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EXTENDED SPECTRUM β-LACTAMASE SHV-12-PRODUCING SALMONELLA FROM POULTRY

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ABSTRACT

Salmonella strains isolated from poultry and poultry products over the period 2005-2006 have been investigated in order to ascertain the presence of Extended Spectrum Cephalosporins (ESC) resistance. Twelve (ESC)-resistant isolates (n=1 S. Enteritidis, n=1 S. Braenderup and n=10 S. Livingstone) were characterized as SHV-12-positive. The multi-drug resistant S. Livingstone SHV-12-producing isolates, untypeable by Pulsed-field gel electrophoresis (PFGE), showed a clonal relationship by Random Amplified Polymorphic DNA (RAPD) analysis. The SHV-12 β-lactamase is reported for the first time in Salmonella enterica strains isolated from poultry in Italy. The results suggest poultry as a source of Salmonella carrying Extended-Spectrum β-lactamases (ESBLs) genes and highlights the need of monitoring animal productions to prevent spreading of (ESC)-resistant strains.
Keywords: Salmonella; Extended Spectrum β-Lactamases; PFGE; RAPD-PCR; poultry.

INTRODUCTION

Non-typhoid Salmonella are among the most important causes of food-borne gastroenteritis worldwide, and poultry is commonly recognized as a reservoir of this zoonotic agent. Salmonella enterica serovar Livingstone has recently become in the last two years the most frequent serovar isolated from poultry in the Veneto Region of Italy and has been also commonly reported in Salmonella isolates from poultry in UK (Liebana et al., 2001). Although human outbreaks of S. Livingstone are relatively uncommon (Old D. C et al., 1994; Guerin et al., 2004), this serovar is potentially zoonotic and genotypic relatedness between human and poultry isolates has been reported in past studies (Old et al., 1995). Salmonella Livingstone (ESC)-resistant has been recently reported in a nosocomial outbreak in a neonatal ward in Tunisia (Bouallègue-Godet et al., 2005). In recent years, we have seen an emergence of Salmonella resistant to ESC (Hasman H. et al. 2005; Arlet G., 2006; Bertrand S., 2006), creating concern because third-generation cephalosporins, such as ceftriaxone, are the drugs of choice for invasive Salmonella infections particularly in children where the use of quinolones are not indicated (Paterson D. L., 2006).

Resistance to these antimicrobials is mainly mediated by Ambler class A Extended-Spectrum β-Lactamases (ESBLs) and plasmid-mediated Ambler class C enzymes. Salmonella (ESC)-resistant are isolated in particular from humans cases but also from animals and food, with poultry as primary food source (Riaño et al., 2006; Hasman et al. 2005; Bertrand at al., 2006). The finding of the same bla genes in Salmonella isolates from foodstuffs and humans suggests a possible source of human infection. However it is also possible that humans act as a reservoir of these genes in animals.
This study reports the characterisation of 12 strains of *Salmonella enterica* carrying a SHV-12 β-lactamase isolated in poultry in Italy in 2005-2006. In particular, the study shows the presence of genetically related clones of *S. Livingstone* SHV-12-producing strains scattered in different farms in the north-east of Italy, which is the main region for poultry production (ISTAT Source [http://www.istat.it]: 38% of national chicken production, 52% of turkey production).

**MATERIALS AND METHODS**

**Bacterial strains.** The Italian National Reference Laboratory NRL for salmonella at Istituto Zooprofilattico delle Venezie (Italy) receives *Salmonella* isolates from animals and foodstuffs for serotyping (approximately 2000 exams/year) through its network comprised of eleven diagnostic laboratories located in three regions of north-eastern part of Italy (Veneto, Friuli Venezia Giulia, Trentino Alto Adige). Moreover, it receives data and isolates for phage typing from all the national official veterinary laboratories. Isolates from poultry are mostly collected in the framework of mandatory salmonella monitoring and control programs according to Reg. 2160/2003. Approximately 2162 isolates from poultry were serotyped in the two years period 2005-2006.

**Serotyping and phage typing.** The isolates were serotyped on the basis of somatic O, phase 1 flagellar and phase 2 flagellar antigens by agglutination tests with commercial antisera (Staten Serum Institut-Denmark) as specified by Kauffmann-White scheme (Popoff, 2001). Phage typing was performed according to the recommendations of Health Protection Agency Service (Colindale, United Kingdom).

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility was assayed on 1840 *Salmonella* isolated from poultry over the period 2005-2006 by the disk diffusion method.
according to the recommendations of CLSI (formerly NCCLS). The following 16 antimicrobial
drugs (Becton Dickinson Microbiology Systems Cockeysville, MD, USA) were tested: colistin
(CL 10 µg), ampicillin (AMP 10 µg), tetracycline (TE 30 µg), kanamycin (KAN 30 µg),
gentamicin (GEN 10 µg), trimethoprim-sulfamethoxazole (SXT 23.75+1.25 µg), nalidixic acid
(NAL 30 µg), chloramphenicol (CHL 30 µg), streptomycin (STR 10 µg), ciprofloxacin (CIP 5
µg), ceftazidime (CAZ 30 µg), amoxicillin/clavulanic acid (AMC 30 µg), cefotaxime (CTX 30
µg), cephalotin (CF 30 µg), enrofloxacin (ENO 5 µg), sulfonamides (SUL 250 µg). All the
isolates that showed resistant and/or intermediate values to cefotaxime and/or ceftazidime were
confirmed for the ESBL phenotype by quantitative E-test special strips (AB, Biodisk, Solna,
Sweden) testing the synergistic effect of clavulanate with ceftazidime, cefotaxime and cefepime.

*E. coli* strain ATCC 35218 and *Klebsiella pneumoniae* ATCC 700603 were used as ESBL-
negative and ESBL-positive controls, respectively. The isolates with ESBL-positive phenotype
were further tested for antimicrobial susceptibility using a commercially available MIC technique
(Sensititre, Trek Diagnostic Systems Ltd, UK) according to CLSI guidelines.

**Molecular characterization of β-lactamases.** Total DNA was extracted with InstaGene matrix
kit (Bio-Rad) in accordance with the manufacturer’s recommendation. The isolates with a ESBL
phenotype were analysed by PCR for the presence of *bla*TEM*, *bla*SHV*, *bla*CTX-M* type genes using
primers and PCR conditions previously described (Liebana et al., 2004; Weill et al., 2004).
Identification of the *bla*SHV genetic variant was done initially by sequencing a 409-bp short
amplicon. Further primers (P-fw 5’ GATGTATTGTGGTTATGCGTT 3’; P-rv 5’
GTGCTRCGRGCCGATAAC 3’) were then designed in order to sequence the entire *bla*SHV
gene. A 30-cycle program with annealing at 60°C was used, resulting in a 1022 bp amplicon. The
purified PCR fragments were sequenced on both strands with the “BigDye terminator v3.1 cycle
sequencing” kit (Applied Biosystems) using the same primers used in PCR reactions. Sequence analysis was performed on an 3100-Avant Genetic Analyzer (Applied Biosystems) and studied using the software SeqScape v2.1.1. The obtained nucleotide sequences and the derived amino acid sequences were compared with those previously described from the GeneBank database www.ncbi.nlm.nih.gov and www.lahey.org/studies/webt.html, respectively.

**Nucleotide sequence accession numbers.** The nucleotide sequence reported in this study have been deposited in EMBL nucleotide sequence database under accession numbers EU137689 (S. Braenderup 1058/10); EU137690 (S. Livingstone 1124/2); EU137691 (S. Livingstone 1262/2); EU137692 (S. Livingstone 654/2); EU137693 (S. Livingstone 919/16); EU137694 (S. Livingstone 1987/3); EU137695 (S. Livingstone 2025/3); EU137696 (S. Enteritidis 2068/8); EU137697 (S. Livingstone 982); EU137698 (S. Livingstone 2030);EU137699 (S. Livingstone 739/3); EU137700 (S. Livingstone 739/4).

**Pulse-field gel electrophoresis (PFGE).** S. Livingstone SHV-12-producing isolates and a control strain (S. Livingstone LEP26 Salm-gene EQA panel) were subjected to PFGE analysis. The genomic DNA was prepared according to the PulseNet method (www.cdc.gov/pulsenet) and digested with the restriction endonuclease XbaI (Roche Diagnostics, GmbH Mannheim, Germany). The *Salmonella enterica* serovar Braenderup H9812 (Pulse Net Standard) was used as the molecular size marker. In order to overcome problems related to DNA degradation, PFGE was also performed with the addition of 750 μM thiourea (Sigma, T7875) to the electrophoresis PFGE buffer and agarose gel as previously described (Liesegang and Tschäpe, 2002; Bouallègue-Godet et al., 2005).

**RAPD-PCR (Random amplification polymorphism).** Sixteen *S. Livingstone* isolates were assayed by RAPD-PCR. Among these, ten were the SHV-12-producing isolates under study. Six
(ESC)-susceptible *S. Livingstone*, isolated from different sources (poultry carcasse, poultry meat and a quality control strain) and in different farms were also included in the study to test the discriminatory ability of RAPD analysis. Total DNA was extracted using the Wizard Genomic DNA purification System (Promega, Medison, WI). The RAPD-PCR reactions were performed using the Ready-To-Go RAPD analysis kit (GE Healthcare, UK) in accordance with the manufacturer’s recommendations. All the samples were assayed with two different primers (primer#1: 5’ GGTGCGGGAA 3’; primer#5: 5’AACGCGCAAC 3’) and the 100 base-pair DNA marker (GE Healthcare, UK) was used as a molecular size standard.

To determine significant differences in the patterns, the reproducibility of results was assessed by repetition of three independent RAPD assays.

The cluster analysis of bands was carried out with InfoQuestFPSoftware (Biorad) using Dice coefficient ($S_d$) and unweighted average pair group method (UPGMA); the position tolerance was set at 1.5%.

**RESULTS**

**Antimicrobial resistance in *Salmonella* from poultry**

In 2005 and 2006, 789 and 1053 *Salmonella* poultry isolates were tested for antimicrobial resistance. Among these, 3 isolates in 2005 (0,4%) and 9 in 2006 (0,9%) were identified as (ESC)-resistant: 1 *S. Enteritidis*, PT6, 1 *S. Braenderup* and 10 *S. Livingstone*. All the strains were isolated from faeces and carcasses of poultry (chicken and turkey) but one from an environmental sample from a farm where animals were found positive as well. Over the period 2004-2006, *S. Livingstone* was the most common *Salmonella* serovar isolated from poultry in north eastern Italy. A marked increase of *S. Livingstone* isolates, rising from 51 (5,70 %) in 2002 to 329 (24,70 %)
in 2006, was noted. The 401 S. Livingstone strains isolated in the period under study were completely susceptible to ciprofloxacin, chloramphenicol, kanamycin and enrofloxacin. Percentages of resistance < 5% were detected for all the other antimicrobials tested. The results of colistin were not included in the analysis of antimicrobial resistance.

The quantitative E-test confirmed ESBLs production in all the twelve strains characterized by resistant or intermediate values to ceftazidime and/or cefotaxime. The results of antimicrobial susceptibility of the ESBLs–producing isolates were confirmed by a microdilution broth assay to determine MIC values (Table 1). All the S. Livingstone isolates showed co-resistance to gentamicin (MIC ≥ 16 μg/ml) and nalidixic acid (MIC ≥ 32 μg/ml); among these, eight strains showed additional resistance to sulfonamides (MIC ≥ 512 μg/ml) and streptomycin (MIC ≥ 64 μg/ml). S. Braenderup was completely susceptible to all additional drugs tested and S. Enteritidis PT6 showed also resistance to Tetracycline (MIC ≥ 64 μg/ml).

Characterization of β-lactamase genes.

The twelve strains showing a ESBL phenotype were screened for the presence of \( \text{bla}_{\text{TEM}} \), \( \text{bla}_{\text{SHV}} \), \( \text{bla}_{\text{CTX-M}} \) genes. All of them only gave positive results for the \( \text{bla}_{\text{SHV}} \) PCR. Sequencing, followed by BLAST searches of the expected 409 bp amplicons, showed 100% of identity with the SHV-12 β-lactamase gene. The amino acid sequences derived from the nucleotide sequences of a 1022 bp PCR product identified a SHV-12 enzyme.

Molecular typing of S. Livingstone. The clonal relatedness of SHV-12 producing S. Livingstone was assessed by PFGE using XbaI. The isolates were not really typeable by PFGE, also adding thiourea to the running buffer and agarose gel; even with this poor quality due to partial degradation of DNA some bands were still identifiable, making some comparisons possible. The use of thiourea not made possible to obtain clear PFGE profiles of the isolates under study. All the strains were subsequently submitted to RAPD-PCR, as an alternative typing method, using
two different primers. The RAPD patterns obtained with primer#5 showed 7-11 bands ranging in
size between 0.3 Kb and 2.0 Kb; the RAPD analysis with primer#1 gave patterns containing 8-9
bands ranging in size between 0.2 Kb and 1.6 Kb. The similarity in cluster analysis was
calculated to be > 85 % in replicate RAPD-PCR for both primers tested. The threshold for RAPD
types identification was therefore set to 85 %. The number of RAPD types generated by cluster
analysis at this level was 3 for RAPD performed with both primer P#1 (A1, B1,C1) and primer
P#5 (A5, B5,C5). When using RAPD, in both case RAPD types A1 e A5 included all the (ESC)-
resistant S. Livingstone isolates (Sd 100%); RAPD types B1 (Sd 100%) e B5 (Sd 93%) included
the three S. Livingstone isolates susceptible to all antimicrobial tested and RAPD types C1 (Sd
100%) e C5 (Sd 94%) included the following S. Livingstone isolates 879/11 (R-type=AMP-SXT-
SUL), 1077/3 (R-type=AMP-CEF-STR-TE-SXT-SUL) and the QC strain LEP26. (Fig 1).

**DISCUSSION**

(ESC)-resistant *Salmonella* strains from poultry, belonging to different serovars, have been
isolated in animals and food in United States and Europe since late 90s. In the USA, poultry and
poultry products are most frequently contaminated with CMY-2-producing *Salmonella* (White et
al., 2001; Chen et al., 2004). However, the epidemiology of ESC-resistant *Salmonella* seems to
be quite different in Europe, where strains isolated from poultry and poultry meat mostly harbour
Ambler class A β-lactamases (Weill et al., 2004; Politi et al., 2005; Hasman et al., 2005).
Some recently published papers point to poultry as an important animal *reservoir* of β-lactamase
genes (Brinãs et al., 2003; Weill et al., 2004; Hasman et al., 2005; Bertrand et al., 2006). Poultry
is also frequently implicated in foodborne outbreaks suggesting a possible route of (ESC)-
resistant *Salmonella* transmission to humans via the food chain.
A marked increase in \( S. \) Livingstone frequency, compared to \( S. \) Enteritidis and \( S. \) Typhimurium, was noted among \( \text{Salmonella} \) isolated from poultry during 2004-2006 in north eastern Italy. This serovar showed susceptibility or low percentage of resistance (< 5%) to all the drugs routinely tested for antimicrobial resistance in our laboratory. The number of ESBL-positive \( \text{Salmonella} \) isolates poultry-associated increased from 3 (0.4%) in 2005 to 9 (0.9%) in 2006 in the Northeast of Italy. All twelve strains under study (\( n=1 \) \( S. \) Enteritidis PT6, \( n=1 \) \( S. \) Braenderup and \( n=10 \) \( S. \) Livingstone) carried a SHV-12 \( \beta \)-lactamase. The multi-drug resistant \( S. \) Livingstone isolates were rough and untypeable by PFGE analysis as previously reported (Murase T. et al., 2004; Liebana et al., 2001). The RAPD-PCR analysis was the method showing the close relatedness of the ten SHV-12-producing \( S. \) Livingstone isolates and made it possible to discriminate among these and two different groups of (ESC)-susceptible \( S. \) Livingstone isolates. This relation suggests a possible common origin of the isolates, e.g., an infected flock of breeders from which the different flocks of broiler originated. This origin could reduce the significance of an increase in the prevalence of (ESC)-resistant isolates, were these strains are related to a possible single outbreak. However, the application of molecular typing methods with a higher level of specificity and reproducibility than RAPD-PCR should be used in order to confirm the clonal origin of the isolates. Although \( S. \) Livingstone human outbreaks are relatively uncommon, the increased prevalence reported demands careful monitoring and possible control actions in order to avoid the dissemination of \( \text{bla} \) genes to other \( \text{Salmonella} \) serotypes of human importance. Concern also arises from the recent sanitary alert for an ESBL CTX-M-2-producing \( \text{Salmonella} \) Virchow clone isolated at first from poultry and subsequently in human gastroenteritis cases in Belgium (Bertrand S. et al., 2006).

The SHV-12 \( \beta \)-lactamase has been recently found to be widespread among ESBLs-producing isolates in clinical settings in Italy (Perilli et al., 2002). Furthermore, studies reporting SHV-12-
producing *Salmonella* and *E. coli* isolated from animals exist to date (Carattoli et al. 2005; Brinăș et al., 2003; Riañô et al., 2006). S. Livingstone ESBL-SHV-12 producing isolates under study showed co-resistance to nalidixic acid, sulfonamides, streptomycin and gentamicin; it is worth noting that this latter resistance is reported rarely in *Salmonella* isolates. Both human and animal SHV-producing isolates have frequently shown additional resistance to these antimicrobials (Brinăș et al., 2003; Riañô et al., 2006; Spanu et al., 2002). Although the use of ESC in poultry farms is infrequent in Italy, sporadic use of ceftiofur in breeder flocks, according to the allowed exception of the Directive 2004/28/EC, should be considered when examining possible reasons for the occurrence of this resistance. Amongst the isolates SHV-12 producing isolates reported in this study a S. Enteritidis was also identified and is a *Salmonella* of public health relevance, representing the serovar most frequently involved in human cases in Europe (The Community Summary Report 2005, EFSA). Careful surveillance and prudent use of antimicrobials in breeding is needed in order to prevent ESBLs-producing *Salmonella* dissemination and human transmission by food chain.

REFERENCES


Figure 1. Dendrograms showing the relatedness among RAPD types of S. Livingstone isolates (ESC)-resistant and (ESC)-susceptible generated by primer#1 (I) and primer#5 (II) (InfoQuestFPSoftware - Biorad).

<p>| | | | | | | | |</p>
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<td>D</td>
<td>E</td>
<td>F</td>
<td>G</td>
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</tr>
<tr>
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<td>.**</td>
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<td>poultry meat</td>
<td>B1</td>
<td>.**</td>
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<td>C1</td>
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</table>
The figures I and II report the following information: A, isolate number; B: collection date; C-D: source of the isolate; E: RAPD-type; F: antimicrobial resistance pattern; G: origin of the sample. Symbols: AMP: ampicillin; EFT: ceftiofur; GEN: gentamicin; NAL: nalidixic acid; STR: streptomycin; SUL-S3: sulfonamides; TE: tetracycline; CEF: cephalothin; SXT: trimethoprim-sulfamethoxazole.

**S. Livingstone ESC-susceptible isolates included in the study.
Table 1. MIC values for the twelve Salmonella SHV-12-producing isolates collected from poultry at NRL for Salmonella (2005-2006).

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<th>CRO</th>
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<td>2</td>
<td>≤0.5</td>
<td>4</td>
<td>≤4</td>
<td>16</td>
<td>2/1</td>
<td>0.12</td>
<td>≥16</td>
<td>≥32</td>
<td>≥8</td>
<td>32</td>
<td>≤0.12/2.38</td>
<td>≤8</td>
<td>≥32</td>
<td>≤32</td>
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<tr>
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<td>4</td>
<td>≤4</td>
<td>32</td>
<td>2/1</td>
<td>0.12</td>
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<td>≥32</td>
<td>≥8</td>
<td>≥512</td>
<td>≤0.12/2.38</td>
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<td>≥32</td>
<td>≥64</td>
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<td>≤4</td>
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<td>2/1</td>
<td>0.12</td>
<td>≥16</td>
<td>≥32</td>
<td>≥8</td>
<td>≥512</td>
<td>≤0.12/2.38</td>
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FOX: cefoxitin; AMK: amikacin; CHL chloramphenicol; TE: tetracycline; CRO: ceftriaxone; AMC: amoxicillin-clavulanic acid; CIP: ciprofloxacin; GEN: gentamicin; NAL: nalidixic acid; EFT: ceftiofur; SUL: sulfonamides; SXT: trimethoprim-sulfamethoxazole; KAN: kanamycin; AMP: ampicillin; STR: streptomycin