Szent István University
Postgraduate School of Veterinary Science

Multidrug resistant *E. coli*: characterization of antimicrobial resistance and virulence genes with molecular epidemiologic approach

PhD dissertation theses

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2011

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Introduction

The main topic of the PhD dissertation is covered by three units structured on the simultanous characterization of antimicrobial resistance and virulence genes of *E. coli* - isolated from different parts of the food chain - in order to answer the same question: whether the widespread use of antimicrobials in animals and humans could also lead to increased dissemination of virulence genes.

Nowadays there is an increasing need for simultaneous detection of antimicrobial resistance pheno-/genotypes and virulence profiles, and for characterization of potential genetic vectors of multiple antimicrobial resistance and virulence determinants in identifying emerging *E. coli* genotypes of potentially increased importance for animals and/or humans. In contrast to pathogenic (clinical) strains, there is a paucity of information regarding the associations between antimicrobial resistance and virulence genes in commensal *E. coli* strains from animals and humans. Furthermore, the molecular epidemiologic aspect of such data collection and -analysis is especially lacking.

Based on the above approach, first we aimed to provide a comprehensive description of antimicrobial resistance and virulence profiles of porcine post-weaning enterotoxigenic *E. coli* (ETEC) strains, representing three middle-European neighbouring countries: Hungary, Austria and the Czech Republic. Characterization of tetracycline resistance plasmids was also attempted, with special regard to those responsible for the transfer of tetracycline resistance genes and to further mobile genetic elements contained.

In the second part of these studies we aimed to review the influence of gentamicin - one of the most frequently used aminoglycoside antibiotic in treating animal and human infections - on the possible simultaneous development of multiple resistance and virulence traits. Therefore, the main objective of this part of the study was to provide a comparative characterization of antimicrobial resistance and virulence genotypes of gentamicin resistant clinical and commensal *E. coli* strains from food animals and humans, in order to enhance our understanding of the zoonotic significance of multidrug resistant (MDR) *E. coli*.

Finally, regarding the increasing concern of plasmid-mediated quinolone resistance not only in human but also in food animals, and the lack of data related to porcine *E. coli* from Europe, we aimed the identification and characterization of *qnrS1* gene in porcine *E. coli* strains derived from a surveillance study on MDR *E. coli* in large piggeries of two, traditionally pork-producing countries such as Romania and Hungary.

Objectives

Based on the above statements the main objectives of the dissertation are clustered in the topics of tetracyclin-, gentamicin- and plasmid-mediated quinolone resistance, and could be defined as follows:

1. Unravelling the role of *tet*(A) tetracycline resistance plasmids in the transfer of antimicrobial resistance (and virulence) genes in multidrug resistant enterotoxigenic *E. coli* (ETEC) strains isolated from pigs with post-weaning diarrhea.

2. Characterization of antimicrobial resistance and virulence genotypes and of potential associations among and between antimicrobial resistance and virulence genes in gentamicin resistant clinical and commensal *E. coli* strains isolated from food animals and humans.

3. Identification of plasmid-mediated quinolone resistance genes and characterization of corresponding plasmids in multidrug resistant commensal *E. coli* strains isolated from feces of healthy pigs.

Materials and methods

Characterization of tet(A) plasmids from multidrug resistant porcine enterotoxigenic E. coli (ETEC) strains

ETEC strains studied here were isolated from cases of porcine post-weaning diarrhea, and the majority of them represented three middle-European neighbouring countries: Hungary (n=16), Austria (n=34) and the Czech Republic (n=17). Further 20 ETEC strains were derived from the USA as well. The antimicrobial resistance phenotype was tested by disc diffusion. ETEC strains phenotypically resistant to tetracycline were subjected to *tet* gene typing, using PCR primers specific to common *tet* genes of *Enterobacteriaceae*.

In order to characterize plasmids responsible for the transfer of tetracycline resistance and of ETEC-specific virulence genes (e*stA*, e*stB*, e*lt*, *f18*, *k88*), a total of 8 *tet*(A) és 12 *tet*(B) $F18^+$ ETEC strains were selected for conjugational plasmid transfer. Parental and transconjugant strains representing successful *tet*(A) and *tet*(B) transfers were selected for plasmid profile analyis and for PCR identification of antimicrobial resistance and virulence genes.

Due to the lack of data regarding plasmids carrying the *tet*(A) gene in $F18^+$ ETEC strains, further analyses were restricted to two *tet*(A)-positive monoplasmidic transconjugant strains (2172/11 and 11732/11), isolated in Hungary and the Czech Republic respectively. Determination of incompatibility (Inc) group of *tet*(A) plasmids was carried out by PCR-based replicon typing (PBRT). Finally, the variable region of the class 1 integron identified in the Hungarian strain was completely sequenced and deposited in the GenBank under accession number JQ313793.

Comparative antimicrobial resistance and virulence genotyping of gentamicin resistant commensal and clinical E. coli strains from food animals and humans

In line with the national antimicrobial resistance monitoring program, a total of 3477 poultry, 1861 porcine and 1794 bovine *E. coli* strains were tested for antimicrobial resistance phenotype in the period between 2004 and 2008. In this study, 12 poultry, 13 porcine and 13 bovine gentamicin resistant (Gen^R) *E. coli* strains were selected from the above larger *E. coli* collection for simultaneous characterization of their antimicrobial resistance and virulence genotypes. As a comparison to food animal isolates, 12 Gen^R *E. coli* strains from humans were also included.

The gentamicin resistant *E. coli* strains were categorized based on the host species and their clinical background: “clinical” strains of *E. coli* were derived from diseased organs of

sick or dead animals or of clinically ill human patients, while the isolates from animal products or from normal feces were defined as ‘‘commensals’’.

Screening for 62 antimicrobial resistance genes, conferring resistance to clinically important antimicrobials and for types of integrases, was performed using a recently developed ‘‘Identibac-AMR’’ PCR-microarray system (ArrayTube™ AMR05). In addition, the identification of 69 virulence genes (plus several subtypes) was undertaken using a similar ArrayTube™ Ec03 PCR-microarray system specific to major virulence gene groups of *E. coli*. Finally, the detection of possible associations among and between antimicrobial resistance and virulence genes was performed by Pearson’s correlation analysis.

Characterization of q*nrS1* plasmids in porcine commensal *E coli* strains

Porcine *E. coli* strains from this study were isolated from the feces of healthy animals, as part of an international surveillance project on molecular characterization of MDR enteric *E. coli* from piglets of one Romanian and one Hungarian large piggery.

The *qnrS* gene was identified in 17 of the MDR *E. coli* strains tested, and all were derived from the Romanian pig farm. Six of the *qnrS* strains were randomly selected for further pheno- and genotyping (including the determination of the *qnrS* gene variant) and for plasmid transfer studies. The antibiotic resistance and genotype of the 6 strains was determined using the AMR05 and Ec03 PCR microarray systems mentioned above. The clonal relation of the six *qnrS1 E. coli* strains derived from the same Romanian farm was established by multilocus sequence typing (MLST).

In order to characterize quinolone resistance plasmids carrying the *qnrS1* gene, conjugation experiments were performed. Parental and transconjugant strains were subjected to plasmid profile analysis followed by the determination of the plasmid type by PCR-based replicon typing (PBRT). The localization of the *qnrS1* gene on IncN plasmid was confirmed by Southern blot hybridization using IncN and *qnrS1* probes. IncN plasmids of the porcine *qnrS1* transconjugants were further characterized by restriction fragment length polymorphism (RFLP), and their restriction profiles were compared with that of the IncN plasmids of human *Salmonella* Kentucky strains used as *qnrS1* IncN plasmid controls.

Finally, the genetic environment of the *qnrS1* gene was analysed, and the nucleotide sequence of 3.6 kb *qnrS1* insert derived from the strain Ec48-1 has been deposited in the GenBank database under accession number JN157839.

Results

Antimicrobial resistance phenotype and tetracycline resistance genes of porcine ETEC strains with different geographical background

Regardless of the geographic origin, majority of the ETEC strains studied shared a common MDR "backbone", most frequently being resistant to sulfamethoxazole (91%), tetracycline (84%) and streptomycin (80%). In general, the prevalence of resistance was lower in ETEC strains from the middle-European countries, as compared to those from The USA.

The tetracyclin resistant phenotype was most frequently due to the presence of the *tet*(B) gene (38%), when *tet*(A) was identified in 26% of the isolates. The prevalence of different *tet* gene types and patterns varied among strains with different geographical background.

Characterization of IncI1 plasmids involved in the transfer of tet(A) gene in porcine ETEC strains

The conjugational plasmid transfer of F18$^+$ ETEC strains from Hungary (Ec2172) and from the Czech Republic (11732) resulted in two *tet*(A)-positive monoplasmidic transconjugant strains: 2172/11 and 11732/71 respectively, where the characterization of *tet*(A) plasmids of ~138 and 106 kb was performed.

Plasmid replicon typing resulted in the identification of IncI1 type plasmids in both strains. Based on the lack of ETEC-specific virulence genes tested here, the above plasmids could be regarded as resistance (i.e. multidrug resistance) plasmids only. According to this, the detection of antimicrobial resistance genes revealed that in addition to the *tet*(A) gene, IncI1 plasmids were involved in the co-transfer of *aadA1* (streptomycin/spectinomycin) and *strA* (streptomycin) genes in the Hungarian strain, while in the Czech 11732 strain we found *tet*(A) associated to the chloramphenicol resistance gene *catA1*.

In the Hungarian F18$^+$ ETEC strain, the *aadA1* gene was part of a typical (*qacEΔ1*$^+$/*sul1*$^+$) class 1 integron, although its *estX-aadA1* cassette array is quite unusual, encoding resistance to streptothricin and streptomycin/spectinomycin respectively.

Antimicrobial resistance pheno- and genotype of gentamicin resistant clinical and commensal E. coli strains from food animals and humans

Regardless of the host species and clinical background, most of these *E. coli* isolates proved to be resistant to tetracycline (84%), ampicillin (82%) and sulfamethoxazole (80%) beside gentamicin. Regarding the prevalence of resistance to third-generation cephalosporins, with significance in human therapy, only 2 animal (bovine) *E. coli* strains

demonstrated resistance to cefotaxime and ceftazidime, whereas resistance to these two drugs among human strains was relatively high (67% and 25%, respectively).

In general, our strains were characterized by large diversity of resistance genotypes. In harmony with the phenotype data, the most common antimicrobial resistance genes associated with Gen^R *E. coli* strains were the bla_{TEM} (ampicillin), *tet*(A) (tetraciklin), *strB* (streptomycin) and *sul1* (sulfamethoxazole) genes. Although the prevalence of the above genes was not related to the clinical background, the number of antimicrobial resistance genes as a whole was significantly higher (p=0.030) in clinical strains as compared to commensal *E. coli* isolates.

Some genes conferring resistance to aminoglycosides (*aac(3)-I*, *ant(2")-Ia* and *aac(6')-Ib*) and phenicols (*catB3*) were almost exclusively found among human isolates.

Virulence genotypes of gentamicin resistant E. coli strains, with some associations among and between antimicrobial resistance and virulence genes

In harmony with data on resistance genotypes, a large variety of virulence patterns have been identified among Gen^R *E. coli* strains tested. The most common virulence gene detected in all groups of *E. coli* was the increased serum survival gene *iss* (in 70% of the isolates), being equally distributed in clinical and commensal strains. The majority of the virulence genes were rarely represented and distributed regardless of the clinical background.

A few strong correlations among and between antimicrobial resistance and virulence genes could be defined. With the exception of the association between the trimethoprim gene *dfrA17* and *aadA4* for streptomycin resistance, which was not related to the host species, other associations of genes were found to be specific to certain hosts. Accordingly, resistance genes *catB3*, *aac(6')-Ib* and $bla_{CTX\text{-}M\text{-}1}$ revealed strong correlation with the SPATE gene *sat* in human strains, whereas the co-existence of the tetracycline resistance gene *tet*(A) and the virulence genes *iroN* and *iss* were detected in *E. coli* strains of poultry origin.

Identification of the qnrS1 gene and the clonal relation of the porcine qnrS1 E. coli strains

In our study, the presence of the plasmid-mediated quinolone resistance gene *qnrS1* was identified and characterized in 6 *E. coli* strains from healthy piglets of a Romanian farm, representing the first report on plamid-mediated quinolone resistance in porcine *E. coli* in Europe. Despite being isolated from the same pig farm, commensal *E. coli* strains from this study there were three different multilocus sequence types (STs) represented: ST48, ST206, and ST542, respectively.

Antimicrobial pheno- and genotype and virulence profile of the qnrS1 E. coli strains

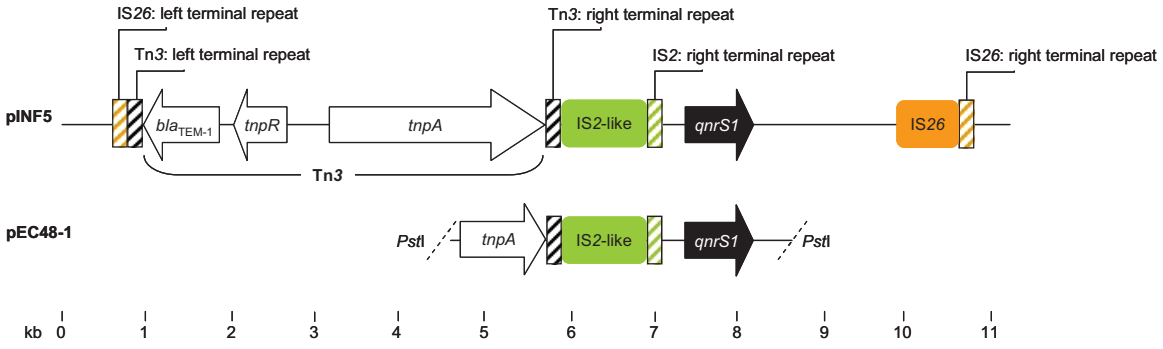
Despite divergent clonality, the six commensal *qnrS1 E. coli* strains showed highly similar antimicrobial resistance phenotypes: in addition to gentamicin resistance, all strains showed resistance to other aminoglycosides as well (kanamycin, streptomycin), but ampicillin and tetracycline resistances were also common. Results of PCR-microarray studies on antimicrobial resistance genes were fully in harmony with the resistance phenotype, detecting the presence of genes for spectinomycin/streptomycin (*aadA1*, *strA*, *strB*), ampicillin (bla_{TEM-1}), and tetracycline (*tet*(A)) resistance.

In contrast to the results on antimicrobial resistance genotype, few virulence genes were detected in two *qnrS1 E. coli* strains only, although they represented different virulence mechanisms.

Characterization of the porcine qnrS1 IncN plasmids, and the genetic environment of the qnrS1 gene

Plasmid replicon typing resulted in the identification of IncN plasmids of ~70 kb as responsible for the transfer of the *qnrS1* gene in porcine commensal *E. coli* strains studied. RFLP results indicated that IncN plasmids from the six porcine *E. coli* strains were very similar in their restriction patterns, but differed from those found in *qnrS1* transformants of human *Salmonella* Kentucky strains used as IncN plasmid control. PCR testing for antimicrobial resistance genes revealed that IncN plasmids were responsible for the co-transfer of aminoglycoside (*aadA1*, *strA*, *strB*), ampicillin (bla_{TEM-1}), and tetracycline resistance (*tet*(A)) genes in addition to the *qnrS1* gene.

Sequence analysis of the 3.6 kb *qnrS1* insert derived from the plasmid pEc48-1 of the transconjugant Ec48-1 revealed that both the up- and downstream regions of the *qnrS1* gene showed 99% homology with the corresponding resistance region of the *qnrS1* plasmid pINF5 (GenBank: AM234722) from a *Salmonella* Infantis strain isolated from chicken (Figure).



New scientific results and theses

The new scientific results and theses of the dissertation could be summarized as follows:

Related to the characterization of *tet*(A) plasmids from multidrug resistant porcine enterotoxigenic *E. coli* (ETEC) strains:

1. In addition to a former study of our group resulting in the complete sequencing of the pTC hybrid plasmid, as a first representant of *tet*(B)-type hybrid plasmids carrying enterotoxin genes, here we characterized the *tet*(A) plasmids of two $F18^+$ ETEC strains isolated from Hungary and from the Czech Republic. As a result, the presence of IncI1 type resistance plasmids responsible for the co-transfer of the *tet*(A) gene and additional resistance determinants were demonstrated for the first time in $F18^+$ ETEC strains.
2. Furthermore, the *tet*(A) IncI1 plasmid of the Hungarian strain carried a class 1 integron, with a variable region, quite unusual among porcine *E. coli*, being composed by *estX-aadA1* gene cassettes encoding resistance to streptothricin and spectinomycin/streptomycin respectively.

Regarding the comparative antimicrobial resistance and virulence genotyping of gentamicin resistant clinical and commensal *E. coli* strains from food animals and humans:

3. We provide the first microarray-based systematic comparative genotyping on clinical and commensal *E. coli* from food animals and humans, suggesting their role as reservoirs of antimicrobial resistance and of virulence genes. Furthermore the co-existence and spread of some genes have been revealed.
4. Contrasting the general concept, some genes conferring resistance to aminoglycosides (*aac(3)-I*, *ant(2")-Ia* and *aac(6')-Ib*) and phenicols (*catB3*), being almost exclusively present among human isolates, confirmed the possibility for existence of human specific pools of these resistance determinants independent of food animal sources.
5. Here we point out that, the commensal *E. coli* strains from food animals are not only important indicators, but – in addition to pathogenic strains – through their numerous virulence and resistance genes, they could also be regarded as potential risk for human health.

Related to the characterization of *qnrS1* plasmids in porcine commensal *E. coli* strains:

6. We described three *qnrS1* porcine MLST clones, which have been previously identified as related mainly to humans, thereby they could be regarded as new clones among animal (ST542) or porcine (ST48, ST206) *E. coli* isolates. Furthermore we provide the first characterization of antimicrobial resistance and virulence genotype of above MLST clones.
7. We also provide first characterization of *qnrS1 E. coli* plasmids of porcine origin, reporting the presence of *qnrS1* IncN plasmids in food animals, as being the first occurence of plasmid-mediated quinolone resistance in porcine commensal *E. coli* strains from Europe.
8. The genetic environment of the *qnrS1* gene showed 99% homology with the corresponding resistance region – related to Tn*3* – of the *qnrS1* plasmid pINF5 from a *Salmonella* Infantis strain isolated from chicken, suggesting the possible transfer of the *qnrS1* gene between *E. coli* and *Salmonella* as a potential risk for human health. Thus our data indicate that in addition to poultry, pigs may also represent a reservoir for the dissemination of plasmid-mediated quinolone resistance gene *qnrS1*.

Publications and abstracts based on the results of the PhD dissertation

Research papers:

1. **Szmolka A.**, Anjum M.F., La Ragione R.M., Kaszanyitzky E.J., Nagy B.: Microarray based comparative genotyping of gentamicin resistant *Escherichia coli* strains from food animals and humans. Vet. Microbiol., (in press) 2011, doi:10.1016/j.vetmic.2011.09.030.
2. **Szmolka A.**, Fortini D., Villa L., Carattoli A., Anjum M.F., Nagy B.: First Report on IncN Plasmid-Mediated Quinolone Resistance Gene *qnrS1* in Porcine *Escherichia coli* in Europe. Microb. Drug. Resist., 17. 567-73, 2011.
3. **Szmolka A.**, Fortini D., Villa L., Carattoli A., Anjum M.F., Nagy B.: In Hungarian: Kinolon rezisztencia plazmidok molekuláris epidemiológiai jellemzése sertés eredetű multirezisztens kommenzalista *E. coli* törzsekben. Magy. Áo. Lapja, (in press) 2011.

Conference abstracts:

1. **Szmolka A.**, Fortini D., Villa L., Carattoli A., Anjum M.F., Nagy B.: First report on IncN plasmid-mediated quinolone resistance determinant *qnrS1* in porcine *Escherichia coli* in Europe. 16th International Congress of the Hungarian Society for Microbiology. Abstract in: Acta Microbiol. Immunol. Hung., 58. 226, 2011.
2. **Szmolka A.**, Anjum M.F., La Ragione R.M., Kaszanyitzky É., Nagy B.: In Hungarian: Gentamicin rezisztens állati és humán *Escherichia coli* törzsek antibiotikum rezisztencia és virulencia genotípusa. Akadémiai beszámolók programfüzete, p. 11., 2011.
3. **Szmolka A.**, Anjum M., Woodward M., González-Zorn B., Tóth Á., Adrián E., Kaszanyitzky É., Nagy B.: Microarray-based analysis of antimicrobial resistance genes in gentamicin-resistant *Escherichia coli* of food, human-, and animal origin isolated in Hungary. MedVetNet 5th Annual Scientific Meeting Abstract Book, p. 58., 2009.

Publications not directly related to the subject of the PhD dissertation

Research papers:

1. Rychlik I., Karasova D., Sebkova A., Volf J., Sisak F., Havlickova H., Kummer V., Imre A., **Szmolka A.**, Nagy B.: Virulence potential of five major pathogenicity islands (SPI-1 to SPI-5) of *Salmonella enterica* serovar Enteritidis for chickens. BMC Microbiol., 9. 268, 2009.
2. **Szmolka A.**, Libisch B., Pászti J., Füzi M., Emody L., Nagy B.: Virulence and antimicrobial resistance determinants of human pathogenic and commensal strains of *Pseudomonas aeruginosa*. Acta Microbiol. Immunol. Hung., 56. 399-402, 2009.
3. Imre A., **Szmolka A.**, Olasz F., Nagy B.: In Hungarian: Szerovarspecifikus plazmidok szerepe a *Salmonella*-törzsek virulenciájában. Magy. Áo. Lapja, 129. 428-440, 2007.
4. **Szmolka A.**, Kaszanyitzky E., Nagy B.: Improved diagnostic and real-time PCR in rapid screening for *Salmonella* in the poultry food chain. Acta Vet. Hung., 54. 297-312, 2006.

Conference abstracts:

1. **Szmolka A.**, Cramer N., Wiehlmann L., Nagy B,: Genomic analysis and clonality of Hungarian bovine and human strains of *Pseudomonas aeruginosa*. Abstract Book, FEMS-Leopoldina-Symposium on Emerging Topics in Microbial Pathogenesis, p.108, 2011.
2. **Szmolka A.**, Imre A., Nagy B.: Colonization, invasion and interleukin induction by the strain *Salmonella* Hadar-18 and its SPI-1 mutants. Abstract CD, Poster Presentations „Microbial pathogens, host susceptibility and response", 3rd Congress of European Microbiologists FEMS 2009, „Microbes and Man - independence and future challenges". 2009.
3. **Szmolka A.**, Imre A., Nagy B.: *In vitro* assessment of virulence of invasive *Salmonella* serovars and by some of their mutants. MedVetNet 4th Annual Scientific Meeting Abstract Book, p. 43-44., 2008.

Acknowledgements

I am grateful to the director and to the coworkers of the Veterinary Medical Research Institute of the Hungarian Academy of Sciences for their help and support.

My first thanks are due to my supervisor **Béla Nagy**, for helping me with his experience, knowledge and encouragement and for providing the intellectual and financial background required for this work. Further thanks are due to **Péter Zsolt Fekete, Tamás Tuboly** and especially for **Ariel Imre**, for supporing me by their useful ideas.

I thank to all coworkers of our research group: **Márta Puruczki**, **Domonkos Sváb**, **István Tóth**, for their patience and advices given during my PhD work. I am especially grateful to **Erika Sajtós** who provided an excellent technical support, and to **Barbara Lestár** as well, for helping me in the analysis of *tet*(A) plasmids.

I am also thankful to **Muna Anjum**, **Alessandra Carattoli** and **Roberto La Ragione**, who supervised and kindly helped me at different stages of the work, when international collaboration was needed. Further thanks to **Erzsébet Adrián**, **Márton Barcsa**, **Miklós Füzi**, **Éva Kaszanyitzky** and to **Ákos Tóth** for kindly providing us the *E. coli* strains. Many thanks are due also to **Margit Király** and **Judit Pászti** for the plasmid profile analysis and for their spirit of collaboration.

This work was supported by the EU FP6 NoE (EuroPathoGenomics, Contract No. 512061) and EU FP6 NoE (MedVetNet., WP29, Contract No. 506122) projects.