ISSN 0015-5497, e-ISSN 1734-9168 Folia Biologica (Kraków), vol. 65 (2017), No 3
https://doi.org/10.3409/fb65_3.143

Evaluation of Urease Activity by the Human *Ureaplasma* Species

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Accepted October 20, 2017 *Published online December 08, 2017* *Published December 21, 2017*

BIERNAT-SUDOLSKA M., BILSKA-WILKOSZ A., ROJEK-ZAKRZEWSKA D., ZAWILIŃSKA B., KOSZ-VNENCHAK M. 2017. Evaluation of urease activity by the human *Ureaplasma* species. Folia Biologica (Kraków) **65**: 143-148.

Ureaplasma species are potentially pathogenic bacteria. *Ureaplasma* can cause inflammation of the genitourinary system, obstetrical complications and can play a role in pathology of the respiratory tract of premature newborns. The aim of this study was to evaluate the urease activity of the human *Ureaplasma* species *in vitro*. Urease plays a key role in *Ureaplasma* cells because its activity is the sole source of ATP production. It is considered that urease is one of the virulence factors of these bacteria. We examined the ammonia product of urea hydrolysis by *U. parvum* and *U. urealyticum* strains in two cell culture systems: A549- human lung carcinoma cells and SiHa – human cervical carcinoma cells and in PPLO broth as a control. Urease activity was assessed by measuring the concentration of ammonia, which was based on the measurement of pH in cell culture medium and PPLO broth. Significantly higher concentrations of ammonia were obtained for *U. urealyticum* as compared with *U. parvum* only in A549 cells (Mann-Whitney U test $P<0.0001$). High ammonia levels observed after inoculation of human cells with *U. urealyticum* suggest higher activity of the urease of this species and may indicate a higher pathogenicity of this species particularly for the human respiratory tract.

Key words: *U. urealyticum*, *U. parvum*, urease activity, pathogenicity of ureaplasmas.

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Ureaplasmas are atypical bacteria, classified within the *Ureaplasma* genus that belongs to the *Mycoplasmataceae* family of the class *Mollicutes*. These are the smallest and the simplest prokaryotic organisms deprived of a bacterial cell wall. They can cause infections of the human urogenital tract and the respiratory system in preterm infants with low birth weight (CASSELL *et al*. 1988a; CASSELL *et al*. 1988b; CASSELL *et al*. 1993; ABELE-HORN *et al*. 1997; HEGGIE *et al*. 2001; KATZ *et al*. 2005; WAITES *et al*. 2005; VISCARDI & HASDAY 2009; SALMERI *et al*. 2012).

Ureaplasmas differ from all other mycoplasmas by possessing urease activity. Urease activity was also demonstrated in a number of other bacteria but ureaplasmas are the only representatives whose growth is dependent on the presence of urea (KENNY & CARTWRIGHT 1977). Urea hydrolysis plays an important role in energy metabolism of ureaplasmas and is their primary energy source. Ureaplasmas produce as much as 95% of their ATP in this reaction.

Ureaplasma urease activity is very high. This enzyme is the major protein component of the cytoplasm (FORD & MACDONALD 1967; MASOVER *et al.* 1977a; MASOVER *et al.* 1977b; ROMANO *et al.* 1980; ROMANO *et al.* 1986; SMITH *et al.* 1993). Beside immunoglobulin A protease, phospholipase A and C, multiple-banded antigen (MBA) and an enzymatic system generating hydrogen peroxide, urease of ureaplasmas is considered to be a virulence factor that plays a role in the pathogenesis of infections with these microbes (KILIAN *et al.* 1984; LIGON & KENNY 1991; ZHENG *et al.*

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1995; KOKKAYIL & DHAWAN 2015). The differences in the activity of enzymes thought to be virulence factors, including ureases, may be decisive for the differences in the pathogenicity of the two *Ureaplasma* species that infect humans. The pathogenic effect of urease results from the generation of ammonia created as a product of hydrolytic decomposition of urea in the following reaction: $H_2N\text{-}CO\text{-}NH_2 + H_2O \rightarrow 2NH_3 + CO_2$

Ammonia released as a result of this reaction has an irritating effect on the mucous membranes of urogenital and respiratory systems which are the targets for adherence and colonization of ureaplasma strains (KONIECZNA *et al.* 2012; KOKKAYIL & DHAWAN 2015; VISCARDI & SUHAS 2015). The concentration of the produced ammonia indirectly reflects the enzymatic activity of urease.

It is not definitively explained whether there are differences in pathogenicity of either species of *Ureaplasma*. Opinions on this subject are divided. Our previous research shows that the less commonly isolated from newborns and adults *U. urealyticum* exhibits higher pathogenicity. Newborns infected with *U. urealyticum* were subject to more frequent and longer therapeutic procedures supporting respiration, needed more frequent surfactant and antibiotic administration (BIERNAT-SUDOLSKA *et al.* 2006b).

The aim of this study was to evaluate the urease activity of the two human *Ureaplasma* species: *U. parvum* and *U. urealyticum* since it is one of the factors which may impact the pathogenicity of these microorganisms.

Materials and Methods

Strains of ureaplasma

The study was conducted on 44 ureaplasma strains, including 22 belonging to *U. parvum* and 22 from *U. urealyticum*. The strains were isolated from tracheal aspirates of neonates hospitalized in the Department of Neonatology, Jagiellonian University Medical College and from the urogenital tract of women with inflammation of the urogenital system diagnosed at the Department of Microbiology, Jagiellonian University Medical College in Cracow. All strains were previously identified by PCR and stored at -70ºC until use in the present experiment.

All ureaplasma strains were cultivated into liquid and solid PPLO (pleuropneumonia like organism) media according to the procedure described by Shepard (SHEPARD & LUNCEFORD 1976). The liquid PPLO medium contained phenol red at a final concentration of 0.002%, to detect an alkaline shift in the medium. The addition of a colour indicator to the liquid medium is necessary because the growth of ureaplasmas does not cause turbidity of the broth. The growth of ureaplasmas in liquid medium was always confirmed by characteristic ureaplasma colonies on PPLO agar. For identification of species of ureaplasmas, species-specific PCR was used according to a previous study (BIERNAT-SUDOLSKA *et al.* 2006a).

Cell cultures and their inoculation with ureaplasma strains

A culture of A549 cells (ATCC CCL-185) derived from human lung was used as a model of respiratory infections which may develop in newborns after vertical transfer of these microorganisms from the infected mother, and SiHa cell culture (ATCC HTB-35) derived from the female genital tract was used to approximate the conditions of genitourinary tract infection.

Cell cultures were maintained in flat tubes in Eagle's (Biovest-France) culture medium supplemented with 10% fetal calf serum and penicillin (final concentration 100 U/ml), which does not affect the proliferation of ureaplasmas, but limited the growth of other bacteria. After inoculation of cell cultures with ureaplasmas, Eagle's medium with 2% fetal calf serum was used. All cultures were incubated at 37ºC in 5% CO_2 in air for 18 hours. For inoculation of cell cultures and PPLO broth as a control of ureaplasmas growth, 0.2 ml of 18 hour cultures of each strain was used. This volume represented 25% of the final volume of PPLO broth and cell culture medium.

Examination of urease activity

The applied method is based on the assumption that the concentration of ammonia formed in the urea hydrolysis reaction can indirectly reflect the urease activity (GLASS *et al.* 2000). The resulting ammonia is clearly more basic (pKa value for ammonia is 9.25) than carbon dioxide (pKa value for H_2CO_3 is 6.35), meaning that the urease-catalyzed reactions are accompanied by solution alkalization and an increase in the pH value. The progress of hydrolysis is monitored using the pH indicators: bromothymol blue, phenolphthalein, phenol red (routinely used in cell culture medium and in broth for ureaplasmas), etc. (VANDEPITTE *at al.* 2003). Thus, we suggested that the progress of the reaction monitored by pH measurement, and the concentration of NH_4OH, calculated on this basis, equal to the concentration of NH_3, can be an indirect measure of the activity of the enzyme. This method has proved useful for assessing the catalytic activity of urease in ureaplasma cultures.

Since the tested ureaplasma strains produced ammonia at nanomolar concentrations (nM), it was assumed, in accordance with the Ostwald's dilution law describing the dependence of the degree of dissociation of poor electrolyte on its concentrations, that NH_4OH, a poor electrolyte, in nanomolar concentrations becomes a strong electrolyte fully dissociated. Assuming the complete dissociation of NH_4OH, it was accepted at the same time that the concentration of hydroxide ions (OH-) derived from the base is equal to the concentration of the base. Hydroxide ion concentration (OH-) derived from ammonium base was calculated on the basis of the measured pH, taking into account (OH-) from the dissociation of water. The pH value of the cell culture medium was measured using a pH-meter (Mettler Toledo). The NH_4OH concentration calculated from the pH measurement was presented in nmol/liter, as the concentration of ammonia. The ureaplasma culture in PPLO broth was used as a control system to provide optimal growth conditions for these bacteria *in vitro*.

The pH was measured in PPLO broth and culture medium from the cells 18 hours after inoculation. The pH was also measured in PPLO broth and culture medium from cells uninfected by ureaplasmas as a control. Concurrently with the inoculation, we carried out a quantitative assessment of each test strain by determining its titter, expressed as CCU/ml (colour changing unit). The results for individual strains ranged from 10^1 to more than 10^9. The number of CCU of the tested microorganisms could, of course, affect the obtained ammonia concentration. To be able to compare the results for all strains of ureaplasmas, the obtained value of ammonia concentration was calculated per 100 CCU/ml for each strain. The values calculated per 100 CCU/ml were the basis for further calculations of the average ammonia levels for both species of ureaplasmas. Measurement of pH in the media of uninfected ureaplasmas took into account in the calculation of the ammonia concentration achieved by each strain of ureaplasma. The results were analyzed statistically using the Mann-Whitney U test and Kruskal-Wallis test. Statistica version 10 was used for the calculations.

Results and Discussion

The mean of concentrations of ammonia calculated per 100 CCU/ml bacteria used for inoculation of A549 and SiHa cell cultures and PPLO broth as a control for both *Ureaplasma* species are presented in Fig. 1 and Fig. 2.

A significantly higher concentration of ammonia was obtained in A549 cells infected with *U. urealyticum* compared with cells infected with *U. parvum* (Mann-Whitney U test P<0.0001) (Fig. 3). Differences in ammonia concentration achieved in SiHa cells infected with *U. parvum* and *U. urealyticum* were not statistically significant.

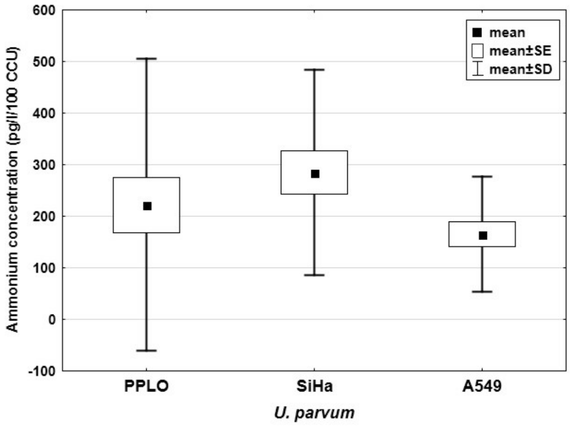

Fig. 1. Ammonia concentrations achieved in the human lung cells (A549), cervical cancer cells (SiHa) and PPLO broth inoculated with the 22 clinical strains of *U. parvum.*

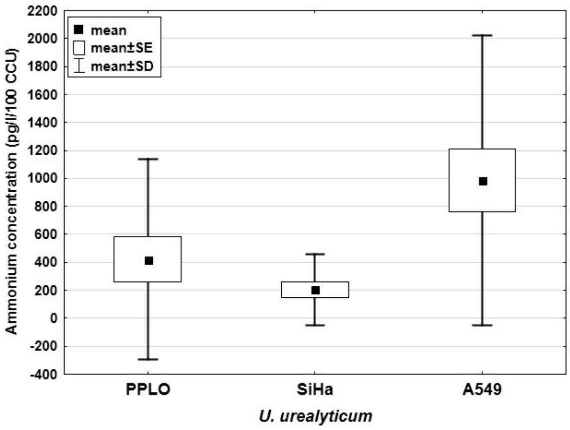

Fig. 2. Ammonia concentrations achieved in the human lung cells (A549), cervical cancer cells (SiHa) and PPLO broth inoculated with the 22 clinical strains of *U. urealyticum.*

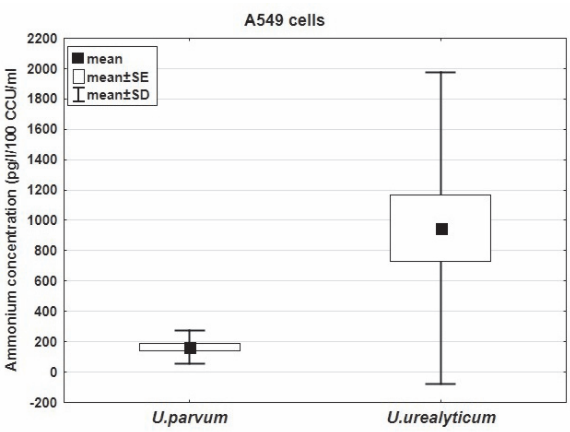

Fig. 3. Comparison of urease activity in the human lung cells (A549) inoculated with the 22 clinical strains of *U. parvum* and *U. urealyticum*.

Urease is produced by many bacteria which are pathogenic to humans. Numerous reports on its role in the pathogenesis of infections with these microbes have been published (SENIOR *et al.* 1980; MOBLEY *et al.* 1991; MOBLEY *et al.* 1995; SANGARI *et al.* 2007; ZHANG *et al.* 2013). It is known that urease is a virulence factor in infections of the urinary tract, including e.g. *Proteus, Klebsiella*, gastrointestinal system, e.g. *H. pylori, Shigella, Yersinia, Brucella* and respiratory tract e.g. *H. influenzae, M. tuberculosis* (MOBLEY *et al.* 1991; COLLINS & ORAZIO 1993; GORDEN & SMALL 1993; JOHNSON *et al.* 1993).

Urease activity can be tested in two ways: by showing the loss of the substrate or the presence/growth of the product formed by the enzyme-catalyzed reaction. There are many methods for the detection of ammonia released by the action of urease, which may be based on vacuum distillation, microdiffusion, steam distillation or electrical conductivity measurement (VAN SLYKE 1927; KATZ 1964; KALTWASSER & SCHLEGEL 1966; RUIZ-HERRERA & GONZALEZ 1969; MCDONALD *et al.* 1972; SHAIK *et al.* 1980).

Various techniques have been applied for the measurement of ammonia concentration, most commonly using the modified method of Bertholet (SMITH et al. 1993; SANGARI *et al*. 2007) or the method of Weatherburn (WEATHERBURN 1967). In contrast to the classical method of Bertholet, we used the method based on pH measurement which allows for an estimation of the urease activity, to assess the concentration of ammonia in the color solution in culture media of A549 and SiHa cells and PPLO broth.

Currently 14 serotypes of ureaplasmas were classified into 2 species *U. parvum* and *U. urealyticum*. The species of *U. parvum* is more common in human infection. It is an open question whether and how much the ureaplasma serotypes or species differ in pathogenicity.

Conflicting reports on this subject appear in the literature (WAITES *et al.* 2005; CULTRERA *et al.* 2006; BIERNAT-SUDOLSKA *et al.* 2006b; ONDONDO *et al.* 2010). Our earlier observations suggested a higher pathogenicity of the *U. urealyticum* species (BIERNAT-SUDOLSKA *et al.* 2006a). It is not known what underlies the differences in pathogenicity. Possibly, they originate from differences at the molecular level. Hence, to address this problem, a variety of aspects should be considered, such as easier horizontal gene transfer, greater variability of surface proteins facilitating escape from the host defence system, higher resistance to drugs or higher activity of enzymes believed to be important virulence factors, like urease, protease and lipase.

We attempted to assess the activity of urease, a key enzyme for ureaplasmas. This enzyme is important for the energy metabolism of ureaplasma cells, but also, by generating ammonia, it produces local cytotoxic effects on the mucous membranes colonized by ureaplasmas. Urease can be lethal to animals after intravenous administration (LIGON & KENNY 1991).

Previous research has shown that this enzyme also allows the survival of bacteria in host tissues (MOBLEY *et al.* 1991; COLLINS & ORAZIO 1993; GORDEN & SMALL 1993; JOHNSON *et al.* 1993; SANGARI *et al.* 2007). Recently, it has been shown that urease also plays a role in respiratory infections. Since urea is translocated to the surface of the airway epithelium and is present in concentrations comparable to the concentrations in the plasma, the presence of urease is also essential for the survival and replication of bacteria causing infections of the respiratory system for example, in infections with *M. tuberculosis* (for survival within macrophages and as the sole nitrogen source in the environment in which they multiply) or *H. influenza* for facilitating the assimilation of nitrogen in the environment of the human respiratory tract (OLIVERA-SEVERO *et al.* 2006; MURPHY & BRAUER 2011; LIN *et al.* 2012).

Urease is targeted by human antibodies in the humoral response, and the presence of anti-urease antibodies correlated with the severity of the disease (FUTAGAMI *et al.*1998). In addition, urease was identified as an immunomodulator of developing inflammatory response (WILSON *et al.* 2000; KONIECZNA *et al.* 2012), thus, it can play a role in pathogenesis, irrespective of the enzymatic activity. This protein acts by several other mechanisms, including the ability to activate macrophages (HARRIS *et al.* 1998), to induce inflammatory mediators (HARRIS *et al.* 1996; TANAHASHI *et al.* 2000; ZHANG *et al.* 2011), apoptosis (FAN *et al.* 2000) and to facilitate survival of bacteria in macrophages (SCHWARTZ & ALLEN 2006; MAKRISTATHIS *et al.* 1998).

In our study, a high concentration of ammonia was demonstrated for *U. urealyticum* in the culture of A549 cells derived from human lung. Our results can suggest a higher urease activity of *U. urealyticum* in the lung tissue and can explain the more severe pneumonia in newborns infected by *U. urealyticum* compared to babies infected by *U. parvum*. ABELE-HORN *et al.* (1997) suggested that *U. urealyticum* was the dominant species, compared to *U. parvum* in patients with pelvic inflammatory disease and might have a greater negative impact on pregnancy, neonatal birth weight, gestational age, and the possibility of preterm delivery. Our results have indicated that urease activity of *U. urealyticum* is higher in cervix cells, which, by analogy to other microorganisms, can promote the survival of these bacteria in the urogenital system and facilitate the ascending infection of particular importance in pregnant women.

We showed a wide disparity between the concentrations of ammonia (Fig. 1 and Fig. 2) observed in strains belonging to both ureaplasma species. These differences most likely result from genetic differences not only between species but also between ureaplasma serotypes. The most recent studies indicate the existence of such differences at the molecular level (PARALANOV *et al.* 2012; ZHANG *et al.* 2013). The various serotypes classified as one species differ from one another to a varying extent. The average level of percent difference in *U. urealyticum* species was 0.62% and 9.5% in *U. parvum* species (PARALANOV *et al.* 2012). In clinical practice, also chimeric ureaplasma strains were isolated, suggesting the existence of recombinant mechanisms also in this group of microorganisms. Perhaps ureaplasmas are a dynamic population, and not a population with stable genotypes, as suggested by the authors of the above paper. At present, clear identification of a gene/group of genes critical for ureaplasma virulence is impossible. It should be remembered that on the one hand, patient's clinical condition depends on the pathogen-related factors, e.g. the presence or lack of the genes responsible for virulence, and on the other, on the defence mechanisms of the host. Future research of *Ureaplasma* biology should focus on the molecular aspects of their diversity, which was postulated, among others by Paralanov (PARALANOV *et al.* 2012).

Conclusions

1. High ammonia levels observed after infection of human culture cells with *U. urealyticum* suggest a higher enzymatic activity of urease of this species.

2. Higher urease activity of *U. urealyticum* in A549 cell culture may indicate a higher pathogenicity of this species particularly for respiratory tract cells and may explain the damage observed in preterm infants vertically infected with *U. urealyticum*.

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