Leakage of emerging clinically relevant multidrug-resistant Salmonella clones from pig farms

Patrícia Antunes¹², Joana Mourão², Nazaré Pestana² and Luísa Peixe²*

¹Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Rua Dr. Roberto Frias, 4200 Porto, Portugal; ²REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, no. 164, 4050-047 Porto, Portugal

*Corresponding author. Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, no. 164, 4050-047 Porto, Portugal. Tel: +351-22-2078972; Fax: +351-22-2003977; E-mail: lpeixe@ff.up.pt

Received 19 April 2011; returned 2 May 2011; revised 5 May 2011; accepted 9 May 2011

Objectives: To assess the presence of multidrug-resistant (MDR) Salmonella with human clinical relevance in pig farms from different regions of Portugal and to analyse their mobile genetic elements associated with antibiotic resistance.

Methods: Seventy-nine samples were collected from six piggeries and analysed for the presence of Salmonella. All isolates were examined for susceptibility to antimicrobial agents and representative isolates for resistance genes and class 1 integrons (PCR/restriction fragment length polymorphism). Clonality was determined by PFGE and multilocus sequence typing (MLST). Plasmid analysis included determination of size, content and characterization of the incompatibility group (rep-PCR and I-CeuI/S1-hybridization).

Results: Thirty Salmonella isolates were recovered from five samples (two manure, two waste lagoons and one animal feed) in half of the piggeries studied. All isolates were resistant to at least one antibiotic (tetracycline) and 97% to at least four antibiotics from different families. In 10 isolates representing different serogroup and resistance phenotype combinations a diversity of resistance genes and integrons was detected. These isolates belonged to the internationally widespread Salmonella Rissen (ST469) and Salmonella Typhimurium DT104 (ST19) clones, as well as to the emerging Salmonella Typhimurium monophasic variant with examples of Spanish (carrying a sul3-atypical integron within IncA/C plasmids, here assigned to ST19) and European (ASSuT phenotype, assigned to ST34) clones.

Conclusions: This is one of the few studies reporting emerging MDR Salmonella clones and the first one detecting Salmonella Typhimurium monophasic variant in the pig production setting. The survival of these strains in manure and waste lagoons is of concern, since these environments might allow spread of MDR bacteria beyond pig farms' boundaries.

Keywords: antimicrobial agents, piggeries, PFGE clones, MLST

Introduction

In recent years, changing trends in salmonellosis and associated serotypes have been observed, with a marked increase in certain multidrug-resistant (MDR) clones of Salmonella Typhimurium and its monophasic variant in different countries.¹² Among these, Salmonella Typhimurium DT104, OXA-30-producing Salmonella Typhimurium clone and Salmonella Typhimurium monophasic variant (4,[5],12:i:-) are the most frequently reported in Europe.²³ The latter, which have recently emerged worldwide, range from pan-susceptible (USA, Brazil) to MDR (Europe) strains and seem to be largely distributed in animal hosts and their derived products (e.g. pork, poultry products, cattle).² Although there is evidence that the animal setting seems to be a reservoir of MDR strains, data concerning the spread of emerging Salmonella MDR clones with features of clinical interest within and off pig farms are still missing. Here, we assessed the presence of MDR Salmonella with human clinical relevance on pig farms from different regions of Portugal and analysed their mobile genetic elements associated with antibiotic resistance.

Materials and methods

Sample processing and Salmonella identification

Seventy-nine samples were collected from six geographically separated Portuguese piggeries (five with intensive and one with extensive production) during 2006 and 2007. They included samples from pigs (n=21; faeces, nostril/surface swabs), feed/medicines (n=22; feed, water, medicine, antiseptics), residues (n=17; swine waste lagoons, residues, feed).
residual waters, manure, septic tank) and piggery facilities (n = 19; water, walls/floors dust, soil). The presence of Salmonella was screened by the conventional method following ISO 6579, which includes two stages of enrichment and plating out in two selective solid media (four plates per sample). Suspected colonies (up to five from each of the four plates) were identified by slide agglutination (Salmonella O poly antisera and serogroup-specific antisera for serogroups B, C1 and D; BD, USA), biochemical tests (API 20 GN; bioMérieux, Marcy l’Étoile, France) and a PCR assay (targeting the invA gene and a DT104/1302 phage type-specific DNA sequence). The serotypes of representative isolates were determined at the National Centre of Salmonella. The Salmonella Typhimurium monophasic variant (4,[5],12:i:-) isolates were confirmed using PCR as previously described.

**Antimicrobial susceptibility testing**

All Salmonella isolates were tested for susceptibility to 10 antimicrobial agents (μg) (amoxicillin (10), gentamicin (10), kanamycin (30), streptomycin (10), ciprofloxacin (5), nalidixic acid (30), chloramphenicol (30), tetracycline (30), sulfamethoxazole (300) and trimethoprim (5)) by the disc diffusion method following CLSI standards. Escherichia coli ATCC 25922 was used as the control strain. Amoxicillin-resistant isolates were further tested for susceptibility to several extended-spectrum β-lactams (ceftazidime, ceftriaxone, cefotaxime, cefepime, cefoxitin, aztreonam and imipenem) and the double disc synergy test (DDST) for ESBL detection was also conducted. From each sample, the selection of representative isolates for further studies was based on serogroup, presence of the invA gene and/or phage type DT104/1302 phage type-specific DNA sequence and antibiotic resistance phenotype.

**Characterization of antimicrobial resistance genes, integrons and plasmids**

Genes coding for resistance to sulfamethoxazole (sul1, sul2 and sul3), tetracycline (tet(A), tet(B) and tet(G)), chloramphenicol (floR, cmlA and catA), amoxicillin (blaTEM, blOPE-1 and blOXA-20), gentamicin (aac(3)-IV), streptomycin (aadA and strA-strB) and trimethoprim (dfrA1 and dfrA12) were searched for by PCR using primers and conditions previously described. The detection and characterization of class 1 integrons was performed by PCR and restriction fragment length polymorphism (RFLP) analysis with TaqI as previously reported. Positive and negative controls were included in all PCRs. Plasmid content and the genetic localization of integrons and sul genes were investigated by S1 nuclease (Takara Bio Inc., Shiga, Japan) digestion of total genomic DNA followed by PFGE. Identification of plasmid incompatibility groups was determined by a rep-PCR typing method including three additional PCR assays for the IncU, IncR and CoI groups. Southern blot hybridization was performed by standard methods using intI1, sul1, sul2, sul3 and rep intragenic probes, following the manufacturer’s instructions (Gene Images Alkphos Direct Labelling System KIt; Amersham G8/GE Healthcare Life Sciences UK Limited).

**PFGE and MLST analysis**

Clonal relatedness among isolates was assessed by PFGE following XbaI digestion of genomic DNA according to the standard 1 day protocol of the CDC. Salmonella enterica serotype Braenderup H9812 (CDC) was used as a molecular size marker. Multilocus sequence typing (MLST) analysis was performed using specific primers to amplify a set of seven housekeeping genes (araC, dnaN, hemD, hisD, purE, sucA and thrA) and sequence type (ST) was assigned according to the MLST database (http://mlst.warwick.ac.uk/mlst/).

**Results and discussion**

Sixty Salmonella isolates were recovered from five positive samples (two manure, two waste lagoons and one animal feed) collected on three pig farms with intensive production (one located in the north and two located in the south of Portugal). Forty-seven isolates were serogroup B, of which 22 were positive for the DT104/1302 phage type-specific region, and 13 were serogroup C1. All isolates were resistant to at least one antibiotic (tetracycline) and 58 out of 60 were resistant to at least four antibiotics from different families (ranging from four to eight antibiotics). Resistances to tetracycline (n = 60 isolates), streptomycin (n = 58), sulfamethoxazole (n = 57) and amoxicillin (n = 56) were the most frequently detected and might reflect the high usage of these antibiotics in food-producing animals. Lesser rates of resistance were observed for the other antimicrobial agents tested; chloramphenicol (n = 22), trimethoprim (n = 19), nalidixic acid (n = 10), gentamicin (n = 8), ciprofloxacin (n = 0) and kanamycin (n = 0). Susceptibility to extended-spectrum β-lactams and absence of extended-spectrum β-lactamases (ESBLs) and AmpC were observed. Because enrichment steps allowed selection of more than one isolate of the same strain from the same sample, 10 isolates representing different combinations of serogroup and antibiotic resistance phenotype were selected for further studies (Table 1). The selected isolates belonged to three Salmonella serotypes (four Rissen, two Typhimurium and four from its monophasic variant 4,[5],12:i:-) and four PFGE clones spread among different farms and samples (Figure 1 and Table 1). Among them a diversity of antibiotic resistance genes were detected. These strains harboured common MDR genetic elements (e.g. integrons) and shared clonal relationships with previously nationally widespread/emerging clones in humans and food products, showing their potential transmission along the food chain and their ability to cause human infections.

More than one resistant genotype or clone was detected within the same piggery, suggesting enrichment of the local metagenome with a broad range of drug-resistant strains and genetic elements, such as integrons. Isolates belonging to the emerging Salmonella Rissen clone (n = 4), detected in the manure of two geographically separated piggeries, were assigned to ST469, only previously reported in the few European isolates of the same serotype allocated in the MLST database (http://mlst.warwick.ac.uk/mlst/). The human acquisition of Salmonella Rissen was previously associated with tourism or international trade outside the EU, but the data of this and a few other studies suggest a more local source of infection with this emerging serotype. Different resistance patterns and the presence of a specific class 1 integrone (dfrA12-orfF-aadA2), located in IncR plasmids (35 kb), were observed, which may provide a selective advantage for this emergent serotype in the animal niche. Two isolates from the widely disseminated clone of Salmonella Typhimurium, DT104, showing the chromosomal located R-type ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline), were detected in environmental samples from one piggery (manure and waste lagoon), suggesting that, beside the classical dissemination routes (animals and foids of animal origin), environmental reservoirs might also play a role in the spread of such strains. Of particular interest was the
Table 1. Characterization of the *Salmonella* clones from Portuguese piggeries

<table>
<thead>
<tr>
<th>Serotype (phage type)/PFGE type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of isolates</th>
<th>MLST – ST (no. of isolates)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Date of isolation</th>
<th>Piggery</th>
<th>Sample</th>
<th>Resistance phenotype&lt;sup&gt;c&lt;/sup&gt;/resistance genes profile (no. of isolates; sample)</th>
<th>Class 1 integron genes (bp)</th>
<th>Class 1 integron and/or sul2 location Chr or PL (kb, Inc)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rissen/N</td>
<td>4</td>
<td>ST469 (n = 2)</td>
<td>2006</td>
<td>B, C</td>
<td>manure B1; manure C7</td>
<td>AMX, STR, SUL, TET, TMP&lt;br&gt;bla&lt;sub&gt;TEM&lt;/sub&gt;, aadA2, sul1, tet(A), df(A12 (n = 2; B1 and C7)&lt;br&gt;AMX, STR, TET, TMP&lt;br&gt;bla&lt;sub&gt;TEM&lt;/sub&gt;, tet(A) (n = 1; B1)</td>
<td>intI1, sul1&lt;br&gt;df(A12, orfR, aadA2 (2000)&lt;br&gt;intI1</td>
<td>PL (35, R)</td>
</tr>
<tr>
<td>Typhimurium (DT104/U302)/A</td>
<td>2</td>
<td>ST19 (n = 1)</td>
<td>2007</td>
<td>E</td>
<td>waste lagoon E32; manure E34</td>
<td>AMX, CHL, STR, SUL, TET&lt;br&gt;bla&lt;sub&gt;PSE-1&lt;/sub&gt;, floR, aadA2, sul1, tet(G) (n = 2; E32 and E34)</td>
<td>intI1, sul1&lt;br&gt;aadA2 (1000)&lt;br&gt;blaPSE-1 (1200)</td>
<td>Chr</td>
</tr>
<tr>
<td>Typhimurium (DT104/U302) 4,[5],12:i:-/O</td>
<td>2</td>
<td>ST19 (n = 1)</td>
<td>2006</td>
<td>C</td>
<td>manure C7</td>
<td>AMX, CHL, GEN, NAL, STR, SUL, TET, TMP&lt;br&gt;bla&lt;sub&gt;TEM&lt;/sub&gt;, cmlA1, aac(3)-IV, aadA2, sul1-sul2-sul3, tet(A), df(A12 (n = 1; C7)</td>
<td>intI1, sul1&lt;br&gt;df(A12, orfR, aadA2 (2000)&lt;br&gt;type III-sul3&lt;sup&gt;g&lt;/sup&gt;</td>
<td>PL (170, A/C)</td>
</tr>
<tr>
<td>Typhimurium 4,[5],12:i:-/Y</td>
<td>2</td>
<td>ST34 (n = 2)</td>
<td>2007</td>
<td>E</td>
<td>feed E2; manure E34</td>
<td>AMX, STR, SUL, TET&lt;br&gt;bla&lt;sub&gt;TEM&lt;/sub&gt;, strA-strB, sul2, tet(B) (n = 2; E2 and E34)</td>
<td>intI1, sul1&lt;br&gt;type III-sul3&lt;sup&gt;g&lt;/sup&gt;</td>
<td>PL (130, A/C)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Clones are designated by capital letters, as previously published.<sup>7,8</sup>

<sup>b</sup>Number of isolates submitted to MLST database.

<sup>c</sup>AMX, amoxicillin; CHL, chloramphenicol; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim.

<sup>d</sup>Chromosomal (Chr) and/or plasmid (PL) location of integrons and the sul2 gene was assessed by hybridization of I-CeuI/S1-digested genomic DNA using intI1, sul1, sul2, sul3 and rep probes.

<sup>e</sup>Structure of the type III sul3 integron: S′CS-estX-psp-aadA2-cmlA1-aadA1-qacH-IS440-sul3.<sup>8</sup>
Emerging MDR Salmonella clones in piggeries

Figure 1. PFGE patterns of Salmonella isolates from piggeries and others previously characterized. 

Acknowledgements

We are deeply grateful to Carla Novais for critical review of this paper prior to submission. We are also grateful to Alessandra Carattoli for kindly providing the positive controls for the IncII, IncE and ColE plasmids, to Centro Nacional de Salmonella (Lisboa, Portugal) for serotyping the strains and CDC for the PFGE protocols and the control strain Salmonella Braenderup H9812.

Funding

This work was partially supported by Fundação para a Ciência e a Tecnologia (FCT), which belongs to the Ministry of Science, Technology and Innovation from Portugal (POCI/AMB/61814/2004).

Transparency declarations

None to declare.

References

2 European Food Safety Authority. EFSA Panel on Biological Hazards (BIOHA2). Scientific opinion on monitoring and assessment of the public health risk of “Salmonella Typhimurium-like” strains. EFSA J 2010; 8: 1826.


