

Isolation and Antimicrobial Testing of *Aeromonas* spp., *Citrobacter* spp., *Cronobacter* spp., *Enterobacter* spp., *Escherichia* spp., *Klebsiella* spp., and *Trabulsiella* spp. from the Gallbladder of Pigs

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Abstract

The presence of Gram-negative bacteria species, other than *Salmonella* spp., in the gallbladder of pigs was examined. Isolated Gram-negative bacteria were assigned to species using the Microgen™ GnA+B-ID Systems. Of the 64 isolated strains 43 were identified as *Escherichia coli*, seven as *Enterobacter* spp., three each as *Klebsiella* spp., *Citrobacter freundii*, *Aeromonas hydrophila* and *Cronobacter sakazakii* and one each as *Escherichia fergusonii* and *Trabulsiella guamensis*. Their antibiograms showed very high resistance to ampicillin, amoxicillin, tetracycline, chloramphenicol and sulfamethoxazole/trimethoprim. It was concluded that the pigs' gallbladder is a reservoir of potentially pathogenic Gram-negative bacteria for pork consumers.

Key words: aztreonam, cholecystitis, doripenem, microflora of gallbladder, potentially pathogenic Gram-negative bacteria

Enteric bacteria have acquired the genetic ability to resist the defence mechanisms of the digestive system, some of which are gastric secretions, hydrochloric acid and bile, variations in pH, low oxygen levels, nutrient limitations and elevated osmolarity (Chowdhury *et al.*, 1996). By this ability, they are either pathogenic or potentially pathogenic for their host. If such bacteria colonize the gallbladder of pigs, they could become pork contaminants and a risk to consumers (Gunn, 2000). Bile is for some bacteria the regulating factor of their survival in the intestinal tract, thus a regulator of gut colonization. They survive the killing effect of bile, but also antibiotics and the host's immune response by forming protective biofilms (Begley *et al.*, 2005; Jensen *et al.*, 2010). Hence, the microflora of the gallbladder is evidence of an animal's intermittent gut colonization by potential pathogens.

Therefore, the objective was to assess Gram-negative bacteria of the pig's gallbladder, as risks to pork consumers.

Swab samples collected at slaughter from 145 randomly selected pigs' gallbladders, originating from

15 finishing farms located in Central Greece were bacteriologically examined.

Samples were enriched in Buffered Peptone Water (BPW Oxoid, England) for 18 ± 2 h at 37°C and subcultured on Columbia Blood Agar (CBA) and MacConkey agar (Oxoid, England). All morphologically different colonies were subcultured on CBA and after 24 h at 37°C, they were examined by Gram's stain. Gram-negative rods were tested for oxidase production (Bactident Oxidase Merck, Germany) and further examined as recommended by Quinn *et al.* (1994). In total, 79 Gram-negative isolates were selected for speciation using the Microgen™ GnA+B-ID (Microgen Bioproducts Ltd, UK) System.

Sixty four non *Salmonella* species were tested against 24 antimicrobial agents using the disk diffusion method, as described elsewhere (Evangelopoulou *et al.*, 2014a). The selection of the antimicrobials was based on their use for treating animal and human infections. They were amoxicillin (30 µg), amoxicillin/clavulanic acid (20/10 µg), ampicillin (10 µg), ampicillin/sulbactam (10/10 µg), aztreonam (30 µg), cefotaxime (30 µg),

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cefoxitin (30 µg), ceftazidime (30 µg), ceftiofur (30 µg), ceftriaxone (30 µg), cefuroxime (30 µg), chloramphenicol (30 µg), colistin (50 µg), doripenem (10 µg), enrofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), penicillin G (10 µg), rifampin (30 µg), sulfamethoxazole/trimethoprim (23.75/1.25 µg), tetracycline (30 µg) and tigecycline (15 µg). Isolates exhibiting resistance to at least three antimicrobial agents belonging to different antimicrobial classes were considered multidrug resistant (MDR) (Schwarz *et al.*, 2010).

Of the 93 (64.1%) bacteria positive gallbladders, 79 Gram-negative bacteria were selected for speciation. Of them 15 were identified as *Salmonella* spp. and 64 were *Escherichia coli* (43), *Enterobacter* spp. (7), three each *Klebsiella* spp., *Citrobacter freundii*, *Aeromonas hydrophila* and *Cronobacter sakazakii* and one

each *E. fergusonii* and *T. guamensis* (Table I). The three *A. hydrophila* isolates exhibited beta-haemolysis after 48 hours of incubation.

High antimicrobial resistance was observed for ampicillin, amoxicillin, tetracycline, chloramphenicol and sulfamethoxazole/trimethoprim (Table I). Somewhat lower was resistance to cephalosporins, quinolones and aminoglycosides, as groups. All isolates, except one (*E. fergusonii*), were resistant to penicillin G and all were susceptible to aztreonam and doripenem used for human treatments. Sixty isolates were considered MDR (Table I).

Of the 79 Gram-negative 15 were *Salmonella* spp. published elsewhere (Evangelopoulou *et al.*, 2014b). Of the remaining, *E. coli* forming the normal intestinal microbiota was the predominant aerobic microorganism identified by the Microgen System. *E. coli* is helpful

Table I
Cumulative results of antimicrobial resistance of Gram-negative isolates recovered from pigs' gallbladder

	<i>Escherichia coli</i>	<i>Enterobacter aerogenes</i>	<i>Enterobacter gergoviae</i>	<i>Citrobacter freundii</i>	<i>Klebsiella pneumoniae</i>	<i>Klebsiella oxytoca</i>	<i>Cronobacter sakazakii</i>	<i>E. fergusonii</i>	<i>Trabulsilla guamensis</i>	<i>Aeromonas hydrophila</i>	Total No of resistant strains
AML	37	3	3	2	1	1	1	0	1	3	52
AMC	12(I)	3	0	1, 1(I)	0	1	0	0	1	3	9
AMP	37	3	3	2	1	1	2	0	1	3	53
SAM	4, 8(I)	1(I)	0	1	0	1	1	0	0	1	8
AZT	0	0	0	0	0	0	0	0	0	0	0
CTX	2(I)	0	0	0	0	0	0	0	0	3	3
FOX	1(I)	0	1(I)	2(I)	0	1	1(I)	0	1	3	5
CAZ	1(I)	0	0	1	0	0	0	0	0	0	1
EFT	1(I)	0	0	1(I)	0	0	0	0	0	3	3
CRO	1	0	0	1	0	0	0	0	0	3	5
CXM	1, 13(I)	3(I)	1(I)	3(I)	0	1(I)	2(I)	0	0	3	4
CT	0	0	0	1	0	1	0	0	0	0	2
C	31	2	0	2	0	2	0	0	0	3	40
DOR	0	0	0	0	0	0	0	0	0	0	0
ENR	2, 2(I)	0	0	1(I)	0	0	0	0	0	1(I)	2
E	39	3	4	3	1	2	3	1	1	3	60
CN	2, 1(I)	0	0	1	0	0	0	0	0	0	3
K	4, 7(I)	0	1(I)	1	0	1	0	0	0	2	8
NA	5, 4(I)	0	0	1	0	1	0	0	0	3	10
P	43	3	4	3	1	2	3	0	1	3	63
RD	21,19(I)	2, 1(I)	4	3	1	2	2, 1(I)	0	1	3	39
SXT	39	2	0	2	0	2	1	0	1	3	50
TE	39	2	0	2	1	2	2	0	1	3	52
TGC	9(I)	2(I)	1(I)	0	1(I)	1(I)	0	0	0	3	3
Total N.	43	3	4	3	1	2	3	1	1	3	64

AML Amoxicillin, AMC Amoxicillin-clavulanic acid, AMP Ampicillin, SAM Ampicillin-sulbactam, AZT Aztreonam, CTX Cefotaxime, FOX Cefoxitin, CAZ Ceftazidime, EFT Ceftiofur, CRO Ceftriaxone, CXM Cefuroxime, C Chloramphenicol, CT Colistin, ENR Enrofloxacin, E Erythromycin, CN Gentamicin, K Kanamycin, NA Nalidixic acid, P Penicillin G, RD Rifampin, SXT Sulfamethoxazole/Trimethoprim, TE Tetracycline, TGC Tigecycline-(I)= Intermediate Resistance

in the development of a normal mucosal immune system, the suppression of harmful bacteria by overtaking their attachment sites and the production of essential nutrients (Canny and McCormick, 2008). However, pathogenic strains could invade the gallbladder and lead to its inflammation (Gunn, 2000). These strains and *Klebsiella pneumoniae*, *Enterobacter* spp. and *Enterococcus* spp. are associated with infectious cholecystitis (Wang *et al.*, 2003; Abeysuriya *et al.*, 2008; Carpender and Gilpin, 2014). Thus, enteric bacteria have developed sophisticated mechanisms not only for resisting the effects of bile salts, but also using favorably this unique environment for their survival, thus becoming under certain circumstances potential pathogens.

Among them could also be *T. guamensis* and *C. freundii*. The two could be confused, due to their phenotypic and antigenic behaviour, with *Salmonella* spp. or *E. coli* (McWhorter *et al.*, 1991; Delgado *et al.*, 2013), if a variety of methods for their differentiation are not used. Specifically, *T. guamensis* does not rapidly ferment lactose or sucrose and produces abundantly H₂S, resembling phenotypically *Salmonella enterica* subspecies *diarizonae* and subspecies *hountanae* (McWhorter *et al.*, 1991). Although its clinical significance for animals is unknown, it could cause diarrhoea, as it does in man, resembling mild salmonellosis. Furthermore, carrier animals could be the source for these occasional human enteric problems.

C. freundii, a commensal microorganism of undocumented clinical importance to animals, is isolated from serious nosocomial infections in man (Nayar *et al.*, 2014). Its pathogenicity is attributed to multidrug resistance (Pepperell *et al.*, 2002), a property observed also in the present investigation (Table I). Evolutionary acquisition of resistance genes affects the course of an infectious disease, the evasion of the immune response and the events of host-pathogen interactions (Delgado *et al.*, 2013).

The same events could be important in human infections caused by multiresistant *Enterobacter* spp., as observed here, a bacterium easily acquiring resistance genes, mainly to beta-lactams, quinolones, tetracycline and chloramphenicol, thus emerging as a public health risk (Thiolas *et al.*, 2005; Boban *et al.*, 2011). The two species isolated here, *Enterobacter aerogenes* and *Enterobacter gergoviae*, are associated to infections of immunocompromised individuals (Boban *et al.*, 2011), as is also *Klebsiella* spp. showing phenotypic and DNA relatedness to *E. aerogenes* (Brisse *et al.*, 2006). *K. pneumoniae*, causing sporadic disease in individual pigs (AHVLA, 2012), is implicated in human pneumonia, urinary tract infections, neonatal septicemia and liver abscesses (Chang *et al.*, 2000; Bleich *et al.*, 2008).

C. sakazakii, previously a species of the genus *Enterobacter*, is reclassified as a new genus within the family

of Enterobacteriaceae (Iversen *et al.*, 2007). Although its pathogenic importance is unknown in animals and adult man, it has been recently implicated in fetal fatal meningitis, neurologic damage, brain abscess, septicemia, etc. (Healy *et al.*, 2010; Joseph and Forsythe, 2011). This association raises questions as to its role with immunocompromised pork consumers.

A. hydrophila, incriminated in a variety of human clinical conditions, such as gastroenteritis, septicemia, cellulitis, myonecrosis, peritonitis, hepatitis, pancreatic abscesses, respiratory, urogenital and eye infections of immunocompromised individuals, is also implicated in infections of poikilothermic animals (amphibians, reptiles and fish) (Janda and Abbott, 2010). However, it is more often isolated from food of plant and animal origin than animal disease (Queiroga *et al.*, 2012). *A. hydrophila* is producing haemolysins, enterotoxin (Ljungh *et al.*, 1981) and is multidrug resistant (Queiroga *et al.*, 2012), as it was observed here. Its isolation from pig gallbladders and its resistance to most of the antibiotics tested here, support its classification as an emerging pathogen for animals and man. Although the above microbes are opportunistic pathogens, their high resistance to commonly used antimicrobials makes them potential pathogens for man, and perhaps, unrecognized causes of reduced animal productivity.

Additionally, the multidrug resistance observed here indicates that pigs are a primary reservoir of multi-resistant bacteria. The use of antimicrobials in food producing helps faecal excretion of highly pathogenic Gram-negative enteric bacteria, such as *Salmonella*, eventually making pork carcasses the source of pork product contamination, thus consumer infections (Friendship *et al.*, 2009).

Observed high resistance above 60% to chloramphenicol, used in the treatment of human salmonellosis, but no longer used in animals in the EU (EVMP, 1994) could result from a variety of reasons. Although non-compliance of farmers is one reason, the number of farms participating (15 farms) is not supportive. Thus it could result from transfer of resistance genes between different bacteria species coding for similar classes of antimicrobials and/be residual caused by the persistence of resistance genes encoded in a microbial resistome (Sommer and Dantas, 2011). Persistent antimicrobial resistance is transferrable not only to pathogens, but also commensal bacteria or opportunistic pathogens, like the above, helping the development of a "superbug" (Thiolas *et al.*, 2005; Shailesh *et al.*, 2012; Frye and Jackson, 2013).

Such events in natural bacteria populations may have important implications in the evolution of bacteria and the means of evading the immune system, thus the outcome of infectious diseases (Delgado, *et al.*, 2013). Hence, pathogenic microorganisms can reside

transiently or permanently in the gallbladder of pigs, making it a reservoir of multidrug resistant Gram-negative bacteria contaminating pork products and infecting consumers.

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