The clinical use of a fast screening test based on technology of capillary zone electrophoresis (CZE) for identification of *Escherichia coli* infection in biological material

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: *Escherichia coli* is a Gram-negative bacterium which is a basic, symbiotic element of the physiological flora of the large intestine of humans and warm-blooded animals. However, in specific cases it may become a very dangerous pathogen (eg, diarrhoea, infection of the urinary tract, lungs, and generalized infections). Its early detection, as a cause of infectious disease, helps to achieve optimal treatment results; however, classical microbiological tests require at least 24 hours from sample taking to diagnosis.

Material/Methods: We present a unique solution based on CZE technologies enabling identification of *E. coli* presence in studied sample within half an hour. Altogether, 30 *E. coli*-infected wounds and ulcerations were examined, comparing the results obtained by classical culture method with the result of capillary zone electrophoresis (CZE) electropherogram.

Results: The method, which does not require any preparation of the sample, achieved 86.7% sensitivity and 85% specificity in the examined clinical material (infections of surgical wounds).

Conclusions: The obtained results enable reliable, very fast testing for *E. coli* as a pathogen.

key words: *Escherichia coli* • capillary zone electrophoresis • infection

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BACKGROUND

E. coli is a Gram-negative bacterium of the Enterobacteriaceae family, and is an important symbiotic member of the physiological flora of the large intestine of humans and warm-blooded animals. Its basic functions in physiological conditions include decomposition of food and production of vitamins of B, K and C groups. It is isolated quite often from soil and water, where it comes from contaminations such as sewage or feces. Its presence is also confirmed in superficial layers of mucosal membranes and skin [1].

There are many strains of these bacteria, differentiated based on somatic, superficial and ciliary antigens of various virulence based on adhesive abilities, capsule structure and production of toxins. Thus, these microorganisms, beneficial in their natural environment, may suddenly become the cause of dangerous infectious diseases: diarrhoea, hemorrhagic enteritis, infections of the urinary tract, surgical wounds, nosocomial pneumonia, and finally, a fatal sepsis.

Early detection of a pathogen, especially a pathogen causing infectious disease of clinically significant manifestation and at an atypical site, may in some cases be essential for selection of appropriate, targeted antibiotic therapy enabling effective treatment or effective prophylaxis of the infection. The optimal diagnostic method, considering the biology of *E. coli* strains, would enable fast identification of bacterial cells in the biological sample (infected tissue, foodstuff, water) without the need to carry out laborious preparatory (incubation) procedures. Unfortunately, current diagnostic methods do not meet basic requirements assuring efficacy of therapy, that is, the time required to obtain microbiological result. Inoculation and phenotypic methods require up to several days of culture, whereas methods based on genetic identification (PCR) available only in a few centres, are very expensive and require specialized equipment. Currently available fast screening strip tests, such as Singlepath® *E. coli* 0157 (Merck) give results in as few as 20 minutes after placing a sample, but require its preparation for many hours (incubation). Obviously, even the quickest test whose purpose is to detect a single specific pathogen, using a guided approach, cannot determine the final clinical diagnosis of an infection, if only because of a potential risk of infection with mixed bacterial flora. To gain clinical significance, such a test should provide the pattern of sensitivity to medication of the detected microorganism.

For several years we have been investigating identification of pathogenic factors of bacterial infections with the use of electrophoresis. The best results were have been obtained by a method of capillary zone electrophoresis. Although current technology allows only a relatively reliable identification of a single pathogen, such as *Escherichia coli*, the construction of the apparatus allows us to combine techniques and to detect conditions, which in future may broaden the spectra of the specific pathogens responsible for mixed infections. The present study evaluates its use under clinical conditions for the first time. We evaluated the diagnostic potential of a method of capillary zone electrophoresis according to methodology of Buszewski et al for screening identification of pathogenic strains of *Escherichia coli*. The above method was used for the first time in clinical conditions for instant microbiological evaluation of biological samples infected with *E. coli*.

MATERIAL AND METHODS

Material

The study enrolled 60 consecutive patients of the Clinic of General Gastroenterologic and Oncologic Surgery of the Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University diagnosed with superficial infection of surgical wounds. The reference method for microbiological diagnosis of *Escherichia coli* and other infections were classical, culture phenotypic studies carried out in the Department of Microbiology of Regional Hospital in Torun. In 4 cases, verification was repeated due to suspicion of coexisting infection in the examined sample after the use of the CZE method.

The infected biological sample was taken from a surgical wound with symptoms of complications in the form of a superficial infection from trophic ulceration of similar character. It was usually a 0.5–1.0 ml sample of a secretion taken in aseptic conditions with a sterile syringe and placed in 1.5 ml of sterile water (Aqua pro injection). When the density or amount of the secretion prevented simple sampling (aspirations to a syringe), the wound was rinsed with 1 ml of water, and then the sample was taken by the method above. The material, poured into sterile, tight, transport test-tubes, was immediately transported to the laboratory and introduced into CZE apparatus directly from the transport container. The methodology of microbiological analyses was based on Buszewski et al. [2–5]. At the same time, with the use of a microbiological spatula, a swab was taken from the wound and subjected to classical microbiological diagnostics. After obtaining the phenotypic result and confirming that only *E. coli* was present in the sample, in the same, sterile way bacterial cultures were taken from a Petri dish and placed in 1.5 ml of sterile water (*Aqua pro injectione*) and then subjected to the same electrophoretic analysis. Clean, standard electropherograms characterizing the presence of individual pathogenic strains of analyzed bacteria were obtained by this method.

Each of the 60 sites from which microbiological test material was taken was catalogued. In 31 subjects these were infected postoperative wounds after resection of the large and the small intestine (group A), in 8 subjects they were trophic ulceration of the lower extremity due to chronic venous insufficiency or ischemia (group B), in 7 patients specimens were taken from infected wounds after urgent laparotomy in the course of peritonitis following perforation of the gastrointestinal tract (group C), in 10 subjects they were from suppurative wounds following other surgical procedures of the alimentary tract without affecting the intestinal continuity (eg, infected necrosis of the pancreas in acute pancreatitis) (group D) and in 4 subjects they were from foot ulceration in diabetic foot syndrome (group E). An example of a wound is presented in Figure 1.

System for capillary electrophoresis

During these tests, the system of measurement consisted of the following elements:

- System HP{3D CE (Agilent Technologies, Waldbronn, Germany) equipped with DAD (*diode array detector*) with diode matrix technology enabling formation of the continuous spectrum of the examined substance in real time.

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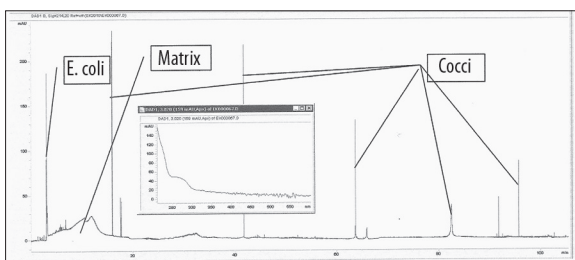**Figure 1.** Example of an infected wound (appendectomy).

Figure 2. Electropherogram of additional strains in the biological sample, in which conventional method primarily detected only presence of *E. coli*. The next inoculation showed co-infection of *Staphylococcus* sp.

The apparatus was connected with working station KAYAK with ChemStation software (Hewlett-Packard) for operating the apparatus and collection of data.

- Fused silica capillaries (Composite Metal Services, Worcester, UK).

Buffer

TBE buffer optimized towards *E. coli* consisted of 4.5 mM Tris/4.5 mM, boric acid/0.1 mM EDTA (disodium ethyleneaminetetraacetate) of pH=8.53 diluted with deionized water in 8: 1 ratio with addition of 0.2 g PEO/40 ml of solution (polymer concentration 0.5%). The polymeric solution was then dispersed in an ultrasound bath for 4 hours at 60°C. Finally, dilution with TBE buffer to the concentration of 0.0125% completed preparation of the final buffer.

RESULTS

Of 60 examined, blind biological samples, isolated infection of *E. coli* was diagnosed in 30 by classical microbiological methods (in 19 subjects from group A, 1 subject from group B, 5 subjects from group C, 5 subjects from group D and no subject from group E), and those samples were selected for further analysis. Other infections were diagnosed in 20 inoculations. Complicated infections accompanying *E. coli* were diagnosed in 10 remaining cases (other bacterial species, fungi) and they were rejected from the examined group.

In the final analyzed group of 30 infections with *E. coli*, in 26 cases pathogen was concomitantly diagnosed within as
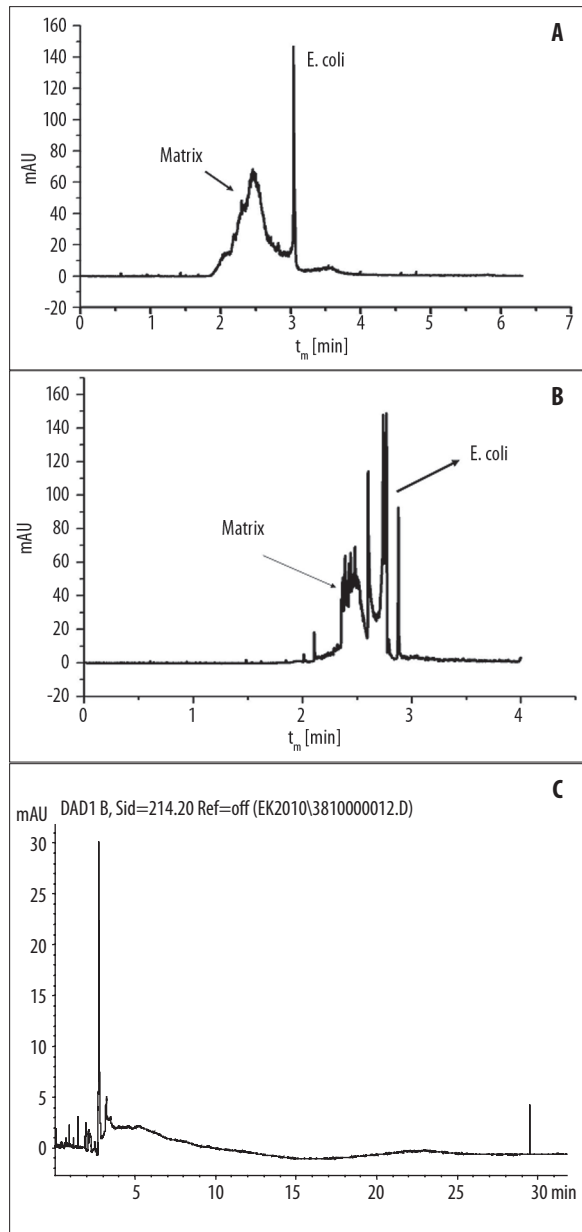

Figure 3. Typical electropherograms of biological sample (**A, B**) and standard (**C**) with presence of *Escherichia coli* (arrow).

few as 45 minutes, based only on pathognomonic electropherogram of the sample. The picture was ambiguous in 4 cases, not allowing a reliable diagnosis. Interestingly, in as many as in 3 of them, based on the character of the obtained CZE diagram, a concomitant infection with cocci forming clusters was diagnosed with a high probability. On the following day classical inoculations from these 3 wounds were repeated. In the obtained, repeated results, concomitant infections with *Staphylococcus sp.* strains (2) or *Enterococcus sp.* (1) were confirmed in all 3 cases (Figure 2). All of these cases were also rejected from further processing for *E. coli* due to the risk of interaction of bacterial cells of various species under conditions of CZE. A similar, but opposite situation was observed in a group of 20 infections with bacteria different from *E. coli*. In these 3 cases, coexistence of signal typical for *E. coli* was recognized by the CZE
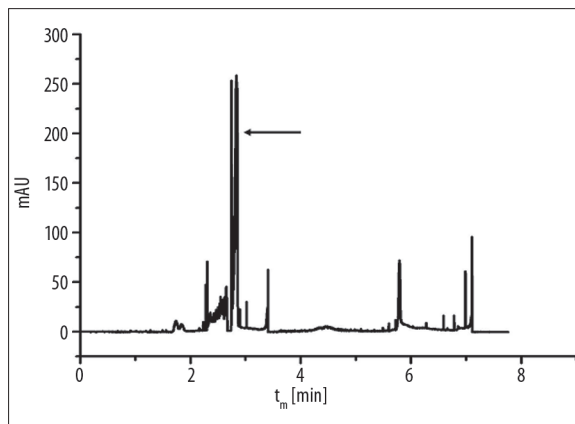
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Figure 4. Selected electropherogram of the biological sample infected with *E. coli* (+++) and *Enterobacter cloacae* (+). Clear, pathognomonic signal of *E. coli* is seen of tm=2.6 minutes.

Figure 5. Spectrum of background (morphotic elements of tissue excretion) – matrix effect.

method; however, it was not confirmed primarily by a reference method, but in this case, subsequent verifying inoculations were not carried out.

Based on the final analyzed 52 (2*26) biological samples of isolated infections, a standard electropherogram that characterized presence of bacterial cells of Escherichia coli was determined (Figure 3).

The typical peak characterizing presence of *E. coli* appeared as a single peak in time range from 2 to 5 minutes within flat-wide, complex and irregular signal (matrix-effect). Such a single high signal in the electropherogram was considered a pathognomonic picture for *E. coli* infection, as well as for cases of complex infections (Figures 3,4). Matrix effect being an irregular, wide signal, contained in 0-15 minutes band, was a picture of sample background, which in this case consisted of solid particles and chemical substances of tissue excretion (eg, epithelial cells, morphotic elements of blood, fibrin, minerals) (Figure 5).

Typical validation methods with respect to the finally selected uniform group of infections were used for determination of basic parameters (sensitivity and specificity) of the developed CZE system as the diagnostic method for detecting presence of *E. coli* rods by screening.

Based on the described, simple comparative analysis, the initial degree of detection of *E. coli* infection was obtained in a biological sample of 86.7%. This sensitivity for CZE set designed in this way was determined at the level of microorganism species. Specificity of the method in the whole examined group reached 85.0%; however, it should be considered that in the group of results considered as non-infected with *E. coli*, some cases could be found which were not detected primarily by phenotypic method (not verified by repeated inoculation).

These parameters may indicate a high quality of the developed screening test already at this stage.
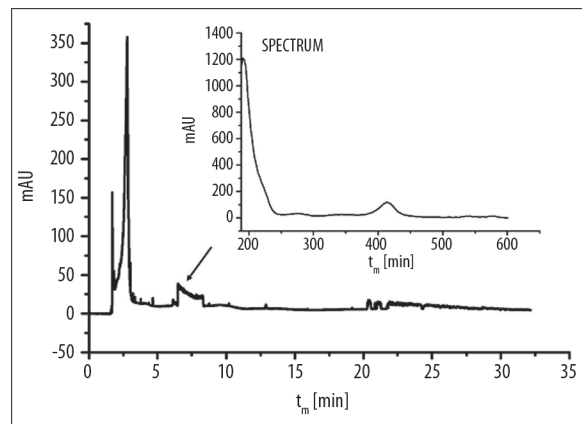
Discussion

Morbidity of *Escherichia coli* is strictly strain-dependent. There are both totally non-virulent strains that are important factors of intestinal homeostasis, and virulent strains causing various infectious diseases. In general, morbidity of these bacteria is revealed after occurrence of some favourable conditions, such as infancy, or a decrease of general and local immunity. In extreme cases, the infection may occur in the systemic form, with multi-organ failure, which is why *E. coli* is the most common factor causing generalized infections in newborns. There are 3 main groups of pathogenic *E. coli*: diarrhoea-causing strains EPEC, strains causing urinary tract infections UPEC, and strains causing sepsis SEPEC and cerebrospinal meningitis NEMEC [6].

In natural conditions, *E. coli* is found in human and animal intestines as well as in soil and water, where it comes from secretion and feces. Its presence in surface water is a commonly-used indicator of contamination. These bacteria may also colonize the skin and mucus membranes of the oral cavity and respiratory system.

Microbiological diagnostics of infections with these bacteria consists of classical inoculation methods and identification of strains with the use of agglutination techniques.

Diagnosis at the level of a strain is sometimes also made by highly specialized PCR methods.

Mean time to obtain a detection result by the traditional method can be from 2 to 7 days [7]. Treatment consists of using initially empirical and then targeted antibiotic therapy.

Starting the antibiotic therapy as soon as possible is very import in the treatment of most severe infections, such as when ensuring that the optimal choice of antibiotic in treatment of the detected bacteria species is based on empirical tables. Sometimes, especially in newborns, hours determine life and death of a patient, but detection of the pathogen causing the disease may not be so quick. Microbiological identification by traditional methods may be difficult – many problems may be encountered that prolong the time to final result. Complicated methods of sampling the material onto transport media and inappropriate conditions of transport

to the microbiological laboratory may cause pre-laboratory errors that make results unreliable. Identification analysis of isolated colonies, depending on selected method, may take, in case of *E. coli*, up to 24 hours. Automation of determinations (eg, Vitek 2) allows this time to be shortened to about 10 hours in the case of Gram-negative bacteria, but a classical method of culture of microorganisms on Petri dish before the test is still required.

Diagnostics at low cellular concentrations may be an additional problem. As few as 10 cells of O157 strain *E. coli* may cause infection, and such a low concentration may be insufficient for identification of a pathogen by currently used methods. In light of these factors, biotechnological and biochemical methods become more and more important.

One of the most commonly investigated methods is combination of PCR and capillary electrophoresis, offering highly sensitive diagnostics for bacteria to be identified based on 16S rRNA amplifications of individual bacterial strains. However, their drawback is time needed, requirement to employ additional specialists and very expensive and complicated laboratory equipment, including genetics and isotope laboratories.

New diagnostic techniques such as capillary electrophoresis-based single-strand conformation polymorphism (CE-SSCP) employ the relatively simple CE method, which significantly simplifies methodology without worsening parameters of the method. The method of microbiological determination based on electrophoresis process and PCR is in some cases widespread, such as during analyses of bacteriological purity of foodstuffs [8]. This method is very sensitive and can be modified, for example by addition of multiplex PCR, in order to isolate electrophoretic signals of certain bacterial strains from a bacterial mixture even in a microscopic sample.

Chip technique is an innovation consisting of miniaturization of a PCR-based model and capillary electrophoresis, analyzing and counting the amplicones specific for certain bacterial strains obtained from just a few cells [9]. Such miniaturized equipment seems to be ideal for mobile microbiological identification, but it is still being developed and is not ready for commercial use.

In our studies we decided to develop a very sensitive method that will meet the requirements of optimal microbiological screening diagnostics, by only slightly decreasing the specificity level with respect to the level of a strain. First stage testing should identify most common infections such as *E. coli*. As a principle, the method should be simple, fast and most important, possible to perform in the place of biological material sampling. Calibration of the method for selected strains of *E. coli* showed that simple morphological analysis of the electrophoretic spectrum of the sample (eg, obtained as a result of ECG evaluation) is sufficient for its identification from a highly complex biological sample (eg, pus). Earlier studies of Buszewski et al on standard models of selected bacterial strains have confirmed that presence of electropherogram signals in their typical place (time to appear) and configuration (mutual order of many peaks) may be of pathognomonic value [3–5]. Our subsequent studies on clinical material show that tests for infections of *Staphylococcus sp.*, *Streptococcus sp.* or *Enterococcus sp.* are equally valuable, and will be published next.

After conducting the first clinical applications, it seems that all assumed characteristics of a good screening test in the CZE method were obtained, but it still requires improvement. At its present stage, it is clearly characterized by:

1. Simplicity – the used equipment enables placing it in any room and it only requires basic service. Analysis does not require preparation of the sample, allowing it to be directly placed into a measuring apparatus immediately after being taken from the examined site.
2. High speed – the time required to identify *Escherichia coli* cells in settled buffer solution TBE+PEO did not exceed 30 minutes. Compared with traditional methods identifying this species of bacteria, this is a crucial result, levelling the time to obtain it with routine, basic blood analyses.
3. Sensitivity and repeatability – based on the analyzed group of patients with surgical wound infections, a 86.7% sensitivity was obtained, as well as in cases of mixtures of bacteria of various species. Specificity of this method was calculated to be 85%. With maintenance of stable parameters of the study, it is a very repeatable method. Moreover, in several cases it detected presence of other bacterial species colonies, thus exceeding sensitivity of routine culture (phenotypic) methods.

The obtained initial results place the above method at the level of a good screening test. Further studies, supported by analysis of greater and more microbiologically complex groups, most probably will allow improvement of the method. Rapid general definition of a pathogen may in the future become a significant factor improving prognosis of most severely ill patients with virulent *E. coli* strains, such as newborns, in whom it is a common cause of bacteraemia and sepsis [10]. Early initiation of empirical antibiotic therapy based on identification of the bacterial species causing the disease will significantly improve the results of treatment, buying time to obtain antibiograms by traditional methods. In each medical centre dealing with treatment of infectious diseases, a detailed table presenting empirical sensitivity to antibiotics should be developed, based on the local and current microbiological statistics; this is of particular importance since increasingly frequent infections with such common bacteria as *E. coli* are characterized by a wide spectrum of resistance to antibiotics [11].

This method is limited at the present stage of development as a parallel and reliable identification of infections caused by mixed bacterial flora and evaluation of their sensitivity to medications is currently impossible. The present research, however, indicates that it is possible to combine tests oriented to other microorganisms, which may partly eliminate the above-mentioned weaknesses.

This method may also be used as an industrial test for evaluation of contamination of food and water, with potential wide use in prophylaxis of bacterial infections, as well as in other, specialized epidemiological studies (eg, for a quick analysis of potential vectors of human infections with *E. coli* 0157: H7, such as molluscs) [12].

It may be possible to improve the sensitivity of the method, reaching for virulence of individual strains, may be isolation with use of both superficial and intracellular CZE-specific cell antigens obtained, for example, by homogenization.

The classical CZE method by itself clearly has great potential for detecting and identifying characteristic, species-specific, bacterial cells.

CONCLUSIONS

These initial results of the clinical use of the method identifying presence of infection with *E. coli* based on electrophoretic signal raises hopes for creating a reliable, very fast and cheap screening test for use both in medicine and industry.

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