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MINIREVIEW

Viral Infection of the Heart: Pathogenesis and Diagnosis

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Abstract

Viral infections of the heart cause serious clinical problems, either as infectious myocarditis, which usually is a consequence of acute infection or as idiopathic dilated cardiomyopathy, resulting rather from a chronic infection. This minireview presents an up-to-date view on pathomechanisms of viral infection of the heart tissues, the role of immune system in controlling infectious process at its various stages and current possibilities of recognizing viral infection of the heart with use of both cardiological and virological methods. Our goal was to present the variety of known viral agents causing heart infection, level of complexity in mutual virus-cell interactions, and consequent clinical scenarios.

Key words: dilated cardiomyopathy, infectious myocarditis, viral infection of the heart tissue, virus-cell interaction

Viral infection of the heart is an important cause of serious clinical problems: infectious myocarditis (MC) in its various forms (fulminant, acute and chronic) and idiopathic dilated cardiomyopathy (iDCM) are crucial consequences of viral infection (Blauwet and Cooper, 2010). From a classical point of view, the group of cardiomyotropic viruses included, first of all, human enteroviruses (HEV), with major agents of acute MC: coxsackieviruses A (CVA) and B (CVB). Group of other viruses thought to be responsible for infections of the heart was rather immutable for decades and consisted of rubella virus (RUBV), influenza viruses A and B (FluAV, FluBV), human adenoviruses (HAdV) and paramyxoviruses (mumps virus – MuV, and parainfluenzaviruses – HPIV) (Dennert *et al.*, 2008). Constant improvement in sensitivity of direct virological diagnostic methods, especially introduction of molecular biology techniques, revealed presence of new viral agents in endomyocardial biopsy (EMB) samples, and thus significantly changed the picture of MC etiology. Nowadays, the most prevalent virus found in EMB samples is parvovirus B19 (B19V). "Classical" cardiomyotropic viruses remain on the list, but increasing role of herpesviruses: human herpesvirus 6 (HHV-6), cytomegalovirus (CMV), Epstein-Barr virus (EBV) and herpes simplex viruses (HSV-1 and HSV-2) was also perceived (Kühl and Schultheiss, 2009; Basso *et al.*, 2013).

According to the current model of acute cytopathic viral infection of myocardium, which is seen in enteroviral and adenoviral diseases, a virus reaches the heart with bloodstream from other, usually primary sites of an infection, in the form of a cell-free virion or within infected leukocytes, mainly lymphocytes or macrophages (Kühl and Schultheiss, 2009). Cardiomyocytes are the most important target cells for these viruses. Infection within myocardium often results in acute inflammation, but may also lead to non-inflammatory damage of cells, and as a consequence, to infection-related cardiomyopathy. Acute MC is dominated by Th1 and Th17 responses (Huber *et al.*, 2002; Yuan *et al.*, 2010), while activation of Th2 immune reactions is necessary for developing of chronic MC with fibrosis and iDCM (Fairweather *et al.*, 2004; Abston *et al.*, 2012). The main opposition between these two processes (Th1 immune response inhibits Th2 reaction and vice versa), is also complemented by important role of Th17 response, which – being a part of Th1-related inflammatory response – is also believed to induce post-inflammatory cardiac remodeling, characteristic for iDCM (Fairweather *et al.*, 2004). Pathophysiological mechanism of enteroviral and adenoviral MC in humans is still not fully understood, but it is suspected, that this is a three-phase process, as was observed in murine models (Kawai, 1999).

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Heart infection begins when virus invades the cardiomyocytes with the participation of a specific receptor. Coxsackievirus uses coxsackie-adenovirus receptor (CAR), which is a junctional protein. The same structure is used by HAdV (Kühl *et al.*, 2003). In the absence of CAR expression on cardiomyocytes, the viral invasion of these cells is impossible (Shi *et al.*, 2009). The decay accelerating factor (DAF, CD55) is a co-receptor for HEV internalization, and αv-type integrins are needed for adenovirus penetration (Bergelson *et al.*, 1997; Stewart and Nemerow, 2007). As long as this stadium is adequately treated, it may not cause significant damage. It can also result in a heart failure or death due to the direct cytopathic effect caused by an active replication (Herzum *et al.*, 1994).

The first, acute stage of an infection, can end with elimination of the virus from the heart and renovation of damaged tissue. The innate immunity is the first line of defense against virus. This conservative system activates the inflammatory process by toll-like receptors (TLRs), especially TLR-3 and TLR-4, which are located in large quantities in the cells of cardiovascular system. TLR signaling is induced by a variety of ligands which are associated with infectious pathogens (Yajima, 2011). It is interesting, that the disruption of TLR-3 is irrelevant to the interferon beta (IFN-β) mRNA expression in the heart, while TLR-3 deficiency suppresses the expression of class-I interferon regulatory factors (IRF) in the dendritic cells (DCs) infected by CVB (Negishi *et al.*, 2008). This suggests a major role of TLR-3 signaling in response to heart infection, however, its role has not been studied specifically in the cardiomyocytes (Yajima, 2011). The receptors mentioned above recognize foreign antigens, trigger the activation of nuclear transcription factors and lead to the production of inflammatory cytokines (Sagar *et al.*, 2012). TLR effects are mediated by few intracellular pathways, especially myeloid differentiation factor-88 (Myd88) (activated by all TLRs, except TLR-3) and TIR-domain-containing adapter inducing interferon-β/TIR-containing adaptor molecule (TRIF/TICAM) (stimulated by TLR-3 and TLR-4). Next step is up-regulation of inflammatory cytokines, by interleukin-associated kinases (IRAK4), TNF receptor-associated factor (TRAF6), TGF-β-activated kinase 1 (TAK1), nuclear factor kappa-light-chain-enhancer (NF-κB), and mitogen-activated protein (MAP) kinases activation. The production of INF-β is also regulated by serine/threonine protein kinase (TBK) and interferon regulatory factor 3 (IRF3) (stimulated by TRIF/TICAM) (Lafyatis and Farina, 2012). The activation of non-TLR sensors like retinoid acid inducible gene I (RIG-I), melanoma differentiation associated protein 5 (MDA5) and Laboratory of Genetics and Physiology 2 (LGP2) is another way to induce a immune response. RIG-I like receptors (RLRs) recognize presence of cytoplasmic double stranded RNA and upkeeps the production of IFN-β. The innate immunity in cooperation with Myd88 adjust inflammation, infiltration and production of cytokines (IL-1, IL-2, IL-6, IL-10, TNF-α, IFN-γ and C-X-C motif chemokine 10 (CXCL10) (Fuse *et al.*, 2005; Yajima, 2011). They are thought to play a major role in defense against viral infection through the attraction and activation of immune cells. IL-6 protects the myocardium in the early inflammatory stage, but persistent activation of IL-6 promotes heart injury through disturbance of viral clearance and impaired rising of circulating TNF-α (Tanaka *et al.*, 2001). Endocrine, paracrine and autocrine influence of cytokines on cardiomyocytes also plays big role in fighting the disease. CVB infection is associated with an activation of Janus kinase – signal transducer and activator of transcription (JAK-STAT) signaling pathway in the heart cells (Yasukawa *et al.*, 2003) and this activation is observed at very early stages of the immune response. IFN-β is very important for limiting viral presence in the heart. Limited expression or absence of this cytokine causes the increase of mortality. The most important features of intracellular reaction to a viral infection of the heart are depicted in Fig. 1. DCs, natural killer (NK) cells and macrophages migrate to the heart in response to the massive cytokine production induced by viral invasion, and minimalize virus propagation, mostly *via* direct cytotoxic effect (Yajima, 2011). First, acute phase of the viral invasion of cardiomyocytes, described above, takes only few days. It can be manifested by fever, weakness, rash, muscle pain and joint pain. It can also be accompanied by symptoms of respiratory or gastrointestinal viral infection.

The second stage of the infection is subacute and lasts weeks to months. It is characterized by more sublimated immune reactions. Signals from the innate immunity system contribute also to the activation of specific T and B lymphocytes, responding to viral antigens. The highest point of antibodies production also occurs in this phase. Antibodies, which are produced to destroy viruses, often react with the structures of human heart and can cause damage of myocardium. The cytotoxic T-cells response is one of the most important mechanisms responsible for the lysis of virus-infected cells as well as for far reaching damage of myocardium. In addition, autoimmune reactions are also observed, when the cytotoxic lymphocytes attack healthy part of the myocardium because of the molecular mimicry. It happens because of the virus-induced cytolysis, when specific cellular antigens from necrotic myocytes such as beta1-adrenergic receptors, myosin or M2 muscarinic receptors are released (Pankuweit and Klingel, 2013). In a murine model, it was demonstrated

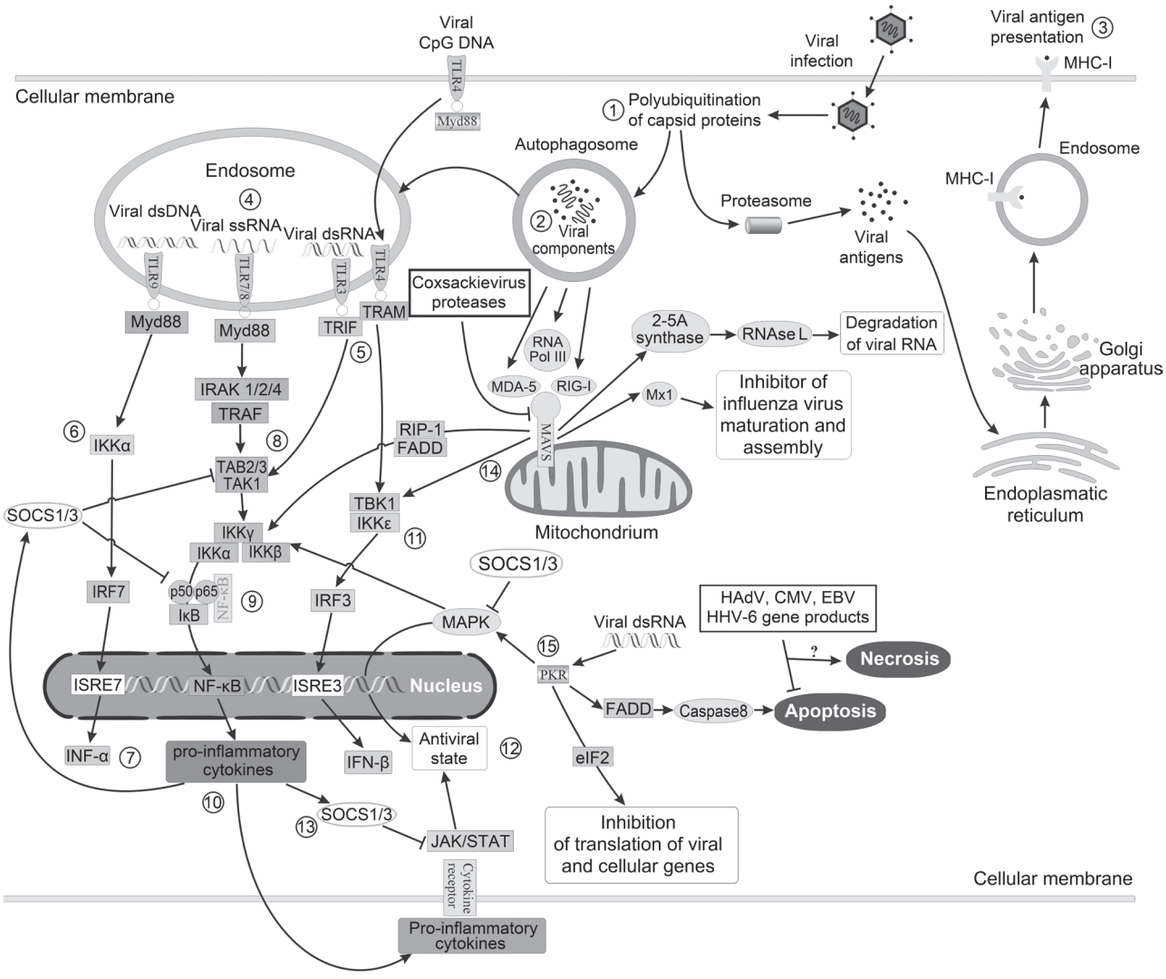

Fig. 1. Major mechanisms activated during viral infection of heart tissue cells.

Directly after viral infection, but also during the entire process of viral replication, viral proteins undergo polyubiquitylation and degradation (1), which leads to trapping of viral components within autophagosomes (2), but also to processing of viral antigens in endoplasmatic reticulum and Golgi apparatus, which, in turn, results in presenting of viral antigens to immune cells in MHC-I context (3). After fusion of autophagosome with endosome, viral DNAs and RNAs are recognized by TLRs (4), from which TLRs 3, 4, 7, 8 and 9 are known to react to viral components. The signal from TLRs, activated by their ligands, is transduced further by Myd88 in case of TLRs 7, 8, 9 and cellular membrane-bound TLR4, or by TRIF (5) in case of TLR3, associated with endosomal TLR4. Signal from TLR9/Myd88 leads to activation of IKKα kinase (6), which phosphorylates IRF7 which results eventually in interferon alpha synthesis (7), while Myd88-transduced signals from TLRs 7 and 8 activate NF-κB pathway *via* IRAK/TRAF-TAB/TAK route (8), where TAK-1 kinase phosphorylates IKKα/β/γ complex, resulting in deactivation of IκB, an inhibitor which retains NF-κB in cytoplasm (9). Released NF-κB migrates to the nucleus, where it initiates expression of over 150 genes, including proinflammatory cytokines and proapoptotic genes (10). NF-κB pathway is also activated by signal from TLR3/TRIF, while interferon beta is expressed in reaction to a signal coming from endosomal TLR4, transduced by TBK/IKKε kinases (11). Autocrine reaction to both type-I interferons and proinflammatory cytokines *via* JAK-STAT results in expression of numerous genes, initiating antiviral state within a cell (12), and this process is negatively controlled by SOCS proteins (13), synthetized in reaction to proinflammatory cytokines. Stimulated by viral components, also MAVS-associated RLRs (14) induce signal cascade involving TBK1- and IKKα/β/γ-depending pathways. Versatile inducer of antiviral response, PKR (15), initiates antiviral state of the cell (*via* MAPK), activates eIF2 and apoptotic process (*via* caspase 8).

that additional, host-depending genetic factors increase the risk of the autoimmune reactions. It is considered that direct viral injury, cytokine context and level of proinflammatory immune reaction together are responsible for determining the severity of MC and probability of drift from acute to subacute stage (Yajima, 2011). The activation of the acquired immunity results in chronic inflammatory response in myocardium and may lead to organ dysfunction due to fibrosis and remodeling of the heart muscle. It can also cause damage due to necrosis and switching on the autophagy of the cardiomyocytes. Recently, it was found that the ubiquitin-proteasome system and lysosome pathways are one of the main factors of the viral infectivity and its inhibition reduces CVB replication in murine (Luo *et al.*, 2003). Fairweather and Rose (2007) showed, that viral genome can be detected in heart tissue during chronic myocarditis. Mechanism of long-term coxsackievirus persistence

in the presence of an intact immune system remains unclear (Yajima, 2011). Patient may present dyspnoea, chest pain, heart palpitations, decreased exercise tolerance, increased sweating and fainting.

Myopathy phase is the next stage of an infection, where generally it is impossible to detect the virus in myocardium. In case of the persistent inflammatory response, the heart may develop iDCM due to the remodeling. Pathogenic role may be played by the antibodies against sarcolemma, myolemma, beta-receptor, acetylcholine receptor, laminin and cardiac conducting tissue. However, the participation of the antibodies against fibrils, stress proteins and intermediate filaments is not entirely clear (Maisch and Pankuweit, 2013). Inflammation may also be followed by release of cytokines (*e.g.* transforming growth factor) and activation of matrix metalloproteinases (*e.g.* urokinase-type plasminogen activator), which predispose to fibrosis through the mothers against decapentaplegic homolog (SMAD) signaling cascade and cardiac dilation (Sagar *et al.*, 2012). Matrix metalloproteinases (MMPs) which can degrade the different components of the cardiac tissue are overexpressed during viral MC and may contribute to tissue remodeling (Li *et al.*, 2009). Additional mechanisms responsible for post-inflammatory remodeling of the heart tissue include enhanced fibrosis as the effect of osteopontin and matricellular protein Cyr61 activity (Pankuweit and Klingel, 2013). At this step, we can observe systolic dysfunction of the left ventricule and walls hypokinesis, often correlating with ECG disorders, especially the ST-T changes. Right ventricular dysfunction is less widespread. Formation of thrombi within the heart cavities and pericardial effusion also can occur.

Furthermore, the presence of the virus, or its components, in cardiac muscle without concomitant local immune response at detectable level, as determined in standard histopathologic examination, can also elicit fibrosis, hypertrophy and degeneration of cardiomyocytes observed in iDCM (Kawai and Matsumori, 2013). Pathological changes in heart muscle can provoke chest pain, tachycardia, irregular heart beating, dyspnea at rest and with effort, fatigue, swelling lower limbs, fainting and hyperhidrosis.

Recently, it has been observed that B19V plays increasing role in the induction of MC in Europe and in the US. Acute infection of B19V is typical for childhood, usually manifests as fifth disease and it is infrequently diagnosed in adults (Bultmann *et al.*, 2003). Erythroid progenitor cells are the main site of B19V replication, however other cell types can be also susceptible for infection. High prevalence of B19V in the hearts of patients with MC has been reported in repeated observations, which strongly suggests connection of presence of the virus with the induction of inflammation. This process is most probably associated with the impact of B19V on the endothelium (Bock *et al.*, 2010). B19V, as an erythrovirus, utilizes P-receptor and coreceptors (integrins, KU80) on the endothelial cells. After the connection with the receptor, virus may enter the cells and pass to the state of persistent infection in the endothelium of various organs, including heart, and in consequence, B19V can be found in cells of venules, small arteries or arterioles of children and adults (Bultmann *et al.*, 2003). Our knowledge about the pathogenetic mechanisms of MC induction by B19V is limited due to the lack of appropriate animal model. Despite this problem, few years ago the first results utilizing murine model for the investigation of B19V recombinant antigen impact on myocardium were published (Pankuweit and Klingel, 2013), revealing that mice treated with a recombinant VP1 protein of the B19V developed myocardial injury. Damage was accompanied by the increasing level of aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatine kinase isoenzyme (CK-MB) in serum (Nie *et al.*, 2010). The second study showed, that mice which received antibodies against B19V VP1 unique region, developed cardiac injury due to inflammation process (Tzang *et al.*, 2011). Treatment by IFN-α or IFN-β almost does not affect to the presence of the B19V in the cells, however cell analyses of infected immortalized human microvascular endothelial cells (HMEC-1) proved, that external administration of IFN-β can obstruct B19V reactivation and improves endothelial cells viability (Schmidt-Lucke *et al.*, 2010).

Summarizing, both viral infection and subsequent reaction of the immune system causes destruction of the heart tissues (Fig. 2). The damage may be a result of direct viral cytopathic effect, inflammation, necrosis and autophagy. We can divide cardiomyotropic viruses into two main groups. First group comprises viruses for which cardiomyocytes are the most important kind of targeted cells (enteroviruses and adenoviruses). Viruses from this group demonstrate strong cytopathic effect. Enteroviruses usually exhibit low risk of persistence, but in some cases their RNA remains detectable in myocardial cells long time after the acute infection. Adenoviruses are very poorly investigated in terms of the pathogenesis of heart infections. Moreover, this picture is also complicated by their long-term presence in lymphoadenoidal tissue, and potential role of this reservoir as a constant source of low-level adenoviral viremia. The viruses from second group, betaherpesviruses, including CMV and HHV-6, infect endothelium and cardiomyocytes, while DNA or antigens of B19V is found, first of all, in endothelial cells. There is a very limited number of data indicating the presence of the B19V DNA in cardiomyocytes of adults and foetuses (O'Malley *et al.*, 2003). Thus, this problem is still open

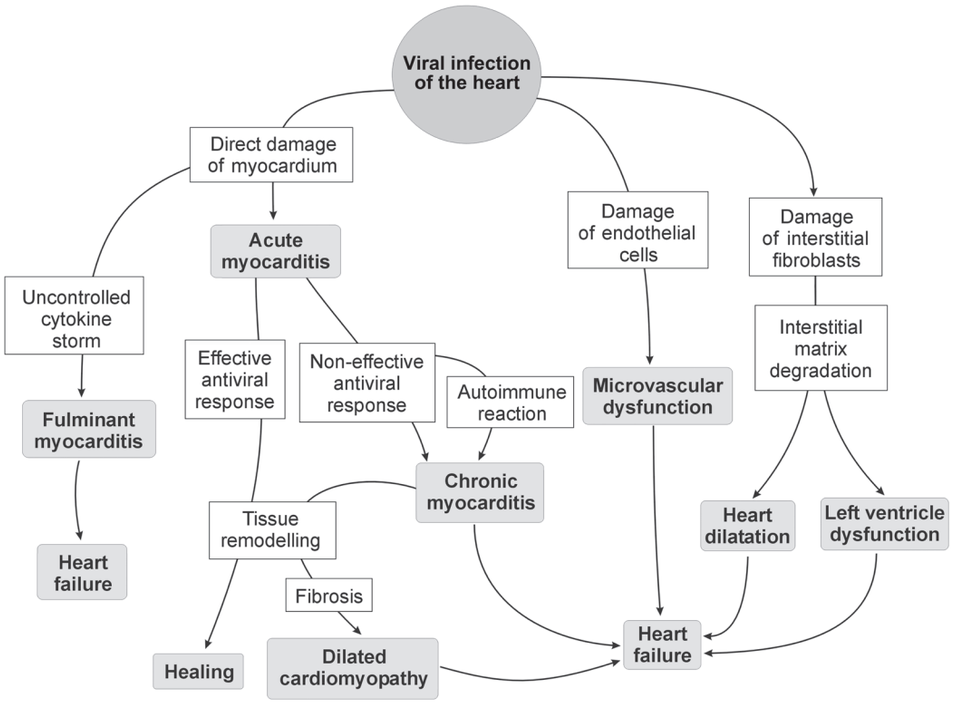

Fig. 2. Potential effects of viral infection of the heart.

Infection of cardiomyocytes, caused by enteroviruses, may result in direct cytopathic effect, resulting in cell damage or death. Prompt activation of intracellular antiviral mechanisms and both innate and acquired immune mechanisms should result in strong local immune response and elimination of both viral particles and infected cells (acute myocarditis). Rarely observed fulminant myocarditis is caused usually by disturbances in control mechanisms of inflammation. Errors in antiviral response, *e.g.* ineffective or delayed innate immune response, weak cytotoxic T-cell response or insufficient antibody production allows the virus to multiply and spread within the heart. Prolonged presence of the virus in cardial tissues may result in constant, but still ineffective infiltration of immune cells, and may lead to chronic inflammation. Further, this dynamic process, accompanied by constant loss of damaged contractile tissue in appearance of fibrosis, may result in dilated cardiomyopathy, and often ends in progressive heart failure, despite introduced treatment. Damage resulting from infection of interstitial tissue, caused first of all by parvoviruses and herpesviruses may result in heart dilatation and left ventricle dysfunction. These viruses are also known to infect the endothelium of local blood vessels, which may result in worsening of clinical picture because of microvascular dysfunction.

to debate, including the question about the ability of B19V to replicate actively in adult cardiomyocyte.

In terms of virological diagnostics, accurate identification of etiologic agent of viral MC or iDCM is one of the most difficult tasks set, and efforts to establish effective diagnostic procedures have been making since late forties of past century (Woodruff, 1980). Virological laboratory diagnosis, including direct methods, specifically virus isolation in cell lines and electron microscopy, considered substantially as more reliable than serological examinations. Despite the unambiguous answer provided in case of isolation of the virus from EMB sample, the low recovery rate of the virus was the main limitation of isolation method. It results in some percentage of false-negative outcomes, very hard to assess without the reference method. Low amount of material obtained during biopsy, sampling inadequacies and technical problems with isolation of the virus from tissues resulted in a weak usefulness of this method, especially in the late course of heart infection (Martino *et al.*, 1994).

The biopsies were initially evaluated only histologically according to the Dallas criteria (Aretz, 1987). The presence of an inflammatory infiltrate with or without necrosis on conventionally stained heart-tissue sections evaluated under light microscopy is required for the histological diagnosis of myocarditis. These criteria are limited by variability in interpretation (39% among pathologists examining the same cardiac tissue samples), lack of prognostic value, and low sensitivity (the Dallas criteria were absent in 50% of the specimens containing PCR-proven viral pathogens), in part due to sampling error (Martin *et al.*, 1994; Mason *et al.*, 1995; Baughman, 2006). For this reason, the solitary use of the Dallas criteria to diagnose myocarditis is poor. To address the shortcomings, in 1999, the WHF and ISFC updated the conventional histological criteria for diagnosis of myocarditis by the introduction of immunohistochemical methods (staining surface antigen, such as anti-CD3, anti-CD4, anti-CD68 and anti-human leukocyte antigens). Criteria for immunohistological diagnosis of myocarditis are specified quantitatively as

14 infiltrating leukocytes/mm^2, preferably T-lymphocytes (*e.g.* CD3) or activated T-cells (CD45ro) (Maisch *et al.*, 1999). Criteria that are based on immunoperoxidase staining, have greater sensitivity and may have a prognostic value. However, many observations confirm, that routine histological and immunohistological analysis is too insensitive to detect myocardial inflammation accurately in acute phase of disease, as well as in chronic phase. Finally, even if conventional histological and immunohistological analysis allow to diagnose inflammation in heart, they do not allow to identify virus and in consequence to use of targeted treatment.

Problems with detection of the viral pathogen in cases of suspected MC or iDCM with use of virus isolation techniques led to searches for alternative diagnostic methods. Before the popularization of molecular biology and methods of direct detection of viral antigens *in situ*, diagnosis was often established on the basis of detection of specific antibodies directed against cardiotropic viruses in patients' sera. Serological investigations utilized paired sera examination, detection of IgM-class antibodies or clinical seroepidemiological studies (Leslie *et al.*, 1989). Utility of serological methods was disputed from the very beginning of their application. In 2011 Mahfoud *et al.* published their observations conducted on the group of 124 patients with symptoms of acute MC. The authors compared results of identification of viral etiologic agent of MC performed with serological (examination of paired sera with fourfold or more antibodies titers increase) and direct methods (detection of viral nucleic acids in EMBs with real-time PCR). It was shown, that the concordance of the results between both methods was 4%, whereas the positive predictive value of serological methods was 9%.

Another diagnostic approach included the isolation of the virus from sites other than EMB (upper respiratory tract, urine or feces) during acute phase of infection in patients with clinical and histopathological changes indicating viral MC (Woodruff, 1980). Diagnostic value of these results in adult patients was not verified with evidence-based medicine standards. However, the wide distribution of viruses with cardiotropic potential in population, along with frequent asymptomatic infections of upper respiratory and gastrointestinal tract, as well as probability of mixed infection with two or more viruses, indicates possibility of isolation of the "bystander" virus instead of pathogen responsible for MC or iDCM.

Contemporary diagnosis leading to identification of etiological agent of viral MC/iDCM relies on direct methods, first of all on detection of viral nucleic acids or antigens in EMB samples, rarely on electron microscopy (Kühl and Schultheiss, 2009). According to the current recommendations, EMB and peripheral blood samples should be investigated, and PCR, real-time PCR (qPCR) and *in situ* hybridization (ISH) are the methods acknowledged nowadays (Cooper *et al.*, 2007; Basso *et al.*, 2013). In extended investigations conducted during last years, genetic material of viruses was detected in 19–94% of examined EMB samples. The results revealed rather coherent group of identified viruses. On the other hand the significant differences in detection frequencies of particular viruses were also observed. In the investigations which embraced wide spectrum of viruses sought after, B19V was the most frequent (found in 2.5–60% of examined EBM samples), followed by HEV (1–33%), HHV-6 (8–30%), HAdV (2–23%), CMV (1–5%) and EBV (0.5–5%) with slight differences between results obtained in patients with MC and iDCM (Bowles *et al.*, 2003; Pankuweit *et al.*, 2003; Mahrholdt *et al.*, 2004; Kühl *et al.*, 2005; Caforio *et al.*, 2007; Bock *et al.*, 2010).

Our growing knowledge about the complex mechanisms triggered during viral infections of the heart, their impact on both short- and long-time consequences affecting patient, along with important change in our consciousness of the most prevalent viral agents responsible for acute and chronic heart damage are one of the most important issues in contemporary cardiology. With revealed futility of serological methods and poor predictive value of classical histopathologic criteria, there is growing need for more precise and reliable diagnostic techniques, allowing rapid and unambiguous diagnosis in cases of suspected virus-related heart damage. This gap has been at least partially filled with the advent of modern direct diagnostic methods, based on molecular biology techniques, but still there is more questions than answers. The most urgent issues include introduction of EMB sampling standardization, assessment of the clinical impact of heart tissue viral load during both acute and chronic infection, and identification of macroscale changes in heart tissue, corresponding at the desired level with virus-driven infectious process. There is also growing number of data, suggesting that deficiency in early antiviral innate intracellular immunity may be an important factor responsible for increased risk of development of the chronic heart diseases resulting from viral infections, what indicates new direction in the search for diagnostic methods. In conclusion, depiction of basic processes accompanying viral infection of the heart, both at macro- and microscale should help in the elaboration and introduction of more efficient diagnostic protocols.

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ORIGINAL PAPER

Gas Gangrene of Different Origin Associated with *Clostridium perfringens* Type A in Three Patients Simultaneously Hospitalized in a Single Department of Orthopedics and Traumatology in Poland

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Abstract

The objective of the study was to perform a comparative analysis of phenotypic and genetic similarity, determination of resistance profiles, detection of toxin-encoding genes and molecular typing of *Clostridium perfringens* isolates originating from patients with gas gangrene. The study encompassed three patients with a clinical and microbiological diagnosis of gas gangrene who were hospitalized in one of the hospitals of the Kujawsko-Pomorskie province in the same period of time between 8th April 2015 and 20th April 2015. The three *C. perfringens* isolates studied had identical biochemical profiles. Two isolates had identical resistance patterns, while the third presented a different profile. Using the multiplex PCR method, all isolates showed the presence of *cpa* gene encoding α-toxin; furthermore, the presence of the *cpb2* gene encoding β2-toxin was confirmed in two isolates. Genotyping with the use of pulsed field gel electrophoresis (PFGE) indicated that the isolates originating from the three studied patients represent three genetically different restrictive patterns which corresponded to three different clones – clone A, clone B and clone C. As a result of the study, it is possible to conclude that the studied patients simultaneously hospitalized in a single Department of Orthopedics and Traumatology developed three different endogenous infections.

Key words: *Clostridium perfringens*, epidemiological investigation, gas gangrene, molecular study

Introduction

Gas gangrene is also known as clostridial myonecrosis (lat. *gangrenea gaseosa*), a bacterial infection caused by anaerobic Gram-positive bacilli of the genus *Clostridium* of a severe clinical course (Sołtys-Bolibrzuch, 2012). *Clostridium perfringens* species is the most commonly isolated etiological factor for gas gangrene (up to 90% of cases) (Knap, 2004; Stasiak *et al*., 2007).

The most common symptoms of gas gangrene are sudden, very severe pain, which responds poorly to painkillers, edema and erythema of the skin and soft tissues, epidermoid cysts filled with blood and serum or sanies, or similar fluid discharges from the wound, crepitus to the touch and a characteristic fetid smell. Furthermore, toxemia-related general symptoms present themselves; also, there are manifestations of the toxic shock syndrome and the multiple organ dysfunction syndrome (MODS) (Sussmann *et al*., 1998; Martirosian, 2007).

A range of extracellular toxins determines *C. perfringens* pathogenesis. They were assigned consecutive letters of the Greek alphabet. Alpha-toxin (α), which is a major toxin, as well as theta-toxin (θ) and kappa-toxin (κ) cause the local and systemic symptoms characteristic of gas gangrene (Stevens and Bryant, 2002).

Among healthy individuals, primarily in elderly patients, the percentage of *C. perfringens* carriage ranges from 6% to 31% (Sobel *et al*., 2005). The frequency of gas gangrene occurrence, according to the literature in English, is estimated to be from 0.1 to 1.0 incidences per one million residents a year and is primarily associated with traumas (Sussmann *et al*., 1998). In the end of the 20th century, in Great Britain, there were 70–80 cases reported a year; compared with 200–400 in the USA, while Polish estimates mention around 100 cases of gas gangrene a year (Knap, 2004).

Due to its high toxicity, which results in invasiveness, the species *C. perfringens* can be considered alert microorganisms in accordance with the regulation of

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the Minister of Health in Poland of 23rd December 2011 concerning the list of alert factors, hospital infections and alert factors records as well as reports on the current epidemiological situation of the hospital (Ministerial Decree, 2011).

Microbiological diagnostic analysis, together with clinical picture, form the basis for diagnosing gas gangrene. The former requires proper sampling of the material for testing, which is either a tissue fragment or sanies from the border of healthy tissues, and when it is impossible to obtain biopsy material, a swab is taken. Biological material makes it possible to prepare a direct smear which should be stained using the Gram stain technique and tested under the microscope for Gram-positive cylindrical forms. It is common for direct smears not to contain leucocytes. It results from lytic activity of lecithinase and theta-toxin. Simultaneously, a culture for aerobic and anaerobic bacteria should be prepared using the biological material, which enables a conclusive confirmation of *C. perfringens* occurrence. Tissue biopsy material taken from the site affected by gas gangrene is subjected to a histopathological test to enable, in a way similar to direct smear, quick preliminary confirmation of the diagnosis. In order to diagnose gas gangrene and implement treatment, it is enough to have a clinical picture of the patient and detect the presence of Gram-positive cylindrical forms in the direct smear, while the *C. perfringens* culture serves to confirm the diagnosis (Sussmann *et al*., 1998; Martirosian, 2007).

Molecular methods employed for bacterial isolates typing make it possible to determine the genetic similarity of species originating either from infections or from hospital environment (van Belkum *et al*., 2007).

For *C. perfringens* the toxin typing is very important because of particular toxin types are associated with the specific clinical course of the disease (Stevens and Bryant, 2002). A multiplex PCR method simultaneously detecting the four major toxin genes (*cpa*, *cpb*, *etx*, *iap*), the enterotoxin gene (*cpe*), and all variants of the beta2 toxin gene (*cpb2)* (van Asten *et al*., 2009).

Pulsed field gel electrophoresis (PFGE), which is recognized as the gold standard in microbial typing, make it possible to compare the genetic similarity of *C. perfringens* isolates, which permits genetically-identical bacterial clones to be selected (van Belkum *et al*., 2007). It is a very helpful tool applied in epidemiological investigation of gas gangrene cases reported in patients hospitalized in the same hospital unit (Maslanka *et al*., 1999; Brzychczy-Włoch *et al*., 2014).

Objective of the study. The objective of the study was to perform a comparative analysis of phenotypic and genetic similarity of *C. perfringens* isolates originating from three patients with gas gangrene simultaneously hospitalized in a single Department of Orthopedics and Traumatology in 2015.

Experimental

Materials and Methods

The study encompassed three patients with a clinical and microbiological diagnosis of gas gangrene who were hospitalized in one of the hospitals of the Kujawsko-Pomorskie province in the time period between 15th April 2015 and 20th April 2015.

Description of the cases

Patient 1. A man aged 53 was admitted to the Department of Orthopedics and Traumatology on 23 March 2015 as emergency inpatient with a fractured left tibial epiphysis due to a fall from a ladder on the day of the admission. On 24 March a plaster splint was applied and traction was used. Due to lower leg swelling, a surgical procedure was postponed. From 23 March to 3 May, the patient received amoxicillin with clavulanic acid 3 × 1.2 g IV. On 2 April, the surgical procedure was performed. Perioperative prophylaxis was provided with cefazolin 2g IV. After 8 days on 10 April, the patient developed a fever. A knee puncture was performed. Bloody fluid was taken from the knee and sent for testing. *Enterococcus faecalis* was cultured aerobically and *C. perfringens* was cultured anaerobically from the material. The targeted therapy was used. Clindamycin was administered 3 × 600 mg IV. On 15 April, wound debridement was carried out and intraoperative material was taken. *C. perfringens* was cultured anaerobically from the material (lab no. 7013); aerobically, there was no growth. On 23 April, clindamycin was discontinued and metronidazole was started at a dose of 0.5 g IV × 8 h until 3 May. Wound irrigation was performed using hydrogen peroxide. Patient isolation was implemented. The material from the patient was collected several times more until 2 May – cultures were negative. Consultation with Hyperbaric Oxygen Center in Gdansk was done. The patient was qualified for treatment as the wound was healing slowly, but the treatment was not applied. April, 24: wound debridement, removal of necrotic sections. Aquacel Ag (ConvaTec Poland) foam dressing. April, 28: VivanoMed (Hartmann) vacuum dressing was applied for about 3 weeks. On 2 June, after 102 days of hospitalization the patient was discharged from hospital in a good condition.

Patient 2. A woman aged 88 was admitted to the Department of Orthopedics and Traumatology on 26 March 2015 after a fall at home. The diagnosis: a closed comminuted fracture of the right femur. Comorbidities: type 2 diabetes, atherosclerotic cardiomyopathy, circulatory failure NYHA III, hypertension, cholecystolithiasis, erosive gastritis. On the day of admission, 3 units of packed red blood cells (PRBCs)

were transfused. On 27 March, a surgical procedure was carried out involving fracture fixation with a stainless-steel popliteal intramedullary nail (Megdal). Perioperative prophylaxis was used – cefazolin 2 g IV. The patient was agitated on 28 April during the night, periodically without contact. April, 30: patient's condition is stable. On 2 April, moderately severe condition. Due to destabilization, reoperation to remove the fixation and an NCB (Zimmer) titanium femur plate was placed. After 24 h, the swelling without symptoms of crepitus of the affected limb. Two units of PRBCs were transfused and material from the wound was collected. On 7 April, ciprofloxacin was administered 2 × 750 mg IV until 13 April. On 9 April, material was taken for testing. There was lack of growth of aerobic flora (there was no culture for anaerobic bacteria). Lack of progress in wound healing. Discharge of a small amount of serum. Octenisept (Schulke S&M) rinsing was employed and Cosmopor (Hartmann) dressings. On 17 April, material for testing was collected. *C. perfringens* (lab no. 7149) was cultured; aerobically, no growth. From 17 April, amoxicillin with clavulanic acid was started 3 × 2.4 g IV in combination with clindamycin 3 × 600 mg IV until 20 April. On the night of 19 April, deterioration of patient's condition. Symptoms of circulatory failure. The patient received oxygen therapy. April, 20, on the 26th hospital day: Patient's death due to multiorgan failure.

Patient 3. A woman aged 76 was admitted to the Department of Orthopedics and Traumatology on 8 April 2015 as emergency inpatient. As a result of a fall at home, she suffered a right thigh injury. Diagnosis: comminuted closed fracture of the femur. Comorbidities: hypertension, ischemic heart disease, type 2 diabetes. On 9 April, supramalleolar traction was employed. On 14 April, MIPO (minimally invasive plate osteosynthesis) thigh fixation surgery was carried out with the use of the NCB (Zimmer) titanium plate. April, 17: deterioration of the patient's condition, temperature increase to 38.9°C, pain in the limb, skin bruising. During the exam, a subcutaneous space was found, which showed symptoms of crepitus upon palpitation. The necrotic skin was incised with evidence of gas release and discharge of a significant amount of turbid dark liquid. Material for microbiological testing was collected. *C. perfringens* (lab no. 7143) was cultured; aerobically – no growth. The resulting fistula was rinsed with hydrogen peroxide. From 17 April, amoxicillin with clavulanic acid was prescribed 3 × 1.2 g in combination with clindamycin 3 × 600 mg IV. In view of the fulminant gas gangrene, on 17 April, a decision was made to amputate the limb, however, the patient did not consent to surgery. On 18 April, after obtaining the patient's consent to amputate the limb, the life-saving procedure was undertaken. During the surgery, CPR was performed several times on the patient. Death occurred on the 10th hospital day during surgery. Final diagnosis: death due to infection with *C. perfringens*.

Microbiological diagnostics. Materials for microbiological examination from the three studied patients were taken using sterile swabs for the Amies (COPAN) activated carbon substrate. Culturing was carried out on the Columbia Sheep Blood Agar (BioMérieux) medium under aerobic and anaerobic conditions as well as on liquid thioglycollate medium with resazurin (BioMaxima). Solid media with the inoculated material were incubated under aerobic conditions at 36°C/48 h and under anaerobic conditions at 36°C/24 h and the liquid medium under aerobic conditions (with a closed cap) at 36°C/48 h. Identification of the cultured bacteria was carried out using the Vitek 2 compact system (BioMérieux). The biochemical patterns for *C. perfringens* isolates were received with the use of API 20A test (BioMérieux).

The following strains of *C. perfringens* were protected for the further studies: from patient 1 – isolate no 7013; from patient 2 – isolate no 7149; from patient 3 – isolate no 7143. *C. perfringens* 12915 ATCC (The American Type Culture Collection) standard was used as reference strain. The strains were stored with the use of Cryobank (BioMaxima) at –70°C.

Protection of epidemic outbreak and epidemiological investigation. On 15 April 2015, the ward and the epidemiological nurse were informed by phone by the Department of Microbiology Diagnostics about the isolation of an alert pathogen. The nurse went to the unit to arrange a method for patient isolation. Since the patient was in the Main Operating Theater, the Octenisept (Schulke S&M) sporicidal agent was used (for debridement and rinsing of the wound).

On 17 April, a meeting was held of the Hospital Infection Control Team and the Head of the Department of Orthopedics and Traumatology on account of culturing an alert pathogen in patient no. 1 (lab no. 7013) and a suspicion in patient no. 3 (lab no. 7143). Intensification of the sanitary regime was established, together with isolation of patient no. 3, application of the Incidin Active (Ecolab) sporicidal agent for surface decontamination. Aniosept activ (Medilab) was used for surfaces and tools in the Main Operating Theater. Equipment was allocated to the isolated patients. An inspection was carried out of the compliance with handling medical waste and bed linen (labeling of notifications according to the procedure in double red bags). The Head of the Main Operating Theater was instructed to execute preventative decontamination of all operating rooms with a sporicidal agent and to support it by fogging the rooms with HyPro technical 6–7% H_2O_2 using the Hyspray (Hymetec) appliance.

On 18 April, after oral information from the Department of Microbiological Diagnostics concerning the suspicion of *C. perfringens* culture in patient no. 2 (lab no. 7149), patient isolation was continued, sanitary regime was intensified, and separate staff was assigned to the isolated patients.

On 19 April, all the patients of the Ward were included into the outbreak – a total of 20 people. Action taken: contact isolation of patients; intensification of sanitary regime; dedication of separate equipment to isolated patients; assignment of staff to care for isolated patients; environmental control after each decontamination procedure.

On 20 April, the Director of the Hospital decided not to perform the scheduled surgical procedures and to execute another general disinfection of the operating rooms and equipment in conjunction with fogging and taking environmental tests, including from the hands of workers in the operating theater, employees and surgical wards.

On 21 April until 29 April, in accordance with the decision of the District Sanitary Inspector, planned admissions to the surgical wards and scheduled surgical procedures were suspended. At that time, decontamination of rooms was carried out together with fogging and all surgical instruments were subjected to reprocessing.

Between 19 and 24 April, daily meetings were held involving the Infection Control Committee and the Hospital Administration with the purpose of discussing the current situation.

Inspection of the decontamination of surfaces and hygienic hand washing was carried out by the contact plate method using Count-Tact (BioMérieux) system with the use of Agar Count-Tact (BioMérieux) radiation-sterilized plates. Incubation of the plates under aerobic conditions at 30°C/72 h.

After the exhaustion of the stock of plates, further examination was carried out using swabs. Materials were collected on a sterile swab (COPAN) and subsequently placed in Tryptic Soy Broth (BioMaxima). Incubation was carried out at 36°C/72 h. Positive liquid cultures were plated on solid media: Columbia Agar, MacConkey Agar and Sabouraud Agar (BioMérieux). Each material was tested for aerobic and anaerobic flora.

Antibiotic susceptibility testing. To determine the drug-resistance profiles, the Kirby-Bauer disk diffusion method was used in which the Columbia Sheep Blood Agar (BioMérieux) and antibiotic disks (Oxoid) were utilized: penicillin – P [1 U], imipenem – IPM [10 μg], amikacin – AK [30 μg], gentamicin – CN [10 μg], tobramycin – TOB [10 μg], netilmicin – NET [10 μg], metronidazole – MTZ [5 μg], chloramphenicol – C [30 μg] and the E-test method enabling determination of MIC (Minimal Inhibitory Concentration) for: ampicillin – AM, clindamycin – DA, doripenem – DOR, doxycycline – DC, ciprofloxacin – CI, vancomycin – VA (bioMerieux). The results were interpreted according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST) version 5.0 (EUCAST, 2015).

PCR multiplex. To isolate DNA, the Genomic Mini Set (A&A Biotechnology) was used according to the manufacturer's protocol. The presence of genes encoding toxins of *C. perfringens* was confirmed using multiplex PCR amplification according to van Asten *et al.* (van Asten *et al.*, 2009) with specific primers (Genomed). The following genes were detected: *cpa* 324 bp (α-toxin); *cpb* 195 bp (β-toxin); *cpb*2 548 bp (β2-toxin); *etx* 376 bp (ε-toxin); *iap* 272 bp (ζ-toxin); *cpe* 485 bp (enterotoxin). The final pictures from electrophoresis were processed using QuantityOne software, as well as GelDoc2000 device (Bio-Rad, USA).

Molecular typing with PFGE. The chosen *C. perfringens* isolates underwent molecular typing using the PFGE method according to the methodology described by Maslanka *et al.* (1999). Chromosomal DNA of bacterial strains was isolated in agarose blocks and then digested with the use of restriction enzyme *Sma*I (MBI Fermentas). Electrophoretic separation was performed on CHEF-DR II (Bio-Rad) machine, while restriction analysis was carried out using GelCompar II (Applied Maths) software with the application of UPGMA clustering method and Jaccard index. The obtained genetic profiles were interpreted according to the guidelines given by van Belkum *et al.* (2007).

Results

The diagnosis of gas gangrene made on the basis of clinical picture in the three patients hospitalized in the Department of Orthopedics and Traumatology of the district hospital was confirmed with microbiological testing, in which *C. perfringens* strains were isolated from the intraoperative clinical materials: patient 1– isolate no. 7013; patient 2 – isolate no. 7149; patient 3 – isolate no. 7143.

The first strain of *C. perfringens* no. 7013 was isolated on 10 April 2015 (patient 1), and reported to the ward and the epidemiological nurse on 15 April. The second and third strains were isolated on 17 April (patient 2 and 3). On 6 May 2015, activities associated with eradicating the outbreak were completed.

During the epidemiological investigation, materials were taken in the amount of 173 from patients of the Department of Orthopedics and 140 from the staff of the Department of Orthopedics, General Surgery, Anaesthesia and Intensive Care, and the Main Operating Theater. 250 materials were also taken from the hospital environment (mainly the ward and operating

Table I
Biochemical profiles obtained with the using of the API 20A test (BioMérieux).

Patient / Isolate no.	Biochemical tests																						
	IND	URE	GLU	MAN	LAC	SAC	MAL	SAL	XYL	ARA	GEL	ESC	GLY	CEL	MNE	MLZ	RAF	SOR	RHA	TRE	CAT	SPOR	GRAM	COCC
Patient 1 7013	–	–	+	–	+	+	+	–	–	–	+	–	–	–	+	–	–	–	–	+	–	+	+	–
Patient 2 7149	–	–	+	–	+	+	+	–	–	–	+	–	+	–	+	–	–	–	–	+	–	+	+	–
Patient 3 7143	–	–	+	–	+	+	+	–	–	–	+	–	+	–	+	–	–	–	–	+	–	+	+	–

"+" – positive reaction; "–" – negative reaction

theater). None of the materials enabled culturing the *C. perfringens* alert pathogen.

With the use of API 20A (BioMérieux) test, biochemical profiles of the isolates tested were established (Table I) and they were identical for the isolates no. 7149 and no. 7143 (the second and the third patient) and different for isolate no. 7013 (the first patient).

Table II
Antibiotic susceptibility for *C. perfringens* isolates.

	Patient 1/ Isolate no 7013	Patient 2/ Isolate no 7149	Patient 3/ Isolate no 7143
The Kirby-Bauer disk diffusion method	Inhibition zone [mm]		
Penicillin	35	35	35
Imipenem	35	35	35
Amikacin	32	24	5
Gentamicin	30	22	5
Tobramycin	30	22	5
Netilmicin	30	22	5
Metronidazole	5	5	5
Chloramphenicol	35	35	35
The E-test method	Minimal inhibitory concentration; MIC [mg/l]		
Ampicillin	0.016	0.016	0.016
Clindamycin	0.064	0.19	48
Doripenem	0.064	0.064	0.064
Doxycycline	0.19	0.125	2
Chloramphenicol	0.25	0.19	0.25
Vancomycin	0.016	0.016	0.016

Using the Kirby-Bauer method and E-test, drug-resistance patterns of the studied isolates were determined (Table II). In the EUCAST version 5.0 for 2015 the criteria for determining susceptibility testing of anaerobic bacteria by disc diffusion method not yet been determined. However, in the case of the first and second patient, the isolated strains no. 7013 and 7149 had the same drug-resistance patterns, while the isolate no. 7143 from the third patient had different antibiotic drug resistance profile. Using the E-test method the susceptibility to ampicillin, clindamycin, doripenem, doxycycline, ciprofloxacin and vancomycin were detected for the study isolates. Only isolate no. 7143 from the third patient was resistant to clindamycin.

When multiplex PCR was applied, all *C. perfringens* isolates originating from the three patients demonstrated the presence of *cpa* gene encoding α-toxin. Moreover, in isolate no. 7149 coming from patient 2 and in isolate no. 7143 from patient 3, *cpb2* gene encoding β2-toxin presence was confirmed (Fig. 1; Table III).

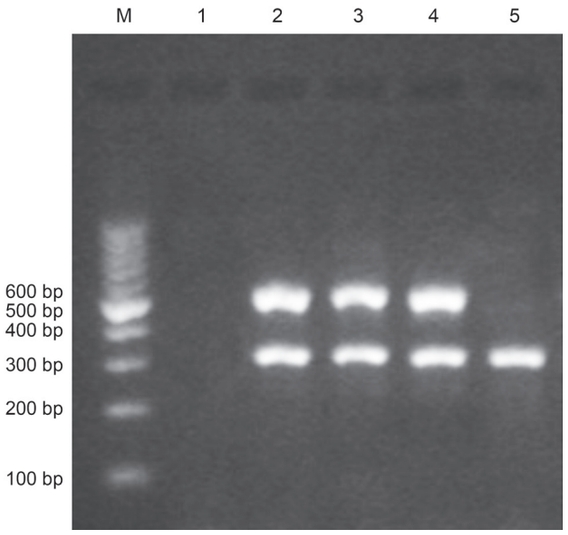

Fig. 1. Detection of genes encoding virulence factors of *C. perfringens* by multiplex PCR method.
M – size marker; 1 – negative control (H_2O); 2 – reference strain of *C. perfringens* ATCC 12915; 3 – *C. perfringens* isolate no. 7143 from patient 3; 4 – *C. perfringens* isolate no. 7149 from patient 2; 5 – *C. perfringens* isolate no. 7013 from patient 1.

C. perfringens isolates genotyping with the use of pulsed field gel electrophoresis (PFGE) indicated that the isolates originating from the three studied patients represent three genetically different restriction patterns and are related to each other in 66.7% (isolates no. 7143 and 7149) and in 46.4% (isolates no. 7143, 7149 and 7013). The isolates studied represented three different

Table III
Occurrence of genes encoding virulence factors in *C. perfringens* isolates.

Gene	Size of the amplification product	Toxin	Patient 1/ Isolate no. 7013	Patient 2/ Isolate no. 7149	Patient 3/ Isolate no. 7143
cpa	324 bp	α-toxin	*cpa*	*cpa*	*cpa*
cpb	195 bp	β-toxin	–	–	–
cpb2	548 bp	β2-toxin	–	*cpb2*	*cpb2*
etx	376 bp	ε-toxin	–	–	–
iap	272 bp	ι-toxin	–	–	–
cpe	485 bp	enterotoxin	–	–	–

PFGE types, which corresponded to three different clones – clone A (isolate no. 7143), clone B (isolate no. 7149) and clone C (isolate no. 7013) (Fig. 2).

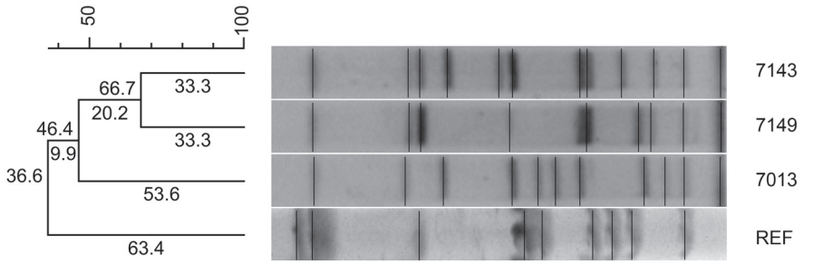

Fig. 2. Analysis of genetic profiles of *C. perfringens* isolates, subjected to DNA digestion by restriction enzyme *Sma*I, carried out with the use of pulsed field gel electrophoresis (PFGE) method and GelCompare II software.
Legend: 7143 – *C. perfringens* isolate from patient 3; 7149 – *C. perfringens* isolate from patient 2; 7013 – *C. perfringens* isolate from patient 1; REF – *C. perfringens* reference strain ATCC 12915.

Discussion

Nowadays, in the USA about 1000 cases of gas gangrene are recorded each year (Abella *et al.*, 2003). In Poland there are no epidemiological data concerning this issue.

The three clinical cases of gas gangrene associated with *C. perfringens* described in this paper, ending with the death of two patients, confirm the fact that gas gangrene is an extremely severe surgical infection associated with high mortality. In our study the mortality rate was 66,7%. Presently, gas gangrene is very rare, however, similar cases were described in Polish literature in the past (Wojdat *et al.*, 2005; Stasiak *et al.*, 2007; Brzychczy-Włoch *et al.*, 2014).

The most important virulence factor of *C. perfringens* is α-toxin encoded by the gene *cpa* which possesses activities of lecithinase and haemolysine. α-toxin causes damage and lysis of cell membrane which has a lethal effect to different tissues. Moreover, this toxin has dermonecrotic properties and inhibits functions of neutrophils (Stevens and Bryant, 2002). Gibert *et al.* (1997) described the new toxin with cytotoxic ability called the β2-toxin (CPB2) encoded by a gene *cpb*2. In a study on patients with food poisoning of *C. perfringens* etiology, 15–20% of isolates had this toxin (Fisher *et al.*, 2005). Probably the synergic interactions of both toxins is of great importance.

With the use of multiplex PCR we detected the presence of *cpa* gene encoding α-toxin in all *C. perfringens* isolates originating from the three patients. Moreover, in two isolates from patient 2 and 3 the *cpb*2 gene encoding β2-toxin presence was confirmed. Described in the literature cases of gas gangrene were associated with the presence of α-toxin (Stevens and Bryant, 2002; Stasiak *et al.*, 2007). It is worth noting that in our study the presence of strains with additional β2-toxin, was associated with severe course of infection and the death of two patients.

Due to the fact that gas gangrene progresses rapidly and combined with irreversible soft tissue changes, the success of therapy very much depends on the time of the diagnosis on the basis of the clinical picture, bacteriological study results and the implementation of treatment. The close cooperation between a microbiological laboratory and a surgical staff is essential in proper management of gas gangrene (Sussmann *et al.*, 1998; Martirosian, 2007).

Crystalline penicillin applied intravenously in high doses (*e.g.* 24 units per 24 h), most often combined with metronidazole, is the most often selected antibiotic in the treatment of gas gangrene. Supplementary effective antibiotics are aminoglycosides, lincosamides *e.g.*

clindamycin, semisythetic penicillins *e.g.* amoxicillin, ticarcillin combined with bacterial β-lactamase inhibitors *e.g.* clavulanic acid, fluoroquinolones and tetracyclines coupled with aminoglycosides (Knap, 2004; Martirosian, 2007).

In three patients described, after isolation of *C. perfringens* from clinical materials, amoxicillin with clavulanic acid in combination with clindamycin were used. However, despite adequate treatment implemented two of the patients died due to infection with *C. perfringens* and multiple organ failure.

In order to limit the epidemic outbreak a number of measures to identify the outbreak and to limit the spread of infection were taken. Unfortunately, in the cases described highlights a very long time (5 days) since the first strain of *C. perfringens* from the first patient were isolated, to forward this information to the Department of Orthopedics and the Hospital Infection Control Team. In the circumstances lacked a rapid response to the isolation of alert pathogen, which was associated with a high risk to other patients and the possibility of spread of the epidemic outbreak to other departments.

Microbiological studies were carried out in patients of the Department of Orthopedics, in the staff of the Department of Orthopedics, General Surgery, Anaesthesia and Intensive Care, and the Main Operating Theater and in the hospital environment. Despite the huge number of materials testing (total 563) in any case *C. perfringens* was not isolated. It indicates that the pathogen was not spread to other patients and did not get to the hospital environment.

The treatment of gas gangrene should be complex. It consists in debridement, employment of empirical and targeted antibiotherapy as well as hyperbaric therapy. Hyperbaric oxygen therapy (HBOT) is effective in limiting the number of toxins produced by *C. perfringens* that cause tissue necrosis in gas gangrene, and coupled with antibiotherapy, it reduces the time needed for treatment and significantly lowers mortality (Sussmann *et al.*, 1998; Fielden *et al.*, 2002; Martirosian, 2007). In the first patients described efforts were made to implement the HBOT but unfortunately it was not applied. Implementation of HBOT certainly would shorten the time of his hospitalization, which was as high as 102 days, after which the patient in good condition was discharged at home.

Antiseptics effective against *C. perfringens* including spores are: 0.1% Octenisept, 10% Betadine or 5% Hibitane (Stasiak *et al.*, 2007). In described cases Octenisept rinsing was employed for debridement and rinsing of the wound.

As shown in the studies, phenotypic characteristics, including the determination of biochemical profiles and patterns of antibiotic resistance is inadequate in differentiating strains of *C. perfringens.* Molecular typing is of paramount importance in epidemiological investigation testing, which tries to detect causes, sources and mechanisms of microorganisms spreading among patients, *e.g.* in a single hospital units (Fleischer and Salik, 2006; Brzychczy-Włoch *et al.*, 2014). Detailed molecular studies using the PFGE method showed that *C. perfringens* strains isolated from the three patients represented three different genetic clones. This demonstrates the different origins of these infections, which was the most likely source of endogenous flora in these patients. Similar results were also described earlier by our team, where there was an infection of *C. perfringens* of various origins in two patients simultaneously hospitalized in a single department (Brzychczy-Włoch *et al.*, 2014). Similar results were also described by Stasiak *et al.* (2007), who presented two patients with severe gas gangrene syndromes of a completely different origin.

Conclusions

As a result of the epidemiological and molecular study, it is possible to conclude that the three studied patients simultaneously hospitalized in a single Department of Orthopedics and Traumatology with diagnosed gas gangrene developed three different endogenous infection.

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ORIGINAL PAPER

Study of Acid Phosphatase in Solubilization of Inorganic Phosphates by *Piriformospora indica*

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Abstract

Phosphorus is an essential plant macronutrient present in the soil. Only a small portion of phosphorus in soil is taken up by plants and the rest of it becomes unavailable to plants as it is immobilized. Phosphate solubilizing microorganisms play a vital role in converting the insoluble form of phosphates to the soluble form. The present paper reports the solubilization of tricalcium phosphate, rock phosphate, single super phosphate, zinc phosphate and aluminum phosphate by *Piriformospora indica* with the production of organic acids as well as acid phosphatase. The amount of phosphate released (4.73 mg ml^{-1}) and titratable acidity (0.12%) was found to be the highest in the case of single super phosphate as compared to other phosphate sources. High performance liquid chromatography (HPLC) revealed the presence of oxalic acid, lactic acid, citric acid and succinic acid in the media. Highest phosphatase activity was observed with the cell membrane extract of the organism in the presence of zinc phosphate.

Key words: *Piriformospora indica*, acid phosphatase, organic acids, phosphate solubilization

Introduction

Phosphorus is an essential plant macronutrient which plays a significant role in the development of root, flowers and seed formation, helps in crop maturity and provides resistance to plant diseases (Khan *et al*., 2009). The functioning of certain key enzymes responsible for the regulation of metabolic pathways is also dependent on phosphorus availability (Tallapragada and Seshachala, 2012).

Most Indian soils do not contain a sufficient amount of available phosphorus which is necessary to maximize plant growth. Though large amount of inorganic phosphates are added to the soil in the form of chemical fertilizers, only a small fraction is utilized by plants and rest is converted to insoluble forms (due to soil pH) which becomes unavailable to the plants (Tallapragada and Seshachala, 2012). Phosphorus combines with iron and aluminum salts present in soil and forms complexes, as a result most of it is fixed in the soil. Acidic soils usually contain inorganic phosphates in the form of iron and aluminum salts where as neutral soil contains calcium phosphate (Gyaneshwar *et al*., 2002). Excessive utilization of chemical fertilizers in order to minimize phosphorus deficiency affects soil fertility and consequently affects crop yield (Nath *et al*., 2012).

A group of soil microorganisms known as phosphate solubilizing microorganisms (PSMs) plays a key role in converting the insoluble form of phosphates to soluble form thus making it available for the plants (Illmer and Schinner, 1995; Whitelaw, 2000). Microorganisms assimilate soluble phosphates and prevent it from adsorption or precipitation (Khan *et al*., 2009). PSMs are divided into two groups (i) Phosphate solubilizing bacteria (PSB) and (ii) Phosphate solubilizing fungi (PSF) (Tallapragada and Seshachala, 2012). Microbial communities involved in phosphorus acquisition include *Pseudomonas striata*, *Pseudomonas* fluorescens, *Bacillus megaterium*, *Bacillus polymyxa*, *Azotobacter* spp., *Burkholderia* spp. , *Rhizobium* spp., *Penicillium* spp., *Aspergillus* spp., *Trichoderma* spp. and various mycorrhizal fungi (Rodriguez and Fraga, 1999; Vyas and Gulati, 2009).

The mechanism of phosphate solubilization takes place via various microbial processes including (i) organic acids production, (ii) proton extrusion and (iii) phosphatase enzyme (Khan *et al*., 2009). Some microbes have potential for mineralization and solubilization of both inorganic and organic phosphorus. PSBs dissolve the soil phosphorus by producing weak organic acids such as gluconic acids, ketogluataric acids, succinic acid *etc*.

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Piriformospora indica, an arbuscular mycorrhiza like fungus plays an important role in the phosphate solubilization. It is known to solubilize different sources of organic phosphates as well as polyphosphates with the help of an enzyme acid phosphatase (66 kDa), present in its hyphal tips. *P. indica* exerts growth promotional effects on various plants by colonizing their roots. It has tremendous applications in the field of plant biotechnology as it acts as a biofertilizer, bioregulator, stimulator and a biocontrol agent (Malla *et al*., 2004).

Solubilization of tricalcium phosphate by diffe rent microorganisms is available in the literature (Agnihotri *et al*., 1970; Nath *et al*., 2012; Tallapragada and Seshachala, 2012). Few reports are also available on solubilization of other phosphate sources such as rock phosphate, zinc phosphate and aluminum phosphate by different microorganisms (Sagervanshi *et al*., 2012; Xiao *et al*., 2013). However, there are no reports available related to solubilization of single super phosphate by any organism.

Many insoluble forms of calcium, iron and aluminum phosphate occur in soil; however, few studies are reported related to the solubilization of aluminum and iron phosphate. Presently, rock phosphate and single super phosphates are being chiefly employed to sustain soil phosphorus level in available form for plants. Also zinc is one of the limiting factors in crop production. Hence the present study was undertaken to study the solubilization of different phosphate sources by replacing tricalcium phosphate(TCP) (present in Pikovskaya's media) with rock phosphate (RP), single super phosphate (SSP), zinc phosphate (ZnP) and aluminum phosphate (AlP) by *P. indica*. Both organic acids and phosphatase enzyme produced by the organism have contributed to the solubilization of phosphates.

Experimental

Materials and Methods

Materials. Tricalcium phosphates, zinc phosphate and aluminum phosphate used in the present study were obtained from Qualigens and S. D. Fine chemicals, Bangalore India. Single super phosphate and rock phosphate were obtained from the University of Agricultural Sciences, Bangalore, India. Molecular weight marker was obtained from Genei Pvt Ltd, Bangalore India.

Microorganism and culture maintenance. The culture of *P. indica* with an accession number of AF014929 USA; was obtained from Prof. Ajit Varma (Amity Institute of Herbal and Microbial Studies, Noida, India). The stock culture was maintained on potato dextrose agar at optimum conditions and stored at 4°C for further studies.

Phosphate solubilization in liquid medium. Phosphate solubilization was performed by inoculating 1×10^6 ml^{-1} in Pikovskaya's broth with five different phosphate sources TCP, RP, SSP, ZnP and AlP at initial pH adjusted to 7 and incubated at 30°C for 15 days. Parameters such as phosphate estimation, drop in pH, titratable acidity, production of organic acid and phosphatase activity were studied.

Phosphate estimation. Amount of phosphate released was estimated according to Fiske-Subbarow method (Fiske *et al*., 1925). The supernatant (0.5 ml) was mixed with 1 ml of 2.5 M sulphuric acid and 2.5% ammonium molybdate. To the above mixture 1 ml of the reducing agent (0.2 g of 1 amino-2-naphthol-4 sulfonic acid and 1.2 g of sodium sulfite in 100 ml distilled water) was added, incubated at room temperature for 10 minutes and the absorbance was read at 650 nm using potassium dihydrogen phosphate as standard.

Titratable acidity. Titratable acidity was estimated by titrating the known amount of culture filtrate with 0.1 M NaOH in the presence of phenolphthalein as an indicator (Nenwani *et al*., 2010). Drop in pH of the media was also recorded using digital pH meter (Elico make).

Organic acid production. Presence of organic acids in the media was detected by inoculating *P. indica* in Reyes basal medium containing five phosphate sources in presence of an indicator bromocresol green. The change in the colour of the media indicates the production of organic acids which was further identified and confirmed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) (Reyes *et al*., 1999; Gadagi and Sa, 2002).

Thin layer chromatography. Presence of organic acids produced in the media was detected by TLC using silica gel. The culture filtrate was reduced to $1/10^{th}$ of its volume prior to spotting. Five different solvent systems: (i) n-propanol: water: ammonia (60 : 20 : 20), (ii) benzene: methanol: acetic acid (90 : 16 : 8), (iii) methanol: 5 mol/l ammonia (80 : 20), (iv) ethanol: water: 25% ammonia (100 : 12 : 16), (v) n-propanol: 2N ammonia (7 : 3); were used to develop chromatograms which were later dipped in 0.4% (w/v) of bromocresol green in ethanol to detect the organic acid (Kraiker and Burch, 1973).

High performance liquid chromatography (HPLC). In order to identify and quantify the organic acid produced in the culture medium containing five different phosphate sources, 20 µl of the culture supernatant was subjected to HPLC analysis (Waters make) using C 18 column with acetonitrile and water in the ratio 7: 3 and pH of 3.5. The flow rate was maintained at 1 ml min^{-1} and detected by UV detector at 210 nm (Mardad *et al*., 2013).

Protein estimation. The concentration of proteins was measured by Lowry's method using bovine serum albumin as a standard (Lowry *et al*., 1951).

Enzyme assay. Acid phosphatase activity was determined by spectrophotometer using para-nitrophenol

(pNPP) as substrate. Extraction of phosphatase enzyme from the cell membrane of *P. indica* was performed according to Malla *et al.* (2004). The protein concentration of the extract was estimated by the Lowry's method. Five hundred microlitres of the crude enzyme extract was mixed with 1 ml of pNPP solution along with 1 ml of sodium acetate buffer pH 5.3. The reaction mixture was incubated at 40°C for 30 minutes. The reaction was stopped by the addition of 2 ml of 0.05 M NaOH and the absorbance was measured at 410 nm. The enzyme activity is defined as the amount of enzyme required to release p-nitrophenol per ml, per minute under standard conditions.

Electrophoresis. SDS-PAGE was performed according to Laemalli (1970) to determine the molecular weight of the protein. Cell membrane extract was loaded into 10% gel along with a molecular weight marker.

Statistical analysis. All experiments were conducted in triplicates. The data obtained from three independent experiments were analyzed using Origin Pro 8.1 version and Microsoft Excel. Each value represents the mean of three independent experiments performed in triplicate. Student 't' test was performed for all experiments.

Results and Discussions

Phosphate solubilization was performed in Pikovskaya's broth with five phosphate sources TCP, RP, SSP, ZnP and AlP. Increase in soluble phosphate was in correlation to decrease in pH, which suggests the role of $[H^+]$ in solubilization mechanism as reported by Kang *et al.* (2002) which was in accordance with our results. Malla *et al.* (2004) has also reported the utilization of complex insoluble phosphates like TCP and RP by *P. indica*. Similar studies on solubilization of TCP, RP and AlP by different microorganisms were reported earlier (Kang *et al.*, 2002; Frankem *et al.*, 2008; Nath *et al.*, 2012). Panhwar *et al.* (2011) reported the solubilization of triple super phosphate by two *Bacillus* spp. PSB 9 and PSB 16.

Phosphate estimation. Figure 1A represents the phosphate estimation plots for all the five substrates. Amount of phosphate liberated was highest with SSP (4.73 mg ml^{-1}) followed by TCP (2.89 $mgml^{-1}$). RP was found to be better compared to ZnP and AlP which released same amount of phosphate.

Titratable acidity and pH. Titratable acidity and pH also followed the similar trend. Percentage of titratable acidity was found to be highest (0.12%) for SSP with a comparative lower pH of 4.12 as depicted in Figure 1B. Titratable acidity of 0.01% for AlP was found to be least as compared to RP and ZnP. These results clearly explain the fact that the phosphate solubilization occurs by synthesized carboxylic acids released by microorganisms, as a result pH of the media decreases. Decrease in pH as well as increase in titratable acidity was observed with all the phosphate sources which are in accordance with Nenwani *et al.* (2010).

Organic acid production. Change in colour from blue to yellow in Reyes basal medium indicated the solubilization of phosphate by organic acid production. The sucrose content in the basal medium might have contributed to the increased production of organic acid as a result change in colour was observed (Reyes *et al.*, 1999). Solubilization of aluminum phosphate in Reyes basal media containing bromocresol green/ bromothymol blue dye by *Pseudomonas striata* and *Penicillium oxalicum* has been reported by Gadagi and Sa (2002).

Thin layer chromatography. Out of five different solvent systems, n-propanol and 2 N ammonia in the ratio 7 : 3 and benzene, methanol and acetic acid in the ratio 90 : 16 : 8 was found to be best solvent system.

High performance liquid chromatography. HPLC analysis of the culture filtrate was performed to identify and quantify the organic acids produced during

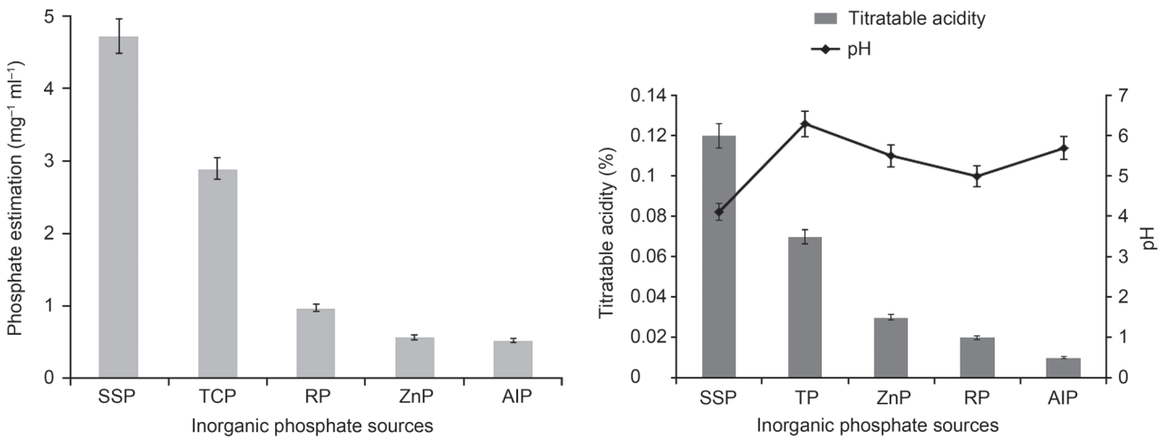

Fig. 1A and 1B. Plots depicting phosphate estimation (1A) and titrable acidity, pH (1B)

Table I
Quantification of the organic acid produced with respect to five different inorganic phosphate sources

Organic Acids	TCP	RP	SSP	ZnP	AlP
Oxalic Acid	0.3 mg ml^{-1}	2 mg ml^{-1}	0.48 mg ml^{-1}	–	0.74 mg ml^{-1}
Lactic Acid	3.8 mg ml^{-1}	0.49 mg ml^{-1}	3.75 mg ml^{-1}	4.02 mg ml^{-1}	–
Citric Acid	–	–	0.29 mg ml^{-1}	–	–
Succinic Acid	–	–	–	–	1 mg ml^{-1}

the solubilization of TCP, RP, SSP, ZnP and AlP. The quantitative difference in the production of organic acids during solubilization of different phosphate sources is as given in Table I. Both oxalic acid and lactic acid were produced during the solubilization of TCP and RP. Similarly with SSP, citric acid was produced along with oxalic acid and lactic acid. Lactic acid alone was produced during the solubilization of ZnP whereas with AlP, both lactic acid and succinic acids were produced. Two or three different unknown acids were also produced during the solubilization of all five phosphate sources.

The lactic acid and oxalic acid produced during the solubilization of phosphate sources varied in their quantity. Acid production during phosphate solubilization appears to be a common event of occurrence and the type of acid produced depends upon the type of phosphate source (Vyas and Gulati, 2009; Mardad *et al*., 2013). Among the carboxylic acids produced oxalic acid, tartaric acid, malic acid, fumaric acid, malonic acid and citric acids are more effective for phosphorus solubilization (Ryan *et al*., 2001). Our results are in accordance with Vazquez *et al*. (2000), who have also reported the production of lactic acid, succinic acid *etc*.

Phosphate solubilization is the combined effect of both drop in pH and organic acid production. Microorganisms secrete different organic acids which dissociates the bound form of phosphates. Phosphate solubilization of TCP involves the acidification of the microbial cells surrounded by proton substitution reaction that releases phosphate and calcium ions (Khan *et al*., 2009). The organic anions and associated protons are effective in solubilizing precipitated forms of soil phosphates such as Al, Fe *etc*. by chelating metal ions that may be associated with complex forms of phosphates or may facilitate the release of phosphates through ligand exchange reaction (Jones, 1998; Omar, 1998).

Enzyme activity. Phosphatase enzymes are believed to be very important in the uptake of phosphorus. Phosphatases have wide specificity which cleaves phosphate ester bonds and plays an important role in hydrolysis of insoluble polyphosphates and organic phosphates. They are also involved in phosphate transport within the cells. Phosphatases produced by plants and microorganisms are presumed to convert insoluble form of phosphates to soluble form there by helping plants to take up phosphate easily. The phosphatase activity of *P. indica* cell membrane extract with ZnP (0.088 Uml^{-1}) was found to be highest followed by RP (0.076 Uml^{-1}), SSP (0.065 Uml^{-1}) AlP (0.045 Uml^{-1}) and TCP (0.022 Uml^{-1}) which explains the importance of the type of phosphate source used (Fig. 2A). The molecular weight of acid phosphatase when analyzed on 10% polyacrylamide gel was 66 kDa (Fig. 2B) similar to the earlier report of Malla *et al*. (2004). According to Calleja *et al*. (1980) cell bound phosphatase is thought to be most important in cleavage and procure-

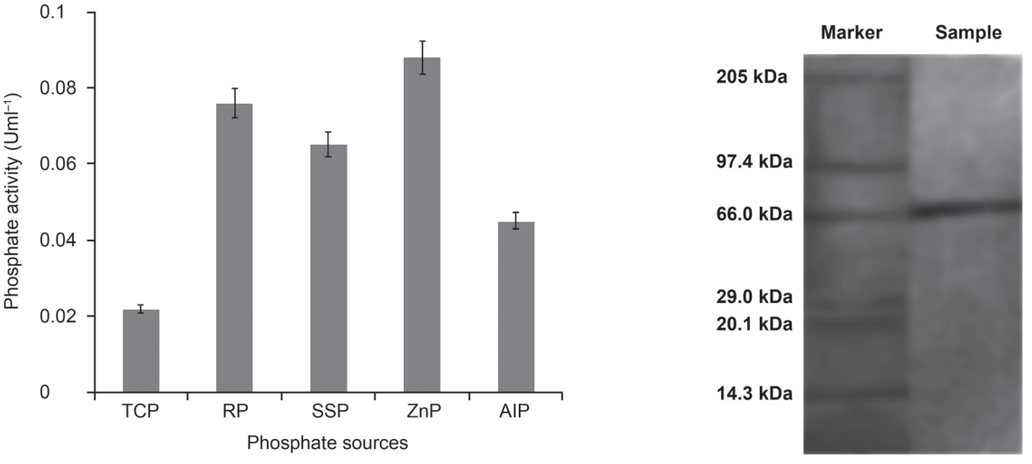

Fig. 2A and 2B. Plots depicting phosphatase activity (2A) and molecular weight of the protein obtained on polyacrylamide gel (2B)

ment of inorganic phosphates. Singh *et al.* (2002) have reported that *P. indica* secretes acid phosphatases to mobilize complex forms of phosphate present in rhizosphere there by facilitating the host plant to have better accessibility to soil phosphorus. Not many reports are available on the phosphate solubilization by phosphatase enzyme.

Phosphate solubilizing microorganisms and plants form a synergistic relationship in nature where in the PSM's provides soluble phosphates to plants and in return plants supply nutrients to microbes in the form of root exudates that promotes microbial growth. Organisms' having potential to solubilize the phosphates increases the availability of soluble phosphate and thereby enhances the plant growth and crop productivity. The mechanism of mineral phosphate solubilization has been a subject of analysis since long time and is still a matter of curiosity (Bagyaraj *et al*., 2000). Organic acids (mono, di and tricarboxylic acids) produced by the phosphate solubilizing microorganisms have been mainly involved in chelating the insoluble complexes of phosphate (Bagyaraj *et al*., 2000). According to Fankem *et al*. (2006) phosphate solubilization is the result of combined effect of decrease in pH and organic acids production. Phosphatase enzymes are likely involved in hydrolysis of insoluble phosphate complexes (Singh *et al*., 2002; Malla *et al*., 2004). Solubilization of phosphate by *P. indica* occurs due to the combined effect of both phosphatase enzyme and organic acid production. However the exact mechanism and correlation between the two needs further study for scientific validation.

Conclusion

The present study reports the solubilization of five different inorganic phosphate sources namely TCP, RP, SSP, ZnP and AlP by *P. indica*. A maximum amount of 4.73 $mgml^{-1}$ soluble phosphate with respect to SSP was solubilized by *P. indica*. Titratable acidity was found to be 0.12% and a drop in pH from 7 to 4.12 was observed with SSP. Organic acids present in the medium were detected and identified as oxalic acid, lactic acid, citric acid and succinic acid by TLC and HPLC analysis. Phosphatase activity was reported in *P. indica* with a maximum activity of 0.088 Uml^{-1} with respect to cell membrane extract of ZnP sample. The molecular weight of the phosphatase was found to be 66 kDa. Organisms such as *P. indica* having the potential to solubilize different sources of inorganic phosphates and the ability to increase the availability of soluble phosphate without the over application of chemical fertilizer enhance plant growth and crop productivity. The above outcome can be explored further and used as an innovative technology in organic farming for better crop productivity.

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ORIGINAL PAPER

Characterization of Endolithic Culturable Microbial Communities in Carbonate Rocks from a Typical Karst Canyon in Guizhou (China)

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Abstract

The endolithic environment is a ubiquitous habitat for microorganisms and a critical interface between biology and geology. In this study, a culture-based method and the phylogenetic analysis based on 16S rRNA and internal transcribed spacer (ITS) sequences were used to investigate the diversity of endolithic bacteria and fungi in two main types of carbonate rocks (namely dolomite and limestone) from Nanjiang Canyon in Guizhou karst area, China. The results of bacterial diversity indicated that all bacteria isolated from dolomite and limestone rocks were divided into 4 bacterial groups, including *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes*. For these two kinds of rocks, *Proteobacteria* was the first dominant group, and *Gammaproteobacteria* occupied the greatest proportion which might be closely related to *Pseudomonas* in phylogeny to be the most dominant genera after isolation. *Actinobacteria* and *Bacillus* bacteria were also widespread in these two kinds of rock environments. There were only 9 and 8 strains of fungi isolated from dolomite and limestone respectively, which all belonged to *Ascomycota*. To the best of our knowledge, this is the first report on diversity of endolithic culturable bacteria and fungi in carbonate rocks in Guizhou karst region. These microorganisms may play an important and unprecedented role in the carbonate rock weathering during the long history of geological evolution.

Key words: endolithic bacteria, endolithic fungi, carbonate rock

Introduction

The endolithic environment, the tiny pore and crack space in rocks, is a ubiquitous habitat for microorganisms and a critical interface between biology and geology (Friedmann, 1982; Walker and Pace, 2007a). Microorganisms native to the endolithic environment must possess adaptive ability and tolerance to severe stresses including rapid temperature variations, oligotrophy, desiccation, and high UV flux (Sigler *et al.*, 2003; Selbmann *et al.*, 2013). Communities of microorganisms that inhabit endolithic environments include autotrophic and heterotrophic bacteria, fungi, algae, and lichens (Sigler *et al.*, 2003). The endolithic microorganisms rarely existed by means of single colony formed by a single species, while they usually grew collectively to form microbial community with complex, various structures and functions (Gorbushina *et al.*, 2004; Gorbushina, 2007). Nowadays, endolithic microorganisms have been observed not only in a variety of extreme terrestrial ecosystems but also in temperate environments (Gerrath *et al.*, 2000; Wierzchos *et al.*, 2006; Horath and Bachofen, 2009; Wong *et al.*, 2010; Tang *et al.*, 2012). They also have been detected inhabiting a variety of rock types such as halite, sandstone, quartz, gypsum, dolomite and limestone (Dong *et al.*, 2007; Gorbushina, 2007; Tang *et al.*, 2012; Roldan *et al.*, 2014).

Nowadays, many investigations of endolithic microbial communities used culture-independent techniques (Sigler *et al.*, 2003; Walker and Pace, 2007b; Horath and Bachofen, 2009; Wong *et al.*, 2010). Although, culture-independent methods can provide important information about the diversity of phylogenetic groups in endolithic environments, pure cultures are still indispensable for the determination of the metabolic activity of microorganisms, which can be significantly different even among closely related phylotypes (Spring *et al.*, 2015). In addition, the culturable microorganisms account for about 0.001 ~ 15% of the total microorganisms of environmental samples (Tamaki *et al.*, 2005). Thus, a considerable part identified by culture-independent method is uncultured microbial species. Pure

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culture method played and plays an irreplaceable role in exploring microbial community structures, especially in the following aspects, such as new species or special functional microbes, *etc* (De Leo *et al*., 2012; Krakova *et al*., 2015). However, to date, no study has examined the endolithic microbial community in Guizhou karst environment using culture-dependent method. This method has the potential to improve our understanding of microbial diversity and facilitate functional studies of endolithic microorganisms.

Guizhou, a province in southwest of China, is one of the three largest developing karst areas in the world. The carbonate rock area is 130,000 km^2, covering 73.8% of total land surface area of the province. The weathering of carbonate rocks influences the geochemical compositions of rocks, soils, the atmosphere, and organisms, and the transfer process of matter and energy in the karst environment (Lian *et al*., 2010). Endolithic microorganisms have been found to be directly and indirectly involved in the weathering of carbonate rocks. Papida *et al*. (2000) found that a mixed microbial population exacerbated physical weathering of carbonate rocks. The endolithic microorganisms play an important and unprecedented role in the carbonate rock weathering during the long history of geological evolution. So, the aim of the present study was therefore to investigate the endolithic microbial diversity in dolomite and limestone rocks in karst area of Guizhou Province using culture-dependent techniques. Combined with the culture-independent method at the early stage (Tang and Lian, 2012; Tang *et al*., 2012), endolithic microbial diversity of carbonate rocks could be reflected more fully. Meanwhile, the research laid the foundation to further explore the carbonate rock weathering by using the microbial resources in the karst endolithic environment.

Experimental

Materials and Methods

Site description and sample collection. Nanjiang canyon (26°56″N, 106°58″E) is a typical karst canyon, which is praised as "Karst Ecosystem Museum". It is located in Kaiyang County of Guizhou Province, southwest China. Details of the site are given in our previous study (Tang and Lian, 2012; Tang *et al*., 2012). There are a lot of exposed carbonate rocks in this area. The dolomite and limestone rocks are weathered and porous. The soil developed on its weathering crust is non-zonal soil with shallow soil layers and no apparent genetic layers. In this area, three Triassic dolomite (D1-D3) and three Triassic limestone samples (L1-L3) up to a depth of ~ 1 cm were collected using a sterile rock chisel and placed in sterile bags on ice. The samples were kept at 4°C until subjected to microbial isolation. All samples were collected in September 2009.

Powder X-ray diffraction. For mineralogy in the dolomite and limestone rock samples, powder X-ray diffraction patterns were obtained using CuKa radiation at 40 kV with an X-ray diffractometer (D/Max-2200/PC, Rigaku). The results revealed 2 dolomite samples to be pure dolomite rocks and one dolomite sample to be mostly dolomite (99%) with trace amounts of anorthose. The 3 limestone samples were mostly calcite (97–99%) containing trace of dolomite and quartz.

Isolation and enumeration of microorganisms. In accordance with the requirements of aseptic technique, the mineral powder of each crush rock sample was scraped. After serial dilution, they were coated on beef extract-peptone agar medium (5 g/l beef extract, 10 g/l peptone and 15 g/l agar) and Martin culture medium (1 g/l K_2HPO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 5 g/l peptone, 10 g/l glucose, 15 ~ 20 g/l agar and 3.3 ml/l 1% streptomycin aqueous solution). Then they were reversely cultivated under constant temperature of 30°C. After 2 ~ 3 days for bacteria or 5 ~ 7 days for fungi, the colony counting could be obtained. The single colonies with different phenotypes, were picked up and the purified strains were transferred to the corresponding agar slants for conservation at 4°C.

DNA extraction, PCR amplification, and sequencing. DNA extraction and PCR amplification were performed using the TIANcombi DNA Lyse&Amp PCR Kit (Tiangen Biochemical technology Co., Ltd., China). The bacterial universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') and fungal universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), were used to amplify the bacterial 16S rDNA and fungal internal transcribed spacer (ITS) sequences, respectively. All of the primers were synthesized by Shanghai Sangong Co., Ltd., China.

All reactions were carried out in a 50 µl reaction mixture, containing 25 µl of 2 × PCR Reagent, 1.25 µl of forward primer and reverse primer (10 µM) respectively and 5 µl of DNA template. PCR was run under the following conditions: initial denaturation at 94°C for 5 min; followed by 30 circles of 1 min denaturation at 94°C, 1 min annealing at 52°C, 2 min extension at 72°C, and a final extension step of 10 min at 72°C. The results of PCR amplification were confirmed using 1% agarose gel, stained with SYBR Safe 10.000 × in DMSO (Invitrogen). PCR products were subsequently purified using PCR purification kit (E.A.N.A. Gel Extraction kit, OMEGA, USA) and subjected to sequencing in an ABI PRISM 3730 automatic sequencer (Shanghai Sangon Co., Ltd., China).

Phylogenetic analysis. DNA sequences were analyzed using Bellerophon (Huber *et al.*, 2004) and CHIMERA_CHECK (Cole *et al.*, 2003) software to remove chimeric artifacts. The sequences were considered to be of the same phylotype if they were ≥97% similar to one another over the region of the 16s or ITS rRNA gene sequenced (Stackebrandt and Ebers, 2006). Then the effective sequences were compared with known sequences in the NCBI database (http://www.ncbi.nih.gov/) by Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990). The sequences were aligned using Clustal X software (Thompson *et al.*, 1997), and phylogenetic trees were constructed with the Mega 4.0 program package using the neighbor-joining method (Kumar *et al.*, 2004). Bootstrap confidence values were obtained with 1000 replicates. The trees were constructed by calculating the Kimura distance (Kimura, 1980).

Nucleotide sequence Accession numbers. The nucleotide sequences reported in this study have been deposited in GenBank under accession numbers JN650544-JN650602.

Results

Isolation and enumeration of microorganisms. Beef extract-peptone agar medium was utilized to isolate bacteria of rock samples. There were 39 strains and 26 strains of bacteria with different phenotypic characteristics in dolomite and limestone respectively. Fungi of rock samples were separated by Martin culture medium, and dolomite and limestone respectively obtained 9 and 8 strains of fungi with different colony phenotypes. It was calculated that the culturable bacteria of dolomite and limestone were about 2.3×10^5 CFU/g and 2.0×10^5 CFU/g by using dilution-plate method; while the culturable fungi were about 7.7×10^3 CFU/g and 3.0×10^3 CFU/g respectively.

Diversity of the bacterial 16S rRNA gene sequences from dolomite rocks. There were 39 effective sequences in total by sequencing 16S rDNA for the isolates of 39 strains of dolomite. The alignment results of similarity indicated that they were classified into 4 phyla, 15 genera and 26 species (Table I). These 39 strains of bacteria showed high similarity with the known 16S rDNA sequences in database, namely 98 ~ 100%.

The sequence analysis of 16S rDNA (see Table I and Fig. 1) illustrates that 39 strains of bacteria from dolomite were classified into 4 major bacteria groups. More than 53.8% of them belonged to *Proteobacteria*, which including 12 strains of *Gammaproteobacteria*, accounting for 30.8%; 5 strains of *Alphaproteobacteria*, accounting for 12.8%; and 4 strains of *Betaproteobacteria*, accounting for 10.2%. In addition, 10 strains were from *Actinobacteria*, and accounted for 25.7%, while 7 strains were *Firmicutes*, accounting for 17.9%. Moreover, one strain belonged to *Bacteroidetes*, accounting for 2.6%.

Gammaproteobacteria, as one subphylum of *Proteobacteria*, accounted for the highest ratio of culturable bacteria in the endolithic dolomite environment so that it was regarded as the most dominant group obtained by isolation. The 10 strains of bacteria in the group, accounting for 25.6%, had close relations with *Pseudomonas* in phylogeny, and the similarity of 16S rDNA sequences was 99%. Thus, the group was the most dominant bacterial genus acquired by isolation. Therein, four strains were closely associated with *Pseudomonas putida*, 1 strain was highly homologous to *Pseudomonas nitroreducens*. In *Alphaproteobacteria*, there were 2 strains which were closely related to *Brevundimonas* and *Sphingobium* respectively and 1 strain had homology of 99% with *Sphingobium*. Besides, 4 strains of bacteria belonged to *Betaproteobacteria*, where 2 strains were *Duganella* and the other 2 strains belonged to *Marseille*.

Actinobacteria was the second dominant group of the culturable bacteria from dolomite. In this group, 4 strains of bacteria had a close genetic relationship with *Arthrobacter*; three of them were highly homologous with *Rhodococcus*; the homology of two strains and *Streptomyces zaomyceticus* (FJ792553) reached 100%; and the rest one was 99% homologous with *Microbacterium oxydans* (HQ202812).

There were 7 strains of bacteria in *Firmicutes*, including 6 *Bacillus*, which were the second dominant genera of the culturable bacteria from dolomite. In this group, three of them were 99% homologous with *Bacillus cereus* (GU969128), two showed high homology with *Bacillus sphaericus* (DQ286315), and one was 100% homologous with *Bacillus simplex* (JF496520). Moreover, DCB46 was 99% homologous with *Paenibacillus* sp. (FJ006903).

Only one strain of bacteria belonged to *Bacteroidetes*, which was 99% homologous with *Dyadobacter* sp. (HQ231938).

Diversity of the bacterial 16S rRNA gene sequences from limestone rocks. 16S rDNA of 26 bacterium isolates from limestone was sequenced to obtain 26 effective sequences in total. The relationships between 16S rDNA sequence of the bacteria isolated from limestone and the most similar sequence in GenBank database are listed in Table II. It can be seen that they were divided into 4 phyla, 13 genera and 17 species. There was a high similarity between the 26 strains of bacteria and the known 16S rDNA sequences in GenBank database. Among them, the similarity of LCB37 was 96% only, while that of the rest was higher than 97%.

As indicated by 16S rDNA phylogenetic analysis, the 26 strains of bacteria isolated from limestone were assigned to 4 major phylogenetic groups (Fig. 2). It can

Table I
Similarity analysis of 16S rDNA sequences of isolates from dolomite rocks

Representative isolate (accession no.)	No. of isolates in OUT*	Nearest type strain (accession no.)	Similarity (%)
Actinobacteria	10		
Arthrobacter	4		
DCB14(JN650552)	2	*Arthrobacter* sp. (HM165265)	99
DCB16(JN650554)	1	*Arthrobacter* sp. (HQ166103)	99
DCB48(JN650561)	1	*Arthrobacter* sp. (FN794213)	99
Microbacterium	1		
DCB18(JN650556)	1	*Microbacterium oxydans* (HQ202812)	99
Rhodococcus	3		
DCB15(JN650553)	1	*Rhodococcus* sp. (JF900087)	99
DCB17(JN650555)	2	*Rhodococcus* sp. (EF612316)	99
Streptomyces	2		
DCB61(JN650569)	2	*Streptomyces zaomyceticus* (FJ792553)	100
Bacteroidetes	1		
Dyadobacter	1		
DCB47(JN650560)	1	*Dyadobacter* sp. (HQ231938)	99
Firmicutes	7		
Paenibacillus	1		
DCB46 (JN650559)	1	*Paenibacillus* sp. (FJ006903)	99
Bacillus	6		
DCB1(JN650544)	3	*Bacillus cereus* (GU969128)	99
DCB23(JN650558)	1	*Bacillus simplex* (JF496520)	100
DCB8(JN650548)	2	*Bacillus sphaericus*(DQ286315)	99
Proteobacteria	21		
Alphaproteobacteria	5		
Brevundimonas	2		
DCB19(JN650557)	1	*Brevundimonas vesicularis* (FJ999941)	99
DCB60(JN650568)	1	*Brevundimonas* sp. (DQ778038)	99
Methylobacterium	1		
DCB49(JN650562)	1	*Methylobacterium sp*. (FN868959)	99
Sphingobium	2		
DCB53(JN650564)	2	*Sphingobium amiense* (JF459961)	100
Betaproteobacteria	4		
Duganella	2		
DCB58(JN650566)	1	*Duganella* sp. (JF778719)	99
DCB59(JN650567)	1	*Duganella zoogloeoides* (NR_025833)	99
Massilia	2		
DCB52(JN650563)	1	*Massilia* sp. (DQ257420)	99
DCB56(JN650565)	1	*Massilia* sp. (AB552860)	98
Gammaproteobacteria	12		
Enterobacter	1		
DCB6(JN650546)	1	*Enterobacter amnigenus* (DQ481471)	99
Pseudomonas	10		
DCB7(JN650547)	3	*Pseudomonas* sp. (FN547413)	99
DCB11(JN650550)	2	*Pseudomonas* sp. (AM934695)	99
DCB13(JN650551)	4	*Pseudomonas putida* (EU118779)	99
DCB74(JN650570)	1	*Pseudomonas nitroreducens* (EU500825)	99
Serratia	1		
DCB5(JN650545)	1	*Serratia fonticola* (HQ407251)	99

* The strains were clustered into operational taxonomic units (OTUs) at a level of sequence similarity ≥ 97%.

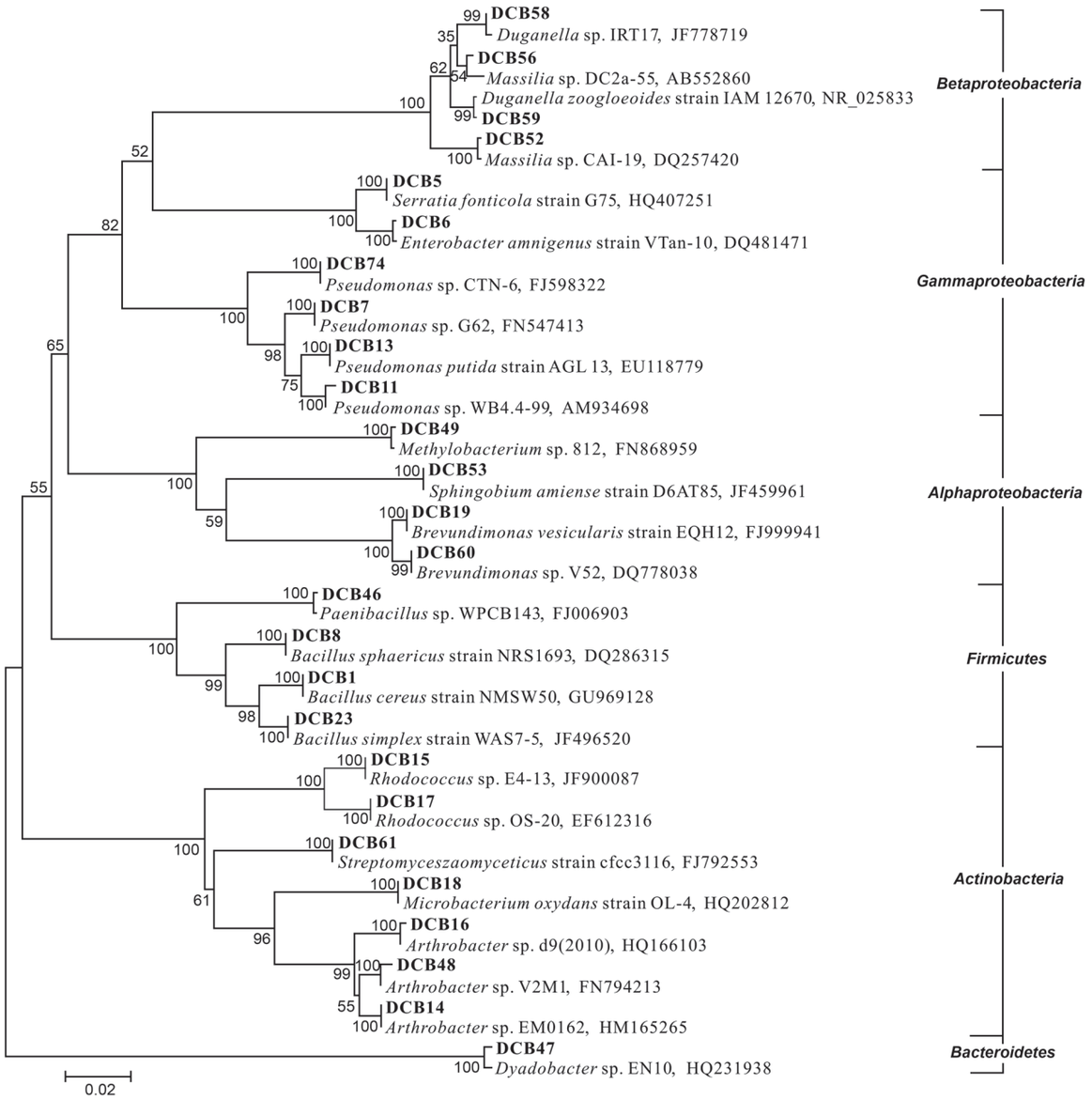

Fig. 1. Phylogenetic relationship based on 16S rRNA gene sequences of culturable bacterial clones isolated from dolomite rocks (in bold type) with closely related sequences from the GenBank database. Neighbor-joining trees; bootstrap values (1,000 replicates) are shown at the nodes

be known that *Proteobacteria* played a dominant role in bacterial diversity and had 18 strains, taking up 69.3%, including 8 *Betaproteobacteria* and *Gammaproteobacteria*, accounting for 30.8% respectively; 2 strains of *Alphaproteobacteria*, accounting for 7.7%. In addition, three strains belong to *Actinobacteria* and three of them were *Bacteroidetes*, which accounted for 11.5%. Moreover, there were 2 strains of *Firmicutes* (7.7%).

As for *Proteobacteria*, 8 of them belonged to *Gammaproteobacteria* subphylum, and all of them were *Pseudomonas*, which was regarded as the first dominant genera of culturable bacteria from limestone. In the subphylum, LCB43 had 100% similarity to dolomite isolate DCB13, which means that they were the same kind of bacteria. In addition, the similarity between them and *P. putida* (EU118779) reached 99%. The other 7 strains of bacteria were 100% similar to *Pseudomonas* sp. (FJ006865), while as for the species of *Pseudomonas*, it was needed to be further identified. There were 8 strains of *Betaproteobacteria*, including 4 *Marseille bacteria*, which were sub-dominant genera of culturable bacteria from limestone. Moreover, 2 *Janthinobacterium*, a *Duganella*, and a *Methylibium* were included. Only 2 strains in *Alphaproteobacteria* showed 100% homology with *Brevundimonas intermedia* (JF915342).

Table II
Similarity analysis of 16S rDNA sequences of isolates from limestone rocks.

Representative isolate (accession no.)	No. of isolates in OUT*	Nearest type strain (accession no.)	Similarity (%)
Actinobacteria	3		
Arthrobacter	1		
LCB65 (JN650582)	1	*Arthrobacter* sp. (HQ231937)	99
Microbacterium	1		
LCB38 (JN650577)	1	*Microbacterium oxydans* (HQ316116)	99
Oerskovia	1		
LCB39 (JN650578)	1	*Oerskovia paurometabola* (HM244951)	100
Bacteroidetes	3		
Flavobacterium	2		
LCB34 (JN650574)	1	*Flavobacterium* sp. (HQ425305)	98
LCB37 (JN650576)	1	*Flavobacterium* sp. (DQ178976)	96
Sphingobacterium	1		
LCB40 (JN650579)	1	*Sphingobacterium* sp. (AM411998)	99
Firmicutes	2		
Bacillus	1		
LCB77 (JN650587)	1	*Bacillus cereus* (GU969128)	99
Staphylococcus	1		
LCB44 (JN650581)	1	*Staphylococcus* sp. (EU855210)	99
Proteobacteria	18		
Alphaproteobacteria	2		
Brevundimonas	2		
LCB32 (JN650573)	2	*Brevundimonas intermedia* (JF915342)	100
Betaproteobacteria	8		
Methylibium	1		
LCB69 (JN650585)	1	*Methylibium* sp. (FJ615290)	99
Duganella	1		
LCB66 (JN650583)	1	*Duganella zoogloeoides* (NR_025833)	99
Janthinobacterium	2		
LCB30 (JN650572)	2	*Janthinobacterium* sp. (FN293043)	99
Massilia	4		
LCB36 (JN650576)	2	*Massilia* sp. (DQ257420)	99
LCB71 (JN650586)	1	*Massilia* sp. (AB552860)	98
LCB67 (JN650584)	1	*Massilia* sp. (FR865960)	99
Gammaproteobacteria	8		
Pseudomonas	8		
LCB43 (JN650580)	1	*Pseudomonas putida* (EU118779)	99
LCB28 (JN650571)	7	*Pseudomonas* sp. (FJ006865)	100

* The strains were clustered into operational taxonomic units (OTUs) at a level of sequence similarity ≥ 97%

Three strains of bacteria in *Bacteroidetes* group were closely related to *Flavobacterium* and *Sphingobacterium*, while 3 strains of bacteria in *Actinobacteria* group were *Arthrobacter* sp., *Microbacterium* sp. and *Oerskovia* sp. respectively. Besides, the similarity between LCB77 of *Firmicutes* group and dolomite isolate DCB1 was up to 100%. They were 99% similar with 16S rDNA sequences of *B. cereus* (EU979024), namely type strain. In addition, an isolate of *Firmicutes* had close relations with *Staphylococcus*.

Diversity of culturable fungi from dolomite and limestone rocks. ITS sequences for 9 strains of fungi isolates from dolomite and 8 strains of fungi isolates from limestone were compared with the sequence

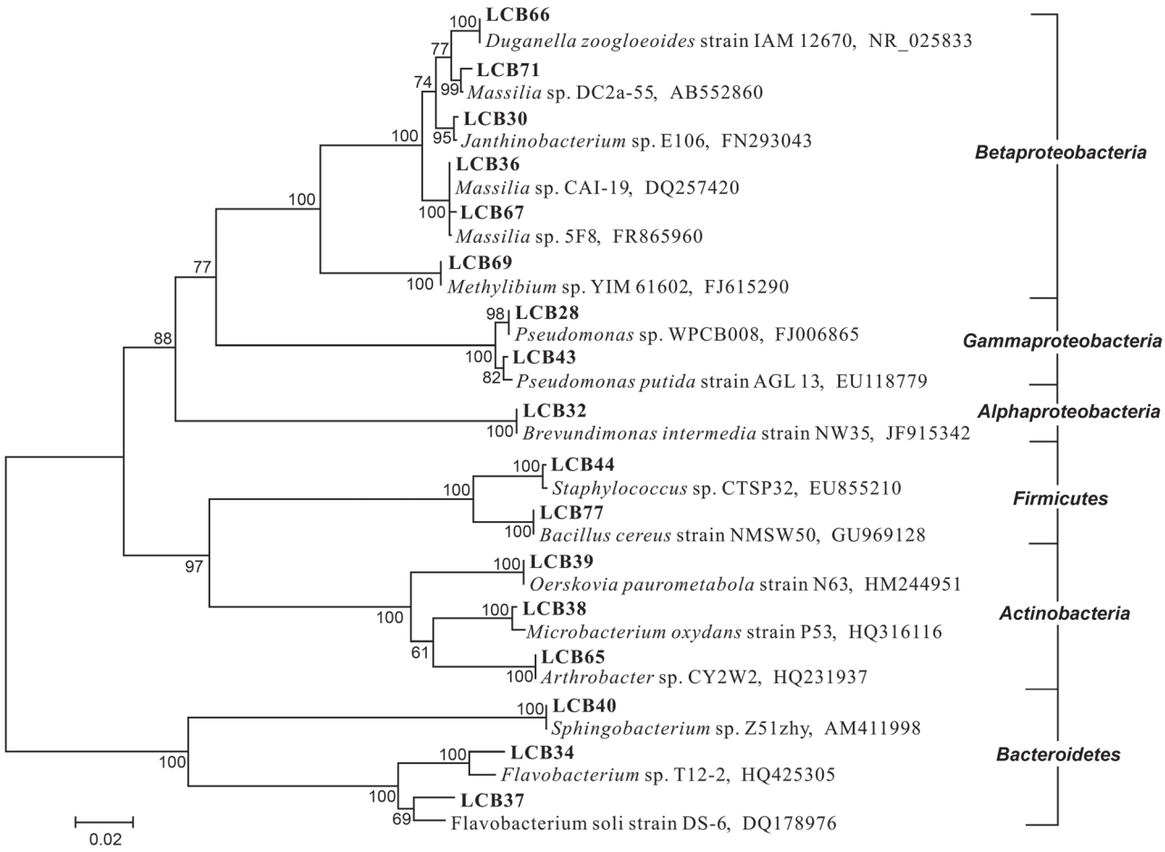

Fig. 2. Phylogenetic relationship based on 16S rRNA gene sequences of culturable bacterial clones isolated from limestone rocks (in bold type) with closely related sequences from the GenBank database. Neighbor-joining trees; bootstrap values (1,000 replicates) are shown at the nodes

of GenBank database to obtain the most similar sequences. Table III shows the relationships between ITS sequences of fungi isolates from dolomite and limestone and the most similar sequences in GenBank database. The results indicated that 9 isolates from dolomite were classified into 1 phylum, 6 genera and 7 species; while the 8 strains of fungi isolates from limestone were divided into 1 phylum, 7 genera and 8 species. They all had high similarity (98 ~ 100%) with the reported ITS sequences of GenBank database, including 12 strains with 100% similarity.

A phylogenetic tree was constructed based on the sequences with high similarity between the reference sequences in database and the strains isolated from dolomite and limestone, as shown in Fig. 3. The results manifested that 9 strains of fungal isolates from dolomite and 8 strains from limestone were *Ascomycota*. Among the fungal isolates from dolomite, 3 of them were *Aspergillus*, which was the genus with the most isolates. There were 2 strains with 100% similarity to a strain of *Aspergillus niger* (EF592173), while the other one had 100% similarity with a strain of *Aspergillus oryzae* (HQ285588). There was also an isolate belonging to *Penicillium*, which was 100% similar with *Penicillium crustosum* (GQ340556). Besides, the other four strains were closely associated with *Aureobasidium pullulans* (JF439462) of *Aureobasidium*, *Myrothecium verrucaria* (GQ131886) of *Myrothecium*, *Purpureocillium lilacinum* (HM439952) of *Paecilomyces* and *Trichoderma* sp. (GQ497170) respectively, and the homology was as high as 99 ~ 100%.

The 8 fungi isolates from limestone belonged to 7 genera of *Ascomycota*, including *Alternaria*, *Bipolaris*, *Chaetomium*, *Cryptococcus*, *Fusarium*, *Penicillium* and *Trichoderma*. Except that there were 2 isolates in *Chaetomium*, the other ones were divided into different genera. From the phylogenetic tree, it can be seen that isolate LCF94 and the fungal isolate from dolomite, namely DCF87 were 100% similar, which suggested that they were the same kind of fungi. In addition, the similarity between them and *P. crustosum* (GQ340556) of *Penicillium* was 100%. Moreover, isolate LCF98 and fungal isolate from dolomite DCF83 showed similarity of 100%. It means that they were the same kind of bacteria and both of them were *Trichoderma*. Therefore, the fungal strains of *Penicillium* and *Trichoderma* were

Table III
Similarity analysis of ITS sequences of isolates from dolomite and limestone rocks.

Representative isolate (accession no.)	No. of isolates in OUT*	Nearest type strain (accession no.)	Similarity (%)
Dolomite samples			
Ascomycota	9		
Aureobasidium	1		
DCF84 (JN650590)	1	*Aureobasidium pullulans* (JF439462)	100
Aspergillus	3		
DCF86 (JN650591)	2	*Aspergillus niger* (EF592173)	100
DCF91 (JN650594)	1	*Aspergillus oryzae* (HQ285588)	100
Myrothecium	1		
DCF88 (JN650593)	1	*Myrothecium verrucaria* (GQ131886)	99
Penicillium	1		
DCF87 (JN650592)	1	*Penicillium crustosum* (GQ340556)	100
Purpureocillium	2		
DCF81 (JN650588)	2	*Purpureocillium lilacinum* (HM439952)	100
Trichoderma	1		
DCF83 (JN650589)	1	*Trichoderma* sp. (GQ497170)	100
Limestone samples			
Ascomycota	8		
Alternaria	1		
LCF97 (JN650600)	1	*Alternaria* sp. (HQ833813)	100
Bipolaris	1		
LCF96 (JN650599)	1	*Bipolaris* sp. (FJ770073)	98
Chaetomium	2		
LCF92 (JN650595)	1	*Chaetomium* sp. (HQ607819)	99
LCF93 (JN650596)	1	*Chaetomium nigricolor* (JF439467)	99
Cryptococcus	1		
LCF99 (JN650602)	1	*Cryptococcus albidus* (AF444355)	100
Fusarium	1		
LCF95 (JN650598)	1	*Fusarium oxysporum* (EF495237)	99
Penicillium	1		
LCF94 (JN650597)	1	*Penicillium crustosum* (GQ340556)	100
Trichoderma	1		
LCF98 (JN650601)	1	*Trichoderma* sp. (GQ497170)	100

* The strains were clustered into operational taxonomic units (OTUs) at a level of sequence similarity ≥ 97%

isolated from samples of dolomite and limestone. It can be seen that the two types of fungi existed in both kinds of rocks studied in the research.

Discussion

As a special habitat, carbonate rock provided a great challenge to the organisms living in it owing to the features of rocks, such as arid environment, nutrition deficiency, great temperature fluctuations, *etc.* However, there were various microorganisms on its surface and in its cracks, which played a crucial role in biogeochemical cycle. Although molecular biological method has been developed during exploring and revealing microbial diversity, it is still necessary to separate and purify microorganisms to understand their ecological functions in biosphere. Thereby, culture-dependent method is still an essential approach to research microbial diversity.

A total of 65 bacterial strains and 17 fungal strains were isolated from dolomite and limestone by using pure culture method. The 39 isolated bacterial strains from dolomite samples were divided into 4 phyla, 15 genera and 26 species; while the 26 bacterial strains of limestone were classified into 4 phyla, 13 genera

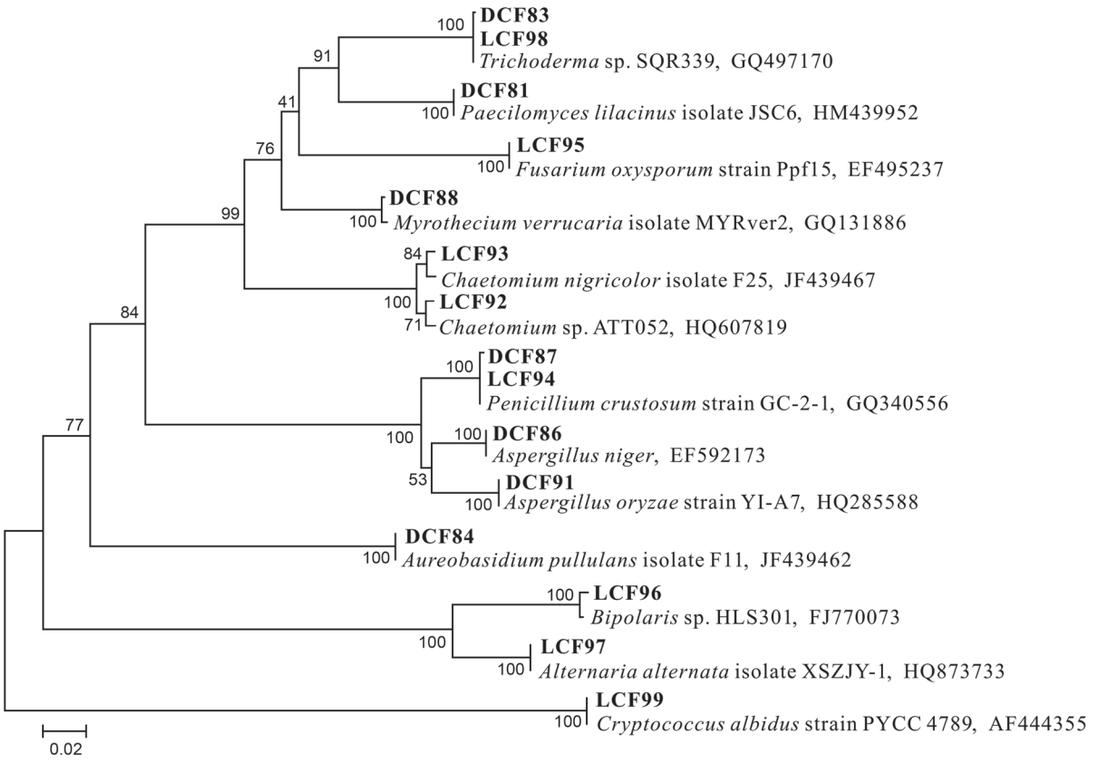

Fig. 3. Phylogenetic relationship based on ITS gene sequences of culturable fungal clones isolated from dolomite (DCF) and limestone (LCF) rocks (in bold type) with closely related sequences from the GenBank database. Neighbor-joining trees; bootstrap values (1,000 replicates) are shown at the nodes

and 17 species. The main phyla of the isolated bacteria from both kinds of rocks were consistent, but there were some differences in species. In addition, the diversity of bacteria was much higher than that of fungi. For these two kinds of rocks, *Proteobacteria* was the first dominant group, and *Gammaproteobacteria* occupied the greatest proportion. Besides, it was closely related to *Pseudomonas* in phylogeny to be the most dominant genera after isolation. *Pseudomonas* is a genus composed of ubiquitous metabolically versatile Gram-negative bacteria, capable to grow in a variety of environmental conditions (Belgini *et al.*, 2014). Some researchers found that *Pseudomonas* also existed in the soil of Antarctic area even in deep sea and it was connected with polycyclic aromatic hydrocarbons (PAHs). Moreover, some *Pseudomonas* even was directly involved in degradation (Ma *et al.*, 2006; Cui *et al.*, 2008). A lot of pseudomonads bacteria were found in the dolomite and limestone rock endolithic environments, but their roles in rock environment were still unclear. The proportion of *Actinobacteria* in two kinds of rocks was greater. Meanwhile, the group is widespread in soil. An explanation for the high fraction of *Actinobacteria* in Guizhou karst region could be due to their strong cell wall, the capability of forming spores, and their high GC-content. These characteristics would allow their survival in harsh environments. Many bacteria belonging to *Bacillus* were isolated from the rock environment. Owing to the kind of bacteria forming the spores, which had powerful resistance to poor environment, they could survive in rock environment. Our results confirmed that members of the *Proteobacteria*, *Actinobacteria* and *Bacillus* are ecologically significant constituents of carbonate rocks. However, future work is needed to determine the functions of these important groups in karst environments.

There were only 9 and 8 strains of fungi isolated from dolomite and limestone respectively, which all belonged to *Ascomycota*. Besides, they were common fungi in environment which suggested that the culturable fungi resources were very limited on the two kinds of rocks. In view of numerous slow growing fungi colonize carbonate rocks, the cultivating time of fungi in the study might be short. With longer periods of time, it is possible that a more realistic and abundant fungal diversity would have been registered. Nevertheless, the roles of these fungi in rock environment have not been reported so far. Hence, it is necessary to study the functions of these microorganisms in karst rock environment, especially the roles in the process of weathering and pedogenesis of rocks by further utilizing these microbial resources.

Combining the research results and our previous report about the endolithic microbial diversity in the carbonate rocks in karst area of Guizhou by using culture-independent method (Tang and Lian, 2012; Tang *et al*., 2012), it was indicated that the microbial diversity of carbonate rocks was abundant. There were autotrophic microorganisms, such as *Cyanobacteria* with nitrogen fixation and photosynthesis, anoxygenic phototrophic *green non-sulfur bacteria*, as well as heterotrophic microorganisms, for instance, *Proteobacteria*, *Acidobacteria*, *Bacteroides*, *Firmicutes* and heterotrophic fungi as well as *Archaea*. The autotrophic microorganisms could be the main primary producer in rock environment to provide nutrients for heterotrophic microorganism by fixing the carbon and nitrogen in the air. However, it was not enough for the numerous heterotrophic groups in rock environment merely depending on the nutrient substances supplied by autotrophic microorganisms (Lian *et al*., 2010). Owing to it tended to be more serious for heterotrophic microorganisms in rock environment, they survived by intercepting the few soil particles and nutrients in air and water except for being symbiotic with autotrophic microorganisms (Sterflinger, 2000; Viles and Gorbushina, 2003; Gorbushina, 2007; Lian *et al*., 2010; 2011). The rock microorganisms were in a relation of mutual cooperation and symbiosis (Lian *et al*., 2010). The main purpose of cooperation between different microorganism groups was to retain water and gain limited trace nutrients to sustain life activity and population continuity (Sterflinger, 2000; Gorbushina *et al*., 2003; Lian *et al*., 2011). Once the environmental conditions, such as temperature and humidity fitted for growth, they would quickly breed, spread and accelerate the weathering of minerals (Chen *et al*., 2014). The rock microorganisms in karst area played an irreplaceable role in the long geological evolution process of weathering and pedogenesis of carbonate rocks. Therefore, the future study is expected to further investigate the microbial community in the carbonate rock environments and deeply recognize it in special habitat so as to profoundly research its biogeochemistry function, such as the weathering and pedogenesis of carbonate rocks by using these microbial resources.

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ORIGINAL PAPER

High Seroprevalence of CMV Among Women of Childbearing Age Implicates High Burden of Congenital Cytomegalovirus Infection in Poland

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Abstract

Cytomegaloviruses are common worldwide, with variable frequency of infections. The infection in pregnancy may lead to pregnancy loss or serious sequelae for the child. To understand the risk posed by CMV in Poland we conducted cross-sectional study on women aged 15–49 basing on existing serum bank. Age dependent CMV incidence, the rates of congenital infection and sequelae were modelled from sero-prevalence, literature and demographic data. The overall anti-CMV IgG prevalence was 81.9% increasing from 74.3% in <30 years old to 94.3% in subjects 45+ years old. The lowest incidence was estimated at the age of 15 and the highest at the age 34 (3.8 and 8.95 respectively/100 women/year). The estimated rate of cCMV varies from 22.4 to 37.2 per 1000 live birth depending on the assumptions made. The proportion of cases due to secondary infection ranged from 34.8% to 49.9% accordingly.

Key words: CMV IgG prevalence, CMV in Poland, congenital CMV infection, congenital CMV sequelae, women of childbearing age

Introduction

Cytomegalovirus (CMV) infections are common with a worldwide distribution. The virus spreads by contact with infected body fluids, usually saliva, urine, blood, or genital secretions (Cannon *et al.*, 2011). After the primary infection (the first infection), despite immune response, CMV establishes a lifelong latency with intermittent reactivations (Sinclair and Sissons, 2006). The infected individual may also experience reinfection by another strain of CMV. During pregnancy, primary or secondary (reactivation or reinfection) maternal infection can lead to transmission through placenta and infecting the fetus, which gives rise to congenital CMV infection (cCMV) (Hyde *et al.*, 2010).

CMV is the leading cause of congenital infections in humans, resulting in pregnancy loss or disability of the child, including sensorineural hearing loss, developmental delay and mental retardation. The consequences of the infection of a pregnant women depend largely on the type of CMV infection. While primary infection during pregnancy has a higher rate of mother-to-fetus transmission and is more likely to cause symptoms at birth, strong evidence exist that in fact the secondary infections account for the most of the disease burden associated with cCMV (Kenneson and Cannon, 2007; Wang *et al.*, 2011; Yamamoto *et al.*, 2013). The risk of acquiring the primary infection during pregnancy depends both on the incidence and on the proportion susceptible among the pregnant women, which can be approximated by the proportion of women who do not have CMV antibodies. In the situation, in which the majority of pregnant women have already experienced a prior CMV infection the majority of *in utero* infections will be due to secondary maternal infections. Hence, these infections will become the main source of the cCMV.

Results of the earlier sero-epidemiological studies showed that prevalence of CMV infection depends on age, origin and socioeconomic status. Adults of high socioeconomic status in developed countries may have an antibody prevalence as low as 40%, while in developing countries more than 80% subjects are CMV positive (Nyholm and Schleiss, 2010). Among women in Europe the seroprevalence level varies from 30% to 90% (Ludwig and Hengel, 2009). The last, representative serosurvey in Poland was carried out in 1979 (Imbs and Rudnicka, 1987). Thirty five years ago in Poland,

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seroprevalence in women aged 15–39 years old was 83.3% and these infections are not under surveillance. The burden of congenital disease remains therefore largely unknown and we are currently not able to quantify the risk of infection and its complications to pregnant women and their children. Our aim was to estimate current seroprevalence of CMV among women of the childbearing age in Poland and estimate the risk of CMV, cCMV and of the long term sequelae related to cCMV in order to better inform pregnancy screening and counselling practices as well as other potential strategies to prevent cCMV.

Experimental

Materials and Methods

Serum samples. Serum samples for testing were selected from a serum bank collected in 2010–2011 (Project NN404 191 636; ethics statements 6/2008 – Committee of Bioethics at NIPH-NIH, 19.08.2008). Serum bank, intended to represent the general population, included 4822 serum samples collected by cluster sampling method, with clusters defined by the hospital unit and specific day within the recruitment period. The selection of 21 hospital units was made randomly from "Register of Healthcare Units" circumscribed to orthopedic and general surgery wards in 5 geographical regions of Poland (lubelskie, mazowieckie, świętokrzyskie, warmińsko-mazurskie and wielkopolskie). Each individual participating in the project was asked to provide written consent to include their anonymized serum sample for future epidemiological investigations of infectious diseases (serological or molecular). Serum samples were collected from all consenting adult patients fulfilling the following criteria: admitted to the hospital because of emergency indications (accidents, injuries) or hospitalized for appendicitis, hernia, or the gallbladder inflammation as described in (Godzik *et al.*, 2012). Sera were aliquoted, catalogued and stored at –70°C. The consent forms are stored within the medical records at the laboratory, which performed diagnostic testing for the initial project. The serum bank is maintained by the Department of Virology, National Institute of Public Health – National Institute of Hygiene and is fully anonymous (http://www.pzh.gov.pl/page/index.php?id=1075&L=1). The bank is accessible for further sero-epidemiological research upon acceptance by the project committee. We studied a total of 712 samples collected from women in childbearing age in the following age groups: < 30 years (218), 30–34 years (105), 35–39 years (133), 40–44 years (114), 45+ years (142).

Laboratory test and quality control. IgG antibodies specific to cytomegalovirus were detected using an indirect enzyme immunoassay (ETI-CYTOK-G PLUS test, DiaSorin, Italy). The antibody level was expressed in international units per millilitre (mIU/ml). For the internal quality control purpose the 10% of tested samples (n = 70) were blindly retested with the same test kit. Internal quality control showed 100% concordant results (61 positive and 9 negative).

Statistical analysis. We used chi-square test and logistic regression to compare the prevalence of CMV across age groups, region and type of residence (urban/rural). Relation by age was investigated both using age group and with the fractional polynomial model, but for simplicity of interpretation age group is shown in the prevalence model. Likelihood ratio test was used to test the effects of covariates in the logistic model.

Estimation of the force of infection and incidence. Assuming life-long immunity (demonstrated by detectable antibodies), negligible mortality due to the CMV infection and stability of the force of infection over time we were able to estimate the age dependent force of infection *l(a)* based on the serology results using the catalytic model:

$$\frac{d}{da} q(a) = -l(a) \times q(a),$$

Where $q(a) = 1 - \pi(a)$ is the proportion of susceptible individuals among the population of age a, $\pi(a)$ is prevalence of antibodies at the age a, so $q(a) = e^{-\int_0^a l(s)ds}$

The force of infection, $l(a)$, can be derived as follows:

$$l(a) = \frac{\pi'(a)}{1 - \pi(a)}$$

The prevalence is modeled with a linear model with link function g and the linear predictor $\eta(a)$:

$$\pi(a) = g^{-1}(\eta(a))$$

In order to ensure a flexible choice of the model for the force of infection we used the fractional polynomial model for $\eta(a)$ with logit and complementary log-log link functions g to model the age dependent prevalence $\pi(a)$ (Shkedy *et al.*, 2006).

For the logit link function we have the following equality for the force of infection:

$$l(a) = \eta'(a) \times \pi(a)$$

We selected best fitting models based on deviance criterion and Akaike information criteria (AIC) for selecting among models with different link function. We restricted the considered models to those estimating positive force of infection. Based on these criteria we selected logistic model with

$$\eta(a) = \beta_0 + \beta_1 \times a^2 + \beta_2 + a^2 \times \ln(a).$$

Substituting the formula for $\eta(a)$ the force of infection is estimated according to the formula:

$$l(a) = (2 \times \beta_1 + \beta_2 \times a \times [2 \times ln(a) + 1] \times \pi(a)$$

The estimated force of infection describes the rate of infections among the susceptible fraction of the population. In order to inform the burden of disease among women of the childbearing age and the possible impact on congenital infections we estimate the rate of infections in the entire population, incidence $I(a) = l(a) \times q(a)$.

Estimation of the burden of congenital CMV infection. We estimated the rate of congenital infection per 1000 births assuming:

- the incidence in pregnancy is not different then among non-pregnant women of the same age and the childbearing age is restricted to 15–49 years
- the rate of transmission to fetus in case of previously seronegative pregnant women is 32% (Kenneson and Cannon, 2007)
- the rate of cCMV due to primary maternal infections, R_{prim} will be estimated by summing mother age specific rates (child bearing age):

$$R_{prim} = \frac{1}{B} \times T_1 \times \sum_{a=15}^{49} D \times l(a) \times q(a) \times B(a)$$

$$B = \sum_{a=15}^{49} B(a)$$

Where $B(a)$ number of births given by mothers aged a, B is the total number of life births D is the time at risk for infection resulting in cCMV, as fraction of year and T_1 the transmission rate to fetus, $T_1 = 32\%$. The calculations based on the number of births (live and dead) registered in 2011, according to the maternal age, $B(a)$ (Central Statistical Office of Poland).

- the rate of cCMV due to secondary infections, R_{sec}, is estimated in two different scenarios:
  - "reinfection model": assuming that the reinfections occur at the same rate as primary infections (force of infection $l(a)$), cCMV is associated only with reinfection and the transmission rate to fetus, T_2, is related to T_1 as followed: $T_2 = 0{,}31 \times T_1$. The cCMV rate per 1000 live births in the "reinfection model", $R_{sec} = R_{reinf}$, is:

$$R_{reinf} = \frac{1}{B} \times \sum_{a=15}^{49} D \times T_2 \times l(a) \times (1 - q(a)) \times B(a) =$$

$$= \frac{1}{B} \times \sum_{a=15}^{49} D \times 0.31 \times T_1(a) \times (1 - q(a)) \times B(a)$$

  - "reactivation model": the cCMV is associated only with reactivation, i.e. not related to the reinfection rate. We further assume that every seropositive woman has the same chance of transmitting the virus to the fetus independently of the incidence of CMV in the population. In particular this proportion will not vary by age. The rate of cCMV, $R_{sec} = R_{react}$ inputted in this model is based on the proportion observed in other countries as proposed by de Vries *et al.* (2013):

$$R_{react} = \frac{P}{1 + \exp(6.15 - 2.44 \times P)}$$

where P is the seroprevalence among pregnant women estimated based on seroprevalence among women of childbearing age weighted by the number of birth by maternal age:

$$P = \frac{1}{B} \times \sum_{a=15}^{49} (1 - q(a)) \times B(a)$$

- the proportion of cCMV infections that are symptomatic infections at birth is $S_1 = 15\%$ for cCMV related to primary infection and $S_2 = 1\%$ for those related to secondary infection (Gardella, 2008). The rate of cCMV symptomatic at birth per 1000 birth, R_{sympt}, was evaluated as:

$$R_{sympt} = R_{prim} \times S_1 + R_{sec} \times S_2$$

- the proportion of long term sequelae is $L_1 = 90\%$ for children with cCMV symptomatic at birth and $L_2 = 10\%$ for those without symptoms at birth (Gardella, 2008). The rate of long term sequelae, R_{seq}, was calculated as:

$$R_{seq} = R_{sympt} \times L_1 + (R_{prim} + R_{sec} - R_{sympt} \times L_2)$$

We considered the following scenarios:

Scenario 1 (no pregnancy loss/reinfection): there is no pregnancy loss associated with CMV infection and the cCMV due to secondary infection is related to reinfections.

Scenario 2 (first trimester pregnancy loss/reinfection): all infections in the first trimester lead to pregnancy loss and thus only the infections in second and third trimester lead to cCMV, and the cCMV due to secondary infection is related to reinfections.

Scenario 3 (no pregnancy loss/reactivation): there is no pregnancy loss associated with CMV infection and cCMV associated with secondary infections is related to reactivations.

Scenario 4 (first trimester pregnancy loss/reactivation): all infections in the first trimester lead to pregnancy loss and cCMV associated with secondary infections is related to reactivations.

For the four scenarios we evaluated the overall cCMV rate, the rate of symptomatic infections at birth and the rate of long term sequelae. The calculations based on the number of births (live and dead) registered in 2011, according to the maternal age (Central Statistical Office of Poland).

The confidence intervals for the force of infection, the cCMV rates, the rates of symptomatic infections and

Table I
Factors independently associated with higher anti-CMV prevalence among women of child-bearing age in Poland.

		No. positive/ No. tested	Prevalence (%)	Adjusted odds ratio	95% C I	p-value (LR test)
Age group (years)	< 30	162/218	74.3	Reference		
	30–34	76/105	72.4	0.96	0.57–1.64	< 0.0001
	35–39	111/133	83.5	1.83	1.05–3.19	
	40–44	100/114	87.7	2.54	1.34–4.81	
	45+	134/142	94.4	5.91	2.71–12.89	
Residence	Rural	265/323	82.0	Reference		
	Urban	317/388	81.7	0.92	0.62–1.37	0.674
Region	Wielkopolskie	137/177	77.4	Ref.		0.2180
	Lubelskie	98/122	80.3	1.35	0.75–2.43	
	Mazowieckie	163/200	81.5	1.38	0.82–2.31	
	Świętokrzyskie	101/116	87.1	1.99	1.02–3.86	
	Warmińsko-mazurskie	84/97	86.6	1.89	0.94–3.81	

long term sequalae were estimated by parametric bootstrap method resampling 1000 times (Normal-based bootstrap confidence intervals, BCI). In this method the model for prevalence was assumed to be correct, the mean adjusted standardized Pearson's residuals were resampled and combined with the model estimated values to create the bootstrapped dataset (Davidson and Hinkley, 1997). Statistical analysis was carried out in STATA version 13.1. The confidence intervals were calculated only taking into consideration variability arising from seroprevalence data with all the literature parameters assumed to be known.

Results

CMV IgG prevalence and estimated incidence of primary CMV infection among women of childbearing age. The overall prevalence of CMV IgG among women of childbearing age was 81.9% (583 out of 712). The number of negative samples was 129 (18.1%). There were no equivocal results. The prevalence was age-dependent and increasing from 74.3% in < 30 years old to 94.3% in subjects 45+ years old.

In multivariable analysis age was the most important predictive factor for CMV prevalence (Table I). Additionally we note regional differences, although the overall contribution of the factor on likelihood ratio test is insignificant. On the other hand the difference between the rural and urban residence was small and insignificant.

Observed and predicted prevalence by age was shown on Fig. 1. It should be noted that for the age range in question there is an almost linear increase.

Figure 2 depicts the incidence of primary infection in the population of the observed level of susceptibility. The lowest incidence is expected at the age of 15 (3.8 per 100 women per year) and the highest during the age 30–40 (the maximum 8.95 at the age 34). For higher ages the incidence then declines due to universal immunity, although the force of infection remains high (data not shown). The estimate of the overall incidence among women of childbearing age is 6.98 per 100 per year (Normal-based 95% Bootstrap CI, BCI, 5.15–8.0).

Estimated burden of congenital CMV infections in Poland. CMV seroprevalence standardized to the age distribution of the pregnant women in 2011 was 75.3% (95% CI 70.7 –79.4%) and the estimated incidence of primary infection in this group – 7.76 per 100 per year (95% BCI 5.68–9.85). The estimated rate of cCMV varies from 22.4 to 37.2 per 1000 live birth depending on the scenario, with non-overlapping 95% confidence intervals for the highest and the lowest esti-

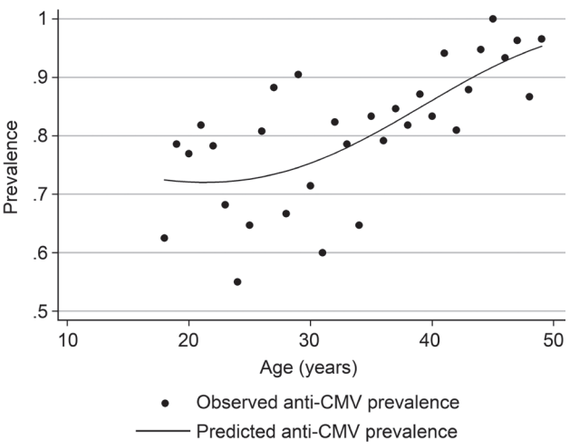

Fig. 1. Prevalence of CMV IgG among women of childbearing age. Predicted prevalence is based on the accepted fractional polynomial model relating the prevalence to the woman's age.

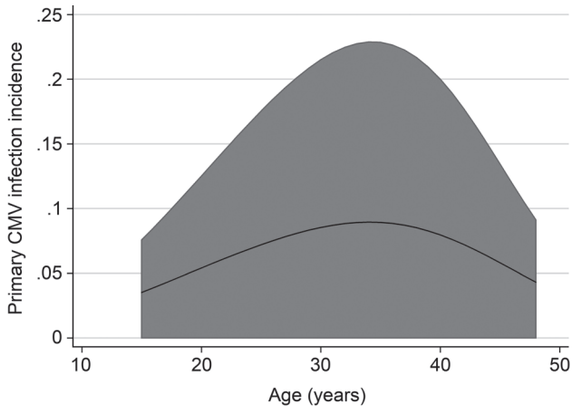

Fig. 2. Estimated annual incidence of primary CMV infection per 100 women of childbearing age.

The incidence is estimated from observed prevalence based on catalytic model for the force of infection combined with fractional polynomial prevalence model. The 95% confidence band (shaded area) is obtained through parametric bootstrap method.

mate (Table II). The difference was especially apparent for older ages of the mother (Fig. 3). These estimates included infections due to primary infection of the pregnant mother at 18.6 per 1000 live births in scenarios 1 and 3, assuming no relation of CMV infection to pregnancy loss, or 12.4 in scenarios 2 and 4 assuming all infections in the first trimester lead to pregnancy loss. The estimates of the number of cCMV rates due to secondary infections were higher in the scenarios 1 and 2, relating the risk of cCMV to the force of infection, which may be viewed as re-infection model. The proportion of cases due to secondary infection ranged from 34.8% to 49.9% accordingly (Table II).

The rate of infections symptomatic at birth ranged across the scenarios from 1.9 to 3 per 1000 births, for all of the scenarios being the highest among women aged 30–35. The rates of long-term sequelae had a similar peak for the scenarios 3 and 4, but for the scenarios 1 and 2 were increasing for older ages of the mother (Fig. 3).

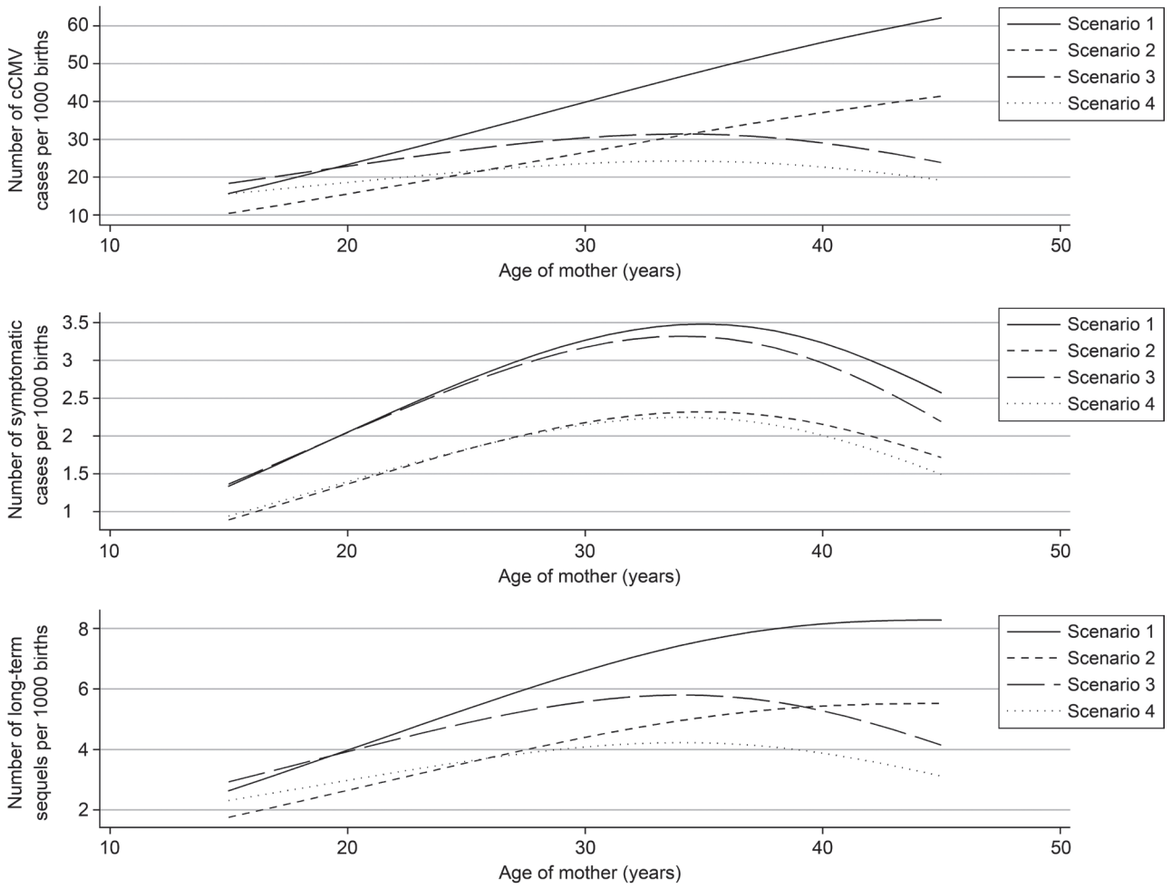

Fig. 3. Predicted rate of cCMV, cCMV symptomatic at birth and long-term sequelae, by mother's age.

The rates of cCMV, cCMV symptomatic at birth and the long term sequelae are estimated from estimated force of infection, estimated prevalence and published values of individual risk for symptoms at birth and development of long term sequelae. The following scenarios were used: Scenario 1. No pregnancy loss due to CMV infection/ cCMV due to secondary infection related to reinfections; Scenario 2. All infections in the first trimester lead to pregnancy loss/ cCMV due to secondary infection is related to reinfections; Scenario 3. No pregnancy loss due to CMV infection/ cCMV due to secondary infections related to reactivations; Scenario 4. All infections in the first trimester lead to pregnancy loss/ cCMV due to secondary infections related to reactivations.

Table II
Estimated rates of congenital (symptomatic and asymptomatic) CMV infection (cCMV) and frequency of long term sequels.

CMV infection sequelae		Observed estimate (normal-based 95% Bootstrap CI)			
		Scenario 1	Scenario 2	Scenario 3	Scenario 4
cCMV cases	Total number	14520 (10766–18274)	9680 (7177–12182)	11155 (9229–13081)	8732 (7456–10008)
	Rate per 1000 birth	37.2 (27.6–46.8)	24.8 (18.4–31.2)	28.6 (23.7–33.5)	22.4 (19.1–25.7)
cCMV cases symptomatic at birth	Total number	1163 (852–1474)	775 (568–983)	1129 (836–1422)	766 (571–961)
	Rate per 1000 birth	3 (2.2–3,8)	1.9 (1.4–2.4)	2.9 (2.1–3.6)	2 (1.5–2.5)
cCMV cases with expected long term sequel	Total number	2382 (1758–3006)	1588 (1172–2004)	2019 (1592–2445)	1486 (1202–1769)
	Rate per 1000 birth	6.1 (4.5–7.7)	4.1 (3–5.1)	5.2 (4.1–6.3)	3.8 (3.1–4.5)
cCMV associated with secondary infection in pregnancy	Total number	7251.8 (5449.3–9054.3)	4834.6 (3632.9–6036.2)	3886.7 (3806.1–3967.4)	3886.7 (3806.1–3967.4)
	Proportion (%)	49.9 (49.2–50.7)	49.9 (49.2–50.7)	34.8 (23.8–45.9)	44.5 (33.7–55.3)

Discussion

We estimate that 81.9% of women of childbearing age in Poland have been infected with CMV, as evidenced by presence of CMV IgG antibodies. The sero-prevalence increased from 74.3% in < 30 years old to 94.3% in subjects 45+ years old, suggesting the decreasing with age pool of women susceptible to primary infection. Accounting for the distribution of the births by mother's age we were able to produce overall estimates of congenital infection burden. Assuming no CMV related pregnancy loss, the rate of cCMV was estimated at 37.2 (95% CI 27.6–46.8) per 1000 births under re-infection model or 28.6 (95% CI 23.7–33.5) under re-activation model. Conversely, if all women infected in the first trimester of pregnancy miscarried, the rates of cCMV would be respectively 24.8 (95% CI 18.4–31.2) and 22.4 (95% CI 19.1–25.7) per 1000 births.

The results of our study showed that seroprevalence in Poland is higher than in the majority western European countries in white women with: 30.4% in Ireland within Irish women (Knowles *et al.*, 2005), 45,6% in The Netherlands (Kordenwal *et al.*, 2015), 45.9% in United Kingdom within white women (Tookey *et al.*, 1992), 47.5% in Germany (Enders *et al.*, 2003), 51.5% in France (Gratacap-Cavallier *et al.*, 1998), 57.4% in Spain (de Ory *et al.*, 2004), 70.7% in Finland (Mustakangas *et al.*, 2000), 72% in Sweden (Engman *et al.*, 2008). It was also observed that seroprevalence in 2010 (81.9%) not much differ from this observed in 1979 (Imbs and Rudnicka, 1987) (83.3%). High prevalence, which is comparable to the estimates from before 35 years as well as little regional differences suggest persistent and universal existence of factors driving CMV transmission in Poland. These factors have not been well studied, but taking into account significant burden of disease more attention should be given to establish the contexts of transmission.

In line with the difference in seroprevalence between women in childbearing age in Poland and in other developed European countries, the rate of cCMV estimated in our model, 22.4 up to 37.2 per 1000 births depending on assumed scenario, is higher from the results of two independent meta-analyses performed by Kenneson *et al.* (2007) and Dollard *et al.* (2007) – 0.64% and 0.7% accordingly. Numerous previous studies noted the positive correlation between the seroprevalence among pregnant women and the rates of cCMV (Kenneson and Cannon, 2007; Ludwig and Hengel, 2009; de Vries *et al.*, 2013). This is explained by the fact that the higher seroprevalence indicates higher force of infection and therefore a higher risk of primary infection in seronegative pregnant women, but also the higher risk of reinfection among the seropositive women.

Incidence estimated based on the seroprevalence cross-sectional sample is low among women below 20 and peaks at the age of 30–35 (Fig. 2). Women in their late 30-ties or early 40-ties are more likely to have contact with small children of their own or in their surroundings and may be therefore exposed to CMV. This is compatible with the observation that having a child was a risk factor for infection (Kiss *at al.*, 2002; Revello *et al.*, 2011). The virus activation from latency is the multifactorial process in which the stress affecting the immunological system could trigger reactivation (Liu *et al.*, 2010). The significant proportion

of burden of cCMV illness was also attributed to secondary infection of pregnant women by other authors (Wang *et al*., 2011). In the developed countries this proportion was even higher than in our study, possibly in relation to the lower force of infection and thus lower rate of primary infections.

Our results, especially if considered by age, were sensitive to the assumptions regarding the role of reinfection/reactivation. While it is known that the role of non-primary maternal infections as a source of congenital infection is very important, it is not known what extent of cCMV are caused by viral reactivation versus reinfection with a different viral strain. Observed epidemiological patterns such as the difference between the rate of cCMV in infants born to low income women (1 to 2 percent) and the rate in those borne to middle or high income women (0.1 to 0.2 percent) cannot be explained solely by reactivation (Boppana *et al*., 2001). Accordingly it was suggested that reinfection plays a crucial role in cCMV development. Further, some studies have demonstrated association of the reactivity to new CMV strains in mothers who were initially seropositive with cCMV (Boppana *et al*., 2001; Yamamoto *et al*., 2010; Ikuta *et al*., 2013). However, our "re-infection model" produces higher overall estimates than "re-activation model", for which the overall estimates rely on frequencies observed in other countries. We therefore conclude that although the age patterns seen in the "re-infection" models are more plausible then those in "re-activation" model the "re-infection" models may in fact overestimate the true number of cCMV. Mixed acquisition models for secondary infection could be also considered. However, at this point without additional data to inform the "mixture" parameters we would not be able to judge whether these models provide more realistic estimated. We therefore presented only the "extreme" models.

Attribution of cCMV associated with secondary infection to re-infection has implications for public health interventions aiming at behavioral change. As the children are the main source of virus and then could be a cause of re-infection in their mothers, avoiding exposure to CMV through behavioral changes, mainly increasing hygiene, should be beneficial for seropositive as well as seronegative pregnant women (Kiss *et al*., 2002; Revello *et al*., 2008; Price *et al*., 2014).

We also observed, that for mothers approaching 40 or older even over 60% of long term sequelae may develop despite the lack of symptoms at birth. This estimate is supported by other studies indicating that the majority of children with cCMV infection who will developed subsequent late-onset hearing loss at the first 6 years of life have a normal hearing at birth and could be missed in newborn hearing screening (Fowler *et al*., 1999). Therefore, to detect the late onset and progression of hearing loss the continued monitoring in population of cCMV infected children should be undertaken (Dahle *et al*., 2000). Similarly as in case of hearing loss, the role of asymptomatic infection at birth may be underestimated in case of other cCMV sequelae as vision impairment and motor/cognitive deficit (Manicklal *et al*., 2013). Given the high birth prevalence of cCMV in Poland the universal screening for cCMV should be considered.

The limitations of our study include that not much data on the important model parameters such as proportion of symptomatic infections and the proportion of long term sequelae are available. Simple numbers available from literature were used for the estimations in this paper, which overestimates our confidence in the results. No reliable information could be found of the rates of pregnancy loss in case of maternal infection and the differentiation between the role of reinfection and reactivation is still not well studied. We therefore considered four alternative scenarios and showed that the results are sensitive to the assumptions made. This allowed us to understand the possible ranges of cCMV rate, although we were not able to produce a single number for cCMV rate estimation.

Conclusion

We demonstrated persistently high burden of cCMV in Poland, higher than in neighboring countries. The reason for this difference is unknown, which should stimulate further research to clarify the level of awareness of the health providers and the pregnant women of the infection (Adler *et al*., 2004; Revello *et al*., 2011; Cordier *et al*., 2012a; 2012b) and inform the public health interventions. While the CMV IgG negative pregnant women should be still counselled about the risk of acquiring CMV, the importance of secondary infections in pregnancy, despite the low risk of it affecting the child on individual level, should be emphasised. Taken into the consideration the role and the impact of secondary infections, the prevention measures, such as vaccine or behavioral interventions, will be potentially beneficial not only to prevent primary infections but also secondary one.

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ORIGINAL PAPER

Genotyping and Clinicoepidemiological Characterization of Rotavirus Acute Gastroenteritis in Egyptian Children

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Abstract

Group A rotavirus (RVA) acute gastroenteritis (AGE) is a common cause of severe childhood diarrhea. The dominant circulating RVA genotypes in a given region may vary between and within the geographic regions and from year to year. Our cross-sectional study was designed to determine the burden of RVA genotypes among children with AGE admitted to referral Children Hospital at Egypt prior to implementation of the vaccine. Stool samples with clinico-epidemiological data were collected from 92 children ≤ 3 years-old with AGE. RVA G and P typing were performed with type-specific primers. RVA was detected in 48.9% of patients. Higher rates of RVA infections, 73.3% were detected in infants < 1 year-old. Breast-fed infants were significantly fewer in RVA positive group ($P = 0.0006$). Non-breast-feeding was a major risk factor for RVA AGE (OR 0.3, $P = 0.02$). RVA diarrhea occurred mostly in autumn and winter months (55.4% and 26.6%) with a significant difference in autumn ($P = 0.0005$) and was associated with vomiting and dehydration (OR; 1.66, $P = 0.021$ & 1.4, $P = 0.03$). RVA genotypes G1P[8] (26.7%), G9P[8] (20%) and G3P[8] (15.6%) were accounting for 62.3% of RVA AGE. G9 was significantly associated with mucus diarrhea, than G1 or G3 which were associated with watery diarrhea ($P = 0.025$). Also, G9 was significantly associated with loose stool for > 5 days ($P = 0.006$) and 54.4% of G9 patients had severe dehydration. The diversity of RVA strains detected in Nile Delta Egypt and emergence of G9 RVA highlight the need to apply vaccines against this genotype in Egypt.

Key words: gastroenteritisin in children, RVA genotypes, clinicoepidemiological characterization in Egypt.

Introduction

The acute gastroenteritis (AGE) caused by Group A rotavirus (RVA) contributes significantly to childhood morbidity and mortality in developing as well as developed countries (Tate *et al*., 2012).

RVA is a double-stranded RNA virus that is classified into 8 serotypes (A-H) based on antigenicity and nucleotide sequence identities of the VP6 intermediate capsid protein and encoding gene (Matthijnssens and Van Ranst, 2012). They exhibit broad genetic and antigenic diversity due to re-assortment among RVA strains and the accumulation of point mutations in the two most external capsid proteins, VP7 and VP4 and have been further categorized in VP4 (P-type) and VP7 (G-type). Currently, there are 35 P-types and 27 G-types with an intratypic variation (Matthijnssens *et al*., 2008; Esona *et al*., 2009; Matthijnssens *et al*., 2011).

Globally, G1, G2, G3, G4, G9 and G12 genotypes are the major RVA circulating genotypes, accounting for over 88% of all strains analyzed worldwide (Santos and Hoshino, 2005). Out of the five, G9 RVAes were the last to emerge and their origin is unclear. Besides humans, G9 RVAes have only been identified from pigs as early as 1980 (Cao *et al*., 2008; Matthijnssens *et al*., 2010). The African RVA Surveillance Network (AfrRSN) reported that G9 RVAes were common after the middle of the 1990s in Kenya, Libya and Tunisia (Mwenda *et al*., 2010).

The most important global strains causing majority of infections are G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8]. The examination of the G- and P-type distribution is necessary as the dominant RVA genotypes that circulating in a given region may vary between and within the geographic regions from year to year (Gentsch *et al*., 2005).

Genotyping is based on analysis of the viral RNA by different methods as type-specific PCR (Gentsch *et al*., 1992), restriction fragment length polymorphism (Iturriza-Go'mara *et al*., 2002), sequence analysis (DiStefano *et al*., 2005), oligonucleotide probes (Lovmar *et al*., 2003).

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RVA infection is highly contagious and not easily controlled by improvements in hygiene and sanitation, as evidenced by similar incidence rates in developed and developing nations (Parashar *et al*., 2006). The only control measure likely to have a significant impact on the incidence of severe disease is vaccination (Matson *et al*., 2010).

WHO recommends surveillance for the burden of RVA disease and circulating RVA strains, before and after inclusion of RVA vaccination in national expanded programs on immunization (WHO, 2009).

The World Health Organization (WHO) has reported that 20–30% of stool samples collected during 2009 from Egyptian children with AGE were positive for RVA (Mansour *et al*., 2013).

In Egypt, there is no well-developed surveillance system for RVA strain identification and the RVA vaccine is not included in the national immunization programs. The private sector only applies RV vaccination (Ortega *et al*., 2009).

Several epidemiological surveys had been done in different Egyptian governorates showing different RVA genotypes with many untypeable strains raising the possibility of spread of additional genotypes (Radwan *et al*., 1997; Naficy *et al*., 1999; Kamel *et al*., 2009). However, there is no data about the RVA strains in Dakhalia Governorate which lies in northern Egypt at the base of the Delta triangle with a population of about 5 million people. The governorate won worldwide fame for hosting specialized medical centers and hospitals that receives patients from all surrounding governorates in Delta.

This pilot study was conducted to evaluate the genotype diversity of RVA strains among children younger than 3 years of age with acute gastroenteritis (admitted to a referral Children Hospital in Dakhalia). We examined the relationship between RVA genotypes and the clinical characteristics of AGE.

Experimental

Materials and Methods

Study population and specimens. The study was conducted at Mansoura University Children's Hospital (one of the main referral hospitals for both urban and rural districts in the Nile Delta) from September 2010 to February 2012 (17 months). The principles outlined in the Declaration of Helsinki were followed and informed consents were obtained from legal guardians after study protocol approval by the local Ethics Committee of Faculty of Medicine, Mansoura University, Egypt. One out of five AGE cases, under the age of 3 years admitted to the Children's Hospital for treatment was randomly selected.

Diarrhea was defined as the occurrence of three or more loose or watery stools in the preceding 24 hours. During this study, 92 acute gastroenteritis patients were included as soon as they were diagnosed by a pediatrician and fresh stool samples were collected from selected patients for RVA detection.

Clinic epidemiologic data including age, sex, source of drinking water, feeding modalities (breast-fed and/or bottle-fed and weaning practice), clinical symptoms such as fever, vomiting and dehydration, and the stool characteristics were recorded for each child.

Detection and typing of RVAes. The stool samples were collected using wide mouthed sterile plastic containers from inpatients within 48 h following their hospitalization (one for microbiological diagnosis and the other for PCR diagnosis).

Microbiological diagnosis (routine stool culture). Cultures for detection of bacteria in stool were performed as follow; about 3–4 loopfuls (1 gm) of stool was added to 20 ml selenite broth (Oxoid) and incubated at 37°C over night then plated out on MacConkey's agar (Oxoid) for 24 hours. The isolated colonies were identified by Gram stain and manual biochemical reaction on Triple Sugar Iron agar (TSI), Christensen's urea agar and Simmon' citrate agar (Oxoid). If no pathogenic bacteria were isolated after 72 hours, the result was deemed negative.

RVA RT-PCR and genotyping. Rotavirus double-stranded RNA (dsRNA) was extracted directly from 10% of fecal suspension of each sample using TRIzol (Life Technologies) and precipitated with isopropanol following the manufacturer's recommendations.

The extracted dsRNA was subjected to G- and P-typing by multiplex reverse transcription-polymerase chain reaction (RT-PCR) with type-specific primers.

Consensus primers Beg9 and End9 were used in the first-round PCR (30 cycles) to amplify the full-length VP7 gene (1,062 bp); cDNA was used in the second-round PCR for G-typing (25 cycles) with primer set aBT1 (G1), aCT2 (G2),aET3 (G3), aDT4 (G4), aFT9 (G9) and primer set FT5(G5), DT6 (G6), HT8 (G8), ET10 (G10), BT11 (G11) (Gouvea *et al*., 1990; 1994).

For P-typing, consensus primers Con2 and Con3 were used in a first-round RT-PCR (30 cycles) to amplify the 876 bp of the VP8 region of the VP4 gene, and the second-round PCR (20 cycles) used primer set 1T-1 (P[8]), 2T-1 (P[4]), 3T-1 (P[6]), 4T-1 (P[9]), 5T-1 (P[10]) (Gentsch *et al*., 1992). All PCR products were analyzed by electrophoresis in 1.2% agarose gels and illumination after staining with ethidium bromide.

Statistical analyses. Statistical analysis was performed using SPSS 19 software for Windows (SPSS Inc, Chicago, Ill). Data were described using mean ±SD for continuous data and frequency (n%) for categorical data. Student's *t*-test was used to compare

means of quantitative data between two groups and one-way ANOVA for multiple groups. For categorical data the Chi^2 or Fisher's exact probability test were applied. *P value* < 0.05 was considered as a level of statistical significance.

Results

A total of 92 children patients with AGE were enrolled during the study period. They were 52 boys, 40 girls; mean age [±SD] was 10.64 ± 7.87 month. Stool specimens were examined for RVA by RT-PCR. RVA was detected in 45 (48.9%) of the cases (RVP group). The other group, RVA negative (RVN), 47 (51.1%), included 34 (37%) patients having bacterial pathogens (*Salmonella* in 20.7%, *Klebsiella* sp. in 4.4%, and *Escherichia coli* in 11.9%), and 13 patients (14.1%) having no detectable pathogen.

Considering the demographic data of studied patients (Table I), the age was comparable between the RVP group and RVN group ($P = 0.92$) and in all age stratifications without statistically significant difference. There were higher rates of RVA infections (33/45; 73.3%) in infants < one year old: [13 (28.9%) in infants aged < 6 months and 20 (44.4%) in infants aged 6–12 months] and the least rates (4/45, 8.9%) were in children > 2 years old. There was no statistical significant association between RVA infection with either sex or residence ($P = 0.2$ and $P = 0.09$).

Among breast fed infants ≤ 12 months of age, the number of RVP cases were significantly lower than RVN cases ($P = 0.0006$). The weaning practice increased significantly the rate of RVP compared with RVN (44.4% *vs.* 23.4 %, $P = 0.047$) (Table I).

RVP diarrhea was found to occur mostly in the autumn and winter months with a significant difference in autumn ($P = 0.0005$) Most RVN cases occurred in summer (65.9 % of cases) with significant difference in their frequency ($P = 0.0001$) than RVP. No RVP cases were detected in spring (Table I).

Regarding the analysis of hygiene factors such as the source of drinking water, 49% of RVP and 31.9% of RVN reported sometimes use of safe water; 40% of RVP and 51.1% of RVN always use it; while 11% of RVP and 17% of RVN did not use this but used tanks for

Table I
Demographic data of studied Egyptian children

Disease characteristics	RVN** N0 (%)	RVP** N0 (%)	Total N0 (%)	*P***
Total	47 (51.1)	45 (48.9)	92 (100)	
Age of child (month) Mean ± SD	10.57 ± 7.7	10.72 ±8.2	10.64 ± 7.8	0.92
< 6	11 (23.4)	13 (28.9)	24 (26.1)	0.67
6–12	17 (36.2)	20 (44.4)	37 (40.2)	0.53
12–24	15 (31.9)	8 (17.8)	23 (25)	0.9
>24	4 (8.5)	4 (8.9)	8 (8.7)	1
Gender				
Male	30 (63.8)	22 (48.9)	52 (56.5)	
Female	17 (36.2)	23 (51.1)	40 (43.5)	0.20
Residence				
Urban	22 (46.8)	13 (28.9)	35 (38)	
Rural	25 (53.2)	32 (71.1)	57 (62)	0.089
Season				
Winter	7 (14.9)	12 (26.6)	19 (20.7)	0. 23
Summer	31 (65.9)	8 (17.8)	39 (42.4)	**0.0001**
Autumn	9 (19.2)	25 (55.6)	34 (36.9)	**0.0005**
Breast feeding (< 12 m)	28	33	61	
Yes	22 (78.6)	11 (33.3)	33 (35.9)	**0.0006**
No	6 (21.4)	22 (66.7)	28 (64.1)	
Weaning	11 (23.4)	20 (44.4)	31 (33.7)	**0.047**
Mothers education: Secondary and above	20 (42.6)	15 (33.3)	35 (38)	0.396
Safe water				
Not always	8 (17)	5 (11)	13 (14.1)	0.55
Yes always	24 (51.1)	18 (40)	42 (45.7)	0.27
Sometimes	15 (31.9)	22 (49)	37 (40.2)	0.136

RVP = RVA positive, RVN = RVA negative

drinking, with no significant difference between both groups (Table I).

With regard to the clinical features of the studied patients (Table II), RVA positive patients showed significant increase in the frequency of reported clinical manifestations than RVN, fever (80% *vs.* 59.6%, $P=0.04$) vomiting (88.9% *vs.* 46.8 %, $P=0.0001$) and dehydration (84.4 % *vs.* 63.8%, $P=0.032$).

The RVP patients had more severe dehydration as compared with RVN group; dehydration was mild in (18.4% *vs.* 76.6%, $P=0.0001$), moderate in (52.6% *vs.* 16.7%, $P=0.0026$), and severe in (29% vs. 6.7%, $P=0.029$) of cases respectively.

Duration of diarrhea with loose stools for ≥ 5 days, (mean ± SD) was shorter in RVN (3 ± 2.5 days) than in RVP (5 ± 3.5 days) ($P=0.58$). The frequency of diarrhea (≥ 5 loose stools/24 hr) was higher in RVP (7 ± 3) than RVN (4 ± 3) ($P=0.36$). As regard vomiting, the duration ≥ 3 days in RVP patients (3 ± 2) was longer than RVN patients (2 ± 1.5) ($P=0.8$). The frequency of vomiting ≥ 5 episodes/24 hr in RVP (5 ±3) was higher than RVN patients (3 ± 2) ($P=0.33$) the difference was statistically insignificant (Table II).

In addition, the results representing delays of medical care were not significantly different between RVP and RVN ones, with a mean period between the onset of the diarrhea and hospitalization of 4.45 and 4.06 days, respectively (data not shown).

Regression analysis (Table III) demonstrated that severity of dehydration is 1.4 times higher in cases of RVA diarrhea (OR 1.4, 95% CI 0.06–2.6, *P* 0.03) than in cases of RVN diarrhea. The presence of vomiting

Table II
Clinical variables of studied Egyptian children

Disease characteristics	RVN** N0 (%)	RVP** N0 (%)	Total N0 (%)	*P***
Total	47 (51.1)	45 (48.9)	92 (100)	
Diarrhea				
Pattern				
Mucus	23 (48.9)	20 (44.4)	43 (46.7)	0.68
Blood	1 (2.1)	0 (0.0)	1 (1.1)	0.55
Watery	40 (85.1)	36 (80)	76 (82.6)	0.59
Frequency (mean± SD)	4 ± 3	7 ± 3	5 ± 3	0.36
Duration (mean± SD)	3 ± 2.5	5 ± 3.5	4 ± 3	0.58
Vomiting				
Yes	22 (46.8)	40 (88.9)	62 (67.4)	**0.0001**
Frequency (mean± SD)	3 ± 2	5 ± 3	4 ± 2	0.33
Duration (mean± SD)	2 ± 1.5	3 ± 2	2.5 ± 1.5	0.80
fever				
Present	28 (59.6)	36 (80)	54 (58.7)	**0.042**
Dehydration	**30 (63.8)**	**38 (84.4)**	**68 (73.9)**	**0.032**
Severe	**2 (6.7)**	**11 (29)**	**13 (19.1)**	**0.0289**
Moderate	**5 (16.7)**	**20 (52.6)**	**25 (36.8)**	**0.0026**
mild	**23 (76.6)**	**7 (18.4)**	**30 (44.1)**	**0.0001**
IVF	40 (97.6)	34 (94.4)	74 (96.1)	**0.048**

RVP = RVA positive; RVN = RVA negative; IVF = Intravenous Fluid

Table III
Relative risk factors associated with RVA diarrhea among studied children.

Factors	*P value*	Odds ratio	(95.0% CI)	
Age (> 10)	0.54	1.08	0.49	2.37
residence (urban)	0.84	1.48	0.48	2.43
Vomiting	**0.021**	**1.66**	**0.744**	**3.7**
Diarrhea duration (> 5days)	0.18	1.95	0.71	5.32
Sex (Male)	0.20	0.602	0.276	1.317
Fever	0.52	1.28	0.59	2.78
Breast feeding	**0.02**	**0.31**	**0.11**	**0.85**
Severity of dehydration	**0.036**	**1.42**	**0.06**	**2.68**
Secondary education	0.52	1.55	0.39	6.09

Table IV
Genotype distribution and the seasonal pattern of studied RVA strains

Genotypes (n)	P						Total	Season					
	P[8]		P[6]		P[4]			Winter		Summer		Autumn	
	No	%	No	%	No	%		No	%	No	%	No	%
G1	12	63.2	1	5.3	6	31.5	**19**	4	21	3	15.8	12	63.2
G9	9	75	3	25	0		**12**	5	41.7	3	25	4	33.3
G3	7	77.8	0		2	22.2	**9**	2	22.2	1	11.1	6	66.7
Mixed G1, G9	1	50	1	50	0		**2**	1	50	0		1	50
G1, G4	2	100	0		0		**2**	0		0		2	100
Untypeable	0		1	100	0		**1**	0		0		1	100
Total	31	68.9	6	13.3	8	17.8	**45**	12	26.6	7	15.6	26	57.8

(odds ratio 1.66, 95% CI; 0.74–3.7 *P* 0.021) was found to be significantly higher in the RVP patients. Children not currently breast fed were at a higher risk of RVA diarrhea (OR 0.3, 95% CI 0.11–0.85, *P* 0.02) than breastfed children.

In the present study, a total of 44 (97.8%) of the 45 RVP samples were found to be G-typed and only 1 (2.2%) was untypeable. As illustrated in Table IV, the genotype distribution of RVA strains showed that the prevalent G genotypes were G1, G9 and G3 accounted for 19 (42.2%), 12 (26.7%) and 9 (20%) of RVP cases respectively. Mixed G-types reflecting dual infections G1+G9 and G1+G4 were detected in 4 (8.9%) of RVP samples. G2, G6 and G8 were not detected. All RVA strains were P-typeable and three P genotypes P[8], P[6] and P[4] were identified, accounting for 68.9%, 13.3% and 17.8% of cases respectively. The untypeable G strain (2.2%) was P[6] genotype. Of the 45 studied strains, G1P[8] (26.7%), G9P[8] (20%), and G3P[8] (15.6%) were the most prevalent strains and caused 62.3% of RVP cases in Northern Egypt. Other strains were detected in lower frequency as G1P[4] 13.3%, G9P[6] 6.7%, G3P[4] 4.4%, and G1P[6] 2.2%.

Genotype and temporal variations were clarified (Table IV) as following; the RVA genotypes G1 and G3 fluctuated with a characteristic seasonal pattern in autumn. Of the identified 12 G9 strains, 5 cases (41.7%) occurred during the rainy winter season and the remaining 7 cases (58.3%) occurred during the summer and autumn.

Clarification of the clinico-epidemiological features among pure genotypes (n = 40) with exclusion of infection caused by mixed genotypes (n = 4) and untyped strain (Table V), there was a significant difference between genotypes regarding gender ($P = 0.041$), G9 was recorded in higher percentage (66.7%) in male children while G3 was higher (77.8%) in female children.

Pattern of diarrhea significantly differed according to different genotypes ($P = 0.025$). G9 was significantly associated with mucus diarrhea in (66.7%), when compared to either G1 or G3 which were associated with higher frequency of watery diarrhea in (76.5% and 85.7%, respectively). G9 was significantly associated with loose stool for more than 5 days when compared to G3 ($P = 0.006$). Although degree of dehydration did not differ significantly between different genotypes, higher percentage of G9 patients had severe dehydration in (54.5%) than patients of other genotypes (17.6% for G1 and 0% for G3) (Table V).

Discussion

Continuous monitoring of RVA infection surveillance and typing of circulating strains remain valuable all over the world before the introduction of RVA vaccination. In this study we aimed to extend the previous RVA genotype studies conducted in Egypt to provide the baseline information to implement the appropriate vaccines.

In this study, most cases of AGE were infants, 66.3% were infants < one year old which is similar to other Egyptian studies (Naficy *et al*., 1999; Amer *et al*., 2007).

The decline of RVA diarrhea occurred with age confirming the role of the immune system in prevention of the RVA infection; 73.3% were in infants < one year old while the least rates (8.9%) were in children more than 2 years old.

Highlighting the protective effect of breast feeding, the breast fed infants were significantly lower in RVP group than RVN group (33.3%, 78.2%, $P = 0.0006$) and the non-breast feeding was a major risk factor for RVA infection (OR 0.3, 95% CI 0.11–0.85, *P* 0.02). This was because the children were introduced to gradual weaning at age of 6 months with semi solid food and infant ready food mixes thus increasing exposure to water and other environmental sources of contaminations.

Studies in Egypt, Germany, Austria and Malaysia emphasis on the positive relationship between breastfeeding and protection against RVA diarrhea (Naficy

Table V
The clinicoepidemiological features among differ

Total (n = 40)	G1 N = 19	G9 N = 12	G3 N = 9	*P* value*
Age of child (months)				
< 6 (n = 13)	6 (31.5)	4 (33.3)	3 (33.3)	0.334
6-12 (n = 15)	4 (21.1)	7 (58.3)	4 (44.5)	
12-24 (n = 8)	6 (31.5)	1 (8.4)	1 (11.1)	
> 24 (n = 4)	3 (15.9)	0 (0)	1 (11.1)	
Gender (n = 40)				
Male (n = 21)	11 (63.8)	8 (66.7)	2 (22.2)	
Female (n = 19)	8 (36.2)	4 (33.3)	7 (77.8)	**0.041**
Diarrhea (n = 36)				
***Pattern**				
Mucus (n = 13)	4 (23.5)	8 (66.7)	1 (14.3)	
Watery (n = 23)	13 (76.5)	4 (33.3)	6 (85.7)	**0.025**
***Frequency**				
(≥ 5 loose stools/24h)	10 (52.6)	9 (75)	4 (44.4)	0.323
***Duration**				
(loose stools for ≥ 5 days)	16 (84.2)	12 (100)	6 (66.7)	**0.006**
Vomiting (n = 37)				
*Duration (≥ 3 days)	17 (89.4)	11 (91.7)	9 (100)	1.000
*Frequency (≥ 5 episodes/24h)	11 (57.9)	8 (66.7)	6 (66.7)	0.918
Fever (n = 33)	15 (78.9)	10 (83.3)	8 (88.9)	1.000
Dehydration (n = 36)				
Severe (n = 9)	3 (17.6)	6 (54.5)	0 (0)	
Moderate (n = 20)	10 (58.8)	4 (36.4)	6 (75)	
Mild (n = 7)	4 (23.5)	1 (9.1)	2 (25)	0.082

et al., 1999; Plenge-Bönig *et al.*, 2010; Prameela and Vijaya, 2012). On the other hand, many studies were controversy to our results as Prasetyo *et al.* (2015), Wobudeya *et al.* (2011), Misra *et al.* (2007), who concluded that the severe dehydration RVA diarrhea in infants ≤ 6 months old who depend exclusively on breastfeeding was not significantly different from those who did not rely on breastfeeding alone. The human breast milk has shown the presence of secretory IgA antibodies and RVA G9P(5) neutralizing capacity. A strong correlation is seen between the level of anti-RVA antibody and the neutralizing capacity of breast milk samples (Santos *et al.*, 2013).

As, the socioeconomic status of some studied patients was poor, their communities have inadequately treated drinking water and 60% of RVP current cases did not use or sometimes used safe water for drinking but used tanks with communal taps, RVP were significantly higher among the weaned infants (44.4%) than RVN (23.4%) ($P = 0.047$). Mandour *et al.* (2013) performed a study on drinking water samples collected from 14 different locations of Dakahlia Governorate and concluded that in some studied areas, water was polluted and not suitable for drinking purpose. Moreover, rural people keep animals in their dwellings and the animals often use the same water source as the humans (Potgieter *et al.*, 2010).

In the present study, a total of 44 (97.8%) of the 45 RVA positive samples were found to be G typed and only 1 (2.2%) was untypeable. The prevalent G genotypes were G1, G9 and G3, causing 42.2%, 26.7% and 20% of RVP cases respectively.

A series of researches targeting RVA genotyping among children had been conducted in different districts in Egypt (Cairo, Behira, Quliobia, Giza, Alexandria, Fayoum and Sharkia). Genotypes G1, G2 and G4 represented the highest prevalence (Radwan *et al.*, 1997; Naficy *et al.*, 1999; Amer *et al.*, 2007; Kamel *et al.*, 2009; Matson *et al.*, 2010). However, in a nearby governorate (Sharkia), Hashem *et al.* (2012) recorded higher prevalence of G1, G9 and G3 (55%, 14.5% and 22.2%) in accordance with our results in the current study.

Interestingly, an increased prevalence of RVA G3 strains has also been reported elsewhere in the world (Fang *et al.*, 2002). A prolonged low incidence of G3 strains in the community might have induced a lack of G3 specific protective immunity, determining its widespread circulation in Dakhalia in the year studied. Also, G3 strains might have advantages over other G types like a more efficient transmissibility.

Other evidence that G1 and G3 are endemic in our locality is the similarity of RV genotypes in our patients to environmental RV types isolated in same area. A local study was done in 2006–2007 by El-Senousy

and El-Mahdy (2009), who examined RVA strains from water treatment plants in (Meet Khamees) and two compact units (Shoha and Mahalet Damana) from Dakhalia Governorate. Although 15.2% of the positive samples were G untypeable and 9.3% were P untypeable, the distribution for RVA strains was 39.3% P[4]G1, 28.6% P[8]G1, 17.6% P[4]G3, 7.1% P[8]G3, 7.1% P[6]G1. Previously, Kamel *et al.* (2009; 2010) found similar RV isolates were circulating in the environment and in the population. This confirms the value of wastewater screening as a tool for assessing RVs circulating in communities with the benefit of detecting types that cause both clinical and subclinical infections (van Zyl *et al*., 2006).

The current study showed higher prevalence of G9 than before in Egypt that nearly increased two folds than the last study done by Hashem *et al.* (2012) from 14.5% to 26.7% and this may be explained by the high frequency in occurrence of re-assortment, at single or multiple gene segments during mixed infections by strains of human-human or human-animal origin (Chouikha *et al*., 2007) and the escape recognition of the less frequent genotypes as G12 or G9 by the host immune system which recognizes the common G1-G4 genotypes.

Similarly, Than and Kim (2013) identified G9 strain in rural health care centers in South Korea, and reported that this genotype was found to be responsible for 7.4% to 39% of rural infections and much lower, only 1% to 3%, in urban hospitals in other Korean studies.

During the last decade, the G9 genotype has emerged as one of the five most common types worldwide. A high prevalence was detected in Ecuador (72–96%), France (55%) and Italy (84%), whereas a lower prevalence was found in Germany (8%) and the United Kingdom (13%) (Van Damme *et al*., 2007; Endara *et al*., 2007).

In our study, genotype G1P[8] was the most prevalent strains and accounted for (26.7%) of RVA cases. Similarly, the results of the preceding studies (1995–2011) indicated that the G1P[8] strain has been detected at a relatively higher frequency and represented 17.1% and 28.6% of RV strains in studies done by Kamel *et al.* (2009) and El-Senousy and El-Mahdy (2009) in Cairo and Dakhalia Governorates of Egypt, respectively. Moreover, Genotype G9P[8] was the most common combinations of G9 strain and found in 20% of studied RVA cases in corroboration with WHO (2008), which confirmed that G9 is combined more commonly with P[8] than other P genotypes (Agócs *et al*., 2014). Globally, P[8], P[6] and P[4] were the most prevalent P genotypes with the frequent combinations, G1P[8], G3P[8], G4P[8], G2P[4], G9P[8] and G9P[6] (Santos *et al*., 2005).

The pattern of RV mixed infections detected in the present investigation (G1/G4 and G1/G9, 8.9%) differed from other studies (G9/G3 and G9/G1). Mixed infection with different RVA strains might reflect co-infections with 2 different RV serotypes or frequent contamination of water resources with RV strains that could facilitate generation of novel RV strains through re-assortment (Unicomb *et al*., 1999).

In the present study, 2.2 % of the RVP samples could not be G genotyped. However, 38.7% of RVP specimens collected from infants in Cairo by Radwan *et al.* (1997) were untypable. Since RVAes genetically mutate it is to be expected that sometimes today's RT-PCR methodologies are unable to identify all types. The natural variation in the primer binding sites of VP7 genes has been documented from different parts of the world (Espinola *et al*., 2008; Rahman *et al*., 2005).

As observed from these results, the overall change in the distribution of RV genotypes over consecutive RV seasons and across different geographical areas observed in various studies may be partly explained by the coexistence of multiple factors such as anti-RVA immunity in children, the differences in methods and study populations, climate and water supply. Also the genetic mutation and the great diversity within RV strains circulating in humans with change in the geographical spread of genotypes highlights the need for continued surveillance to establish which RV strains are circulating in a community at a given time.

The present study found a clear seasonal pattern of acute RV gastroenteritis that peaked in autumn and winter, with only 17.8% of cases in summer and no RV cases in spring that nearly similar to those observed by Amer *et al.* (2007) and Hashem *et al.* (2012), who reported a marked seasonal peak during the cold months of the year (October-February) with low prevalence in spring and summer.

It is conceivable that when there is a seasonal pattern, children may become infected at later ages, because they have not been continuously exposed to the virus. Indeed, seasonality of RV has been observed in different parts of the world; thus, it would be better for future studies to analyze samples from a "season" rather than a "calendar year".

None of the genotypes exhibited a distinct seasonal pattern. The G1 genotype was the most prevalent genotype in autumn (48%) while in winter G9 was the most prevalent one (41.7%). G3 fluctuated all the year with a seasonal pattern in autumn (24%). To our knowledge no previous reports discussed this issue. Instead of this, it was discussed as year-to-year variations in strain circulation However, the surveillance period of this study is short and long-term observations are required to confirm this pattern.

While, RVP showed significant increase in the frequency of reported clinical manifestations, fever, vomiting and dehydration than RVN ($P=0.04$, $P=0.0001$, $P=0.032$ respectively), the multiple regression analysis confirmed that only vomiting and dehydration are major associated presentations of RVA AGE (OR; 1.66, $P=0.021$ and 1.4, $P=0.03$ respectively).

Considering the influence of RVA genotype on the clinical features of AGE, we found that G9 was associated with sever gastroenteritis manifested as mucus diarrhea with loose stool more than 5 days associated with vomiting causing severe dehydration with significant difference than G1 and G3 gastroenteritis that presented mostly with watery diarrhea.

Previous attempts to correlate RVA genotypes and clinical manifestations of diarrhea are limited but no consistent patterns have emerged. An Indian study of hospitalized RVA patients found that G1 caused more severe disease and more severe dehydration than G9 strains (Bahl *et al*., 2005). In the Vizzi *et al.* (2011) study, more than half of the children, who shed G3P[8]/NSP4-E1 and G2P[4]/NSP4-E2 strains showed a severe diarrhea. Linhares *et al*. (2006) reported that circulating RVA belonging to the genotype G9 [8] caused more-severe disease especially under the age of 5 months; the age group targeted for RVA immunization.

Several theories have been put forward to explain these discrepancies. It is known that RV diarrhea can be more severe at younger ages or the newly introduced strains into a community may cause more severe disease due to the lack of pre-existing immunity. Also, there may be year-to-year variations in virulence of particular serotypes or genotypes (Bahl *et al*., 2005; Linhares *et al*., 2006; Schael *et al*., 2009).

Improvements in water supply, hygiene or sanitization are unlikely to decrease the spread of this disease so that vaccines have been identified as the prime means to reduce morbidity and mortality from RVA gastroenteritis (El-Senousy *et al*., 2013). The two available RVA vaccines include the globally common G and P genotypes mimic the protection against severe illness provided by natural infection (Vainio *et al*., 2009).

The main limitation in this study was the low number of samples collected during the surveillance period because not all eligible children were enrolled due to difficulties to obtain consent from all parents. In addition, only one children center hospital participated in the study.

Since the climate varies considerably in different parts of upper and lower Egypt, the differences in temperature and humidity may affect both seasonality and distribution of RVA strains within Egypt. However, limited data are available on the correlation between different genotypes of RVA gastroenteritis, temperature and humidity during various seasons in Egypt.

Conclusion

Our study provides background review to the policy makers before implementation of RVA vaccines in Egypt. It confirms the current burden of RVA AGE in infants and children and shows up the diversity of RVA strains circulating in Dakhalia and neighboring areas in North Egypt. Such variations may be eased by overcrowding population and poor living conditions. The emergence of G9 necessitates the urgent consideration of G9 moiety in RVA vaccines considered for use in Egypt. Year-to-year and geographic variation in the distribution of RVA genotypes underlines the importance of active strain surveillance during several consecutive seasons and supports the need for a vaccine that can provide effective protection against the common RVA types.

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ORIGINAL PAPER

Molecular Characterization of Enteroviruses Isolated from Acute Flaccid Paralysis Cases in Poland, 1999–2014

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Abstract

Enteroviruses (EVs) are among viral pathogens that can cause acute flaccid paralysis (AFP). This study represents an overview of EVs isolated through AFP surveillance in Poland between 1999 and 2014. The presence of enteroviruses was studied in stool samples that were collected from 747 AFP cases and their asymptomatic contacts. Fifty five (6.12%) cases of AFP were associated with enterovirus isolation. Out of the 55 positive cases, 40 were associated with detection of enterovirus in patient, and 15 with detection of EV in healthy contact, without positive detection in paralytic patient. Polioviruses were isolated from 35 AFP cases. The results of this study showed that about 43.6% of positive AFP cases were found in association with the isolation of non-polio enteroviruses (NPEV). A total of 12 different types of the species B were detected (CVA9, CVB1, CVB3, CVB4, CVB5, E3, E4, E9, E11, E13, E30), and one additional isolate represented the species enterovirus A (EV71). Among the 12 serotypes of species B, CVB3 and CVB5 were more frequently detected than others, representing 40% of the characterized isolates, followed by CVB4 (16%), E4 (8%), and E11(8%). Phylogenetic analysis revealed that strains from Poland had the closest genetic relationship with isolates previously identified in Europe (France, Finland, Denmark, Moldova) but also in other parts of the world (Tunisia, China, USA), suggesting wide distribution of these lineages. The paper provides information about NPEV circulation in Poland in the past 16 years, about its association with the AFP and it indicates the need for monitoring NPEV circulation even after the eradication of poliomyelitis.

Key words: acute flaccid paralysis, coxsackieviruses, enteroviruses, poliovirus

Introduction

Acute flaccid paralysis (AFP) is a clinical syndrome characterized by rapid onset of weakness, which has many infectious and non-infectious causes, including viruses, bacteria, toxins, and systemic disease. AFP is the most common sign of acute polio, nationwide acute flaccid paralysis surveillance is the gold standard for detecting cases of poliomyelitis. This syndrome is also associated with a number of other viral pathogens, including non-polio enteroviruses (NPEVs), flaviviruses and adenoviruses, among others (Ooi *et al*., 2003; Saad *et al*., 2005). Several NPEVs have been isolated from cases associated with paralytic disease, including coxsackieviruses (types A 2–11, 14, 16, 21 and 22; and types B 1–6); echoviruses (types 1–4, 6, 7, 9, 11, 14, 16–19 and 30) and enteroviruses (types 68, 70, 71, 77, 89–91, 93, 99, 102, 104) (Shaukat *et al*., 2013; Angez *et al*., 2015).

Human enteroviruses (polioviruses and non-polio enteroviruses) are members of the *Picornaviridae* family, a large and diverse group of small RNA viruses characterized by a single-positive-strand genomic RNA. They are classified into four species: enteroviruses A, B, C and D. More than 100 serotypes are described. They affect millions of people worldwide each year, and are often found in the respiratory secretions and stool of an infected person. Peak EV transmission occurs in the summer and fall in temperate climates. Enteroviruses enter the host by crossing the intestinal mucosa. The outer capsid, which is composed of major structural proteins VP1-VP3, is responsible for host-receptor binding and viral entry into the host cell. Important serotype-specific neutralization epitopes are located on the most external and immunodominant protein VP1 (Halim and Ramsingh, 2000). Molecular methods based on the amplification and sequencing of the VP1 coding region have been used for determination of serotype and for molecular characterization of enteroviruses.

Diagnosis of enterovirus associated with acute flaccid paralysis cases is based on classical virological procedures. Tests for virus isolation are performed on two adequate stool specimens collected within 14 days of

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onset of paralysis, and 24–48 hours apart, from each AFP patient aged less than 15 years. The serotype of strains isolated by this method is identified with neutralizing assay using type-specific antisera (WHO, 2004).

The most common cause of AFP, virus polio, is nearly completely eradicated as a result of global immunization efforts. During the last years, greater attention has been focused on non-polio enteroviruses, which can also cause a polio-like paralytic disease. Several cases of paralysis were reported with coxsackieviruese in Scotland (Grist and Bell, 1984). EV 71 has caused epidemics of acute flaccid paralysis in recent years (especially in Asia), often in association with hand, food and mouth disease (Liu *et al*., 2000). EV 68, which primarily causes respiratory infections, is another serotype that may cause AFP. Paralysis cases associated with enterovirus 68 have been almost unseen, until now (Lang *et al*., 2014). These cases illustrate the importance of appropriate screening for polio and non-polio enteroviruses.

The aim of this study was the molecular characterization of non-polio enteroviruses isolated from AFP cases registered in Poland in the period 1999–2014, identification prevalent serotypes, and determination of their genetic diversity.

Experimental

Materials and Methods

Virus isolation in cells. In this study, stool samples were obtained from 747 children (< 15 years) with AFP and from their close contacts, during a 16-year period (1999–2014). Stool samples were processed according to the standard procedure recommended by WHO (WHO, 2004). Samples were inoculated onto 2 cell lines, human rhabdomyosarcoma (RD) and a mouse cell line carrying the poliovirus receptor (L20B). RD and L20B cells were cultivated in minimal essential medium (MEM) supplemented with 10% foetal bovine serum (FBS). A volume of 200 µl of sample was inoculated into tubes with RD and L20B cells. The tubes were incubated at 36°C and were examined daily. After 7 days, the tubes were frozen and thawed and repassaged, and another 7-day examination was performed. Each specimen underwent two passages in RD and L20B cells. The development of EV-like cytopathic effect was monitored during 14 days post inoculation. Isolates were initially characterized by a neutralization assay using poliovirus specific antisera and pooled antisera against most NPEVs (National Institute for Public Health and the Environment, The Netherlands). All detected NPEVs were sequenced.

RNA isolation and RT-PCR. Viral RNA was extracted from NPEV positive cell culture supernatant using QIAamp Viral RNA Mini Kit (Qiagen) following the manufacturer's instructions. Extracted RNA was amplified by a combined RT and first round PCR using Superscript III (Invitrogen) followed by a second amplification reaction with nested primers for species B VP1 sequences and PCR cycling times and temperature as previously described (Leitch *et al.*, 2009). One sample, negative for species B, was amplified with primers for species A VP1 sequences according enterovirus surveillance guidelines (WHO, 2015). Amplified products were analysed in 1.5% agarose gels, GelRed-stained, and examined under a UV DNA transilluminator.

Sequencing and sequence analysis. The resulting DNA templates were processed in cycle sequencing reaction with a BigDye 3.1 according to manufacturer's protocol. The product of sequencing reaction was run in an automated genetic analyser (Applied Biosystems, model 3730). The resulting sequences were manually edited using BioEdit program and examined to identify the closest homologue using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences of isolated strains (region VP1) were aligned with the reference strains. Molecular and phylogenetic analyses were conducted using MEGA version 6.06. Sequences have been assigned GenBank accession numbers.

Statistical analysis. To identify statistical significance, a Student's *t*-test analysis was performed online (http://www.physics.csbsju.edu/stats/). Results with p values of less than 0.05 were considered significant.

Results

A total of 747 cases of AFP were reported in Poland (Fig. 1) between 1999–2014. Stool specimens were collected from patients with paralysis and their close

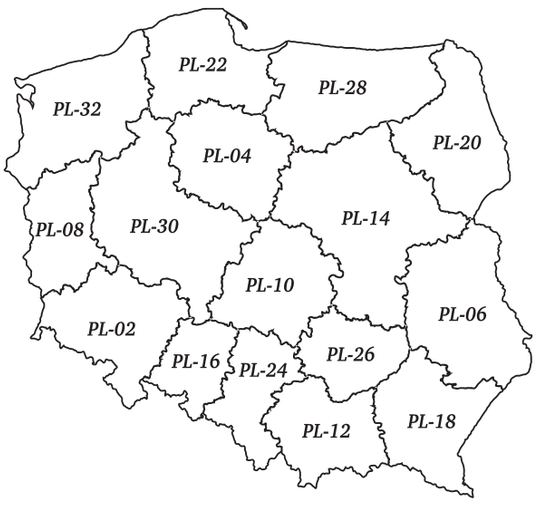

Fig. 1. Map of Poland reflecting study area.

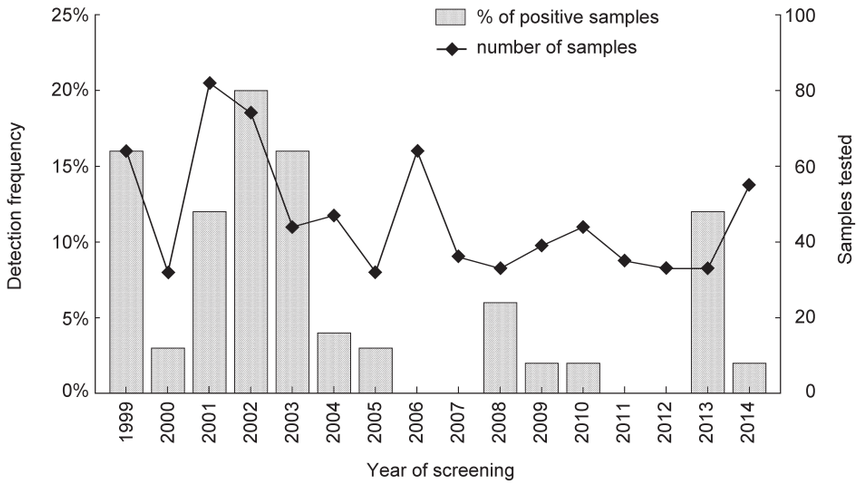

Fig. 2. Detection frequency of enterovirus (polio and non-polio) in patients with acute flaccid paralysis, 1999–2014.

contacts and sent to the National Polio Laboratory in Warsaw. Fifty five (6.12%) cases of AFP were associated with enterovirus isolation from patients or their close contacts. Out of the 55 positive cases, 40 were associated with detection of enterovirus in patient, and 15 with detection of EV in healthy contact, without positive detection in paralytic patient. Out of the 40 positive specimens from patients with paralysis, 23 were identified as PVs, 14 as NPEVs, and 3 were identified as a mixture of PV and NPEV. Out of the 15 cases with positive enterovirus detection in healthy contacts, 8 were identified as PVs, 6 as NPEVs and 1 as a mixture of PV and NPEV.

The percentage of positive AFP cases varied from year to year, between 0% (2006, 2007, 2011, 2012) and 20% (2002). Figure 2 shows the annual distribution of EV infection cases, confirmed by virus isolation.

During the 16-year study from 1999 to 2014, polioviruses were associated with 35 AFP cases. All PV positive samples produced a cytopathic effect (CPE) both in human rhabdosarcoma (RD) and human poliovirus receptor CD155 expressing recombinant murine (L20B) cells. These isolates were identified as PV by neutralization test, including 5 PV1, 6 PV2, 13 PV3 and 3 mixtures of PV1+PV2+PV3, 5 mixtures of PV1+PV2, 3 mixtures of PV1+PV3. PV isolates were forwarded to the Regional Reference Polio Laboratory. Sequencing and molecular analysis of VP1 coding region in Robert Koch Institute revealed that they were all Sabin strains. No wild polio viruses were identified. From 2000 to 2003, one dose of inactivated polio vaccine (IPV) and five doses of oral polio vaccine (OPV) were recommended in the national immunization program. In 2003, all children received 2 doses of IPV and 3 doses of OPV. Since 2004, three doses of IPV and one dose of OPV have been used in Poland. From 1999 to 2003, the number of polio positive AFP cases (31) was significantly higher than from 2004 to 2014 (4) ($p = 0{,}001$). The average annual incidence of vaccine-associated paralytic poliomyelitis (VAPP) in 2004–2014 was 0,36 cases per year.

The results of this study showed that about 43.6% of positive AFP cases were found in association with the isolation NPEV. From the total collected AFP samples, 25 NPEVs were isolated. Serotyping revealed 15 CVB, 1 CVA9, 1 E3, 2 E4, 1 E9, 2 E11, 1 E13, 1 E30 and one additional isolate remains untyped. The most common was CVB (15/25, 60%). The percentage non-polio positive AFP cases, during the study period, ranged between 0% (2006, 2007, 2009, 2010, 2011, 2012) and 9.1% (2013). There was no significant difference in non-polio enterovirus positive cases between two periods of analysis (1999–2003 and 2004–2014) ($p = 0.213$).

The sequences of isolated strains (region VP1) were compared with all the enterovirus sequences available in GenBank, and the virus isolates to be identified were assigned the serotype of the strain that gave the highest identity score (Table I). The nucleotide identity values for the 25 NPEVs ranged from 90% to 99%. The results show a wide diversity of species EV-B types. A total of 12 different types were detected (CVA9, CVB1, CVB3, CVB4, CVB5, E3, E4, E9, E11, E13, E30), 10 from AFP cases and 5 from contacts. Three types (E4, CVB3 and CVB5) were detected in cases and contacts, while other isolates were found mainly in one group. Compared to other types, CVA9, CVB1, CVB4, E9, E11, and E30 were significantly associated with AFP (Table I). Among the 12 serotypes, CVB3 and CVB5 were more frequently detected than others, representing 40% of the characterized isolates, followed by CVB4 (16%), E4 (8%), and E11 (8%). Among the 25 NPEVs, one type of species EV-A was identified. Enterovirus 71 isolated

Table I
Identification of 25 non-polio enterovirus isolates by BLAST comparisons.

AFP strains from Poland			Highest scoring strains				
No/Region/year of isolation Accession number	Specimen source	Strain	Name	Accession number	% sequence identity	Country and date of collection	Specimen
136/PL14/1999 KU189231	contact	CVB5	COPH59346_DNK_02 CF1107P_FRA_99	HF948072.1 HF948032.1	97% 94%	Denmark 2002 France 1999	stool throat
348/PL10/1999 KU189232	contact	CV B5	COPH59346_DNK_02 CF1107P_FRA_99	HF948072.1 HF948032.1	96% 94%	Denmark 2002 Francja 1999	stool throat
356/PL14/1999 KU189233	case	CVB5	COPH59346_DNK_02 2000/CSF/KOR	HF948072.1 AY875692.1	96% 93%	Denmark 2002 Korea 2000	stool CSF
390/PL14/1999 KU189234	case	E11	Jena 799/2002 S53.98.TUN1998	DQ092796.1 HQ674721.1	95% 98%	German 2002 Tunisia 1998	– stool
616/PL04/2000 KU189235	case	CVB3	Macocy Nancy	JQ040513.1 JX312064.1	99% 99%	China 2007 USA 1949	– stool
722/PL16/2001 KU189236	case	CVB4	CVB4_CF1561_FRA00 08fra96	HF948089.1 AF160024.1	98% 96%	France 2000 France 1996	throat sewage
1024/PL04/2001 KU189237	contact	E4	DuToit	AJ241424.1	99%	France	–
1026/PL08/2001 KU189238	contact	CVB5	Faulkner	AF114383.1	99%	USA 1954	stool
1028/PL08/2001 KU189239	case	E4	DuToit	AJ241424.1	99%	France	–
1038/PL24/2001 KU189240	case	CVB4	P234pak92 6653net94	AF160018.1 AF160022.1	95% 95%	Pakistan 1992 Netherlands 1994	– –
1084/PL18/2001 KU189241	contact	CVB5	Jena804/2002 CF516_FRA_00	DQ092797.1 HF948033.1	98% 97%	Germany 2002 France 2000	– CSF
1130/PL30/2001 KU189242	case	CVB4	P234pak92 16723net98	AF160018.1 AF160027	95% 95%	Pakistan 1992 Netherlands 1998	– –
1152/PL26/2002 KU189243	contact	E13	E13_CF711_FRA00 E13_CF1612_FRA00 S3(1)–1	HF948099.1 HF948100.1 AB501332.1	98% 98% 96%	France 2000 France 2000 Japan 2002	stool CSF river water
1414/PL18/2002 KU189244	case	E9	DM E9_CF315022_FRA04	AF524867.1 HF948096.1	96% 95%	Netherlands 1997 France 2004	– throat
1500/PL12/2002 KU189245	case	CVB3	CBV3-18219-02 2679	AY89673.1 KJ489414.1	99% 94%	Moldova 2002 France 1993	– –
1595/PL20/2002 KU189246	contact	CVB3	CBV3-18219-02 2679	AY89673.1 KJ489414.1	99% 94%	Moldova 2002 France 1993	– –
1718/PL06/2003 KU189247	case	CVB1	1167438_pmMC 05.316.1153	JN797615.1 FJ868324.1	90% 91%	Switzerland 2010 Australia 2005	– –
1792/PL02/2004 KU189248	contact	CVB3	CVB3_CF193061_FRA05 CVB3_CF3109_FRA01	HF948087.1 HF948084.1	96% 96%	France 2005 France 2001	throat urine
1864/PL14/2005 KU189249	contact	E3	LR31G7 E3_CF182018_FRA05	FJ766334.1 HF948092.1	96% 95%	Greece 2005 France 2005	– –
2048/PL24/2008 KU189250	case	CVB3	E2012174 EV_851023_ADAM_NL08	JX027614.1 KC893469.1	98% 99%	Taiwan 2012 Netherlands 2008	– –
2055/PL12/2008 KU189251	case	CVB4	CF185005/CSF_4.64_FRA12 364004-06	HG793672.1 AM711078.1	93% 93%	France 2012 France 2006	CSF CSF
2235/PL12/2013 KT 809227	case	E30	ZJ/WZ-30/11 C24/GD/CHN/2012	JX0282231 KM034795.1	99% 98%	China 2011 China 2012	CSF CSF
2239/PL28/2013 KU189252	case	EV71	EV71/XY4/2011 JB143090060	JX0750098 KC866721.1	99% 98%	China 2011 China 2009	– stool
2249/PL12/2013 KU189253	case	CVA9	A108/YN/CHN/2009 CVA9_Alberta_2010	KM890277.1 JQ837914.1	93% 92%	China 2009 Canada 2010	feces CSF
7667/PL24/2014 KU189254	case	E11	44626_RU_OMS_2012 CF216070/CSF_4.20_FRA12	KP090684.1 HG793710.1	97% 96%	Russia 2012 France 2012	– CSF

from AFP case in Poland had the closest genetic relationship with C4 isolates previously identified in China in 2009 and 2011.

To investigate the genetic relatedness of EV-B isolates in Poland to globally circulating types, we performed phylogenetic analyses of partial VP1 sequences with the prototypes representing different types available in the GenBank. Phylogenetic tree was constructed from the aligned nucleotide sequences by using the neighbor-joining method. VP1 sequences obtained from a total of 24 NPEV isolates formed monophyletic clusters with their prototype strains according to their serotype. The tree in Fig. 3 compares the sequences of 72 isolates, 24 from our region, 48 from other countries, and from prototype isolates and previously published sequences in GenBank. Phylogenetic analysis revealed that strains from Poland had the closest genetic relationship with isolates previously identified in Europe (France, Finland, Denmark, Moldova) but also in other parts of the world (Tunisia, China, USA) (Fig. 3).

In the VP1 tree, several serotypes showed subclustering into various groups. Two distinct genogroups were observed for CVB4 and E11. CVB5 and CVB3 demonstrated three subclusters. The other enterovirus serotypes existed as a single genotype throughout our study period.

A total of 5 CVB3 strains were isolated during the AFP surveillance in 1999–2014. Homologous comparison revealed 78.0% to 98.3% VP1 nucleotide sequence similarities among themselves, and 78.2% and 99.4% with prototype strain Nancy. A total of 5 strains of CVB5 were detected during the study period. Homologous comparison revealed 77.0% to 98.9% VP1 nucleotide sequence similarities among themselves, and 77.1% to 99.9% with prototype strain Faulkner. The comparison of CVB4 strains revealed 89.9 to 98.9% VP1 sequence similarities among themselves, and 82.3% to 83.3% with prototype strain J.V.B. Benschoten.

Discussion

The Global Polio Eradication Initiative has succeeded in reducing the circulation of wild poliovirus. Europe was declared polio-free in 2002. The last indigenous WPV in Poland was isolated in 1984 (Jarząbek *et al.*, 1992). However, AFP surveillance is still of great importance in monitoring re-emergence of WPV poliomyelitis and emergence of vaccine-derived polioviruses. During AFP surveillance in Poland from 1999 to 2014, polioviruses were associated with 35 (4.7%) cases of AFP. The findings confirmed all cases as Sabine-like polioviruses. The isolation of these strains indicates vaccine-associated paralytic poliomyelitis. All 3 serotypes of Sabine polioviruses were found with type 3 being the dominant. This serotype frequently causes of VAPP (Vaccine Associated Paralytic Poliomyelitis) (Kapusinszky *et al.*, 2010). The higher rate of PV detection during the period 1999–2004 likely results from the extensive use of OPV up to 2004. During the past 11 years, OPV has been used only as a booster dose after a sequential schedule of IPV. The risk of VAPP varies by doses of OPV vaccine. Most studies have shown that two doses of IPV induce protective levels of antibodies in 90% or more recipients, which should reduce the incidence of VAPP after OPV administration.

In addition to PVs, 25 NPEVs have been isolated during the AFP surveillance conducted in Poland from 1999 to 2014. The result of our study suggested that about 3.2% of AFP cases are found to be associated with isolation of only NPEV. According to the guidebook issued by the WHO, during 1967–1970, just less than 1% of the patients who were infected with NPEVs showed paralysis. The WHO reported a same percent of non-polio enteroviral AFP during 2000–2003. Scottish researchers reported isolation of coxsackie virus in 15.6% patients with paralysis during 1956–1973 (Grist and Bell, 1984). Australia showed that 15% of AFP in 1963 caused by NPEVs. Nigeria has also reported that 14.6% of AFP during 2002–2003 was caused by NPEV (Mehrabi *et al.*, 2011). Reports of NPEV isolation rate among AFP cases in Asian countries varied from below 10% in Malaysia and the Philippines (Apostol *et al.*, 2012), to as high as 30% in India from 2007 to 2009 (Kapoor *et al.*, 2001). The annual NPEV isolation rate in most tropical countries typically exceeds 10%, but the variability of findings influenced by a number of factors, including the season of the year, elevation, or population hygienic levels.

Isolation of NPEVs from AFP cases is common worldwide, but clinically it is difficult to determine their association with paralytic disorders. Although detection of one of the NPEV types during the course of AFP may not provide a proof of a causal relationship, the etiological role of NPEV associated with this syndrome also may not be ruled out.

The predominance of EV-B species found in this study was similar to that of the recent reports described for NPEV strains from hospitalized children and AFP cases from India, Nigeria and Philippines (Laxmivandana *et al.*, 2013; Oyero *et al.*, 2014). In total 12 different types were detected, 6 serotypes of echoviruses, 1 serotype of CVA and 4 CVB serotypes. The enteroviruses reported in this study (CVA9, CVB1, CVB3, CVB4, CVB5, E3, E4, E9, E11, E13, E30) have been earlier isolated in different countries from patients with AFP. Among the 12 serotypes, CVB were more frequently detected than others, representing 60% of the characterized isolates. CVB and E6, E9, E11 have been also found to be prevalent in AFP cases from India

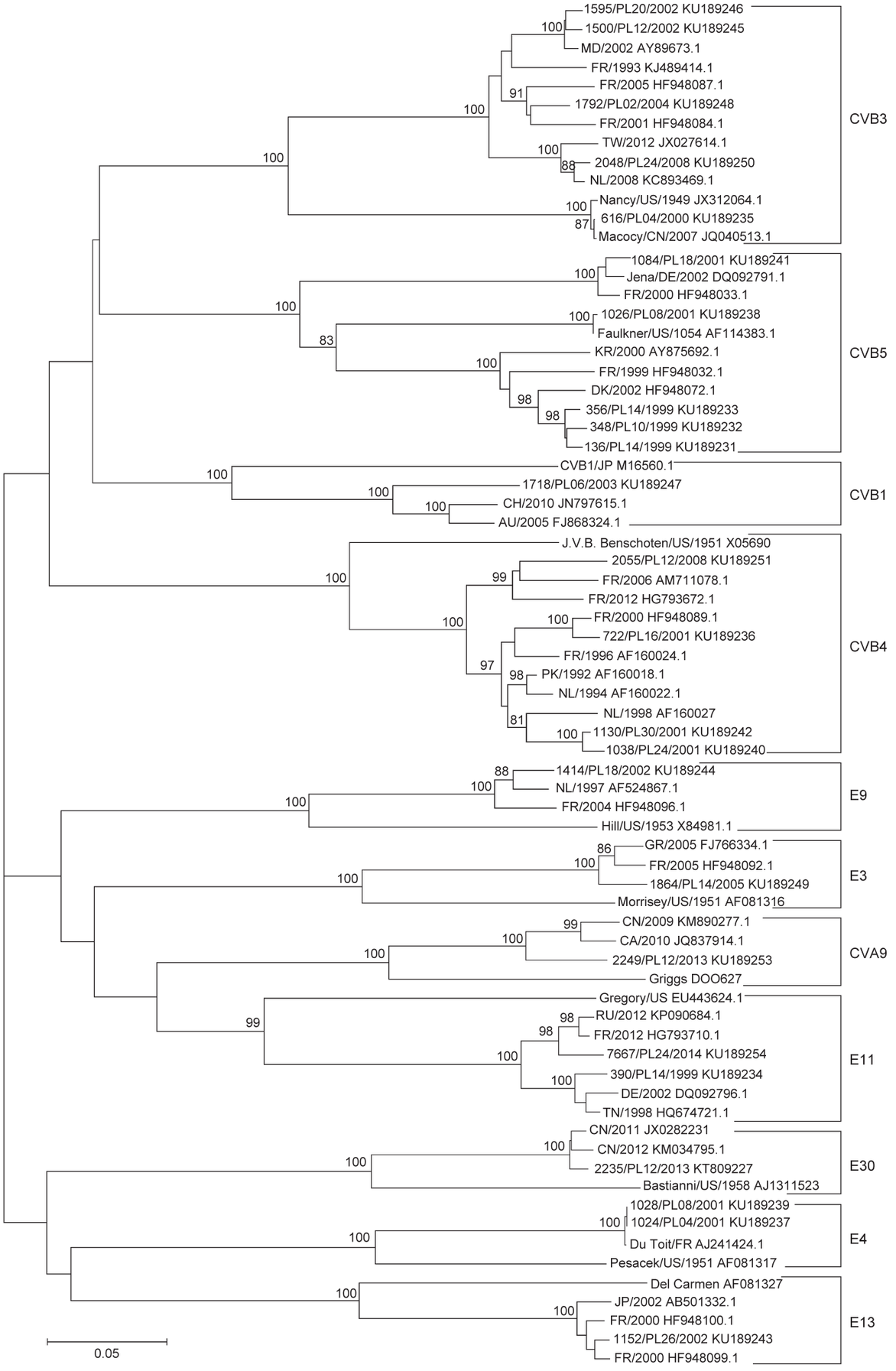
1595/PL20/2002 KU189246
1500/PL12/2002 KU189245
MD/2002 AY89673.1
FR/1993 KJ489414.1
FR/2005 HF948087.1
1792/PL02/2004 KU189248
FR/2001 HF948084.1
TW/2012 JX027614.1
2048/PL24/2008 KU189250
NL/2008 KC893469.1
Nancy/US/1949 JX312064.1
616/PL04/2000 KU189235
Macocy/CN/2007 JQ040513.1
CVB3
1084/PL18/2001 KU189241
Jena/DE/2002 DQ092791.1
FR/2000 HF948033.1
1026/PL08/2001 KU189238
Faulkner/US/1054 AF114383.1
KR/2000 AY875692.1
FR/1999 HF948032.1
DK/2002 HF948072.1
356/PL14/1999 KU189233
348/PL10/1999 KU189232
136/PL14/1999 KU189231
CVB5
CVB1/JP M16560.1
1718/PL06/2003 KU189247
CH/2010 JN797615.1
AU/2005 FJ868324.1
CVB1
J.V.B. Benschoten/US/1951 X05690
2055/PL12/2008 KU189251
FR/2006 AM711078.1
FR/2012 HG793672.1
FR/2000 HF948089.1
722/PL16/2001 KU189236
FR/1996 AF160024.1
PK/1992 AF160018.1
NL/1994 AF160022.1
NL/1998 AF160027
1130/PL30/2001 KU189242
1038/PL24/2001 KU189240
CVB4
1414/PL18/2002 KU189244
NL/1997 AF524867.1
FR/2004 HF948096.1
Hill/US/1953 X84981.1
E9
GR/2005 FJ766334.1
FR/2005 HF948092.1
1864/PL14/2005 KU189249
Morrisey/US/1951 AF081316
E3
CN/2009 KM890277.1
CA/2010 JQ837914.1
2249/PL12/2013 KU189253
Griggs DOO627
CVA9
Gregory/US EU443624.1
RU/2012 KP090684.1
FR/2012 HG793710.1
7667/PL24/2014 KU189254
390/PL14/1999 KU189234
DE/2002 DQ092796.1
TN/1998 HQ674721.1
E11
CN/2011 JX0282231
CN/2012 KM034795.1
2235/PL12/2013 KT809227
Bastianni/US/1958 AJ1311523
E30
1028/PL08/2001 KU189239
1024/PL04/2001 KU189237
Du Toit/FR AJ241424.1
Pesacek/US/1951 AF081317
E4
Del Carmen AF081327
JP/2002 AB501332.1
FR/2000 HF948100.1
1152/PL26/2002 KU189243
FR/2000 HF948099.1
E13
0.05

Fig. 3. Phylogenetic tree depicting the relationships between partial capsid gene sequences of 24 Polish NPEVs isolated from 1999 to 2015 and 48 sequences from GenBank. Each strain is referenced by its geographical origin and its accession number. The tree was constructed by the neighbour-joining method and evaluated with 1000 bootstrap pseudoreplicates. Only bootstrap values ≥ 80% are indicated. In the analyses, genetic distances were calculated with Kimura 2-parameter algorithm. Analyses were conducted in MEGA 6.06 (Tamura *et al*., 2013).

and other countries (Kapoor *et al*., 2001; Bahri *et al*., 2005; Dhole *et al*., 2009). Most of the detected serotypes of NPEV during AFP surveillance in Poland were also found in sewage. E3, E11 and CVB were the predominant serotypes detected in raw sewage in Poland in 2011 (Wieczorek *et al*., 2015).

The enteroviruses reported in this study were also found in stools of healthy persons. In Ghana, CVB, E13 and E7 were detected in healthy school children (Odoom *et al*., 2013). Echovirus 30, E33, E12, E25 and CVA were isolated from healthy family members in Mongolia (Kuramitsu *et al*., 2005) and E6, E11, E9 and CVB were found among healthy children in India and Egypt (Dhole *et al*., 2009; Attoh *et al*., 2014). Healthy children are known to be the major reservoir for enteroviruses in the community.

All the typing results were confirmed by phylogenetic analysis including VP1 sequences of the EV prototype isolates. Analysis of VP1 gene sequences of NPEV strains is widely carried out to determine the EV species and genotypes that circulate in the population. For most enterovirus serotypes, a clear correlation between genetic variability and pathogenic phenotype has not yet to be established. The same virus strain may induce either a clinical disease or an asymptomatic infection. Phylogenetic analysis revealed that strains from Poland had the closest genetic relationship with isolates previously identified in Europe but also in other parts of the world.

This is the first report of the occurrence of EV-71 in Poland. The Polish strain was the closest related to sublineage C4 strains isolated in China. The C4 subgenotype has predominantly been identified in large outbreaks of hand, foot and mouth disease in Asia, where severe cases and a rather high mortality rate have been reported. In 2004 the C4 subgenotype was detected for the first time in Europe (Mirand *et al*., 2010).

The limitation of this study include lack of true controls as only contacts of AFP cases were tested, which are likely to share exposures and consequently viral flora, with cases. In addition, stool is not the optimal specimen for enterovirus disease association studies due to the high proportion of asymptomatic infections and because disease generally affects other organ systems. The study has some limitations for NPEV surveillance as the focus of the WHO is to detect PVs. The two cell lines recommended for PV isolation (RD and L20B) are less suitable for isolation of NPEVs. Some EV strains that cannot be propagated in RD may have been underdetected and the growth of the other EV types may have been favored (Hosoya *et al*., 2002). Until now, no cell line has been identified to isolate all the existing NPEV types.

This study suggests that NPEVs, particularly those within the species EV-B may play a contributing role in the etiology of AFP in the country, highlighting the fact that as polio eradication is closing up, improved efforts should be focused on surveillance of NPEVs. After the eradication of poliomyelitis, AFP cases negative for wild poliovirus, but positive for NPEV will continue to be detected. The results of this study indicate that characterization of NPEV isolates could provide better understanding of the epidemiology of NPEV causing paralysis.

Acknowledgments

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ORIGINAL PAPER

Antistaphylococcal Activity of Selected Thiourea Derivatives

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Abstract

Five of thiourea derivatives were prepared using as a starting compound 3-(trifluoromethyl)aniline, 4-chloro-3-nitroaniline, 1,3-thiazol-2-amine, 2*H*-1,2,3-triazol-4-amine and commercial isothiocyanates. All compounds were evaluated *in vitro* for antimicrobial activity. Derivatives 2 and 3 showed the highest inhibition against Gram-positive cocci (standard and hospital strains). The observed MIC values were in the range of 0.5–8 μg/ml. The products effectively inhibited the formation of biofilms of methicillin-resistant and standard strains of *Staphylococcus epidermidis*. Inhibitory activity of thioureas 2 and 3 against *Staphylococcus aureus* topoisomerase IV was studied. The examined compounds were nongenotoxic.

Key words: antistaphylococcal activity, anti-biofilm activity, genotoxicity, thiourea derivatives

Introduction

Staphylococci belong to the generally present, most important biofilm-formed pathogens and are responsible for a large number of serious nosocomial infections acquired after surgery or hospital (Leclercq, 2009; Agarwal *et al*., 2010). Methicillin resistant strains (both, coagulase-positive and coagulase-negative) often show resistance to many other antibiotics and chemotherapeutics, such as fluoroquinolones, macrolides, tetracyclines or glicopeptides. *Staphylococcus aureus* produces numerous virulence factors, *e.g.* hemolysin, exotoxin, enzymes (hyaluronidase, lipase, nuclease), surface proteins (that promote attachment to host proteins such as laminin and fibronectin) or enterotoxins (responsible for food poisoning) (Heczko *et al*., 2014). This bacterium can generate various types of infections, such as severe skin, subcutaneous tissue and bone infections, scalded skin syndrome, pneumonia, endocarditis, as well as can even cause severe sepsis (Chambers and DeLeo, 2009). Another, commonly present *Staphylococcus* species – *Staphylococcus epidermidis* – has the ability to produce extracellular mucous and is able to adhere to a variety of surfaces, *e.g.* joint implants, vascular lines or artificial heart valves (Arciola *et al*., 2005; Mack *et al*., 2006). Colonization of such biomaterials or medical devices can generate difficult-to-combat local and systemic infections (Otto, 2008; 2009).

Bacterial biofilm constitutes a complex multidimensional structure formed by cells conglomerated with one another and with the base. It is composed of extracellular mucous produced by bacteria, proteins, polysaccharides, nucleic acids and water (Costerton *et al*., 1999). Biofilm can be formed by microorganisms belonging to one or different species and can develop on abiotic surfaces or on living tissues (Costerton *et al*., 1999; Donlan and Costerton, 2002).

The cells of micro-organisms living in the biofilm are, in comparison to planktonic forms, more resistant to antibiotics and chemotherapeutics used in pharmacotherapy (Høiby *et al*., 2010) as well as antiseptics or disinfectant (Bridier *et al*., 2011). This causes numerous therapeutic problems, especially in combating chronic infections. The formation of the biofilm by bacteria constitutes also an essential clinical problem linked to infections associated with medical devices, *e.g.* bone implants, catheters or vascular lines (Donlan, 2001; Maki *et al*., 2006).

Effective methods of fight down bacterial biofilm are still missing. It is extremely important to seek new

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compounds having antibacterial activity which would be effective in combating biofilm. They could be used, for instance, to cover surfaces of biomaterials or protective clothing, to prevent micro-organisms from settling on them and thus making it impossible for the difficult-to-eradicate biofilm to form (Meng *et al*., 2013).

The thiourea fragment represents an important synthon which is responsible for numerous biological activities, such as antimicrobial (Struga *et al*., 2010; Vega-Pérez *et al*., 2012), antiviral (Ranise *et al*., 2003), anticancer (Saeed *et al*., 2010), cytotoxic (Vega-Pérez *et al*., 2012) and anti-inflammatory (Keche *et al*., 2012) properties. In many cases, antistaphylococcal potency of this class of compounds is the result of the type II topoisomerase inhibition, that includes topoisomerase IV and DNA gyrase (Basarab *et al*., 2013; Bielenica *et al*., 2015).

As a part of our research program of rapidly assemble novel bioactive compound we have synthesized five disubstituted thioureas. The compounds with various substituents at the thiourea moiety (aryl, heteroaryl and benzyl) were evaluated for their antimicrobial, as well as investigated as potential inhibitors on biofilm formation of Gram-positive pathogens. The mechanism of their action through topoisomerase IV inhibition was proved.

Experimental

Materials and Methods

Chemistry. 3-(Trifluoromethyl)aniline, 1,3-thiazol-2-amine, 2*H*-1,2,3-triazol-4-amine, 4-chloro-3-nitroaniline were supplied from Alfa Aesar. Isothiocyanates were purchased from Alfa Aesar or Sigma Aldrich. Acetonitrile, chloroform and methanol were supplied from POCh (Polskie Odczynniki Chemiczne SA). All chemicals were of analytical grade and were used without any further purification. Before using, dry acetonitrile was kept in crown cap bottles over anhydrous phosphorus pentoxide (Carl Roth). The IR spectra were obtained on Perkin Elmer Spectrum 1000 spectrometer in KBr pellets. The NMR spectra were recorded on Varian VNMRS 300 Oxford NMR spectrometer, operating at 300 MHz (^{1}H NMR, relax. delay 1.000 sec, pulse 30.0 degrees) and 75.4 MHz (^{13}C NMR, relax. delay 3.700 sec, pulse 45.0 degrees, Waltz-16 modulated). Chemical shifts (δ) were expressed in parts per million relative to tetramethylsilane used as the internal reference. Mass spectral ESI measurements were carried out on Waters ZQ Micro-mass instruments with quadruple mass analyzer. The spectra were performed in the negative ion mode at a declustering potential of 40–60 V. The sample was previously separated on a UPLC column (C18) using UPLC ACQUITYTM system by Waters connected with DPA detector. Flash chromatography was performed on Merck silica gel 60 (200–400 mesh) using chloroform eluent. Analytical TLC was carried out on silica gel F254 (Merck) plates (0.25 mm thickness).

Synthesis of thiourea derivatives (1–5). A solution of amine derivative (3-(Trifluoromethyl)aniline, 4-chloro-3-nitroaniline, 1,3-thiazol-2-amine, 2*H*-1,2,3-triazol-4-amine) (0.01 mol) in acetonitrile (25 ml) was treated with appropriate isothiocyanate (0.011 mol) and the mixture was refluxed for 8 h. Then solvent was removed on rotary evaporator. The residue was purified by column chromatography (chloroform: methanol; 9.5:0.5 vol.). The final product was crystallized from acetonitrile.

1-(1-phenylethyl)-3-[3-(trifluoromethyl)phenyl] thiourea (1). Yield 75%, white powder, m.p. 113–115°C. ^{1}H NMR (300 MHz, DMSO) *δ*: 9.66 (s, 1H, NH), 8.42 (d, J = 7.8 Hz, 1H, H_{arom}), 8.03 (s, 1H, NH), 7.66 (d, J = 8.4 Hz, 1H, H_{arom}), 7.51 (t, 1H, J = 7.8 Hz, H_{arom}), 7.41–7.33 (m, 5H, H_{arom}), 7.30–7.23 (m, 1H, H_{arom}), 5.55–5.51 (m, 1H, CH), 1.48 (d, J = 6.9 Hz, 3H, CH_3). ^{13}C NMR (75.4 MHz, DMSO) *δ*: 179.80, 143.60, 140.58, 129.50, 128.94 (q), 128.34 (2C), 126.88, 126.24 (2C), 126.05, 124.08 (q), 119.91, 118.59, 52.60, 21.83. HRMS (ESI) calc. for $C_{16}H_{14}F_3N_2S$ [M – H]$^-$: 323.0830, found: 323.0834.

1-(3,4-dichlorophenyl)-3-[3-(trifluoromethyl) phenyl]thiourea (2) has been synthesized as described previously (Bielenica *et al*., 2015).

1,3-bis(4-chloro-3-nitrophenyl)thiourea (3). Yield 40%, yellow powder, m.p. 176–178°C. ^{1}H NMR (300 MHz, DMSO) δ: 10.47 (s, 2H, NH), 8.31 (d, 2H, J = 2.4 Hz, H_{arom}), 7.79 (dd, 2H, $J_1 = J_2$ = 2.4 Hz, H_{arom}), 7.74 (d, 2H, J = 8.4 Hz, H_{arom}).^{13}C NMR (75.4 MHz, DMSO) δ: 180.03, 147.02 (2C), 139.16 (2C), 131.50 (2C), 128.73 (2C), 120.16 (2C), 120.09 (2C). HRMS (ESI) calc. for $C_{13}H_8Cl_2N_4S$ [M – H]$^-$: 385.6756 found: 385.6759.

1-(2,3-dichlorophenyl)-3-(1,3-thiazol-2-yl)thiourea (4). Yield 68 %, white powder, m.p. 167–169°C. ^{1}H NMR (300 MHz, DMSO) δ: 12.5 (s, 1H, NH), 10.21 (s, 1H, NH), 7.75 (dd, 1H, $J_1 = J_2$ = 1.5 Hz, H_{arom}), 7.52 (dd, 1H, $J_1 = J_2$ = 1.5 Hz, H_{arom}), 7.44 (d, 1H, J = 4.2 Hz, H_{arom}), 7.37(d, 1H, J = 3.6 Hz, H_{arom}), 7.06 (t, 1H, J = 8.1 Hz, H_{arom}). ^{13}C NMR (75.4 MHz, DMSO) δ: 180.03, 159.17, 138.31, 137.57, 137.15, 131.77, 128.32, 124.46, 119.82, 112.90. HRMS (ESI) calc. for $C_{10}H_7Cl_2N_3S_2$ [M – H]$^-$: calc. 303.9390 found: 303.9399.

1-(4-chloro-3-nitrophenyl)-3-(1*H*-1,2,4-triazol-3-yl)thiourea (5). Yield 68 %, white powder, m.p. 234–236°C. ^{1}H NMR (300 MHz, DMSO) δ: 14.05 (s, 1H, NH), 11.96 (s, 1H, NH), 11.50 (s, 1H, NH), 8.57 (s, 1H, CH=); 8.11(d, 1H, J = 2.4 Hz, H_{arom}), 7.92 (dd, 1H, $J_1 = J_2$ = 2.7 Hz, H_{arom}), 7.75 (d, 1H, J = 8.7 Hz, H_{arom}). ^{13}C NMR (75.4 MHz, DMSO) δ: 177.58, 156.81, 148.92,

146.91, 142.93, 138.87, 131.41, 129.30, 120.66. HRMS (ESI) calc. for $C_9H_7ClN_6O_2S$ $[M-H]^-$: calc. 296.9969 found: 296.9967.

Biological evaluation

Antimicrobial studies. Antimicrobial activities of the thiourea derivatives were tested *in vitro* against six reference Gram-positive strains: *S. aureus*: NCTC 4163, ATCC 25923, ATCC 6538, ATCC 29213, *S. epidermidis*: ATCC 12228, ATCC 35984, and against a series 16 of clinical methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis* (MRSE) strains. Microorganisms were obtained from the collection of Department of Pharmaceutical Microbiology, Medical University of Warsaw, Poland.

Minimal Inhibitory Concentration (MIC) of tested compounds were examined by the twofold serial broth (Mueller-Hinton Broth medium, Becton Dickinson) dilution methods using 96-well microtitre plates (Medlab Products) according to CLSI guidelines (CLSI, 2012). Concentrations of tested compounds in liquid medium ranged from 0.0625 to 256 μg/ml. The final inoculum of all strains studied was about 10^5 cfu/ml. MIC values were read after 18 h incubation at 35°C. Minimal bactericidal concentration of the compounds (MBC – 99.9% killing of the final inoculums) were determined by subculturing 10 μl of suspension from each negative well (no visible bacterial growth) from the MIC test, onto TSA plates and incubated at 37°C for 24 h (CLSI, 1999). Ciprofloxacin was used as the reference antibacterial drug.

Biofilm inhibitory assay. Three thiourea derivatives (2, 3, 5) and Ciprofloxacin (as reference antibacterial drug) were studied for their ability to inhibit the formation of staphylococcal biofilm. Selected methicillin-resistant clinical strains of *Staphylococcus* (four *S. aureus* and four *S. epidermidis*) and two reference *S. epidermidis* strains were used in this assay. The clinical strains were isolated from blood of hospitalized patients. *S. epidermidis* ATCC 35984 was used as high biofilm-producer (positive control), *S. epidermidis* ATCC 12228 was used as low-biofilm producer (negative control). Ciprofloxacin was the reference antibacterial agent.

Inhibition of bacterial biofilm formation was screened using the method, described previously (Stefańska *et al.*, 2015). All strains were cultured overnight in Tryptic Soy Broth medium supplemented with 0.5% glucose (BTL, Poland). The solution of tested compounds in above medium was mixed (1:1) with the bacterial inoculums (10^7 cfu/ml) in sterile 96-well polystyrene microtiter plates (Karell-Medlab, Italy) and incubated at 37°C for 24 h. The final concentrations of tested compounds ranged from 1 to 16 μg/ml.

The positive control (biofilm formation) was bacterial culture in TSB-glucose; a negative control was the medium devoid of the bacteria. After incubation, medium was removed from wells and washed twice with sterile PBS buffer (phosphate-buffered saline) to take out the non-adherent bacteria. Adherent bacterial cells, which usually formed biofilm on wells surface, were uniformly stained with 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT; 0.5% in PBS) and incubated for 2 hours at 37°C. After incubation, the solution was removed and bacterial biofilm was solubilized by DMSO (Merck) with glycine buffer (pH 10.2) and mixed 15 minutes at room temperature. The solution absorbance (A_{554}) was measured at 554 nm using a spectrophotometer PowerWave XS (BioTek).

The biofilm-inhibition results were interpreted from dose (concentrations) – response graphs.

Due to the transparency image on the graphs shows the results for selected four clinical and two reference strains. Results in the graphs are averages of four repetitions ± the standard error of the mean. The standard deviation values were very low, is they are sometimes invisible in the figures.

Genotoxicity. DNA-damaging activity of salinomycin and its derivatives was tested by *rec*-assay using two genetically modified *Bacillus subtilis* strains: M45 (rec^-) and H17 (rec^+) (Saide and Kada, 1976; Kada *et al.*, 1980). *B. subtilis* M45 is devoid of the recombinant – based DNA repair mechanism and is much more susceptible to genotoxic substances compared to *B. subtilis* H17 strain. Tested compounds were dissolved in DMSO and 10 μl of each solution were dripped onto sterile filter paper discs (Whatman No 3MM) to load 256 μg of a given compound per 9 mm disc. Discs were placed on the surface of Nutrient agar plates (BTL, Poland) inoculated with 100 μl of bacterial culture overnight and incubated for 24 h at 35°C. After incubation the growth inhibition zones were measured. NOQ (4-nitroquinoline N-oxide) was used as the reference genotoxin in concentration 2 μg per disc.

Results of the genotoxicity test were read after 18 h of incubation at 35°C by comparing the diameter of the inhibition zone for the *B. subtilis* M45 (rec^-) strain with that observed for the *B. subtilis* H17 (rec^+) strain.

Inhibition of bacterial topoisomerase IV

Decatenation assay. The assay was performed using *S. aureus* topoisomerase IV decantation kit (Inspiralis). Kinetoplast DNA (kDNA) was the substrate for topoisomerase IV. 1 U of topoisomerase IV decatenated 200 ng of kDNA, in the dedicated decantation assay buffer supplied by the manufacturer. Enzyme activity was detected by incubation for 30 min at 37°C in

a total reaction volume of 30 µl and in the presence of different concentrations of the test compounds. The reactions were terminated by adding an equal volume of STEB buffer (40% sucrose, 100 mM Tris-HCl pH 8, 1 mM EDTA, 0.5 mg/ml bromophenol blue), followed by extraction with 1 volume of chloroform/isoamyl alcohol (24:1).

Then, 20 µl of the aqueous phase of each sample was loaded onto a 1% agarose gel. Electrophoresis was conducted in Tris-acetate-EDTA buffer for 1.5 h at 80 V. Gels were stained with ethidium bromide and visualized under UV light in a transilluminator (ChemiDoc MP, Bio Rad).

Results

The preparation of 5 thiourea derivatives was accomplished according the reactions described in Scheme 1.

The first step was to determine the antibacterial activity of tested thiourea derivatives against standard and clinical methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis* strains (MRSE). Moreover derivatives were tested for their genotoxicity, ability to inhibition biofilm formation by various clinical *Staphylococcus* strains and activity of selected compounds against bacterial topoisomerase.

Antibacterial activities of tested compounds were expressed by minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). The results were listed in Tables I–II. Two derivatives, 2 and 3 showed very potent anti-staphylococcal activity. For the compound 2 MIC values were from 0.5 to 1.0 µg/ml and MBC values ranged from 4 to 8 µg/ml. For compound 3 MIC values ranged from 2 µg/ml to 8 µg/ml and MBC values was 256 µg/ml and above. For the reference antibacterial agent – Ciprofloxacin MIC values ranged from 0.125 µg/ml to 0.5 µg/ml and MBC values ranged from 0.5 µg/ml to 2 µg/ml for standard *Staphylococcus* strains. For clinical methicillin-resistant isolates Ciprofloxacin MIC values ranged from 0.5 µg/ml to 64 µg/ml and MBC values ranged from 4 µg/ml to above 256 µg/ml.

Only for the compound 2 the bactericidal activity was observed at a low concentration (4–8 µg/ml). Compounds 1 and 3 showed only bacteriostatic effect (MBC values much higher as compared to the MIC values). Compound 4 and 5 showed weak activity against tested staphylococcal strains.

Sometimes antimicrobial activity of various compounds is directly connected with their genotoxicity. This effect is not profitable to microbiologically active compounds. Genotoxicity of thiourea derivatives was tested by *rec*-assay using two genetically modified *Bacillus subtilis* strains: H17 (rec^+) and M45 (rec^-). *B. subtilis* M45 is more susceptible to genotoxic substances (for example NOQ) compared to *B. subtilis* H17 strain. As shown in Table III there was no significant difference in the diameter of the inhibition zones for both bacterial strains. Examined compounds are nongenotoxic *in vitro* for the tested bacterial cells.

Three of obtained compounds were tested for their ability to inhibit the staphylococcal biofilm formation. Four selected clinical isolates (four *S. aureus* and four *S. epidermidis* isolated from blood of hospitalized patients) and two standard *S. epidermidis* strains (positive and negative control) were used in this study. The biofilm inhibitory activity of derivatives 2 and 3

Table I
In vitro activity of tested compounds against planktonic cells of standard and clinical methicillin-resistant *S. aureus* strains.

No. *S. aureus*	**1** MIC (MBC)	**2** MIC (MBC)	**3** MIC (MBC)	**4** MIC (MBC)	**5** MIC (MBC)	Ref* MIC (MBC)
NCTC 4163	16 (>256)	0.5 (4)	4 (256)	64 (>256)	64 (>256)	0.25 (0.5)
ATCC 25923	16 (>256)	0.5 (4)	8 (>256)	64 (>256)	64 (>256)	0.5 (2)
ATCC 6538	16 (>256)	0.5 (4)	8 (>256)	32 (>256)	64 (>256)	0.25 (1)
ATCC 29212	16 (>256)	0.5 (4)	4 (256)	64 (>256)	128 (>256)	0.5 (2)
452/11	32 (>256)	0.5 (4)	2 (256)	64 (>256)	128 (>256)	32 (>256)
462/11	16 (>256)	1 (8)	2 (256)	64 (>256)	256 (>256)	64 (>256)
514/12	16 (>256)	1 (8)	8 (>256)	64 (>256)	64 (>256)	32 (256)
522/12	16 (>256)	1 (8)	4 (>256)	32 (>256)	64 (>256)	64 (>256)
572/12	32 (>256)	0.5 (4)	8 (>256)	32 (>256)	256 (>256)	64 (>256)
573/12	16 (>256)	1 (8)	2 (256)	64 (>256)	128 (>256)	32 (256)
585/12	16 (>256)	0.5 (4)	4 (>256)	32 (>256)	128 (>256)	64 (>256)
586/12	16 (>256)	0.5 (4)	2 (256)	64 (>256)	256 (>256)	64 (>256)

Ref* – Ciprofloxacin (reference antimicrobial drug), MIC – minimal inhibitory concentration (µg/ml), MBC – minimal bactericidal concentration (µg/ml).

Table II
In vitro activity of tested compounds against planktonic cells of standard and clinical methicillin-resistant *S. epidermidis* strains.

No. *S. epidermidis*	**1** MIC (MBC)	**1** MIC (MBC)	**1** MIC (MBC)	**1** MIC (MBC)	**1** MIC (MBC)	Ref.* MIC (MBC)
ATCC 12228	32 (>256)	1 (8)	4 (256)	64 (>256)	128 (>256)	0.25 (1)
ATCC 35984	32 (>256)	1 (8)	4 (256)	32 (256)	64 (>256)	0.125 (0.5)
409/11	16 (>256)	1 (8)	2 (>256)	32 (>256)	128 (>256)	2 (32)
455/11	16 (>256)	0.5 (4)	2 (>256)	64 (>256)	128 (>256)	32 (256)
459/11	16 (>256)	0.5 (4)	4 (>256)	32 (>256)	64 (>256)	32 (256)
469/11	16 (>256)	0.5 (4)	4 (>256)	64 (>256)	128 (>256)	8 (64)
517/12	16 (>256)	1 (8)	2 (>256)	32 (>256)	128 (>256)	32 (256)
519/12	16 (>256)	1 (8)	2 (>256)	32 (>256)	64 (>256)	0.5 (4)
526/12	32 (>256)	0.5 (4)	4 (>256)	64 (>256)	128 (>256)	4 (32)
528/12	16 (>256)	1 (8)	4 (>256)	64 (>256)	128 (>256)	32 (256)

Ref* – Ciprofloxacin (reference antimicrobial drug), MIC – minimal inhibitory concentration (μg/ml), MBC – minimal bactericidal concentration (μg/ml).

Table III
Genotoxicity of tested compounds in *rec-assay.*
Concentration of tested compounds – 256 μg per 9 mm disc.
*NOQ (4-nitroquinoline N-oxide) – reference genotoxic agent, 2 μg per 9 mm disc

Tested compound	Diameter of growth inhibition zones (mm)	
	Bacillus subtilis H17 (rec^+)	*Bacillus subtilis* M45 (rec^-)
1	12	12
2	na	na
3	14	13
4	na	na
5	na	na
NOQ*	12	24

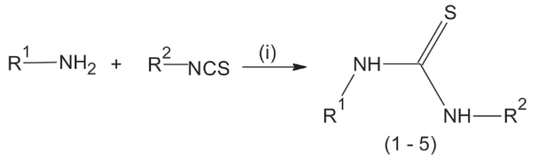
na – no growth inhibition zone

were higher than the reference agent – Ciprofloxacin (Fig. 1–4). The more active compound 2 inhibited biofilm formation in the range from 40% to above 90% in concentration 1 μg/ml (MIC for planktonic cells) (Fig. 1). The same concentration of compound 3 caused inhibition of bacterial biofilm formation in 30% to 50% by 5 of 10 tested strains (Fig. 2).

Biofilm inhibitory activity of tested thiourea derivatives 2 and 3 is the result of inhibiting of multiplication of bacterial cells, in consequence they prevent from biofilm formation and reduce its amount. In the presence of the compound 2 the lower amount of staphylococcal biofilm was produced and it was more susceptible to the mechanical damage and removal of the staining process, as shown in the Figure 5.

The last step was the investigation of mechanism of the antimicrobial activity. Considering the results in the *in vitro* antibacterial assay, we have investigated the inhibitory effect on topoisomerase IV of compounds 2 and 3, showed highest therapeutic potential against strains of *S. aureus* and *S. epidermidis*, including clinically relevant resistant isolates. Compounds 2 and 3 were used in wide range of concentrations (32 μg/ml, 8 μg/ml, 2 μg/ml, 0.5 μg/ml, 0.25 μg/ml).

The affinity of the selected compounds towards bacterial type II topoisomerases, such as topoisomerase IV from *S. aureus* was analysed. The results obtained



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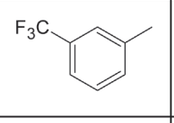
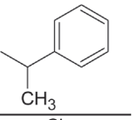
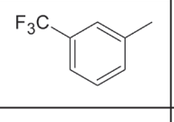
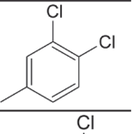
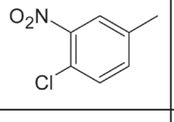
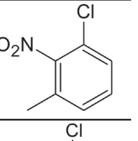
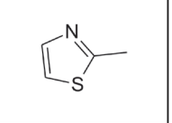
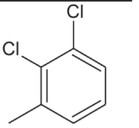
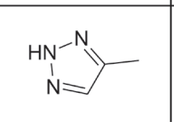
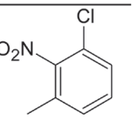
1		
2		
3		
4		
5		

Scheme 1. Synthesis and structure of tested compounds. Reagents and conditions: (i) MeCN, reflux 8 h.

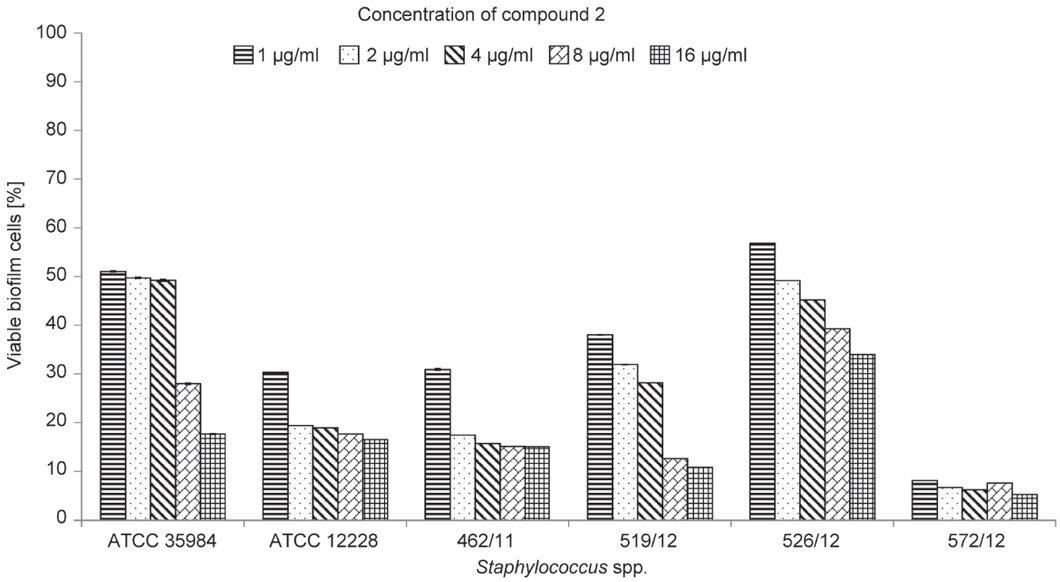


Fig. 1. Inhibitory effect of compound 2 for biofilm formation by standard and selected hospital methicillin-resistant *Staphylococcus* spp. strains.
All presented results are mean from experiments performed in quadruplicate ± S.D.

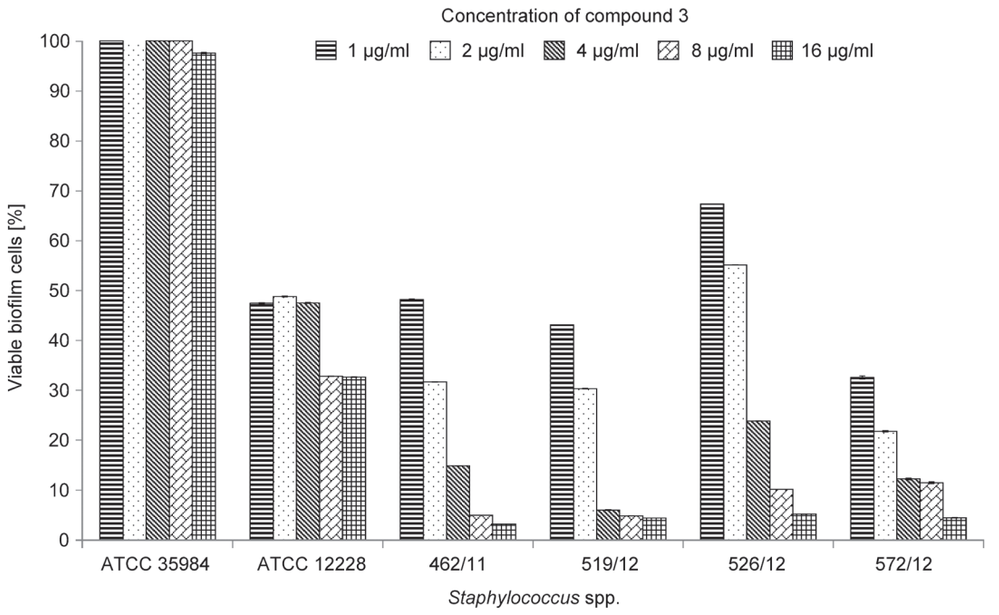


Fig. 2. Inhibitory effect of compound 3 for biofilm formation by standard and selected hospital methicillin-resistant *Staphylococcus* spp. strains.
All presented results are mean from experiments performed in quadruplicate ± S.D.

demonstrated that synthesized compound 2 applied at concentration 32 µg/ml was equally active as Ciprofloxacin, however the compound 3 did not show inhibitory potency towards *S. aureus* topoisomerase IV (Fig. 6). For 3 the inhibition of bacterial type II topoisomerases in not the sole factor responsible for the antibacterial activity.

Discussion

When the effect of the substituent at thiourea nitrogen (N1) was evaluated, it was found that the functionalities could be valued in order of their decreasing influence as follows: 3-trifluoromethylphenyl > 4-chloro-3-nitrophenyl > 1,3-thiazol > 2*H*-1,2,3-tria-

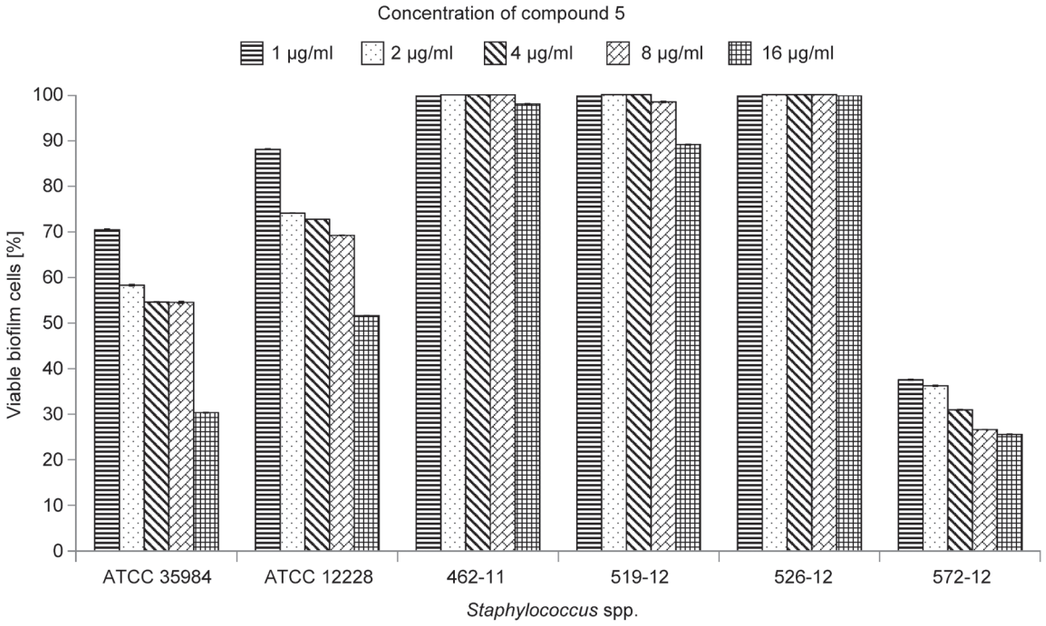


Fig. 3. Inhibitory effect of compound 5 for biofilm formation by standard and selected hospital methicillin-resistant *Staphylococcus* spp. strains.
All presented results are mean from experiments performed in quadruplicate ± S.D.

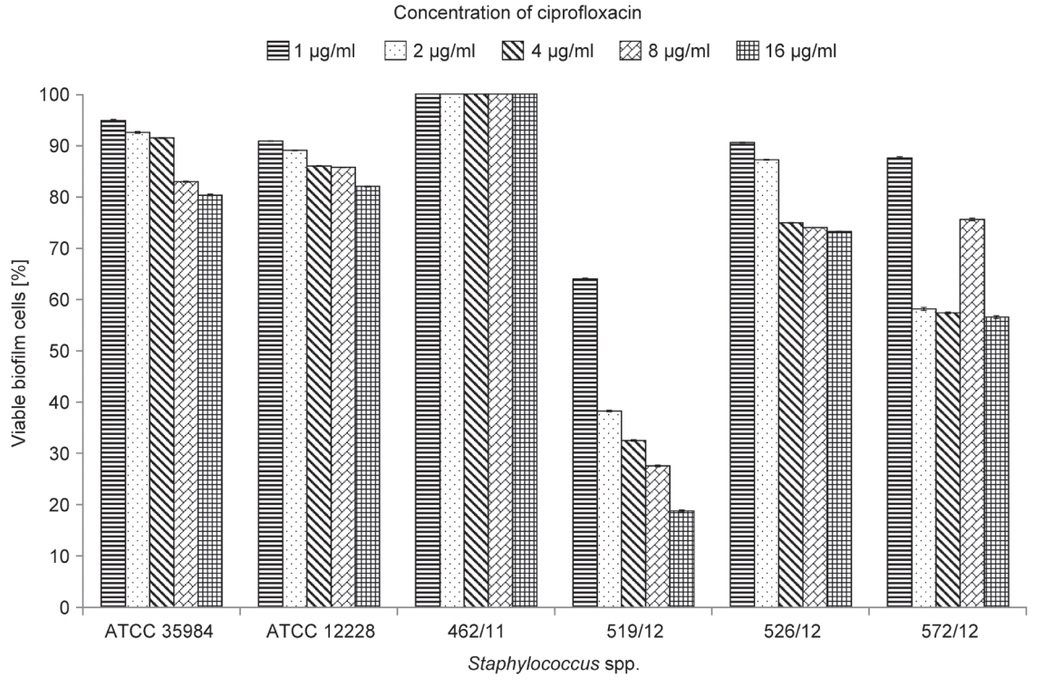


Fig. 4. Inhibitory effect of ciprofloxacin for biofilm formation by standard and selected hospital methicillin-resistant *Staphylococcus* spp. strains.
All presented results are mean from experiments performed in quadruplicate ± S.D.

zol. Interesting was activity comparison of activity of compound 1 and 2 because only substituent connected to N2 nitrogen atom was changed. Antimicrobial activity decreased when between thiourea and aryl substituent alkyl group was introduced. Only compound 2 with trifluoromethylphenyl and 3,4-dichlorophenyl connected to thiourea moiety possessed bactericidal activity (Tables I, II). The rest of tested compounds

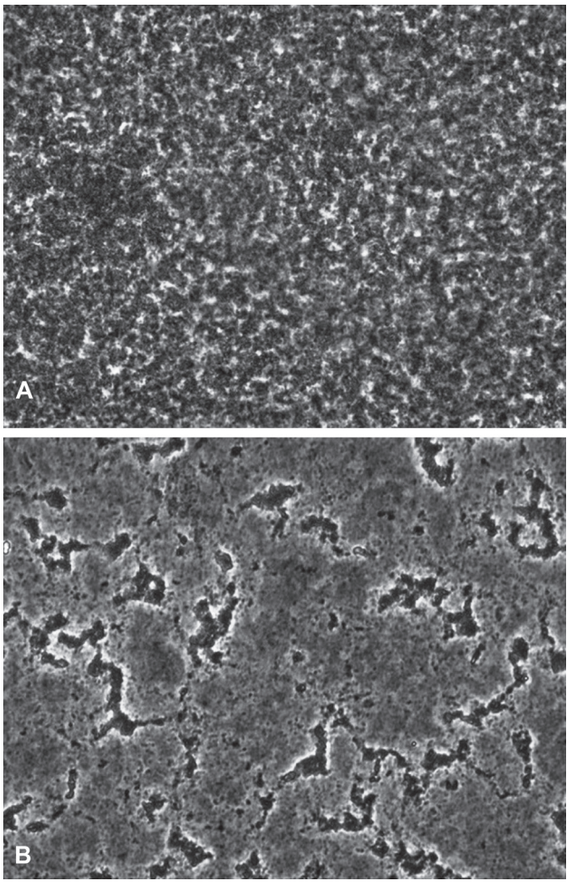


Fig. 5. Biofilms of *S. epidermidis* ATCC 35984 growth for 24 h on polystyrene microtiter plates: A – in TSB medium supplemented with 0.5% glucose; B – in TSB medium supplemented with 0.5% glucose in the presence of compound 2 in concentration 1 μg/ml. The digital images were visualized using the phase-contrast inverted microscope Eclipse TS 100F Inverted Routine Microscopes (Nikon, USA) equipped with the DeltaPix Invenio 5SCII camera using DeltaPixInSigh software.

showed bacteriostatic effect (MBC values much higher compared to the MIC values).

3-(Trifluoromethyl)aniline, 4-chloro-3-nitroaniline, 1,3-thiazol-2-amine, 2*H*-1,2,3-triazol-4-amine were subjected to the reaction with isothiocyanates to obtain thiourea derivatives (Scheme 1). To assure structural variability, different aryl, heteroaryl and aryl connected by methylene group at thiourea moiety were introduced. Whereas unsubstituted 1,3-diphenylthiourea exerts no relevant antimicrobial activity (Cunha *et al*., 2007), howewer its various structural modifications improve the biological effectiveness of a compound (Mishra and Batra, 2013). Literature survey reveals that incorporation of halogen atom(s) within the molecule is one of the most effective strategies to enhance its biopotency, bioavailability and lipophilicity. The fluoro-containing arylthiourea compounds show better activity as compared to other analogues (Suresha *et al*., 2011), however fluoro-methyl substituent on the benzene ring also improve antimicrobial potency (Bielenica *et al*., 2015). According to other authors findings (Chikhalia and Patel, 2009; Saeed *et al*., 2009; 2010; Faidallah *et al*., 2013), the antibacterial and antifungal efficacy depends on the presence of such electron withdrawing (NO_2, Cl, CF_3) substituent at C-2 and C-4 position of the phenyl ring.

In this work in order to assure structural variability, different aryl substituents connected to N1 and N2 atom of thiourea moiety were introduced and the biological activity depended on the structure of new thiourea derivatives.

Potent Gram-positive antibacterial activity of several analogs of thiourea, urea (Ehmann and Lahiri, 2014) and thiosemicarbazide derivatives (Siwek *et al*., 2011) is explained by an inhibition of the catalytic site of bacterial type II topoisomerases, in particular DNA gyrase and topoisomerase IV. Topoisomerase IV is a bacterial type II topoisomerase that is essential for proper chromosome segregation and is a target for quinolone-based antimicrobial agents, such as Ciprofloxacin and Levofloxacin (Fournier *et al*., 2000).

Fluoroquinolones stimulate topoisomerase IV-mediated DNA cleavage both by increasing rate of DNA scission and by inhibiting relegation of cleaved DNA. As a result, quinolones inhibit the overall catalytic activity of topoisomerase IV by interfering with enzyme-ATP interactions (Anderson *et al*., 1998). Presented preliminary results showed that thiourea-derived compounds presented in this study were able to inhibit the activity of bacterial topoisomerases, such as *S. aureus* topoisomerase IV.

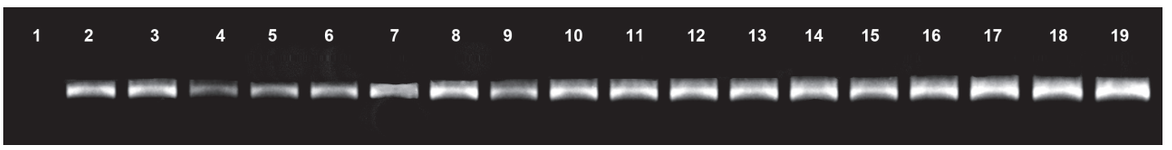


Fig. 6. The effect of studied compounds on topoisomerase IV in decatenation assays: 1 – control assay without enzyme; 2 – *S. aureus* topoisomerase IV control; 3 – *S. aureus* topoisomerase IV control with DMSO; 4 – Ciprofloxacin (CFX) (96 μg/ml), 5 – CFX (32 μg/ml), 6 – CFX (8 μg/ml), 7 – CFX (4 μg/ml), 8 – CFX (2 μg/ml); 9 – compound 2 (32 μg/ml), 10 – comp. 2 (8 μg/ml), 11– comp. 2 (4 μg/ml), 12 – comp. 2 (1 μg/ml), 13 – comp. 2 (0.5 μg/ml), 14 – comp. 2 (0.25 μg/ml); 15 – comp. 3 (32 μg/ml); 16 – comp. 3 (8 μg/ml), 17 – comp. 3 (4 μg/ml), 18 – comp. 3 (1 μg/ml), 18 – comp. 3 (0.5 μg/ml).

It is known, that 3-(trifluoromethyl)phenyl]thiourea derivatives, close analogs of 1 and 2, are not cytotoxic against normal (HaCaT) cells (Bielenica *et al*., 2015). They do not considerably decreased viability and have no visible influence on the mortality of tested cells. Similar tests for thiourea compounds presented in that paper will be conducted in the near future. To exploit their antibiofilm properties, the title compounds could be used to cover the surface of biomaterials or medical devices.

Acknowledgments

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Conflicts of interest

All authors declare, that there are not any potential conflicts of interest.

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SHORT COMMUNICATION

Expiration Date Prediction of Biocontrol Agent Prepared with *Bacillus subtilis* B579 Using the Accelerated Aging Method

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Abstract

The expiration date of biopesticidal products is an essential feature of their use and storage. In the present work, the expiration date of biocontrol agent was predicted using the accelerated aging method. The available bacteria in *Bacillus subtilis* B579 biocontrol agent were $3.7 \pm 0.2 \times 10^{11}$ CFU/g. It is calculated that the expiration date of the agent was about 17 months at 25°C. During this period, the available bacteria retained more than 90% of the value in the initial product. Thus, this work suggests the expiration date of biocontrol agents composed with spores could be estimated using the accelerated aging method.

Key words: *Bacillus subtilis*, accelerated aging, biocontrol agent, expiration date

Plant diseases caused by soil-borne pathogenic fungus, such as *Fusarium* and *Pythium*, affect vegetables and crops growth and yield worldwide, and even result in significant economic loss (Noble and Coventry, 2005; Asad *et al*., 2014). A synthetic chemical fungicide has long been used for reducing the incidence of soil-borne plant diseases. However, it causes soil, water and air pollution, and is harmful to the health of people (Pieters and Vlietinck, 2005). With increasing attention to environmental protection and the increasing market demand for organic products, biological control exhibits excellent performance (Brannen and Kenney, 1997; Zheng *et al*., 2013).

Some antagonistic microorganisms, such as *Bacillus*, *Pseudomonadaceae*, *Agrobacterium* and *Pasteurella*, can produce antimicrobial compounds which have been proved to be quite effective in controlling various plant diseases (Shrestha *et al*., 2014). Among them, *Bacillus subtilis* is one of the most effective microbes in controlling plant diseases (Nagórska *et al*., 2007). It has been reported that *B. subtilis* can not only inhibit the growth of pathogenic fungi by producing various lytic enzymes and antibiotics, but also induce systemic plant resistance by increasing the activities of several enzymes related to plant defense (Baysal *et al*., 2008; Choudhary and Johri, 2009; Santoyo *et al*., 2012). Furthermore, it is easy to cultivate and stabilize in the environment by forming spores (Lalloo *et al*., 2009). These characteristics provide *B. subtilis* with potential advantages over the other antagonistic microorganisms in the production and preservation of biocontrol agents (Chen *et al*., 2010b; Nagórska *et al*., 2007). It must be recognized that the efficacy of biocontrol agent prepared with antagonistic bacteria largely depends on the count of viable bacteria. One of the persistent challenges is assuring acceptable stability in the development of a biocontrol agent. The allowable level of loss of viable bacteria in biocontrol agent should be generally less than 10%.

In our previous work, a biocontrol bacterium, *B. subtilis* B579, which exhibited obvious growth inhibition effect against pathogens, *Rhizoctonia solani*, *Fusarium gramirum*, *Fusarium solani*, *Fusarium oxysporum* and *Phytophthora capsici*, was obtained (Chen *et al*., 2010a; Chen *et al*., 2013). In the present study, the relation of viable bacteria to the storage period and temperature of biocontrol agent of *B. subtilis* B579 was calculated using the accelerated aging method.

The strain *B. subtilis* B579 was maintained on a slant of LB agar medium at 4°C. Seed medium was comprised of (g/l) peptone 10, glucose 5, yeast extract 5, beef extract 5 and NaCl 5 (pH 7.0). Fermentation medium used for batch cultivation was comprised of

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(g/l) yeast extract 6, soluble starch 11, glucose 6, soy bean meal 13.9, beef extract 8.9 and maize meal 12.6 (pH 7.0). The seed culture was performed in a 250 ml Erlenmeyer flask containing 30 ml of seed medium at 37°C and 180 rpm for 12 h. A 7 l bioreactor (Baoxing Biotech Ltd, Shanghai, China) with a working volume of 5 l was used for *B. subtilis* B579 batch fermentation with 5% inoculums volume from seed culture. Cultivation temperature, aeration rate and the agitation rate were maintained at 37°C, 2 l/min and 500 rpm, respectively. The pH was adjusted with 1 mol/l NaOH when required. The fermentation was stopped after 26 h cultivation, and the fermentation broth with a cell concentration of $1.1 \pm 0.1 \times 10^{10}$ CFU/ml was obtained. The sporulation ratio was about 90%, which is the ratio of spores to the total cell concentration measured according to Chen *et al.* (2010b).

The fermentation broth was treated by centrifugation at 8000 × g for 20 min. The cell pellet was washed with deionized water, and then resuspended in 2 l deionized water. The dextrin, sucrose and sodium glutamate were added as thermal protectants to the concentrations of 12.5 g/l, 12.2 g/l and 3.7 g/l, respectively. Diatomite (particle size 20–60 μm) was added to 10.0 g/l as the carrier. The biocontrol agent of *B. subtilis* B579 was prepared by spray drying with entrance temperature of 120°C, outlet temperature of 70°C, pump rate of 60 ml/min and the atomization controlled at an air pressure of 0.1 MPa. For assaying the concentration of available cells, 100 μl of an appropriate dilution of biocontrol agent was spread on solid LB media with 3 replicates, and then the colony forming units were counted. The viable cell count of the agent was $3.7 \pm 0.2 \times 10^{11}$ CFU/g.

The cylinder-plate method was used to verify the inhibitory effect of biocontrol agent against *F. oxysporum* (Nagórska *et al.*, 2007). The pathogen was incubated in the centre of PDA plates at 28°C for 2 days. Then, 200 μl of fermentation broth, biocontrol agent, negative solution (agent without *B. subtilis* B579) or sterile deionized water was added into the oxford cup. The concentrations of biocontrol agent and negative solution were 1 g/l. The fermentation broth was diluted to 3.7×10^{11} CFU/l which was the same as that of biocontrol agent solution. After 2 days incubation at 28°C, their inhibition effects were compared.

The antagonism of biocontrol agent (1 g/l) against *F. oxysporum* was determined on PDA plates using the dual plate assay method (Tang *et al.*, 2010). Sterile deionized water served as a control. Fungal radial growth was measured with vernier calliper. The fungitoxicity was recorded in terms of percentage growth inhibition which was determined as $[(Dc - Dt)/Dc] \times 100\%$. Where Dc was the average increased diameter of the fungal colony in control, and Dt was the average increased diameter of the fungal colony in treatment (Amini *et al.*, 2010). Three replications were carried out for each treatment.

The expiration date of the biocontrol agent of *B. subtilis* B579 was predicted by the accelerated aging method (Waterman and Adami, 2005). The expiration date was defined as the time when the survival cell number of the agent was 90% of the initial cell number.

The inactivity of *B. subtilis* B579 cells follows the first order kinetics process. Under certain temperature, the equation can be represented as the equation (1).

$$\text{Log } N_t = \log N_0 - k_T \cdot t/2.303 \quad (1)$$

N_0 is the initial cell concentration of agent, and N_t is the concentration of survived cells at time t. k_T is the rate constant under the temperature T. The accelerated test was conducted at temperatures of 50°C, 60°C, 70°C, 80°C and 90°C. At each temperature, the survived cell number was detected at 0 day, 1 day, 2 day, 3 day, 4 day, 5 day, and 6 day, respectively. The k_T at each temperature could be obtained based on the linear regression between log N_t and t.

Arrhenius exponential law is the theory evidence of accelerated aging test, of which the logarithmic form can be expressed as equation (2).

$$\text{Log } k_T = (-Ea/2.303\ R)\cdot(1/T) + \log A \quad (2)$$

Where, k_T is the rate constant calculated from equation (1) at temperature T, Ea stands the activity energy, and R is gas constant. A regression equation could be obtained based on the linear relation between log k_T and 1/T. For example, the k_{25}, the rate constant at 25°C, could be calculated from log k_{25}. Thus, the period for 10% cell inactivity of the agent at 25°C could be calculated according to the equation (1), namely, the expiration date of the biocontrol agent.

As shown in Fig. 1, the inhibition zones of biocontrol agent, fermentation broth, negative solution and sterile deionized water were 10.0 ± 0.7 mm, 11.3 ± 0.6 mm, 0.5 ± 0.1 mm and 0 mm (no inhibition), respectively. The negative solution which was used for agent preparation showed little inhibitory effect on *F. oxysporum*. The inhibition zone of biocontrol agent was a little less than that of fermentation broth because some lytic enzymes such as chitinase and β-1, 3-glucanase were produced during the fermentation (Chen *et al.*, 2010a). This result indicated that the B579 biocontrol agent could effectively inhibit the growth of plant pathogen *F. oxysporum*. The inhibition effect of $72.4 \pm 4.2\%$ against *F. oxysporum* was obtained at 1 g/l of the biocontrol agent.

Temperature is one of important factors determining the survival of *B. subtilis* B579 in the agent. In this study, temperatures were set as 50°C, 60°C, 70°C, 80°C and 90°C, separately, to investigate the expiration date of biocontrol agent using the accelerated aging method. The number of survival cells in the agent at

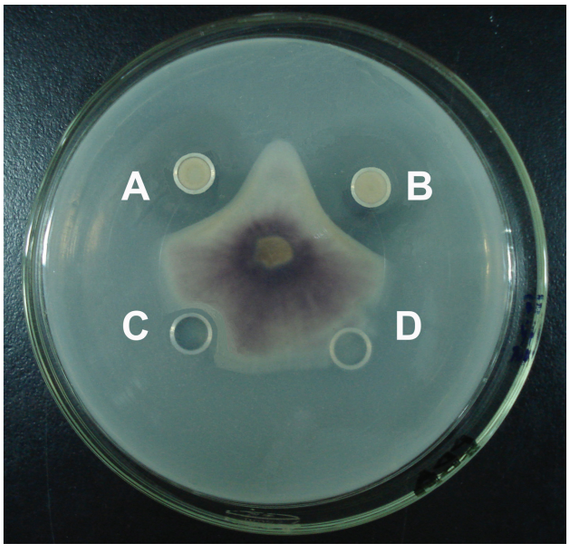

Fig. 1. Comparison of the inhibitory effect.
A – biocontrol agent of *B. subtilis* B579 (1 g l^{-1}, 3.7×10^{11} CFU l^{-1});
B – fermentation broth of B579 (diluted to 3.7×10^{11} CFU l^{-1});
C – negative solution (1 g l^{-1});
D – sterile deionized water.

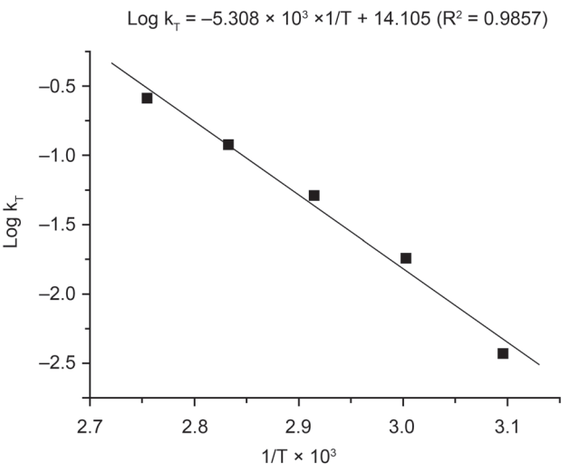

Fig. 2. The regression equation of Log k_T with 1/T.

variable temperatures was detected, and the results are listed in Table I. The inactivity rate of cells in the agent increased with the increase of temperature. The regression equation under different temperature conditions was obtained as shown in Fig. 2. The rate constant at temperature 25°C, K_{25}, was calculated as 2.042×10^{-4} d^{-1}. Therefore, $t_{0.9}$, *i.e.* the time when the number of surviving cells was 90% of the initial agent, was calculated as 514 d according to the equation (1). The expiration date of the *B. subtilis* B579 biocontrol agent was predicted as 17 months at 25°C.

The accelerated aging method is usually used for predicting the expiration date of drugs in a relatively short time (Waterman and Adami, 2005). To our knowledge, few studies have been reported on the expiration date prediction of biocontrol agent prepared with antagonistic bacterial cells. Commonly, the inactivity of bacteria cells is different from that of chemical drugs because most bacteria are sensitive to heat. However, spores of *B. subtilis* are heat resistant, and are in a state of metabolic dormancy (Setlow, 1994). Luckily, the inactivity of agent containing *B. subtilis* B579 spores follows the first order kinetics process. The R^2 of regression equation under 50°C, 60°C, 70°C, 80°C and 90°C was 0.9811, 0.9913, 0.9847, 0.9768 and 0.9733, respectively. Thus, the inactivity kinetics of biocontrol agent prepared with spores could be analyzed using Arrhenius equation. The present work suggests that the expiration date of biocontrol agent prepared with spores of antagonistic bacteria could be predicted with the accelerated aging method which is effective and timesaving.

Nevertheless, additional work is required, since the inhibition effect of fermentation broth against *F. oxysporum* was a little more than that of biocontrol agent with the same cell number. Besides chitinase and β-1, 3-glucanase, some antibiotics (Iturin A and Surfactin) that were thermal stable were identified from fermentation broth of *B. subtilis* B579 by the method of HPLC-MS (date not shown). Therefore, a more efficient biocontrol agent, containing antibiotics that are produced during the fermentation, might be obtained by spray drying of fermentation broth.

Table I
Cell survival of *B. subtilis* B579 biocontrol agent under different temperature conditions

Temperature	Time (days)						
	0	1	2	3	4	5	6
	Logarithm of relative cell concentrations						
323 K (50°C)	2.000	1.999	1.998	1.995	1.994	1.993	1.990
333 K (60°C)	2.000	1.995	1.987	1.979	1.973	1.961	1.954
343 K (70°C)	2.000	1.983	1.969	1.937	1.906	1.890	1.875
353 K (80°C)	2.000	1.977	1.931	1.891	1.832	1.763	1.695
363 K (90°C)	2.000	1.949	1.833	1.747	1.659	1.464	1.333

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SHORT COMMUNICATION

Evaluation of the Levels and Quality of Microbial Contamination in Medical Emergency Departments in Comparison to Other Workplaces

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Abstract

Work in Hospital Emergency Departments (HEDs) exposes both the emergency ward staff and patients to infectious and in other way harmful biological agents. The results of this study shows the presence of pathogenic bacteria isolated by three different methods. It revealed 9.8% of pathogens detected by imprint method, 10.5% of pathogens by swabbing method, 17.6% and 22% in HEDs corridors and rooms, respectively, by air sampling method. In control workplaces (offices) pathogenic bacteria reached the level of 6.5% and 14.7% by imprint method and swabbing, respectively. The relatively low level of contamination by bacteria in HEDs may depend on the effectiveness of Standard Protective Precautions in the studied hospitals.

Key words: harmful biological agents, Hospital Emergency Department, antimicrobial resistance, microbiological monitoring surface and air samples

In accordance with the Act of 8 September 2006 on State Emergency Medical Services, the Hospital Emergency Department (HEDs) is a hospital ward whose main task is to provide comprehensive health services to adults and children in cases of sudden or life-threatening injuries or illnesses (isap.sejm.gov.pl) (ISAP, 2011). HEDs activities are focused mainly on stabilizing the patients, providing an initial diagnosis and transporting the patients to specialised wards for further treatment.

The character of HEDs work exposes both the emergency ward staff and patients to the presence of dangerous biological agents; however, the level of microbiological risk is not only related to the specifics of HEDs' functioning but it is also to the manner of performing the medical procedures by healthcare professionals. Followed correctly and in accordance with sanitation standards, medical procedures ensure security for both patients and medical personnel at the initial stage of treatment. The level of microbiological safety is further enhanced by ensuring that hospital environment is free of microorganisms capable of causing infection.

Pathogenic microorganisms in hospital environments such as HEDs are located generally on the surfaces of medical devices, as well as on the surfaces in direct contact with patients (walls, bed frames, medical devices switches, sinks *etc.*) and may pose the risk of hospital acquired infections (HAIs). The most common routes of transmission of potential pathogens are: direct contact with infected hospital personnel and contact between patients (cross transmission). Inaccurate cleaning of rooms or incorrect disinfection of medical equipment contributes to the spreading of pathogens through the devices routinely used in diagnosis, treatment and rehabilitation (Fiedotow and Denys, 2006). Bacteriological monitoring of hospital environment enables detection and differentiation of the colonizing and infective bacterial flora and provides the basis for effective empiric antibiotic therapy and eradication of the microorganisms from the ward environment (Maszkiewicz, 2007).

Systematic surveillance of infections, mainly by identifying the etiological agents of HAIs, enables identification of the most commonly found microorganism,

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posing a threat not only to the patient but also to the whole hospital environment (Heczko and Wójkowska-Mach, 2009).

This study used a random sampling strategy in HEDs environment and in a control workplaces (offices) not exposed to contact with harmful biological agents. The samples were taken from hospital surfaces (floors, walls, tables) and irregular, hard to access or frequently used surfaces (ventilation grilles, keyboards, medical device buttons, pens *etc.*) that may pose a risk of possible transfer of pathogenic microorganisms.

Environmental samples for this research were collected in the autumn and winter of 2014. All materials were sampled from areas with normal work system in HEDs in the presence of patients, visitors and medical personnel. Sampling apparatus was placed at height of around 0.8–1.2 m in a representative location of the examined spaces. The examined areas were varied in relation to the capacity and numbers of beds. All rooms were equipped in gravity ventilation systems. In total, 90 samples were collected in 10 Hospital Emergency Departments, including 20 air samples (HED's rooms and corridors) and 30 imprint samples; the last were taken in order to assess the total number of microorganisms. The remaining 40 samples were collected by swabbing high risk surfaces, *e.g.* door handles, keyboards, badges, pens. A similar sampling algorithm was adopted in the analysis of microbiological contamination of control workplaces, where a total of 80 samples were collected, 70 samples from surfaces (30 – imprints, 40 – swabs) and 10 air samples. In total, 170 environmental samples were collected from HEDs and offices. The biological material from imprinting samples Count-Tact® 3P™ Agar-CT3P (bioMérieux, France), together with samples collected using swabs, was inoculated on selective culture media and prepared in accordance with the generally accepted microbiological diagnostic procedures.

Air sampling was carried out with the assistance of a Coriolis Recon apparatus (Bertin, France); this technic collects a large amount of biological particles (0.5–10.0 μm) in a liquid sample at a flow rate of 6 m^3 (for 10 min) and allows preparation of a highly representative sample for further microbiological analysis.

Air samples were filtered through a 0.22 μm filter (Merck Millipore, Poland) using the filtering apparatus vacuum pump P504 Millipore (Merck Millipore, Poland). The filter was placed on the surface of Columbia agar medium with 5% sheep blood (Graso Biotech, Poland), and the culture of the biological material was incubated at 37°C for 24 h; the following stages were carried out in accordance with the generally accepted diagnostic scheme. Identification and testing the strains of bacteria for susceptibility to various groups of antibiotics was carried out using an automated system Vitek 2 Compact (bioMérieux, France) following the manufacturer's instructions. For confirmation of antimicrobial resistance of bacterial isolates the disc diffusion method was used according to actual EUCAST recommendation (www.korld.edu.pl/spec-rekomendacje-eucast.php). Controls for the study were conducted using following reference strains: *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603, *Streptococcus pneumoniae* ATCC 49619.

Statistical evaluation of the obtained results by three methods was done by Mann-Whitney test because the analysed data have not passed Shapiro-Wilk normality test (GraphPadPrism, USA). The difference is statistically significant at $P < 0.05$.

Harmful biological agents are a significant problem in occupational medicine and environmental health. Identification and characterization of the agents in the hospital environment makes it possible to conduct a reliable risk assessment of biological hazards to medical emergency staff in HEDs (Kramer *et al.*, 2006). The standards of assessment of the microbiological hygiene status of contact surfaces in medical institutions were suggested by the US Department of Agriculture (< 5 cfu/cm^2 and < 1 cfu/cm^2 for indicator organisms: *S. aureus*, *Clostridium difficile*, vancomycin-resistant *Enterococcus* (VRE), multidrug resistance (MDR) Gram-negative *Bacilli*, *Salmonella* spp., *E. coli* O157) (Dancer, 2004). According to these standards, the status of contact surfaces contamination (floors, walls, tables) in the examined HEDs falls within the permissible norm (Table I). All examined surfaces in HEDs *vs.* offices, except floors, revealed statistically significant differences. In similar studies concerning the levels of biological contamination of small scale medical equipment *e.g.* stethoscope (Shiferaw *et al.*, 2013), a significant degree of micro-contamination was revealed (micro-contamination ≥ 20 cfu per membrane; the equivalent of approx. 2 cfu/cm^2). In the light of the above, the hygiene status of the examined contact surface in HEDs seems to be satisfactory. It may be the result of correct implementation of the Standard Protective Precautions and effective disinfection and decontamination procedures (Dancer, 2004). Microbiological contamination of air in HEDs rooms and corridors was on average at the level of 2.1×10^1 cfu/m^3 and 3.8×10^1 cfu/m^3, respectively. The mean value of air micro-contamination in offices was 1.4×10^1 cfu/m^3 (bacteria) (Table I).

According to the research of the past few decades, micro-contamination of air in hospital rooms varies depending on the sterility class of the examined hospital rooms *e.g.* from 7.0×10^1 cfu/m^3 for bacteria and fungi as the highest acceptable concentration in

Table I
Total number of bacteria from tested sites

Tested sites	Imprint samples (cfu/25cm^2)						
	HEDs; n* = 30			Offices; n* =30			P value**
	Mean values ± SD	Median values	Min.–Max.	Mean values ± SD	Median values	Min.–Max.	
Tables	$8.7\times10^1\pm8.4\times10^1$	5.5×10^1	18 – 260	$2.6\times10^1\pm2.9\times10^1$	1.3×10^1	2 – 90	0.0147
Floors	$1.3\times10^2\pm1.0\times10^2$	1.1×10^2	16 – 300	$8.4\times10^1\pm9.8\times10^1$	4.4×10^1	4 – 300	0.1502
Walls	$3.5\times10^1\pm3.4\times10^1$	1.9×10^1	6 – 100	$1.0\times10^1\pm7.5\times10^0$	1.0×10^1	0 – 20	0.0310
Total	$8.6\times10^1\pm8.6\times10^1$	6.2×10^1	6 – 300	$4.0\times10^1\pm6.6\times10^1$	1.6×10^1	0 – 300	0.0009
Air samples (cfu/m^3)							
	HEDs; n* = 20			Offices; n* = 10			
Air	$2.8\times10^1\pm1.9\times10^1$	2.8×10^1	2 – 70	$1.3\times10^1\pm1.2\times10^1$	8.0×10^0	3 – 42	0.0365

* n = number of samples ** – calculated with Mann-Whitney test

a neurosurgical wards to 7.0×10^2 cfu/m^3 as the highest acceptable concentration in treatment rooms (Górny, 2004). Other examples of microbiological assessment analyses are presented by Rubino (1995), according to whom, the level 0–2.3×10^1 cfu/m^3 is pronounced as very good and the value $>3.75\times10^2$ cfu/m^3 marks a highly unsatisfactory level of microbiological contamination (Charkowska, 2003).

According to new surveys the trends of microbiological air contamination are for an improvement of quantitative and qualitative status of bio-aerosols of the hospital environments (Ekhaise *et al*., 2008; Hoseinzadeh *et al*., 2013; Mirzaei *et al*., 2014).

Microbiological air quality is frequently monitored in sterile hospital rooms (where the risk of infection is highest) however, there is little data about air microcontamination of hospital corridors. Some research was presented by Park *et al*. (2013), who registered the air microbiological contamination in hospital corridors at the level 7.2×10^2 cfu/m^3 for the bacteria. The values found here are much higher than the ones registered in "clean rooms" (*e.g*. operation rooms or haematology, pulmonology, obstetrics and gynaecology wards). The hospital wards with the highest level of sterility such as surgical and transplant units present the microbiological contamination in the range from 0.01–1×10^1 cfu/m^3 (Park *et al*., 2013).

However, in our research, both HEDs rooms and corridors represent the microbiological contamination at the level of 2.7×10^1 cfu/m^3, which places them within the limits of accepted standards (Gołofit-Szymczak *et al*., 2013). Also in the research conducted by Augustowska and Dutkiewicz (2006) concentrations of bacteria in the air assumed values from 1.0 to 9.6×10^1 cfu/m^3.

Relatively low concentrations of microorganisms detected in the hospital environment (contact surfaces and air) may result from improved levels of hygiene in hospitals due to infrastructure development (air conditioning and highly efficient HEPA filters) and implementation of modern and highly effective sanitary procedures. Another preventive measure reducing microbiological contamination in the hospital environment is the implementation of hospital infection control teams. This study, carried out in the winter, achieved quantitative results similar to the values observed by other researchers who conducted their tests in the same season (NSI, 2009; 2010; 2011).

In assessing the microbiological quality of samples, this research found mainly non-fermentative Gram-negative *Bacilli* (NFGNB) and *Stenotrophomonas maltophilia*, naturally resistant to many broad-spectrum antibiotics (*e.g*. β-lactams including all carbapenems) and coagulase-negative *Staphylococci* (CoNS) with constitutive macrolide-lincosamide-streptogramin B ($cMLS_B$) and macrolide-streptogramin B (MS_B) phenotypes (Table II).

These bacteria may constitute a considerable risk of HAIs due to their resistance to a large number of antibiotics and antimicrobial therefore cause treatment problems; the bacteria may be classified as potentially pathogenic agents. In the examined contact and swab samples collected from HEDs, NFGB were prevalent. In air samples, besides the mentioned NFGNB *Acinetobacter* spp. and *S. maltophilia* (mainly isolated from HEDs corridors) large numbers of CoNS with constitutive MLS_B (especially in HEDs rooms) were found. In the tested air samples from HED corridors mainly NFGNB were found; in HEDs rooms CoNS with constitutive MLS_B were isolated. In hospital offices contact and swab samples contained mainly CoNS; in the air samples no pathogens were detected (Table II). Comparison of the proportional presence of pathogenic bacteria isolated by three different methods revealed 9.8% of pathogens detected by imprint method and 10.5% of pathogens by swabbing method; and in relation to air sampling 17.6% and 22% for HEDs corridors and rooms, respectively. In offices pathogenic bacteria reached the level of 6.5% and 14.7% for imprint method

Table II
The percentage of potential pathogens in relation to the number of all isolates

Places	Samples	NFGNB (non-fermentative Gram-negative *Bacilli*)		CoNS (coagulase-negative *Staphylococci*)	
		Species	%	Species	%
HEDs	Imprints (n = 30)	**B. cepacia*	2.0	[1]*S. saprophyticus*	3.9
		S. maltophilia	2.0		
		A. lwoffii	2.0		
	Swabs (n = 40)	*A. lwoffii*	2.8	Not detected	
		A. haemolyticus	2.8		
		A. radioresistens	2.8		
	Air (n = 10) Corridor	*S. maltophilia*	10.0	Not detected	
		***A. baumannii cplx*	5.0		
		A. lwoffii	5.0		
	Air (n = 10) Rooms	Not detected		[2]*S. haemolytcius*	2.6
				[2]*S. hominis*	2.6
				[3]*S. lentus*	2.6
Offices	Imprints (n = 30)	*A. lwoffii*	11.4	*S. hominis*	2.9
		A. baumannii	2.0	*S. haemolyticus*	2.9
				S. warnerii	2.9
	Swabs (n = 30)	Not detected		*S. warnerii*	6.6
				S. hominis	6.6
	Air (n = 10)	Not detected		Not detected	

* isolate with ESBL resistance phenotype (alert-pathogen), **multidrug resistance alert-pathogen, n – number of samples, [1] 1 isolate with MS_B and 1 isolate with $cMLS_B$ and methicilin-resistant coagulaso-negative *Staphylococci* MRCNS, [2] isolate with $cMLS_B$, [3] isolate with MRCNS and $cMLS_B$

and swabbing, respectively. In the air samples pathogenic microorganisms were not detected.

Widespread usage of antibiotics, both in hospitals and ambulatory treatments, has led to the selection of pathogens with varied phenotypes of antimicrobial resistance *i.e*. alert-pathogens. Among the pathogens isolated from contact and air samples collected in HEDs, this study detected two multidrug-resistant *Acinetobacter baumannii* (MRAB) and producing extended sepctrum of β-lactamases *Burkholderia cepacia* (ESBL+) (Table II). According to the Ordinance of the Polish Ministry of Health, December 23rd, 2011 (List of alert pathogens, Attachment 1) these belong to alert pathogens (isap.sejm.gov.pl).

Literature concerning microbiological quality assessment discusses mainly research conducted in wards requiring long-term hospitalising *e.g.* intensive care, internal, haematology, urology, ophthalmology and surgical wards (Kępa *et al*., 2012; Nourmoradi *et al*., 2012; Paluchowska *et al*., 2012a; 2012b; Guzek *et al*., 2013; Hoseinzadeh *et al*., 2013; Seweryn *et al*., 2014). In this study, the bacteriological quality analysis of isolates from hospital environment (HEDs) correlates with the profiles of potentially pathogenic species determined by other researchers in similar tests. For example, in other studies the main bacterial isolates responsible for microbiological contamination of contact surfaces in hospitals (floors, medical tables, couches, washbasins) were methicillin-resistant *S. aureus* (MRSA), VRE, *Clostridium difficile*, multidrug-resistant *Acinetobacter* spp., *Pseudomonas* spp., *Enterococcus* spp., and additionally Enterobacteriaceae ESBL+ (*E. coli, Enterobacter* spp., *Salmonella* spp. and *Klebsiella* spp.) (Kramer *et al*., 2006; Garcia-Cruz *et al*., 2012; Weber *et al*., 2013; Seweryn *et al*., 2014).

According to Paluchowska *et al.* (2012b) the largest proportion of HAIs caused by alert-pathogens is registered in intensive care wards, burns units, internal, haematological and surgical wards. The pathogens isolated most often were multi-drug resistant, NFGNB (mainly *A. baumannii* and *P. aeruginosa*) which are recognised as the most problematic to control and eradicate (Paluchowska *et al*., 2012b). CoNS and *Enterococcus* spp. are the key factors in HAIs and they are mostly isolated form surgical, haematological and oncology wards (Guzek *et al*., 2013; Nourmoradi *et al*., 2012; Hoseinzadeh *et al*., 2013). Additionally MRCNS and NFGNB isolated from urology wards may constitute sources of infection (Kępa *et al*., 2012).

The results quoted above show that the diversity of microorganisms detected in this study is comparable

with the standard trends. In quality testing of control samples alert-pathogens were not detected; however, potentially pathogenic strains (mainly CoNS) were found. The relatively low level of contamination by bacteria in HEDs may attest to the effectiveness of implementing the standard protective precautions in the examined hospitals. In quality assessment of HEDs the main bacterial strains found were NFGNB and CoNS; the presence of the bacteria may result from person to person transmission or introduction of pathogens from outside the hospital.

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SHORT COMMUNICATION

The Usefulness of Biosynthetic Vascular Graft Omniflow II and Autologous Veins for the Treatment of Massive Infection of Dacron Vascular Graft with *Enterococcus faecalis* HLAR

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Abstract

Infections of vascular grafts are the most severe complications in vascular surgery. We present the case of a 73-year-old male with infection of a dacron prosthesis with a strain of *Enterococcus faecalis*. The patient was treated with replacement of a full prosthesis with a combined graft constructed from Biosynthetic Vascular graft Omniflow and autologous veins. This graft is recommended for implantation in patients with a higher risk of infection. Our case is one of the first reported usage of this kind of graft in the aortic region and in a 2 years observation period no recurrence of infection was observed.

Key words: *Enterococcus faecalis*, abdominal aortic surgery, Omniflow II, vascular graft infection, vascular prosthesis

Infections of synthetic vascular grafts are undoubtedly the most severe complications in vascular surgery and despite the continuous advancement of the medical science, the results of treatment of such complications have not improved. The percentage of amputations and deaths among these patients continues to remain at a high level, reaching 20–30% (Samson *et al*., 1988). The risk of an infection of a graft in aorto-femoral section has been described as 0.5–3% (Swain *et al*., 2004) and it is extremely closely related to the place of implantation. The likelihood of a graft infection is moreover increased by a number of factors such as extended time of an operation, the presence of different catheters and drains, intra- and postoperative chyle leak, coexisting infections, immunodeficiency conditions, advanced age, malnourishment, diabetes mellitus or chronic immunosuppression. The lack of observance to the principles of aseptic techniques also has an impact.

The most common etiological factors are *Staphylococcus* strains which are responsible for more than 53% of all aorto-femoral infections. They produce glycocalyx, which on the one hand allows adhesion to the graft surface and on the other constitutes a component of biofilm providing protection against phagocytosis, antibodies and antibiotics (Bandyk *et al*., 1991). Gram-positive cocci of *Enterococcus* sp. cause an infection to a much lesser extent in grafts in the aorto-femoral location. Several decades ago they were considered relatively pathogenic – little virulence and minor percentage of infections combined with their common occurrence in a human environment. The wide implementation of cephalosporins, to which Enterocci has a natural resistance, has led to their domination in gastrointestinal microbiota, which resulted in a higher rate of severe infections caused by these bacteria among hospitalized patients. Moreover, long-term application of a combination of penicillin and high concentrations of aminoglycosides in treatment of endocarditis caused by enterococci has led to the formation of HLAR strains. In turn, the extensive use of vancomycin in hospital therapy (for prevention and treatment of MRSA infections) with time resulted in the evolution of VRE strains (with varying degrees of resistance to vancomycin and teicoplanin). Of course, there are other determinants that are responsible for the pathogenicity of enterococci, including the ability to produce cytolysin (hemolysin), gelatinases, a serine protease, collagen binding protein (Ace) and fibrinogen (SagA), aggregation substance (AS), the extracellular surface protein (Esp), the cell wall adhesin (protein Efa), peroxides, hyaluronidase, lipoteichoic acid, DNase, lipase and polysaccharide capsule. The presence of these factors results in

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Fig. 1. Biosynthetic vascular graft Omniflow II

Fig. 2. Bifurcated prosthesis created by connecting two arms Omniflow II

the ability of adhesion, colonization of the graft's surface and formation of protective biofilm. The dominant species among enterococci causing these infections are strains of *Enterococcus faecalis*, which comprise 70–80% of the strains (Bronk and Samet, 2008).

A major dilemma faced by a surgeon in view of a vascular graft infection is the selection of treatment. Currently it is commonly agreed that the removal of the infected graft and a concurrent vascular reconstruction is the best method. While surgical removal of the infected graft does not raise concerns in most cases, the reconstruction stage is associated with the necessity of selecting the optimal location of the reconstructed vascular bed (extra-anatomical bypass or *in situ* bypass graft) together with the material necessary for its formation (autologous vessels, cadaver homograft, silver-coated grafts or antibiotic-soaked vascular grafts).

The authors also encountered the problems presented above based on the case of a 73-year-old patient with a massive infection of a dacron aortobifemoral vascular graft. The patient was initially operated on due to symptomatic Leriche syndrome with ischemia of both lower extremities stage III°, according to the Fontaine's scale (20–30 m intermittent claudication, rest pain, especially severe at night). The patient received a 18/9 mm bifurcated dacron graft. The upper anastomosis was performed with the aorta below the origin of the inferior mesenteric artery and the lower fixations on both sides over femoral arteries bifurcation. In the early postoperative period (day 8), a massive leak of serosanguineous fluid appeared from the wound located in the right groin, and afterwards also from the left groin. During the next days, the content leaking from the wound gradually changed its character to purulent. Throughout the whole postoperative period the patient did not present any fever and inflammatory markers were not specific for massive infection (leukocytosis 7000–9500/mm^3, CRP 33–37 mg%).

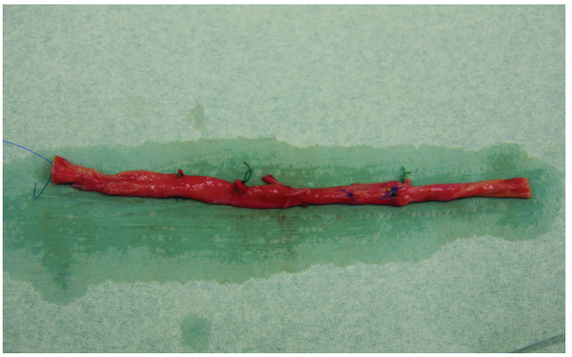

Fig. 3. One of two femoral veins harvested from the patient

Microbiological examination of the wound in left groin detected *E. faecalis* HLAR. Abdominal and Pelvic Computed Tomography revealed the presence of inflammatory infiltrate around both branches of the graft and an abscess in the distal anastomosis area on the right side, with no evident signs of infection within the aortic segment of the graft. It was decided that the patient should be reoperated. During the operation a total lack of the ingrowth healing process of the primary graft and features of infection (purulent fluid around the graft), also in the vicinity of the proximal anastomosis to the aorta, were noticed. Dacron prosthesis was entirely removed and replaced by a combined aortobifemoral graft constructed from biosynthetic vascular graft Omniflow II and two femoral veins harvested from the patient (Fig. 1–3). To this end a straight section of the Omniflow II graft (8 mm in diameter and 20 cm in length) was cut intraoperatively into two equal parts and the walls were incised longitudinally (2–2,5 cm section). Afterwards the two parts were anastomosed with each other (side to side) in the previously mentioned incisions to give a bifurcated prosthesis with size 16/8 mm. Then, reversed 18/19-cm-long segments of both femoral veins obtained from the patient were sutured to the

branches of the graft (end-to-end). The combined biosynthetic-autologous prosthesis constructed that way was implanted in place of the removed aortobifemoral graft. In the first hours after surgery, acute ischemia of the left lower limb occurred in the course of thrombosis of the left branch of implanted bypass, which demanded surgical thrombectomy. Further hospitalization proceeded without any significant complications and the patient was discharged from the hospital. It should be noted that throughout the entire period of the hospital treatment a constant antibiotic therapy was used, despite obtaining negative cultures from the fluid surrounding the aortic section of the removed graft (however pathogens such as *E. faecalis*, which are capable of creating bacterial biofilm coating implanted materials are very rarely detected in the inflammatory exudate). Control Computed Tomography Angiography was performed 3 months after reoperation, which indicated complete regression of the inflammatory infiltrate described in the previous test and the presence of significant stenosis (80% on the right side and 85% on the left side) in anastomoses of the Omniflow II prosthesis with autological femoral veins. Due to the progressive symptoms of ischemia of both lower limbs related to the gradual progression of these lesions, the patient was qualified for a planned endovascular procedure. An effective angioplasty of both anastomoses of bioprosthesis with femoral veins was performed, complemented by bilateral implantation of stents. Satisfactory hemodynamic effect and complete regression of reported symptoms were achieved. In the nearly 2-year ambulatory follow-up the patient did not show any signs of recurrence of infection within the vascular system or blood circulation disorders of the lower limbs.

In spite of conviction of the need for complete removal of a vascular graft in case of infection, attempts of conservative treatment (antibiotic therapy) or surgery limited to the direct focus of infection are still reported. This results from the often-isolated nature of an infection and the relatively high risk of complications that may occur during the removal of a prosthesis and reconstruction of a vascular bed. The scope of these operations may involve the removal of necrotic tissues, evacuation of inflammatory fluid reservoirs, irrigation drainage, local antibiotic therapy, coverage of bare sections of prostheses with well-vascularized muscle lobes and in selected cases segmental resection of the infected part of the graft with the reconstruction of the defect. In the described case the infection involved the entire graft, therefore its total elimination was the only option. Another argument in favour of such a procedure was the fact that the infection was caused by intestinal bacteria (*E. faecalis*), as a full recovery in such cases can only be achieved through the complete elimination of artificial material (Seabroock,1990). Because of the severe symptoms of ischemia of the lower limbs prior to the primary surgery, simultaneous vascular reconstruction was necessary.

Revascularization at the site of infection, because of the high risk of re-infection of the new bypass, requires the implantation of biological material (autologous vessels or cadaver homograft) or synthetic grafts more resistant to infections (antibiotic-soaked or impregnated with silver). The latter are mainly used in the case of deficiency of the biological material. In fact, results obtained in recent years have shown in fact no antimicrobial activity of prostheses impregnated with silver and a high risk of their recurrent infection (Batt *et al.*, 2008). On the other hand, experimentally proven effectiveness of grafts coated with antibiotics to prevent intraoperative infections does not resolve doubts about their long-term antibacterial effect, resulting from the gradual progressive decrease of local concentration of antibiotics and increasing antibiotic resistance (Lew and Moore, 2011). The use of homografts, with significantly higher resistance to reinfections, is inextricably linked to their limited supply (requiring access to a tissue bank), gradual degeneration (due to the immunological rejection reactions), and retrograde changes (which are a consequence of conservation by freezing), resulting in true aneurysms, spontaneous rupture of the graft wall, anastomotic bleeding and thrombotic complications, as well as the risk of transmission of viral diseases (Verhelst *et al.*, 2000). Admittedly, the use of immunosuppression prolongs the proper functioning of allogeneic grafts, but requires continuous application (Pupka *et al.*, 2004). A way to circumvent these problems may be the use of autologous femoral veins, which provide all of the benefits associated with the use of biological material (the least vulnerability to superinfection, the ability to fully grow into a focus of infection) in the simultaneous absence of risks specific to the allografts (Dirven *et al.*, 2015). The disadvantages of this method include extended operation time and unfortunately common disproportion between the available resource of veins and the length of the prosthesis, which they ought to replace. The first one loses its importance due to improvements in surgical technique (Budtz-Lilly *et al.*, 2014), however to overcome the other, an additional source of material for the reconstruction of the vascular bed is required (with properties as close as possible to transplant tissue). The authors decided to use, as a supplement of the patient's femoral veins, a composite biosynthetic vascular graft Omniflow II. Its complex structure is created by a polyester mesh endoskeleton, which is the endurance element providing mechanical resistance and which is a kind of scaffolding for a biological component and the covering coating of ovine collagen allowing overgrowth of the graft wall by the vessels and tissues of the recipient.

Omniflow's II high ability of incorporation into the host tissue has been known for over 20 years (Werkmeister *et al*., 1995), which makes it an alternative to autologous material during the preparation of the vascular graft (Dünschede *et al*., 2015) or arteriovenous dialysis access (Palumbo *et al*., 2009). Until recently, due to the lack of manufacturer's recommendations for Omniflow II implantation within the foci of infection, its use in the treatment of vascular graft infections was avoided. In 2012, Töpel *et al*., presented the first positive results of the use of these prostheses (in two cases combined with saphenous vein) for replacement of an infected graft located below the inguinal ligament (Töpel *et al*., 2012). Other authors have arrived at similar findings (Fellmer *et al*., 2014; Wiltberger *et al*., 2014).

In 2014, Neufang took advantage of these bioprostheses, combined with autologous superficial veins, to create vascular spans allowing to bypass long-segment obstructions of the arteries of the lower limbs. In the described cases, Omniflows II were the proximal parts of those spans (Neufang *et al*., 2014). So far the only work devoted to reconstructions using Omniflow II grafts in the aortofemoral segment was published in 2015 (Krasznai *et al*., 2015). Most authors highly evaluated the bioprosthetic Omniflow II in terms of their resistance to reinfection and degenerative changes. Despite the high resistance to infection, the authors limited the scope of the use of the mentioned prosthesis to the proximal section of aortobifemoral graft only. This was due to the presence of purulent fistulas in both groins, which were the locations of distal anastomoses. The authors concluded that the use of a bypass comprising even small amounts of synthetic material, in such conditions can cause a reinfection. The applied method of anatomical revascularization, using a combination of prosthesis Omniflow II with bilateral venous grafts allows the rational use of the existing stock of autologous vessels, as well as the simultaneous use of biosynthetic material in areas of reduced risk of infection. At the same time, however, it should be noticed that the combined character of the grafts carries the risk of potential stenoses in multiple anastomoses and requires regular clinical and radiological controls to provide their early identification.

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SHORT COMMUNICATION

Survival of Bacteria in Respiratory Protective Filters

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Abstract

The aim of this study was to estimate the susceptibility of *Staphylococcus hominis* bacteria to the bacteriostatic agent in respiratory protective filters. Four types of filter media of different characteristics were tested. The number of bacteria was estimated by a culture-based method. It was proved that in treated filters the number of *S. hominis* was significantly reduced, even below the detection limit, while in untreated material bacteria were able to grow and multiply up to 100-fold within 8 hours. There was no correlation between filter structure and changes in the number of bacterial cells.

Key words: respiratory protective filters, biostatic agent, treated filters

Coal excavation is still an activity that needs people involvement. Miners working underground are exposed to many environmental factors which cause harm to health or life. The presence of fumes, mists, gases such as carbon monoxide and dioxide, nitrogen monoxide, hydrogen sulphide and dust of different particle fractions requires the use of different preventive and protective measures (Wells, 1998). One of them is the reduction of dust particle generation using water spray systems.

Additionally, coal miners are obliged to use individual protective respiratory equipment, *i.e.* respiratory protective masks or respirators. The presence of dust particles of the respirable fraction in concentration exceeding limit values causes such serious diseases as "black lung", coal workers' pneumoconiosis, and chronic obstructive pulmonary disease (Donoghue, 2004). Lung illnesses are among the most serious of all diagnosed occupational diseases in the Polish mining industry in recent years (Labour Protection Council, 2007).

The application of respiratory protective masks is regulated by relevant standards, for example EN 136 or EN 140 and the Regulation of the Minister of Economy (EN 136:1998/AC:2003; EN 140:1998/AC:1999; Regulation of the Minister of Economy, 2002).

Air filters used in protective masks applied by coal miners are exposed to relatively high temperature and humidity. It was noticed that the relative humidity of the exhaled air exceeds 95%. Moreover, high amount of water is perspired by the coal workers – even more than 1litre of sweat per hour (Gierlotka, 2002). It was proved that at favourable temperature and humidity and in the presence of available source of carbon and energy bacteria deposited on the filters are able to colonize the filter material (Hugenholtz and Fuerst, 1992).

The aim of the research work was to study the influence of biostatic agent on bacterial growth in air filters used by coal miners in personal protective equipment under conditions similar to those occurring underground.

Respiratory protective filters classes P1 and P2 of different structure and characteristics were used in the studies (see Table I). They are applied in protective, dust absorbing masks used by coal-miners. The filters were treated with a biostatic agent (silver salts), added by the manufacturer after the production process. Untreated filters were used as a control. Treated filters were marked as A_t (treated A filter), B_t, C_t and D_t. Filters A, C, D consisted of three layers. The surface layer-coarse filter contained thick fibres of polyamide (PA), polyethylene (PE), polypropylene (PP) (substance 150–200 g/m^2). The next one, main filter of high efficiency, was made of pure PP fibres of the diameter below 1 μm (substance 60–100 g/m^2). The third, PP layer was a main filter cover (substance 15 g/m^2). Filters signed as B were made of PE and PA fibres and had only one layer (substance 200 g/m^2).

The bacterial strain used in the study was the Gram-positive *Staphylococcus hominis* which is a harmless

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Table I
Characteristics of filters tested.

Filter class	Filter kind	Nr of layers	Woven material	Substance (g/m^2)
P1, P2	A, A_t	1 – surface layer	PA[a], PE[b], PP[c]	150–200
		2 –main filter	PP	60–100
		3 – main filter cover	PP	15
P1	B, B_t	1– main filter	PE, PA	200
P2	C, C_t	1 – surface layer	PA, PE, PP	150–200
		2 – main filter	PP	60–100
		3 – main filter cover	PP	15
P1, P2	D, D_t	1 – surface layer	PA, PE, PP	150–200
		2 – main filter	PP	60–100
		3 – main filter cover	PP	15

[a] PA – polyamide, [b] PE – polyethylene, [c] PP – polypropylene

commensal on human skin. The strain was identified with the API Staph biochemical tests (bio-Merieux). In order to simulate conditions in which filters are used by coal miners a suspension of the bacterial cells was made in 0.1% solution of peptone (10^5–10^6 cfu/ml), samples of treated and untreated filters were inoculated with the sprayed bacterial suspension and incubated under static conditions (no air flow) at 39 ± 1°C and relative humidity (RH) 97 ± 2%. The number of bacteria was estimated by a culture-based method at the beginning of the experiment and after 8, 24 and 48 hours. Fragments of filters were measured and weighed and, then placed in 100 ml of 0.08% sterile sodium pyrophosphate solution containing 0.0005% of polyoxyethylenesorbitan monooleate (Tween 80). Before and after incubation bacteria were washed out of the filters within 40 min in a water bath shaker, plated on nutrient agar and incubated at 37°C for 2 days. The number of bacteria was given as cfu/cm^2 (colony forming units per cm^2 of the filter). Additionally, *S. hominis* growth in filters within first 8 hours was characterized by generation time.

It was observed that in A treated filters total decay of bacteria appeared within 8 h of incubation. In untreated filters bacteria were present within the whole time of the experiment (48 h). In P1 filters number of bacteria increased over fourfold within 8-hour incubation, from 2.3×10^4 cfu/cm^2 to 1.0×10^5 cfu/cm^2 and eightfold after 24 h. Generation time of *S. hominis* was 3 h 15 min. In P2 filters the number of bacteria decreased slightly within 8 h from 1.7×10^5 to 1.2×10^5 cfu/cm^2 and stayed at the same level to the end of the experiment. Initial humidity of A filters was 66–67% and A_t 68–75%.

In B treated filters the number of *S. hominis* was reduced 1.6 times after 8-hour contact with antimicrobial agent. After 24 h only 50 cfu/cm^2 were found.

In untreated filters the number of bacteria increased 100-fold and reached 1.3×10^5 cfu/cm^2 within 8 h. Generation time was 2 h 24 min. Initial humidity of B filters was 80%, B_t 85%.

C treated filters did not sustain bacterial growth; the number of *S. hominis* in C_t systematically decreased. The most significant reduction of bacterial cells was observed within 8 h (from 2×10^3 to 62 cfu/cm^2). After 48 h bacteria were not detected in the filtering material. In untreated C filters the number of bacterial cells maintained at the level of 10^4–10^5 cfu/cm^2. Initial humidity of C filters was 88% and C_t 75%.

In the case of D treated filters bacteria were detected only 8 h after filters inoculation but their number was significantly reduced (approx. 2–3-fold). In untreated filters the number of *S. hominis* stayed at the level 10^4 cfu/cm^2 (P1, P2). The highest increase in bacterial number (approx. 6-fold) was noticed in P1 untreated filters within the first 8 hours of incubation. *S. hominis* generation time reached 3 h 8 min. Initial humidity of D filters was 85% and D_t 81%.

The studies indicated that *S. hominis* is able to not only survive but also multiply in untreated material. Sufficient water content (66–88%), favourable temperature (39 ± 1°C) and available nutrients stimulated bacterial growth in filters A, B and D. The most significant increase in number of bacterial cells was noticed in B filters, 100-fold, and in A and D filters (P1) 4.3-fold and 6-fold, respectively. Only in untreated C and D (P2) filters the number of *S. hominis* decreased (1.3 and 1.1-fold, respectively) but still remained at a high level, 10^3–10^4 cfu/cm^2. Generation time for *S. hominis* in untreated filters ranged from 144 to 195 min when water content was 80% and 68–75%, respectively. The generation time in the experiment was much longer than observed in laboratory cultures grown under optimum conditions where it usually reaches 20–25 min. Probably, this was an effect of incubation temperature (39 ± 1°C) which was higher than the optimum (35 ± 1°C) and availability of peptone as the only source of nutrients.

The phenomenon of bacterial growth in untreated woven filters was studied by Karwowska *et al*. (2003). They observed that bacterial strains of different Gram characteristics (*Pseudomonas fluorescens*, *Micrococcus roseus*, *Bacillus subtilis*) multiplied in air filters, which was correlated with the increase in water content in the material. Also Kemp *et al*. (2001) observed significant growth of microorganisms in untreated air filters during the first 2 weeks of the experiment duration.

The application of biostatic agent resulted in significant reduction of bacterial cells in the filters tested. In the case of C_t the number of *S. hominis* decreased 32 fold, from 2×10^3 cfu/cm^2 to 62 cfu/cm^2 within 8 hours and bacteria were not detected in filters after 48 hours. In A_t filters bacteria were not found after 8 hours. This was correlated with the lowest water content (68–75%) if compared to D_t and B_t filters: 81 and 85%, respectively. In D_t filters the number of bacteria decreased below the detection limit within 24 hours.

All of the filters tested were made of both hydrophobic – PP, PE and slightly hydrophilic polymers – PA (moisture absorption 4.5%). Nevertheless, the hydrophobic properties of fabrics were not a protection against bacterial growth. For this reason antibacterial treatment has been introduced by manufacturers. The most popular antimicrobial agent is silver. Silver ions react with thiol groups in vital enzymes inactivating them or interact with DNA (Matsamura *et al*., 2003). The efficacy of $AgNO_3$ to inactivate Gram-positive and Gram-negative bacteria in air filters were described by Miaskiewicz-Peska and Lebkowska (2011). Nevertheless, it was proved that silver particles were released from filtering materials, regardless of whether they were water or air filters (Foss Manufacturing, 2004), (Miaskiewicz-Peska and Lebkowska, 2011).

Recently, in the era of nanotechnology a trend of application of antimicrobial nanoparticles is being observed. Rai *et al*. (2009) described use of silver nanoparticles in textile impregnation against microbial growth and cited Duran *et al*. (2007), who reported such treated cotton fabrics activity against pathogenic *Staphylococcus aureus* bacteria. Nevertheless, there are more and more reports concerning health implications of nanoparticles and their harmful impact on the aquatic ecosystems (Łebkowska and Załęska-Radziwiłł, 2011; Marcato and Duran, 2011). It was proved that these particles can be released from textiles and access the human body by inhalation or contact with the skin. Moreover, their nanoscale size provides a very large surface area, which can cause the generation of oxyradicals (Moore, 2006).

The results for the pairs of filters (treated and untreated with biostatic agent) tested in the research revealed the effectiveness of antimicrobial finishing. Undoubtedly, the information is of benefit to filter manufacturers – they gained proof that the application of biostatic agent reduces bacterial growth under the conditions the filters are designed to be used. Nevertheless, the application of antimicrobial nanoparticles in personal respiratory masks should be preceded by hazard identification, followed by appropriate steps to eliminate harmful impact on human health and the natural environment.

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SHORT COMMUNICATION

Detection of Polioviruses in Sewage Using Cell Culture and Molecular Methods

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Abstract

The work presented here demonstrates the utility of a two-step algorithm for environmental poliovirus surveillance based on: preselection of sewage samples tested for the presence of enteroviral genetic material-RT-PCR assay and detection of infectious viruses by cell culture technique (L20B for polioviruses and RD for polio and other non-polio enteroviruses). RD and L20B cell lines were tested to determine their sensitivity for isolation of viruses from environmental samples (sewage). Finally, we wanted to determine if sewage concentration affects the results obtained for RT-PCR and cell cultures.

Key words: cell lines L20B and RD, environmental surveillance, poliovirus, sewage

Polioviruses (PVs) are small (30 nm in diameter), non-enveloped, icosahedral-shaped capsid viruses belonging to the *Picornaviridae* family. PVs possess an approximately 7.5-kilobase (kb) positive-sense single-stranded RNA genome. Poliovirus is a causative agent of *poliomyelitis*, commonly known as polio (Landsteiner and Popper, 1909). Polioviruses are transmitted by the fecal-oral route, they multiply in the gastrointestinal tract and are excreted in large numbers in the feces of infected persons, whether or not they are symptomatic. Virus infects sensitive cells of lymphoid tissue in the mouth, nose and throat. The incubation period lasts from 2 to 35 days. It leads to a transient viremia and the virus spreads to the reticuloendothelial system without causing clinical symptoms (Sabin, 1956; Bodian and Horstmann, 1965; Melnick, 1996). Most natural infections of humans end at this stage with a minor disease comprising nonspecific symptoms such as sore throat and fever. In very rare cases, 1–2% of infected individuals, the virus enters the central nervous system (CNS) and replicates in motor neurons within the spinal cord, brain stem, or motor cortex. All individuals infected with wild poliovirus or vaccinated with live, attenuated oral polio vaccine (OPV) excrete large number of virus particles in faeces for periods of up to several weeks. The rationale for environmental surveillance is based on the characteristic poliovirus excretion pattern. Viruses cannot replicate outside the host cell and therefore cannot multiply in the environment, however, they can survive and remain infectious in the environment for varying lengths of time, depending on the immediate conditions. For this reason environmental poliovirus surveillance (ENV) is recommended by the WHO (WHO, 2003).

ENV is based on detection of the presence of the virus in sewage samples by a variety of laboratory methods for concentration, separation and identification. Environmental surveillance has been used successfully in monitoring enteric viruses circulation and in assessing the extent or duration of epidemic poliovirus circulation in a specific population (AW and Gin, 2010; Kern *et al*., 2013; Parasidis *et al*., 2013). ENV currently plays an important role in the monitoring of circulating vaccine-derived polioviruses (VDPVs). In recent years, VDPV strains were isolated from sewage in Israel, Finland, Egypt, Greece, Switzerland and Slovakia in the years 1984–2010 (Vinje *et al*., 2004; Kew *et al*., 2004; 2005; Pavlov *et al*., 2005; Dedepsidis *et al*., 2007). VDPV strains are derived from attenuated vaccine-virus contained in oral polio vaccine (OPV). OPV strains can mutate in VP1 region during their replication in the human intestine, and some mutations may result in recovery of the capacity for higher neurovirulence. These neurovirulent revertants may cause paralysis in

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humans and develop sustained circulation (cVDPV). On rare occasions, if a population is seriously under-immunized, VDPVs can continue to circulate for an extended period of time.

In 2007 in Switzerland (certified by WHO as polio-free), testing of sewage samples revealed the presence of wild poliovirus (WHO, 2007). Wild polioviruses were also isolated from sewage samples in Egypt (2012) and Israel (2013) in the absence of reported AFP cases. The list of countries routinely employing ENV includes the Czech Republic, Egypt, Estonia, Finland, India, Japan, Latvia, the Netherlands, New Zealand, Pakistan, Russia, Slovakia and Switzerland. Our previous study confirmed that sewage is a rich source of enteroviruses circulating in the community (Wieczorek *et al.*, 2015). In Poland, systematic environmental poliovirus surveillance is planned. Accordingly, the aim of this study was to develop and optimize a method for the preparation of wastewater samples collected in Poland. The present study was also conducted to develop a two-step algorithm for ENV based on: 1) preselection of sewage samples tested for the presence of enteroviral genetic material-RT-PCR assay, 2) detection of infectious viruses by cell culture technique (L20B for polioviruses and RD for polio and other non-polio enteroviruses). Thus, we would obtain information about the sensitivity of RT-PCR assay for the detection of enteroviruses in sewage. RD and L20B cell lines were tested to determine their sensitivity for isolation of viruses from environmental samples (sewage). Finally, we wanted to determine if sewage concentration affects the results obtained for RT-PCR and cell cultures.

Sewage samples (pooled, homogeneous, tested negative for enteroviruses): raw (100%), diluted in PBS (80%, 50%, 20%) and PBS (0%) were contaminated with reference laboratory poliovirus strain – PV Sabin type 1 ($TCID_{50} = 10^{-8.2}$/1 ml calculated by Spaerman-Kärber formula). Virus decimal dilutions from 10^{-1} to 10^{-7} were used to contaminate sewage samples. Raw sewage was used as negative control. All samples were processed according to the protocol described earlier (Zubriggen *et al.*, 2008). The RT-PCR assays were performed using Pan-enterovirus primers for enterovirus detection based on the WHO manual (WHO, 2004). Viral RNA was extracted from 140 µl of sewage concentrate using Qiagen Viral RNA Kit following to the manufacturer's instructions. A volume of 200 µl of sewage concentrate was inoculated into tubes with L20B cells and RD cells. Each specimen underwent three passages according to WHO procedures (WHO, 2004).

In order to determine its sensitivity, RT-PCR was used for the detection of enteroviral RNA in sewage samples: raw (100%), diluted in PBS (80%, 50%, 20%) and PBS only (0%). Previously, samples were contaminated with PV Sabin type 1. The results of the RT-PCR assay are shown in Table I. The highest dilution of PV suspension, showing a visible band in RT-PCR in all three replicates of a sample, was taken as the detection limit. The amplicon was visible up to 10^{-5} dilution for each concentration of sewage. Viral genetic material was detected in concentration of ~0.5 $TCID_{50}$. The number of positive results and probabilities range from 9 to 10 and 0.60 to 0.67 respectively for each concentration of sewage. The concentration does not significantly affect the amplification results.

The cell lines: L20B for polioviruses and RD for polio and other non-polio enteroviruses were tested to determine their sensitivity for isolation of infectious viral particles from the environmental samples. The results of poliovirus isolation in L-20B and RD cells are

Table I
Detection of poliovirus genetic material in sewage samples by RT-PCR.

RT-PCR						
Poliovirus suspension	Sewage concentration					No of positive results (A)
	100%	80%	50%	20%	0%	
10^{-3} (50 $TCID_{50}$)	+ + +	+ + +	+ + +	+ + +	+ +	14 P(A) = 1
10^{-4} (5 $TCID_{50}$)	+ + +	+ + +	+ + +	+ + +	+ +	14 P(A) = 1
10^{-5} (0,5 $TCID_{50}$)	+ + +	+ + +	+ + +	+ + +	+ +	14 P(A) = 1
10^{-6} (0,05 $TCID_{50}$)	0 0 0	0 0 +	0 0 0	0 0 +	0 0	2 P(A) = 0.14
10^{-7} (0,005 $TCID_{50}$)	0 0 0	0 0 0	0 0 0	0 0 0	0 0	0 P(A) = 0
No of positive results (A) and probability P(A)	9 P(A) = 0.60	10 P(A) = 0.67	9 P(A) = 0.60	10 P(A) = 0.67	6 P(A) = 0.60	

"0" – negative RT-PCR result, "+" – positive RT-PCR result

Table IIA
Poliovirus isolation from sewage samples in L20B cells.

L20B						
Poliovirus suspension	Sewage concentration					No of positive results (A)
	100%	80%	50%	20%	0%	
10^{-3} (2000 $TCID_{50}$)	+ + +	+ + +	+ + +	+ + +	+ +	14 P(A) = 1
10^{-4} (200 $TCID_{50}$)	+ + +	+ + +	+ + +	+ + +	+ +	14 P(A) = 1
10^{-5} (20 $TCID_{50}$)	0 + 0	+ + 0	+ + +	+ + +	+ 0	10 P(A) = 0.70
10^{-6} (2 $TCID_{50}$)	0 0 +	0 + +	+ + 0	0 + +	0 0	7 P(A) = 0.5
10^{-7} (0,2 $TCID_{50}$)	0 0 0	0 0 0	0 0 0	0 0 0	0 0	0 P(A) = 0
No of positive results (A)	8 P(A) = 0.53	10 P(A) = 0.67	11 P(A) = 0.73	11 P(A) = 0.73	5 P(A) = 0.50	
Probability	P(A) = 0.63					

"0" – negative result, "+" – positive result

Table IIB
Poliovirus isolation from sewage samples in RD cells.

RD						
Poliovirus suspension	Sewage concentration					No of positive results (A)
	100%	80%	50%	20%	0%	
10^{-3} (2000 $TCID_{50}$)	+ + +	+ + +	+ + +	+ + +	+ +	14 P(A) = 1
10^{-4} (200 $TCID_{50}$)	+ + +	+ 0 +	+ + +	+ + +	0 0	14 P(A) = 0.79
10^{-5} (20 $TCID_{50}$)	0 0 +	0 0 0	0 0 +	0 0 +	0 0	3 P(A) = 0.20
10^{-6} (2 $TCID_{50}$)	0 0 0	0 0 0	0 0 0	0 0 0	0 0	0 P(A) = 0
10^{-7} (0,2 $TCID_{50}$)	0 0 0	0 0 0	0 0 0	0 0 0	0 0	0 P(A) = 0
No of positive results (A)	7 P(A) = 0.47	5 P(A) = 0.33	7 P(A) = 0.47	7 P(A) = 0.47	2 P(A) = 0.20	
Probability	P(A) = 0.39					

"0" – negative result, "+" – positive result

shown in Tables IIA and IIB, respectively. The highest dilution of virus suspension showing cytopathic effect in all three replicates of a sample (raw sewage, 100%) was 10^{-4} (200 $TCID_{50}$). The probability of poliovirus isolation in cell lines was 0.63 (L20B) and 0.39 (RD).

The highest sensitivity of poliovirus isolation in L20B cells was observed in 80%, 50% and 20% sewage at 10^{-6} dilution of the virus suspension (~2 $TCID_{50}$). For 80%, 50% and 20% sewage samples the number of positive results and probabilities range from 10 to 11 and P(A) = 0.67 to P(A) = 0.73, respectively.

The highest sensitivity of poliovirus isolation in RD cells was observed in 100%, 50% and 20% sewage at 10^{-5} dilution of the virus (20 $TCID_{50}$) and was ten times lower than the values obtained for L20B cell line. For 100%, 50% and 20% sewage samples the number of positive results and probability was A = 7 and P(A) = 0.47 respectively.

The efficiency of poliovirus isolation from control samples, containing only PBS, was significantly lower than the other samples and the probability of isolation in L20B and RD cells was P(A) = 0.5 and P(A) = 0.2 respectively.

Environmental surveillance is an effective approach in investigating the circulation of polioviruses and other human enteroviruses (HEV) in the population.

Both Finland and Israel have used ENV as the main approach to PV surveillance for decades (Hovi *et al*., 2010). The World Health Organization recommends regular testing environment for the presence of poliovirus as an important complement to the new strategy for Global Polio Eradicaction Initiative (GPEI). Criteria for considering environmental surveillance as a supplementary approach in GPEI were published in WHO Guidelines, which contain principles for selecting sampling sites, propose methods for sample processing and suggest possible program responses to PV detection in sewage (WHO, 2003).

In this paper we used the protocol based on concentration of sewage samples by precipitation. A rapid, efficient and inexpensive method was developed to concentrate viruses from sewage. The method consists of adsorbing the viruses to silicon dioxide (SiO_2) in the presence of 0.5 mM $AlCl_3$ and adjustment of the pH to 3.5. Because of its simplicity, the protocols based on silicon dioxide, becomes more often used in environmental virology, especially for large volumes of analyzed samples (Katayama *et al*., 2002; Kocwa-Haluch and Zalewska, 2002; Li *et al*., 2010). Furthermore Zubriggen *et al*. (2008) showed a higher efficiency of virus recovery using a precipitation technique (60%) than the two phase separation method (30%) recommended by the WHO (2003).

In this study we showed the high sensitivity of RT-PCR assay for detection of enteroviruses in sewage. The number of detected virus particles at 10^{-5} dilution was ~0.5 $TCID_{50}$. The RT-PCR assay gave identical results to those obtained in our previous study (Witek and Wieczorek, 2009). Viral RNA was extracted directly from the poliovirus suspension and identical sensitivity of RT-PCR assay was observed. The data suggest high efficiency of virus recovery from sewage samples. Our results also indicate that all steps of analytical process (*e.g.* preparation of sewage sample, amplification procedure) were properly selected.

Cell culture technique, however, in spite of some undeniable problems, is still the gold-standard method for virological surveillance of polioviovirus. According to WHO protocol two poliovirus-sensitive cell lines (RD, L20B) are recommended for virus detection from clinical and environmental samples (WHO, 2004). The results of the study show that L20B cells (~2 $TCID_{50}$ for 100% sewage) can provide greater sensitivity for detection of polioviruses than RD cells (~20 $TCID_{50}$ for 100% sewage). Similar results have been reported by other authors (Wood and Hull, 1999). Likewise, compounds of sewage that are toxic for cells can affect the probability of isolation of poliovirus in different cell lines. Certain substances have a stronger cytotoxic effect on RD cells than L20B cells (Al-Khayat and Ah mad, 2012).

The sewage concentration affected the results obtained for PV isolation in cell lines. The efficiency of virus isolation was lower in control samples containing only PBS. There is evidence that virus survival is enhanced in polluted waters, presumably as a result of some protective effect that the viruses may receive when they are adsorbed onto suspended solid particles in sewage (Feachem *et al*., 1983). The lack of these particles could reduce virus ability to survive outside the host organism.

Preparation of sewage samples according to the protocol based on silicon dioxide and sodium chloride seems to be very promising. Therefore, it is important to consider the limitations of this method such as concentration of sewage and its toxicity. Environmental toxicity and inhibitory factors often interfere with both molecular and cell culture assays. Molecular assays such as direct RT-PCR are sensitive and allow lower quantities of the original sample to be analysed. On the other hand, RT-PCR may provide inaccurate estimates of infectious virus contamination in the environment because they detect both inactivated viruses as well as infectious viruses. Cell culture assay is the only reliable method that will allow the detection of infectious virus in that case. In conclusion, our preliminary data suggest applying two-step algorithm for environmental monitoring: RT-PCR-based detection step and isolation of infectious viruses in cell cultures.

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