NmcA, Sme, IMI-1 and SFC-1 which are chromosomally encoded, as well as the clinically common plasmid encoded KPC enzymes. Carbapenemases of this class are inhibited by clavulanic acid. Class B carbapenemases (Bush group 3) comprise the integron-encoded VIM-types, the IMP-, GIM-1, SPM-1- and SIM-types of enzymes, and the plasmid encoded NDM-1 carbapenemase. These metallo- β-lactamases are inhibited by EDTA but

not by clavulanic acid. Class D (Bush group 2d) consists of OXA-48 type carbapenemases, which are plasmid encoded, and not inhibited by EDTA and not or only weakly inhibited by clavulanic acid. Due to the heterogeneity of carbapenemases, their highly variable substrate spectra and their coexistence with other β-lactamases, the detection of carrier strains is a major technical challenge, which is why the actual prevalence of carbapenemase-producers remains unknown (Nordmann *et al*., 2011).

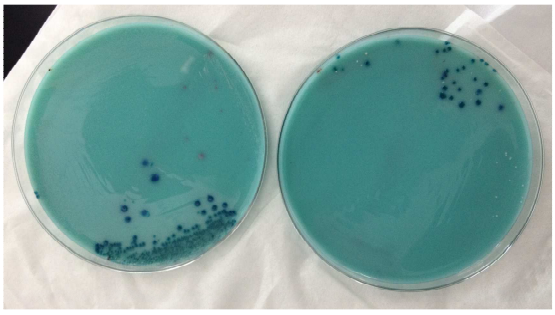
Even carbapenem or related antimicrobials are not licensed for the use in animals, carbapenem resistant bacteria, after being introduced into the animal population, could be selected by the use of other (licensed) beta-lactam antibiotics. Recent reports prove that the intestinal flora of pigs (Fisher *et al*., 2012) and cattle (Poirel *et al*., 2012) constitute a possible reservoir of carbapenemase producers. Because of possible transmission of resistance genes from livestock via the food chain into the human community, these findings are particularly alarming and constitute a crucial public health issue. As there are no novel β-lactams in development, early identification of carbapenemase producers in humans as well as in animals is of utmost importance.

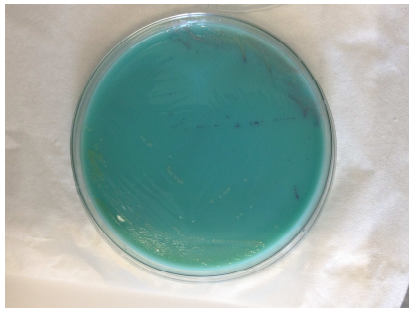
Materials and Methods

To screen for the occurrence of carbapenemase-producing *Enterobacteriaceae* in food-producing animals fecal samples were collected from March to May 2012 from 460 individual healthy food-producing animals at slaughter in Switzerland: 200 fattening pigs, 150 cattle (75<12 months; 75> months) and 110 sheep (97<12 months; 13>12 months). To prevent sample clustering, only one animal per farm was sampled. The farms were geographically distributed in the north western, central and north eastern part of Switzerland. Sampling was done with one swab per animal at a big slaughterhouse (on average 250 pigs per hours, 150 cattle per hour, 60 sheep per hour). Afterwards, the swabs were put into stomacher bags, transported under chilled conditions to the laboratory and processed within 3 hours. Furthermore, 99 herd-level pooled fecal samples of chicken were collected at the entry of a big poultry slaughterhouse (on average 50000 animals per day) from the crates of 99 poultry flocks (approximately 6000 chicken per flock) distributed throughout Switzerland. These samples were sent directly from the slaughterhouse to the laboratory.

Each sample (about 1 gr) was incubated for 24 hours at 37 °C in 10 ml of EE Broth (BD, Franklin Lakes, USA) for enrichment. The enriched fecal sample (10 µl) were inoculated onto Brillance CRE agar (Oxoid, Hampshire, UK) and incubated at 37 °C for 24 hours under aerobic conditions.

Figures 14-15. The two-chromogen system differentiates *E. coli* (Figure 15: pale pink colonies) from the *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter* (KESC) group (Figure 14 on the left: blue colonies).





From each sample with growth on Brillance CRE agar, all colonies with different colony morphology were picked and subcultured on sheep blood agar (Difco laboratories; 5% sheep blood, SB055, Oxoid) at 37 °C for 24 hours. Oxidase-negative isolates were thereafter subjected to identification by API ID 32 E (bioMérieux, Marcy l' Etoile, Fance).

The isolated strains were subjected to antimicrobial susceptibility testing on Mueller-Hinton agar plates using E-Test strips containing imipenem alone and in combination with EDTA (bioMérieux, Marcy l' Etoile, France) in order to gain minimal inhibitory concentrations (MIC) for imipenem as well as preliminary discrimination between serine and metallo- β-lactamases. Moreover, for all strains PCR assays for detection of bla_{MBL} genes (bla_{VIM}, bla_{GIM}, bla_{SPM}, bla_{SIM}, bla_{NDM-1}) as well as bla serine-carbapenemase genes (bla_{IMI}, bla_{KPC}, bla_{OXA-48}, $bla_{OXA-23-like}$, $bla_{OXA-40-like}$, $bla_{OXA-51-like}$, $bla_{OXA-58-like}$) were performed. DNA was extracted by standard heat lysis protocol. Thereafter, specific primer sets (custom-synthesized by Microsynth, Balgach, Switzerland) and the PCR conditions described were used (Woodford, 2010; Nordmann *et al.*, 2011). Positive controls were integrated. Resulting amplicons wee purified using the PCR Purification Kit (QIAGEN, Courtaboeuf, France) according to the manufacturer's

recommendations. Custom-sequencing was performed by Microsynth (Balgach, Switzerland) and the nucleotide and protein sequences were analyzed with Codon Code Aligner V. 3.7.1.1. For database searches the BLASTIN program of NBCI (http://www.ncbi.nlm.nih.gov/blast/) was used.

Results and Discussion

Fecal samples of 200 pigs, 150 cattle, 110 sheep, and 99 pooled fecal samples from crates of 99 poultry flocks were investigated to determine the occurrence of carbapenemase-producing *Enterobacteriaceae* in food-producing animals in Switzerland. From 16 samples (3 sheep, 5 cattle and 8 puoltry) colonies growing on Brillance CRE agar were found. From these samples 20 colonies were subcultured and subjected to an oxidase-test.

Sixteen out of the 20 colonies were oxidase negative and used for further identification. Three isolates turned out to be *Stenotrophomonas maltophilia*, 4 to be *Acinetobacter baumanii*, 4 to be *E. coli* and 5 to be *Citrobacter freundii*. All isolates belonging to the *Enterobacteriaceae* (*E. coli* and *C. freundii*) were selected for further characterization. Minimal inhibitory concentrations for imipenem ranged between 0.19 and 1.5 μg/ml. Moreover, E-test strips containing imipenem in combination with EDTA were used. This double synergy test is a simple method to detect metallo- β-lactamases (MLB). None of the tested isolates produced MLB's. Moreover, PCRs for all *bla* genes tested were negative for all 9 strains.

A technical aspect worth being discussed is why these non-carbapenemase-producing isolates were able to grow on Brillance CRE agar. Three of the four *E. coli* isolates turned out to be ESBL-producers (data not shown), which was a plausible reason for their growth capacity on Brillance CRE agar. For the *C. freundii* strains, it could be shown that the (relatively small) amount of imipenem supplemented to the Brillance CRE agar was responsible for an induction of their chromosomal AmpC β-lactamase. This induction gave rise to a reversible augmentation of the MIC of imipenem from 0.19 to up to 1.5 μg/ml and thus

allowed growth on CRE agar. Subculturing such induced bacteria on blood agar caused their reduced susceptibility to drop back to the normal MIC level of 0.19 μg/ml.

Based on the results of this study, no evidence for the occurrence of carbapenemase-producing *Enterobacteriaceae* in food-producing animals in Switzerland needs currently to be postulated. Further studies, however, covering all regions of Switzerland are necessary to get a complete picture and to assess future trends.

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