SUMMARY

Key words: *antibioticresistance, Enterobacteriaceae, ESBL, CTX-M,*

The doctoral thesis entitled *„Prevalence of Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBL) isolated from broilers' caecum and their implications for public health"* has a total of 227 pages and is structured as required in two parts: the first part containing the literature review and the second part, the personal research.

The first part, entitled ‘’*The present stage of knowledge*” includes 57 pages and synthesizes over five chapters, main bibliographic database of literature regarding general, biochemical and metabolic characteristics of the Enterobacteriaceae family, the bacteriological diagnostic of Enterobacteriaceae, classification of beta-lactams and their mechanisms of action, the classification of extended spectrum beta-lactamases and the description of molecular biology techniques used to identify markers of antibiotic resistance.

Part II intended for personal researches, summarizes 141 pages and contains 9 chapters that describe the purpose and objectives of the thesis, results of the phenotypic and molecular tests performed for the identification of extended-spectrum beta-lactamases-producing strains and genes involved in the development of resistance to beta-lactams and quinolones and genetic diversity of identified strains. Each research chapter contains: the material and the work method, the results and the discussion upon them and also the partial conclusions.

The paper is illustrated with a number of 57 figures, 31 tables and is based on 196 references.

Chapter VI contains *The aim and purpose of the research*. The main hypothesis tested is whether broilers can be considered a source of ESBL - producing Enterobacteriaceae to humans via food chain.

The main objectives of this research were:

• Isolation and identification of the Enterobacteriaceae strains producing extended-spectrum beta- lactamases (ESBL)

• Antibiotic susceptibility tests for the isolated strains

• Phenotypic tests for ESBL- producing strains identification

• Sequencing of the PCR-positive samples to identify the type of ESBL-genes present in the strains, isolated from broilers, AI slaughterhouse workers and human clinical cases

• Identify the types of plasmids present in the Enterobacteriaceae isolated from our samples

• Development of bacterial conjugation experiments to see if the ESBL genes are readily transmitted through the plasmids present in the isolated bacterial strains

• Study of genetic diversity of strains of *Escherichia coli* isolates, using RAPD and MLST techniques.

Chapter VII- Research regarding isolation and identification of Escherichia coli, Klebsiella spp and Proteus spp strains. For isolation of the Enterobacteriaceae strains, 127 caecal samples from broilers slaughtered in two abattoirs and 55 fecal samples from workers in one of the slaughterhouse were collected. Of the 127 samples collected from the broilers, 92 strains of Enterobacteriaceae were isolated, and from samples taken from workers, 22 strains were isolated. 38 strains of *Escherichia coli* and *Klebsiella spp* isolated from human clinical cases were also included in this study to conduct comparative molecular investigations.

Identification of the species *Escherichia coli* was achieved by molecular-PCR assay, using *uidA* gene. Of the 114 strains of Enterobacteriaceae tested for gene *uidA,* 109 were confirmed as belonging to the species *Escherichia coli.* The strains negative for the gene *uidA* were further investigated using *Trek Diagnostic System*, and were identified as *Proteus mirabilis*- 2 strains, *Klebsiella oxytoca* -2 strains and *Klebsiella pneumoniae*-one strain.

Of the 152 strains of Enterobacteriaceae investigated in this study, most belonged to the species *Escherichia coli*, 89.47% followed by 7.23% *Klebsiella pneumoniae, Klebsiella oxytoca* 1.97% and 1.37% *Proteus mirabilis*.

Chapter VIII Researches regarding antibiotic sensitivity of the isolates. All strains were tested in our study to determine antibiotic sensitivity profile to 14 antibiotics. These were: ampicillin (AMP), amoxicillin/clavulanic acid (AMC), cefpodoxime (CPD), cefotaxime (CTX), ceftazidime (CTX), cefoxitin (FOX), imipenem (IMP), gentamicin (CN),

streptomycin (S), tetracycline (TE) rifampicin (RD), nalidixic acid (NA), ciprofloxacin (CIP).

Determination of antibiotic resistance profile was performed by disc diffusion method. The percentage of strains with multiple resistance to antibiotics for the entire collection was 77.63%. Isolates from broilers and isolates from human clinical cases showed high levels of resistance to several classes of antibiotics tested, 92% from broilers and 100% from human clinical cases. For the isolates from AI workers, the percentage of multiple resistance strains was 40.9%.

Resistance profiles obtained from broiler and clinical cases strains were very similar, with the exception of cefoxitin. Of all the antibiotics tested , imipenem (carbapenem) was the only antibiotic to which all 152 strains were sensitive.

Chapter IX Researches regarding phenotypic identification of Enterobacteriaceae producing extended-spectrum beta-lactamases. Confirmation of extended-spectrum β-lactamase producing was performed by the combined discs method. This method uses simple cephalosporin discs and cephalosporins potentiated with clavulanic acid. Cephalosporins used were: cefotaxime 30 µg, cefotaxime/clavulanate 30µg/10 µg, ceftazidime 30µg, ceftazidime/clavulanate 30µg/10µg, cefpodoxime 10µg and cefpodoxime/clavulanate 10µg/1µg.

Were considered ESBL positive, the strains where the inhibition zone diameter was > 5 mm in the presence of clavulanate versus cephalosporin discs without clavulanate. Each of the 114 strains was tested in order to identify ESBL phenotypes using the combined discs. The prevalence of ESBL - producing strains ranged between 42.5 % in AI broilers 32.5% in AII broilers and 7.27 % in AI workers.

Chapter X - *Research regarding the identification of AmpC / ESBL genes involved in the occurrence of resistance to beta-lactam antibiotics*, sought to identify 6 types of AmpC genes and 4 types of ESBL genes. Working protocol included simplex and multiplex PCR assays and gene sequencing. The sequences obtained were then analyzed using *gap4* and *preGap* computer software and subjected to a *BLAST* analysis in order to determine their identity, as compared to existing sequences in the *GenBank* database.

One-way ANOVA demonstrated that there were significant statistical differences among the three groups of samples analyzed: $F(2.15) = 3.82$, $p = 0.046$

From broiler isolates we identified genes belonging to the families: TEM (39/92) SHV (30/92), CTX-M Group 1 (27/92), CTX-M Group 9 (5/92), CIT-M (48/92) and ACC

(1/92). For the strains isolated from AI workers, genes involved in the development of resistance to beta-lactam identified were: TEM (8/22) SHV (9/38), ACC (1/22) and OXY-6 (2/22). The genes for resistance to beta-lactam identified in clinical isolates from human cases were: TEM (19/38) SHV (9/38) OXA (24/38) CTX-M Group 1 (30/38) CTX-M Group 9 (3/38), and CTX-M group, 2 (1/38). After sequencing the samples we noticed a difference in the prevalence of ESBL genes in the two slaughterhouses. Therefore in the AI unit *blaCTX-M-3* had the highest prevalence, while in the AII unit, highest prevalence was registered for *blaCTX-M-15*. CTX-M-14, a beta-lactamase belonging to CTX-group M-9, was found only broilers from AI unit, and it had a prevalence of 4.87%.

For ESBLs, other than CTX-M, the highest prevalence had SHV-12. For beta-lactamases of the TEM family, most of the results were TEM -1, but this enzyme is not placed in the beta- lactamase extended spectrum category.

In isolates from AI workers, the ESBL genes were identified as *blaSHV-12, blaSHV-108* and *blaOXY-6.*

In isolates from human clinical cases, the highest prevalence was recorded for *blaCTX -M-15* 78.94 %, followed by *blaOXA-1*, 63.15%.

Chapter XI Research regarding the identification of plasmid -mediated genes , qnrA , qnrB , qnrS and aac (6')-ib-cr, involved in development of resistance to quinolones . As most often strains of Enterobacteriaceae, resistant to beta-lactam antibiotics, are also fluoroquinolone -resistant, for this group of antibiotics, investigations have been carried out both by disc diffusion and molecular techniques.

For the *qnrA* gene we did not identify any positive strains. *QnrB* and *qnrS* recorded very low prevalence between 2.17% and 9.09 %.

The gene *aac(6')-Ib-cr* was identified in all three categories of samples, but its prevalence ranged from 3.26% from broilers 4.54% from AI workers, up to 63.15% in the isolates from human clinical cases.

One-way ANOVA showed that there is no statistically significant difference between categories of samples analyzed*: $F(2,9) = 0.77$, $p = 0.491$*.

In human clinical cases, *aac-(6')-ic-cr* gene, was associated with *blaCTX-M-15 blaTEM-1*and *blaOXA-1*, this gene association being described also by other similar studies.

Chapter XII-Research regarding the diversity of plasmids present in the isolated strains. Plasmids are the main tool in disseminating a large variety of genes responsible for

the emergence of antibiotic resistance. Based on the type of replicon, *Carattoli A., 2005*, imagined a method to identify plasmids, based on replicon typing PCR (PBRT)

To examine whether the ESBL genes are readily transmitted through plasmids present in the strains isolated, we performed conjugation experiments. The conjugation method used nutrient broth-conjugation, and the strain used as the recipient strain was *E. coli Hb 101-streptomycin-resistant.*

PBRT showed that for most of the strains tested, multiple replicons have been present. Twelve out of the 18 replicon investigated, were identified, of which P, F, FIA, FIB, I and N were identified in all 3 categories of samples. From strains isolated from broilers, most often replicons identified were I1 (64/92) and F (62/92). For strains isolated from human clinical cases, replicons with the highest prevalence were F, FIA and FIB 55% (21/38), I1 having a relatively low prevalence of 11% (4/38).

For 7out of 14 strains selected for conjugation experiments, the presence of *blaCTX-M* genes was confirmed in transconjugates. The most commonly involved in the transfer of genes were plasmids from the group F and I1.

Chapter XIII - Research regarding the genetic diversity of ESBL-producing E coli strains. A rapid protocol based on multiplex PCR has allowed the classification of *E. coli* strains in one of the four major phylogenetic groups, A, B1, B2 and D. The technique is based on a triplex PCR using a combination of two genes *Chua, yjaA* a chromosomal DNA fragment-*TspE4C2.*

The clonal group *O25-ST131* has been identified worldwide, and appears to be responsible for the spread of *blaCTX-M-15* and the fluoroqinolone resistance genes. *(Mora, A. et al., 2010) E coli O25 ST131* is responsible for the occurrence of various diseases in humans, this clone was identified recently and companion animals, broilers and food products (poultry meat). *(Ewers, C. et al., 2010, Rogers, B. A. et al., 2011).* For the identification of *E. coli* strains belonging to serotype *O25* we used Simplex PCR, using the protocol previously described by *Clermont, O. et al., 2008*, and to identify *ST131* clone we used a multiplex PCR protocol.

To analyze the genetic diversity of *E. coli* isolated from the three sources we used two methods: RAPD and MLST. RAPD was carried out using two different primers in two separate PCR reactions, the results being analyzed using the *Simpson's index of diversity*.

MLST analysis is based on the identification of nucleotide sequences for a total of seven housekeeping genes that are subject to minor and very slow modifications in terms of

polymorphism and diversity. The seven fragments of interest are amplified by PCR and then sequenced. After analyzing all sequences, we obtained a combination of seven allele numbers corresponding to the gene variants, which is used to obtain an allelic profile or sequence type (ST).

The 7 genes investigated were: *adk, fumC, gyrB, icd, mdh, purA* and *recA*. The method used for sequencing was *in-house sequencing* and sequence analysis program used was *ChromasPro 1.7.5*. The sequences obtained were then subjected to analysis by comparison with the sequences in *E. coli* MLST existing database, in order to establish their identity. *(http://mlst.ucc.ie)*

Phylogroup A recorded the highest prevalence within our collection of *E. coli* investigated, with a prevalence of 33,5%, followed by phylogroup D 20.9%, B2 19.5% and B1 15.3%.

Escherichia coli O25-ST-131 was identified only among isolates from human clinical cases. RAPD analysis revealed a high genetically diversity of *Escherichia coli* strains isolated from broilers- from 32 strains analyzed, resulting 29 different profiles. 27 strains of *Escherichia coli* isolated from human clinical cases investigated and we obtained 12 profiles RAPD profiles, emphasizing a higher degree of genetic relatedness of the human pathogenic strains present in the hospitals environment and even persistence of certain clones causing nosocomial infections.

MLST analysis confirmed the results of RAPD regarding genetic diversity of *Escherichia coli* strains investigated in this study. Therefore from 13 broiler isolated strains we obtained 12 different sequence types, while for 11 isolates from human clinical isolates we obtained 6 types of sequence types.

Chapter XIV - Final conclusions. Limitations of the study and research perspectives in this chapter are structured the main issues drawn from the investigations in the form of 29 final conclusions.