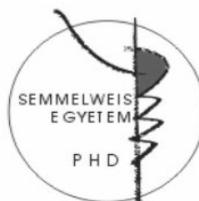


Pathogenetical characterization of *Escherichia coli* strains isolated from human infections

Doctoral (Ph.D.) thesis

Mag Tünde

Semmelweis University, School of Ph.D. Studies
Pathology Interdisciplinary Doctoral School



Consultants:

Ferenc Rozgonyi, MD, DSc

Dóra Szabó, MD, PhD

Opponents:

Béla Kocsis, MD, CSc

Pál Miheller, MD, PhD

Chairman of the Examination Committee:

Károly Rácz, MD, PhD

Members of the Examination Committee:

Gábor Veres, MD, PhD

Levente Emődy, MD, professor emeritus

Budapest
2009

1. Introduction

Majority of *Escherichia coli* strains are considered as non-pathogen but certain groups of them can cause severe enteric and extraintestinal diseases. The diarrhoeal outbreaks caused by *E. coli* mean a significant public health problem primarily in the developing countries; however they are often the case in the developed world as well. Although the mortality and morbidity rates are decreasing with the improvement of hygienic conditions, but the epidemiological significance of the clinical picture and potential serious complications constantly justify the development of laboratory diagnostic.

The *E. coli* strains causing enteric diseases are categorized by the main symptoms, their virulence-factors, and the pathomechanisms, such as enteropathogen *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), verotoxin-producing *E. coli* (VTEC), enteroaggregative *E. coli* (EAaggEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC) and the diffusely adherent *E. coli* (DAEC). *E. coli* strains responsible for urogenital infections (UPEC), and sepsis or meningitis of the neonates (MAEC) belong to the group causing extraintestinal outbreaks. The most important common property of the pathogen isolates is the possession of virulence-factors that are

encoded on a variety of mobile genetic elements such as on plasmids, bacteriophages, transposons and on pathogenicity islands. The harboured adhesins and toxins enable the colonisation of the intestinal mucosa, differently from the non-pathogen members of the normal intestinal flora. As a result the pathogens may cause wide range of enteric infections.

The *E. coli* strains causing diarrhoea were among the first pathogens that were detected by the newly developed molecular methods. In these days the PCR (polymerase chain reaction) would be the most frequently used and the most reliable method to separate the pathogens from the normal intestinal bacteria.

In the course of my work PCR based methods were introduced by means of human diseases associated *E. coli* strains, which were collected in the National Reference Laboratory for Enteric Aerob Bacteria (NERL). Due to these assays now we are able to detect genes encoding different virulence-markers. Furthermore the PCR-RFLP based technique was also brought in to identify and type the enteric pathogens. In a retrospective study all the verotoxin producing human *Escherichia coli* strains isolated in Hungary from 2000 to 2006 were characterized with the newly adapted methods.

2. Aims

1. Introduction of a microplate-agglutination method in place of the traditional tube-agglutination.
2. Adaptation of molecular methods for detecting the human pathogen *E. coli* strains as well as for identification and typing of their virulence-factors.
3. Determination of two significant verotoxin2 subtypes with PCR-RFLP method.
4. Characterization of verotoxigenic *Escherichia coli* (VTEC) strains isolated in Hungary from 2000 to 2006; analysis of geno- and phenotypes, compared to the data of the neighbouring countries.
5. Serotyping of ESBL-producing *E. coli* strains isolated from 2005 to 2007 and comparison the results with the serotypes of the worldwide spread clones.

3. Methods

Microplate agglutination test for determination of *E. coli* O groups

This assay was introduced following the principle of the traditional Widal-tube agglutination as described previously (Orskov, 1984) by the strains collected in the National Reference Laboratory for Enteric Aerob Bacteria (NERL). Polystyrene microtitre plates with 96 “V” wells were used instead of classic glass tubes, that resulted in saving of high amounts of antisera. The antigen and antibody reacted in proportion 1:1, and the evolved aggregates were stained with gentian violet.

Adaptation of molecular methods for detecting the human pathogen *E. coli* strains

First of all the representative virulence-factors (VF) were selected for detecting the different *E. coli* pathogroups. The PCR examinations identified the genes encoding VF's of the strains collected and identified in the National Reference Laboratory for Enteric Aerob Bacteria. After determination of the certain phenotypes, the toxin-production was tested by verotoxin ELISA (RidaScreen) and the

toxins' type and titre were assessed by VTEC-RPLA (Oxoid). The genes, gene clusters or regions required for the expression of virulence-markers can be detected with the adapted PCR techniques, such as the genes of intimin (*eae*), enterohaemolysin (*ehlyA*), enteroaggregative heat-stable toxin (*astI*), ETEC heat-labile enterotoxin (*elt*), ETEC heat-stable enterotoxin (*est*) and the invasion-related plasmid (*Ipa*). The newly adapted method for typing of the flagellar (H) antigen was an PCR-RFLP (restriction fragment length polymorphism)-based method, which completed the classic tube-agglutination assay.

Determination of two significant verotoxin2 subtypes with PCR-RFLP

PCR-RFLP was performed with verotoxin-positive *E. coli* strains collected previously. This typing method is a useful tool for identifying the hypervariant region of verotoxin 2 (PCR with primers GK3-GK4). The amplification products were digested with restriction endonuclease enzymes *Hae* III and *Fok* I (Promega) to differentiate the clinically relevant *vt2* subtypes. The *vt2* types of all the VTEC strains isolated from 2000 were determined by this method.

Characterization of verotoxigenic *Escherichia coli* (VTEC) strains isolated in Hungary from 2000 to 2006

Some VTEC suspicious *E. coli* strains were used for that purpose, which were sent to the National Reference Laboratory for Enteric Aerob Bacteria between 2000 and 2006. The verocytotoxin was detected by verotoxin ELISA (RidaScreen) to confirm the toxin-production. The serotypes of the isolates were identified by tube-agglutination and the toxins' titer was detected by VTEC-RPLA after the phenotypical assays. Antimicrobial susceptibility of the isolates was tested by disk diffusion method in accordance with the 'CLSI' standards and guidelines. The virulence markers were detected by PCR assays, while we used a PCR-RFLP based method for differentiation between the *vt2* variants. The most significant intimin subtypes are the EHEC (mostly O157:H7) associated $\gamma 1$ and the EPEC associated $\beta 1$. Pulsed-field gel electrophoresis was carried out after DNS extraction from the strains according to the standardized *Escherichia coli* O157:H7 protocol of the CDC PulseNet. DNA relatedness and the analysis of cluster were calculated on the basis of the Dice coefficient.

Serotyping of ESBL-producing *E. coli* strains isolated from 2005 to 2007

For determination the serotypes of ESBL-producing strains we received some isolates from the ESBL Reference Laboratory, after the confirmation of ESBL-production. The confirmation, as well as the determination of phylogenetic group and the O25b-ST131 clonal analysis were performed by PCR examinations.

4. Results

Microplate agglutination test for determination of *E. coli* O groups

The results of the reactions on microtitre plates and in tube correlated in every case. This method proved to have the same sensitivity as the classical method. Moreover the microplate agglutination had further advantages: it requires far less reagents and space, the implementation is easier, and the reaction can be performed in a less total volume and under more controlled conditions. The new method is low-priced and more reliable on the whole.

Adaptation of molecular methods for detection of human pathogen *E. coli* strains

Comparative examinations confirmed that the PCR assays had adequate sensitivity and specificity for detection the virulence-markers of the pathogen *E. coli* strains responsible for severe human diseases, moreover we were capable to differentiate the diarrheagenic *E. coli* strains from the nonpathogenic members of the normal flora by the use of these methods. The classical phenotypic assay for invasiveness was the Serény (guinea pig keratoconjunctivitis) test, while the sensitivity of the new PCR examination proved to be the same. Therefore the animal test was exchanged for this molecular method. Previously a differential culture medium (TSB agar with washed sheep erythrocytes) was used for detection of enterohaemolysin, but after doing the comparative tests, the PCR - which was more sensitive and less subjective - was chosen for detection this marker. I have adapted and optimized the PCR based detection of the genes of *ast1*-, *est*-, *elt*-, *eae*-, *eae/β1* and *eae/γ1* by applying of international control strains. The restriction pattern of the flagellar gene's typing PCR-RFLP method was also a valuable tool for typing of non-motile (NM) and non-typeable (NT) H antigens.

Determination of two significant verotoxin2 subtypes with PCR-RFLP

The PCR-RFLP analysis has been used to distinguish *vt2c* from *vt2d-O₁₁₈* subtypes, which show high level of genetic and functional homology. 90% of the GK3-GK4 PCR positive strains carried the *vt2c* toxin type while only 10% possessed the *vt2d-O₁₁₈* toxin type. The results could not be compared with other classical methods' outcomes, because there were no other methods available for toxin-typing before. Currently we don't use it in routine diagnostics only in retrospective studies.

Characterization of verotoxigenic Escherichia coli (VTEC) strains isolated in Hungary from 2000 to 2006

Among the investigated VTEC isolates the O157:NM proved to be the most prevalent representing almost half (45%) of the strains, besides three strains belonged to the O26 group, and two to the O146 serogroup. All other O groups were represented by only one strain and these proved to be O15, O43, O75, O87, O96, O98 and O109 respectively. Thirty percent of the isolates were able to ferment D-sorbitol. All but one of the O157 strains was unable to utilize D-sorbitol. Most of the O157 isolates were obtained from children's

samples (63%). HUS evolved only in two patients both infected by O26:H11. Out of the investigated strains 76% were positive for the *eae* gene, among them the O157 strains were positive for $\gamma 1$, and two O26 strains produced $\beta 1$ intimin. 61% of the strains carried the *ehlyA* gene, and five non-O157 strains had enteroaggregative heat-stable toxin (*east*) gene as well. Six different genotypes were observed for the verocytotoxin genes, in the following combinations: *vt1* (33%), *vt1vt2c* (33%), *vt2c* (18%), *vt1vt2* (6%), *vt2d* (6%) and *vt2* (3%). The O157 isolates had *vt1* and *vt2c* genes in high frequency, especially in combination: *vt1vt2c*. Patients infected by *vt2* producer strains presented HUS in two cases. The non-O157 strains most frequently possessed the *vt1* gene (64%) and less frequently had *vt1vt2* (14%), *vt2d* (14%) or *vt2* (7%) genes.

The majority of the strains (76%) were resistant to at least one of the antimicrobial agents tested. Resistance patterns ranged from resistance to ampicillin only (30%) to resistance to four different antibiotics (sulphonamid, streptomycin, tetracycline, kanamycin). None of the isolates showed ESBL-phenotype. With the exception of two strains the patterns of the O157 strains showed more than 80% similarity to each other, suggesting that they belong to the same genetic cluster and four non-O157 strains also formed a small cluster.

Serotyping of ESBL-producing *E. coli* strains isolated from 2005 to 2007

The results showed that two groups, the O25 (25%) and the O15 (8%) appeared in significant high numbers among the ESBL-producing strains collected from 2005 to 2007. Random PCR assay confirmed that the members of the world-wide spread O25b-ST131 clone were present among the O25 isolates.

5. Conclusions

Microplate agglutination test for determination of the *E. coli* O groups

Several *Escherichia coli* Reference Laboratories are using the microtitre plate agglutination method for the identification of O serogroups. The introduction of microplate test means simplification in the methodology in contrast to tube-agglutination. It is necessary to test every antisera with all antigens, and to allocate the homologous titer and to remove all the cross-reacting antigens. Following these adaptation tests the microtitre plate agglutination method can be applied in the laboratory diagnostics as well.

Adaptation of molecular methods for detection some human pathogen *E. coli* strains

The pathogen *E. coli* strains originating from stool samples were detected on culture media or by serological methods previously, occasionally the pathogenicity of them was confirmed with animal test. All these assays are expensive, time consuming and laborious; therefore the DNA based molecular biological methods would be more effective. The aims of my investigation were to adapt rapid and reliable methods for detection of pathogen *E. coli*, which are required for taming of epidemics and treatment of patients.

The typing methods and retrospective studies help us to recognize the major epidemiological trends, and to develop a successful surveillance system.

Determination of two significant verotoxin2 subtypes with PCR-RFLP

The relationship between the verotoxin types and the severe diseases are proven and well known. The *vt2*, *vt2c* and *vt2d-O₁₁₈*-producing *E. coli* strains are responsible for the most serious outbreaks. The laboratories may decide about what PCR assays they do prefer. In my judgement the determination of VTEC isolates' verotoxin-type

may be the most significant examination for the prognosis of the clinical picture.

Characterization of verotoxigenic *Escherichia coli* (VTEC) strains isolated in Hungary from 2000 to 2006

In our retrospective study 33 VTEC strains –isolated over a seven-year period- were investigated. In agreement with the literature data, O157 was found to be the most frequently identified VTEC serotype. None of the isolates showed ESBL-phenotype, and resistance to five or more drugs was not found among the isolates. The combination of verocytotoxin genes were detected according to the literature data likewise the most severe outbreaks were due to *E. coli* strains producing typical toxintypes . In agreement with the literature data the O157 strains were positive for γ 1-while the O26 strains produced β 1 intimin. The genetic variety observed among most of our O157 isolates by PFGE demonstrates that they do not have a single clonal origin.

It has to be mentioned that the number of the Hungarian human VTEC strains isolated yearly is rather low compared to those isolated in other European countries of similar size and inhabitants as Hungary. Besides the numbers of reported VTEC cases rose in the European Member States during 2000-2005 while in Hungary it

reduced year by year. Most of the non-O157 VTEC strains seem to be lost and the real number of the cases must be surely higher than detected. Due to the deficiencies of the routinely used methods the verotoxigenic *E. coli* strains must be underrepresented in Hungary. The results of the serotyping may be more indicative. Certain tendencies emerged, such as descending number of samples, low diversity of serotypes and low incidence. Our aim is to change this situation based on a considerably improved surveillance system.

Serotyping of ESBL-producing *E. coli* strains isolated from 2005 to 2007

Determination the serotypes and phylogenetic groups of ESBL-producing strains showed that the O25 serogroup were represented in prominently high number in Hungary similarly to other countries' data. Much of the O25 isolates were confirmed by PCR to belong to the worldwide- spread O25b-ST131, CTX-M ESBL harboring clone from B2 phylogenetic group. These data proved to be a highly result in respect of public health as well.

6. List of publication

Related to the thesis

Papers

Mag T (2004) A Shiga toxin termelő *Escherichia coli* törzsek molekuláris tipizálási lehetőségei. Mikrobiológiai Körlevél 2004/4 IV.

Sonnevend Á, Al Dhaheri K, **Mag T**, Herpay M, Kolodziejek J, Nowotny N, Usmani A, Sheikh FA, Pál T (2006) CTX-M-15-producing multi-drug resistant enteroaggregative *Escherichia coli* in the United Arab Emirates. Clin Microbiol Infect, 12: 582-585. IF: 3,554

Kiss R, Szita G, Herpay M, Csikó Gy, Pászti J, **Mag T**, Szita J, Bernáth S (2009) The isolation of verocytotoxin-producing *Escherichia coli* (VTEC) strains from improperly pasteurised cow's milk samples. Acta Alimentaria (in press) IF: 0,441

Mag T, Nógrády N, Herpay M, Tóth I, Rozgonyi F. (2009) Characterization of verotoxin-producing *Escherichia coli* strains isolated from human patients in Hungary over a 7-year period. EJCMID, (in press) IF: 2,866

Citable congress abstract

Mag T (2004) A Shiga toxin-termelő *Escherichia coli* törzsek (STEC) molekuláris tipizálási lehetőségei tekintettel az Stx2 altípusaira. Magyar Mikrobiológiai Társaság Nagygyűlése, Keszthely 2004. 10.

Mag T (2004) A Shiga toxin termelő *Escherichia coli* törzsek (STEC) molekuláris tipizálási lehetőségei tekintettel az Stx2 altípusaira. SOTE, PhD Napok, Budapest 2005.04.

Mag T (2005) *Escherichia coli* pathocsoportok. OEK Bakteriológiai főosztály munkaértekezlet, Budapest 2005. 06.

Mag T (2005) *Escherichia coli*, mint kórokozó - molekuláris vizsgálatok, tipizálási lehetőségek. Referáló előadás, SOTE, Mikrobiológiai Intézet 2005. 04.

Pál T, Sonnevend Á, Al-Dhaheri K, Herpay M, **Mag T**, Usmani A, Kolodziejek J, Nowotny N (2006) Shiga toxin-producing *Escherichia coli* isolated from one-humped camels (*Camelus*

dromedarius) European Congress of Clinical Microbiology and Infectious Diseases (16.ECCMID) , Nizza, 2006.04.01-04. (poszter)

Not related to the thesis

Papers

Lukács J, Bognár Cs, Szántai E, **Mag T.** (2001) Legionella átfertőzöttség

Magyarországon. Focus Medicine. III. évf/2. 31-34.

Bognár Cs, Kádár M, Senoner Zs, Lukács J, Szántai E, **Mag T,**
Tarján E (2001)

Legionellózis Magyarországon. Focus Medicine 2001. III. évf/ 2. 27-30.

Citable congress abstract

Lukács J, Bognár Cs, Szántai E, **Mag T.** (2001) Legionellózis: szeroepidemiológiai vizsgálatok eredményei. Szeroepidemiológia 2000. OEK Tudományos ülés Budapest, 2001.03.06.

Mag T, Herpay M, Hegedűsné Richter Zs (2004) A yersiniák által okozott fertőzések immunszerológiai diagnosztikájának fejlesztése. Biomedica Symposium 2004.03.

Mag T (2007, 2008) Pestis, brucellózis, tularémia és malleus molekuláris biológiai diagnosztikája. Veszélyes kórokozók diagnosztizálása, Továbbképzés, OEK