Aerococcal infections - from bedside to bench and back

Senneby, Erik

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Aerococcal infections

- from bedside to bench and back

Erik Senneby

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Abstract

The genus *Aerococcus* comprises eight species of Gram-positive cocci. Due to difficulties in species identification of aerococci in the past, the incidence and clinical importance of aerococcal infections has been underestimated. We evaluated the usefulness of MALDI-TOF MS as a species identification method for aerococci and found that it had a high sensitivity and specificity. Two population-based studies on aerococcal bacteruria were performed. *A. urinae* and *A. sanguinicola* were isolated in approximately 1 % of all urine samples in Skåne. The patients were of old age and gender was almost equally distributed between men and women. Almost 80 % of patients with aerococcal bacteruria had symptoms of a urinary tract infection. Several antibiotics, including nitrofurantoin and amoxicillin, could serve as treatment options. Resistance against ciprofloxacin was recorded, especially in *A. sanguinicola* isolates. In a retrospective population-based study on aerococcal bacteremia, 77 patients were identified during a three-year period (*A. urinae* n=49, *A. sanguinicola* n=13, *A. viridans* n=14 and *A. christensenii* n=1). The *A. urinae* and *A. sanguinicola* patients were old (median age >80 years), predominately of male gender and underlying urological diseases were common. A majority of the *A. urinae* and *A. sanguinicola* patients had severe sepsis. Five patients with *A. urinae* bacteremia were diagnosed with infective endocarditis. *A. viridans* seemed to be a contaminant in most cases. The aerococcal isolates were sensitive to betalactam antibiotics and vancomycin. Two studies were conducted regarding virulence mechanisms. We demonstrated that *A. sanguinicola* isolates produced biofilm and activated platelets, two potentially important virulence factors. *A. urinae* isolates were subjected to masspectrometry-based proteomics and whole genome sequencing. Two cell wall-anchored proteins with LPXTG-motifs, with unknown functions, dominated the surface and genes encoding these proteins were localized in a conserved locus on the aerococcal chromosome.

Key words: Aerococcus, bacteremia, urinary tract infections, MALDI-TOF MS, surface proteome, genes encoding surface proteins

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Erik Senneby

LUND
UNIVERSITY

Erik Senneby

Division of Infection Medicine

Department of Clinical Sciences

Faculty of Medicine

Lund University

Biomedical Center, B14

Tornavägen 10

221 84 Lund

Sweden

Email: erik.senneby@med.lu.se

Faculty of Medicine
Department of Clinical Sciences, Lund

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I. **Erik Senneby**, Bo Nilson, Ann-Cathrine Petersson, Magnus Rasmussen.
Matrix-assisted laser desorption ionization-time of flight mass spectrometry is a sensitive and specific method for identification of aerococci.
Journal of Clinical Microbiology, 2013 Apr;51(4):1303-4

II. **Erik Senneby**, Birger Eriksson, Erik Fagerholm, Magnus Rasmussen.
Bacteremia with *Aerococcus sanguinicola*: Case Series with Characterization of Virulence Properties.
Open Forum Infectious Diseases, 2014 May 23;1(1):ofu025

III. **Erik Senneby,** Linn Göransson, Sofia Weiber, Magnus Rasmussen.
A population-based study of aerococcal bacteraemia in the MALDI-TOF MS-era.
European Journal of Clinical Microbiology and Infectious Diseases, 2016 May;35(5):755-762.

IV. **Erik Senneby**, Ann-Cathrine Petersson, Magnus Rasmussen
Epidemiology and antibiotic susceptibility of aerococci in urinary cultures.
Diagnostic Microbiology and Infectious Disease, 2015 Feb;81(2):149-51

V. Mohammad Oskooi, Torgny Sunnerhagen, **Erik Senneby**, Magnus Rasmussen.
A prospective observational treatment study of aerococcal urinary tract infection.
Journal of Infection, 2018 Apr;76(4):354-360.

VI. **Erik Senneby**, Björn Hallström, Johan Malmström, Christofer Karlsson, Magnus Rasmussen.
Proteomic and genomic profiling of *Aerococcus urinae* surface proteins identifies two quantitatively dominating cell wall-anchored proteins.
Manuscript 2018

Additional articles not included in the thesis

Erik Senneby, Ann-Cathrine Petersson, Magnus Rasmussen.
Clinical and microbiological features of bacteraemia with *Aerococcus urinae*.
Clinical Microbiology and Infection, 2012 Jun;18(6):546-50.

Hanne Pedersen, **Erik Senneby**, Magnus Rasmussen.
Clinical and microbiological features of *Actinotignum bacteremia*: a retrospective observational study of 57 cases.
European Journal of Clinical Microbiology and Infectious Disease, 2017 May;36(5):791-796

Abbreviations

UTI	Urinary tract infection
PYR	Pyrrolidonyl aminopeptidase
LAP	Leucin aminopeptidase
IE	Infective endocarditis
MALDI-TOF MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
AST	Antibiotic susceptibility testing
CFU	Colony-forming units
PCR	Polymerase chain-reaction
ASB	Asymptomatic bacteriuria
MIC	Minimum inhibitory concentration
TEE	Transoesophageal echocardiography
NAD	Nicotinamide adenine dinucleotide
EUCAST	The European committee on antibiotic susceptibility testing

Abstract

The genus *Aerococcus* comprises eight species of Gram-positive cocci. Due to difficulties in species identification of aerococci in the past, the incidence and clinical importance of aerococcal infections has been underestimated. The aims with this thesis were to describe aerococcal infections from a bacteriological and a clinical perspective and to describe potential aerococcal virulence mechanisms. We evaluated the usefulness of MALDI-TOF MS as a species identification method for aerococci and found that it had a high sensitivity and specificity. Two population-based studies on aerococcal bacteruria were performed. *A. urinae* and *A. sanguinicola* were isolated in approximately 1 % of all urine samples in Skåne. The patients were of old age and gender was almost equally distributed between men and women. Almost 80 % of patients with aerococcal bacteruria had symptoms of a urinary tract infection. Several antibiotics, including nitrofurantoin and amoxicillin, could serve as treatment options. Resistance against ciprofloxacin was recorded, especially in *A. sanguinicola* isolates. In a retrospective population-based study on aerococcal bacteremia, 77 patients were identified during a three-year period (*A. urinae* n=49, *A. sanguinicola* n=13, *A. viridans* n=14 and *A. christensenii* n=1). This corresponds to an incidence of approximately 14 cases per 1,000,000 inhabitants per year for *A. urinae*. The *A. urinae* and *A. sanguinicola* patients were old (median age >80 years), predominately of male gender and underlying urological diseases were common. A majority of the *A. urinae* and *A. sanguinicola* patients had severe sepsis. Five patients with *A. urinae* bacteremia were diagnosed with infective endocarditis. *A. viridans* seemed to be a contaminant in most cases. The aerococcal isolates were

sensitive to betalactam antibiotics and vancomycin. Two studies were conducted regarding virulence mechanisms. We demonstrated that *A. sanguinicola* isolates produced biofilm and activated platelets, two potentially important virulence factors. *A. urinae* isolates were subjected to masspectrometry-based proteomics and whole genome sequencing. Two cell wall-anchored proteins with LPXTG-motifs, with unknown functions, dominated the surface and genes encoding these proteins were localized in a conserved locus on the aerococcal chromosome.

Aims with the thesis

The overall aim with this thesis is to describe aerococcal infections from both a bacteriological and a clinical perspective and to describe potential aerococcal virulence mechanisms.

The specific aims are:

- to evaluate MALDI-TOF MS as a species identification method for aerococci.
- to describe the epidemiology and clinical presentation of aerococcal urinary tract infections and to investigate treatment options for such infections.
- to describe the epidemiology and clinical presentation of aerococcal invasive infections and to determine the sensitivity of invasive isolates to commonly used antibiotics.
- to investigate potential virulence factors of *Aerococcus sanguinicola* with focus on biofilm formation and induction of platelet aggregation.
- to describe the surface proteome and genes encoding surface proteins in *Aerococcus urinae*.

Aerococci – emerging pathogens?

There has been a steady increase of urine and blood cultures positive for aerococci for the years 2009-2017 in Skåne (Figure 1 and 2). The total number of urine and blood cultures is presented in Figure 3.

Such a striking increase naturally raises questions regarding the cause (or causes). In the final chapter of the thesis (Conclusion and futures perspectives), the subject will be discussed further in an effort to explain how come such an extraordinary increase has occurred.

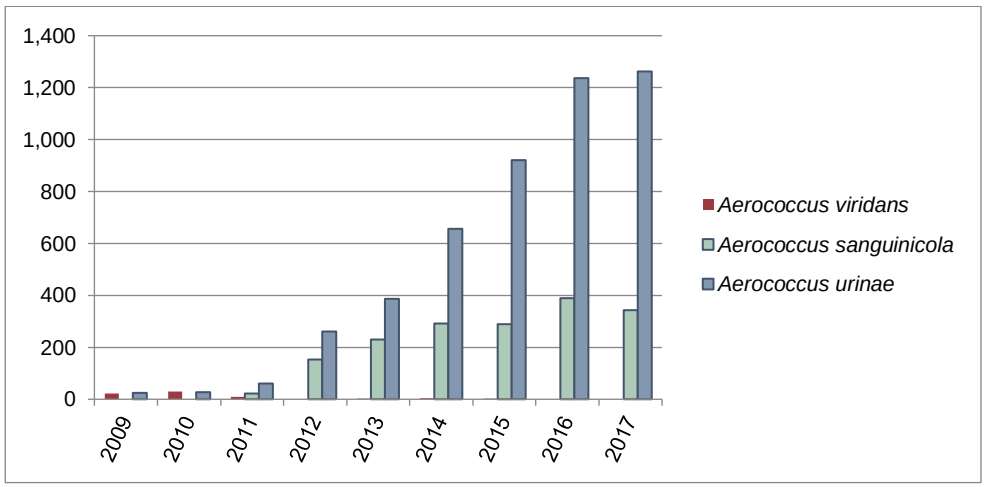

Figure 1.
Number of urine cultures that grew aerococci in Skåne for the years 2009-2017.

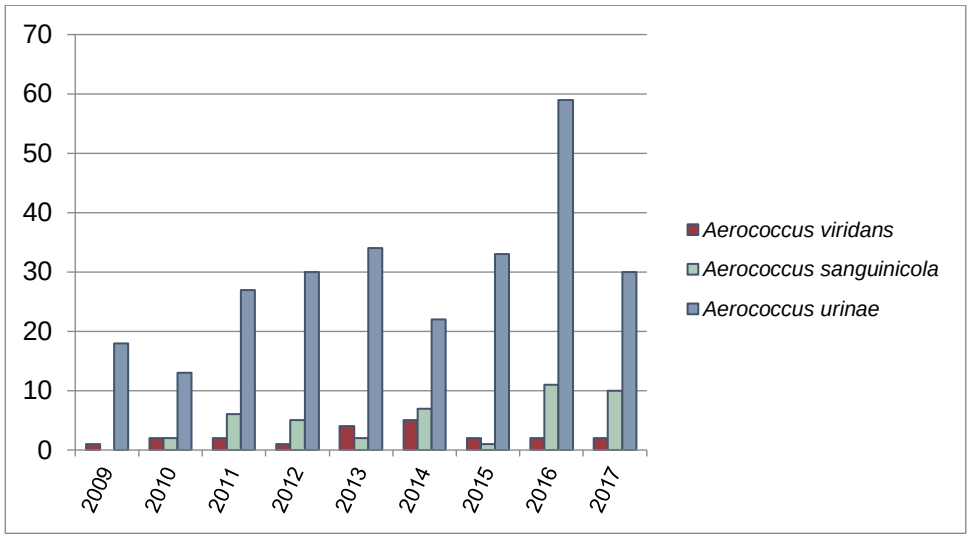

Figure 2.
Number of blood cultures that grew aerococci in Skåne for the years 2009-2017.

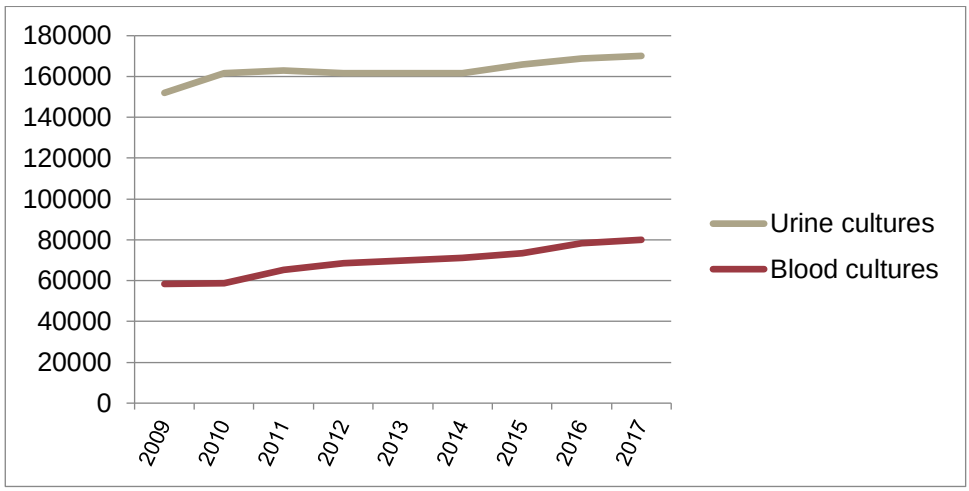

Figure 3.
Total number of urine and blood cultures for the years 2009-2017 in Skåne.

1. Introduction to *Aerococcus*

The genus *Aerococcus* is constituted by eight species – *A. viridans, A. urinae, A. sanguinicola, A. christensenii, A. urinaehominis, A. urinaeequi, A. suis* and *A. vaginalis.*

Aerococci are facultative anaerobic, Gram-positive and catalase-negative cocci that belong to the phylum Firmicutes. Aerococci grow in clusters and resemble staphylococci in Gram stain (Figure 4A), but colonies on blood agar are small and greyish, and sometimes green pigmented, with alpha-hemolysis – features they share with alpha-hemolytic streptococci (Figure 4B).

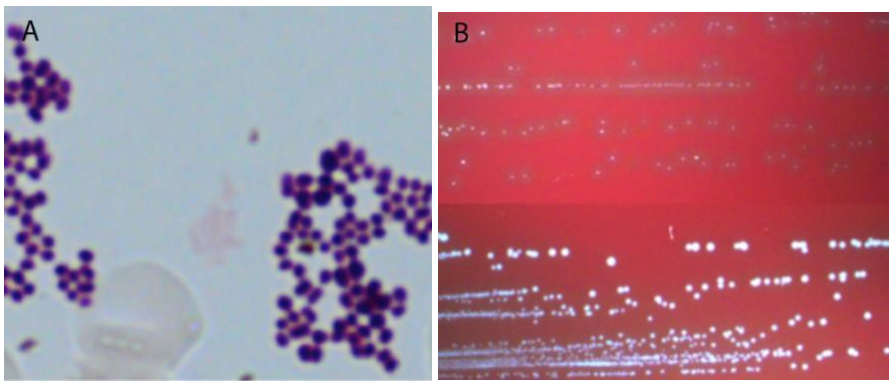

Figure 4.

A) The appearance in light microscopy of Gram-stained *A. urinae* from blood culture broth. B) Morphology of aerococcal colonies on blood agar. The upper part of the picture shows *A. urinae* colonies and the lower part shows *A. sanguinicola* colonies. The bacteria were isolated from blood cultures and incubated for 24 h in 5 % CO_2.
Courtesy of Magnus Rasmussen.

2. A historical overview of the genus *Aerococcus*.

Taxonomic confusion and dead lobsters.

In a publication from 1953, Williams *et al.* proposed a new bacterial genus called *Aerococcus* (6). The organism described was isolated from air and dust in crowded rooms - such as offices, schoolrooms and shoe factories - and from streets in London. The characteristics of *Aerococcus* were presented in the study:

"*The most characteristic features of the organisms are ability to grow in the presence of 40 % bile and to produce greening on blood agar. They are catalase-negative, and do not show chain-formation in culture*".

Since aerococci share features with both staphylococci (appearing in Gram-stain as tetrads or clusters) and streptococci or enterococci (the colony morphology on blood agar), and also due to the fact that no previous reports were present, the authors concluded that aerococci must have been misclassified in the past. They also speculate about the explanation to this confusion.

"*This is partly due to the fact that many of the tests by which we distinguish aerococci from enterococci are of relatively recent introduction*".

It is rather remarkable that we, Senneby *et al.*, in a publication 60 years later draw a very similar conclusion regarding difficulties in species identification of another aerococcal species, namely *Aerococcus urinae* (3). The species name of the organism described by Williams was later proposed to be *Aerococcus viridans*.

Although *A. viridans* did not receive attention as a cause of human disease, the story took an interesting and rather unexpected turn. Several studies in 1947 had described a microorganism causing the deadly disease gaffkemia in American lobster (7-9). The bacterium, initially called *Gaffkya homari,* was thought to enter breaches of the lobster exoskeleton in over-crowded tanks due to fighting or poor handling and then cause a systemic sepsis-like disease, which could lead to detachment of limbs and death (10). In 1960, Deibel *et al.* investigated the relatedness between *A. viridans* and *Gaffkya homari* and found that they were not easily distinguishable from each other (11). They also made the remark, as

Williams did, that these bacteria displayed similarities with species in the genus *Pediococcus*. In following publications, the term *Aerococcus-Pediococcus-Gaffkya*-group was used (12). Eventually, the genus *Gaffkya* was rejected by the bacteriological community based on several publications, such as those by Kelly *et al.* and Steenbergen *et al.* in the 1970s (13, 14). The lobster disease gaffkemia is since attributed to the species described by Williams *et al.* in 1953 - *Aerococcus viridans*. Gaffkemia has had a huge economic impact on the commercial lobster industry, as the disease cause significant post-harvest fatalities, and is still the main motive for research on *A. viridans*.

Aerococcus-like organisms and human disease.

During the following decades, *A. viridans* remained the only species within the genus *Aerococcus*. Studies reporting aerococci as a cause of human disease remained scarce, even though some studies mentioned the presence of aerococci in clinical cultures. For instance, Parker *et al.* studied streptococci and systemic infections and collected streptococcal-like isolates during a 3-year period in the 1970s (15). A total number of 719 isolates were collected from patients that suffered from a systemic infection, of which 7 were classified as "aerococci".

In the late 1980s Christensen *et al.* described human infections caused by so-called *Aerococcus*-like organisms (ALO) in Denmark (16). The isolates came from urine cultures from 29 patients suffering from urinary tract infection (UTI). A phenotypic comparison between the ALO strains and strains from related organism, including *A. viridans*, was performed. This comparison was based on colony morphology, biochemical reactions and antibiotic susceptibility patterns. The authors concluded that ALO was easily distinguishable from the *A. viridans* strains and the tested streptococci with this approach. Approximately 0.8% of the examined urine cultures during the study period grew ALO. In 1991, Christensen *et al.* expanded their investigations of this newly recognized pathogen in a study where 64 cases of UTI from other parts of Denmark were described (17). In this study, additional biochemical tests were evaluated to discriminate between ALO and *A. viridans*. The different types of aerococci differed in their production of pyrrolidonyl aminopeptidase (PYR) and leucin aminopeptidase (LAP). Thus, the authors had shown that two phenotypic tests were available to separate ALO from *A. viridans*. In another study from the same year two cases of invasive disease caused by ALO were reported by Christensen *et al.* (18). The taxonomic confusion regarding ALO was finally dispersed in 1992 when Aguirre *et al.* performed 16S rRNA gene sequencing of five ALO strains (19). The authors proposed that ALO should be classified as a novel aerococcal species – *Aerococcus urinae*. During the

following decades, several studies on *A. urinae* reported cases of invasive disease, such as bacteremia and infective endocarditis (IE) (20-26) and UTI (27-29).

… and additional aerococcal species

In 1999, a new aerococcal species was identified - *Aerococcus christensenii* (30), named after the Danish clinical microbiologist J.J. Christensen. The two strains that were characterized were isolated from human vaginal samples. Two years later, a forth aerococcal species was defined – *Aerococcus sanguinicola* (the name "*sanguicola*" was initially proposed) (31). The strain was isolated from a human blood culture (hence the name, meaning blood dweller in Latin). Facklam *et al.* published a phenotypic description of *A. sanguinicola*, including antimicrobial susceptibility results (32). Six cases of *A. sanguinicola* bacteremia were reported by Ibler *et al.* (33). Cattoir *et al.* and Shelton-Dodge *et al.* reported cases of *A. sanguinicola* UTI (28, 29).

In 2001, Lawson *et al.*, suggested that *Aerococcus urinaehominis* should constitute a novel aerococcal species (34). This proposition was based on both phylogenetic and phenotypic analysis of an aerococcal strain isolated from human urine. In 2005, *Pediococcus urinaeequi* was reclassified as *Aerococcus urinaeequi*, as suggested by Felis *et al.* (35). In 2007, an organism isolated from swine specimens was designated *Aerococcus suis* (36). In 2014, the latest contribution to the genus *Aerococcus* saw the light of day as *Aerococcus vaginalis* was proposed as a new species (37). This organism was isolated from the vaginal mucosa of a Japanese beef cow. No cases of clinical disease in human caused by *A. urinaehominis*, *A. urinaeequi*, *A. suis and A. vaginalis*, have been reported.

3. Methods in clinical bacteriology

In this chapter, methods in clinical bacteriology that are of special importance regarding identification of aerococci will be described. Historically, species identification of bacterial isolates has much relied on visual inspection of colonies on agar plates. Even though the arsenal of species identification methods has expanded since the first agar plates were developed in the 19th century, categorizing bacteria based on colony appearance still remain a fundament in clinical bacteriology. Different characteristics, such as colony size, form and colour and number of colonies, can be used to discriminate between groups of bacteria.

Gram staining

The Danish pathologist Hans Christian Gram developed the Gram stain method in 1884 (38, 39). The method is still used today, although somewhat modified, and allows one to separate Gram-positive bacteria from Gram-negative bacteria. Bacterial colonies are smeared on to a glass slide and fixated with heat. Crystal violet is poured over the glass, and after rinsing with water, Lugol's solution is added. In the next step, Ethanol is poured over the glass slide and finally, bacteria are counterstained with safranin (the counterstaining step was not performed by H.C. Gram in the original method). This procedure will stain some bacteria purple, which are denoted Gram-positive bacteria, and some bacteria pink or red and these are called Gram-negative bacteria. The difference in colour uptake is explained by differences in the thickness of the cell wall in the two groups of bacteria.

MALDI-TOF MS

The possibility of using mass spectrometry as a method for species identification of microorganisms was investigated in 1975 by Anhalt *et al.* (40). In this study, mass spectra profiles for Gram-positive bacteria (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and several Gram-negative bacteria (for instance *Pseudomonas aeruginosa* and *Salmonella* species) were presented. In the 1980s and 1990s, technique improvements led to the development of Matrix-Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS) and it was evaluated as a tool for rapid identification of bacterial isolates (e.g. (41-43)). However, the real breakthrough for MALDI-TOF MS as a useful method in clinical practice was not seen until the 2010s, when it was commercialized and made accessible for non-experts in mass spectrometry (44-46). MALDI-TOF MS was shown to rapidly and accurately identify bacteria to a low cost. It was the beginning of a paradigm shift in clinical microbiology.

MALDI-TOF MS is a mass spectrometry method that utilizes the fact that microbes have unique protein contents. The work-flow for MALDI-TOF MS is summarized in this section, and is in large based on what is described in (47). Colonies from agar plates are smeared onto a designated spot on a metal plate with a sharp object (for instance a tooth pick) and a small amount of a special matrix-solution is added to the bacteria. The plate is left to dry for a couple of minutes. This is referred to as the direct smear method. Some microorganisms also require a few microliters of formic acid before the matrix solution is added. Bacteria from blood culture bottles (non-solid medium) can also be transferred to the metal plate after bacterial pellets are produced through the use of a certain protocol. Briefly, this includes the use of lysis buffer, ethanol, formic acid and acetonitrile with centrifugation steps in between (48). The plate is inserted into the instrument, which is controlled by a computer. Many samples can be analyzed in the same session. When the procedure is initiated, a laser fires short bursts at the spots to be analyzed, and parts of the bacterial-matrix-complex are ionized and vaporized. Charged ions of different sizes are generated. An electric potential difference is applied and ions are accelerated through a vacuum space towards a detector. Even though separation is in theory based on mass-to-charge ratio (m/z), since the charge of the analytes is essentially the same, separation is by molecular weight. Ions with small mass travel faster than ions with large mass. The smallest ions will hit the detector first, followed by progressively larger analytes. Finally, a "mass fingerprint" based on the protein content is generated (Figure 5). The mass range of the molecules analyzed by MALDI-TOF MS is typically 2-20 kDa. A computer software compares the mass spectrum of an organism to mass spectra profiles in a database and suggests a genus and a species name, accompanied by a score describing how certain the identification is considered to be. For example, a score

value >2.0 is considered to be reliable to the species level. The method allows identification of microorganisms within minutes.

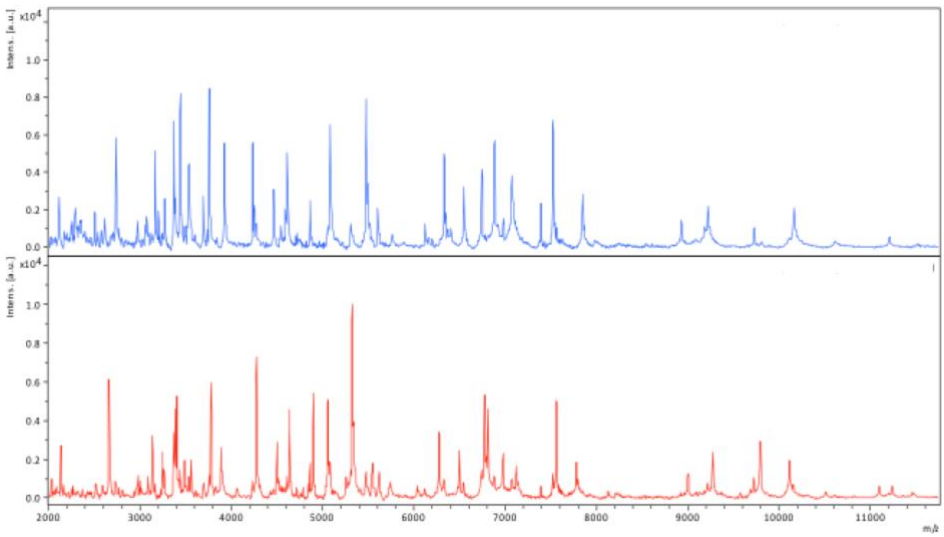

Figure 5.
MALDI-TOF MS masspectra of *A. urinae* (blue) and *A. sanguinicola* (red). Courtesy of Bo Nilson, Clinical Microbiology, Lund.

Urine culture procedures

The two clinical microbiology laboratories in Malmö and Lund used the same routine procedures for urine cultures for the years 2009-2015. In 2015, the two laboratories merged into one laboratory, located in Lund, which since has served the entire health care system in the Region of Skåne (with a population of approximately 1.3 million inhabitants).

The current routine work-up of urine samples has principally been the same since 2009 regarding the set-up of plates and criteria used for assessment of cultures. In mid-2015, the fully automated microbiology specimen processor WASPLab (Copan, Italy) was implemented in the routine diagnostic work in Lund. This robotic system moves the urine samples through the whole process, from sampling of urine with an inoculation loop, streaking the plates and incubating them, to creating digital images.

Routinely, between 5-8 ml of urine is collected from a patient and sent to the laboratory in a clean plastic tube. The tube enters the WASPLab and 10 µl of urine is streaked onto half of a plate containing blood agar and colistin and nalidixic acid, and 10 µl of urine is streaked onto the other half containing Uriselect 4 agar (Bio-Rad Laboratories AB, Sweden). This plate is called the biplate. Urine is also inoculated onto a Müller-Hinton (MH) agar plate and six antibiotic disks are applied for antibiotic susceptibility testing (AST) with the disk diffusion method. The antibiotics tested are mecillinam, ampicillin (amoxicillin is interpreted from ampicillin), cefadroxil, nitrofurantoin, trimethoprim and ciprofloxacin. The plates are incubated for at least 16 hours in 35° C in ambient air. The images is viewed on a screen and assessed by the laboratory workers who perform further work-up manually when necessary. For instance, plates with growth of bacteria that demands species identification with MALDI-TOF MS or fastidious bacteria that require Müller-Hinton fastidious (MH-F) agar plates for AST are picked out. When aerococci are suspected, both these measures are required. The MH-F plates are incubated over-night in 35° C in 5 % CO^2. Most of the usual findings, such as a non-resistant *Escherichia coli*, require no further work than visual inspection of the biplate and the antibiogram. The Uriselect 4 agar is chromogenic and growth of different bacterial species generates specific colours. For instance, *E. coli* is pink, *A. urinae* is white and *A. sanguinicola* is turquoise (Figure 6).

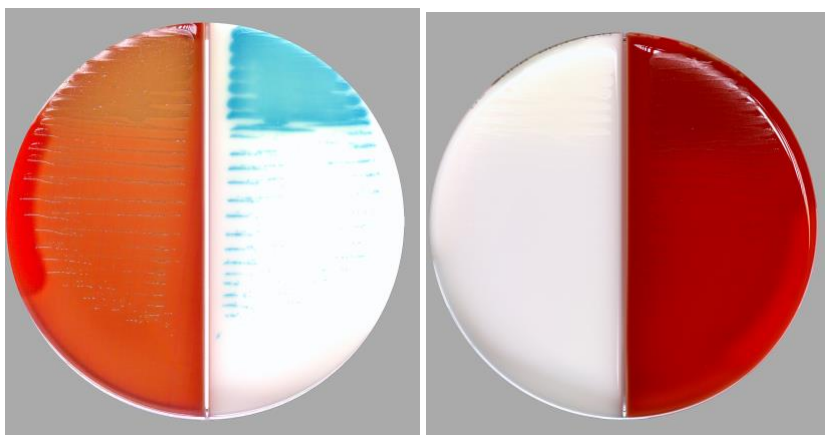

Figure 6.
Colonies of *A. sanguinicola* (left picture) and *A. urinae* (right picture) on biplates. Courtesy of Martina Christensson, Clinical Microbiology, Lund.

Figure 7.
Growth of *A. urinae* on a MH-F-plate with six antibiotic disks. Courtesy of Martina Christensson, Clinical Microbiology, Lund.

Quantification of growth

Quantification of growth is needed when assessing a urine culture. The number of colony forming units per liter (CFU/l) is estimated by counting colonies. Since the urogenital flora easily contaminates urine during sampling, a certain level of growth is required to yield significant growth. Different cut-offs are used for different bacterial species. For instance, in most cases, $\geq 10^6$ CFU/l is required for *E. coli*, which is equivalent to 10-99 colonies in a 10 µl urine specimen. For aerococci, $\geq 10^7$ CFU/l and growth of no more than one additional species is required to yield a positive urine culture. Cultures with polymicrobial growth (> two species) or cultures with $< 10^6$ CFU/L, is in most cases considered not to be significant.

A standard urine cultures is incubated in ambient air. This is not optimal for aerococci, which grow better in 5 % CO_2. This may have influenced the sensitivity of the urine culture method to detect aerococci in urine. The requirement of $\geq 10^7$ CFU/l may also have influenced the sensitivity. Measuring the level of white blood cells or pro-inflammatory cytokines in urine could possibly enhance the method, since information about the inflammatory response could help to resolve issues of contamination or colonization.

Blood culture procedures

The two clinical microbiology laboratories in Malmö and Lund used the BacT/Alert blood culture system (bioMérieux, Marcy l'Etoile, France) for the years 2009-2014. In December 2014, it was replaced by the BACTEC FX blood culture system (Becton Dickinson, Franklin Lakes, USA).

For adult patients, it is recommended that two blood cultures are drawn at the same time, from two different punctures. One blood culture generally consists of one aerobic and one anaerobic bottle. The recommended amount of blood for one bottle is 8-10 ml (thus a total volume of 30-40 ml is recommended) (47). The volume of blood drawn is of great importance and many observations have been made regarding the relationship between the volume cultivated and the likelihood of recovering bacteria (49-51). The blood culture bottles are incubated in 35-37^{o} C for 5 days. A chemical detector is sensing the production of CO_2 and this is measured every ten minutes. When a bottle yields growth, the system notifies the user and the bottle is removed from the incubator manually. Routinely, the initial step is Gram staining of the blood culture broth, followed by inoculation of a set of agar plates in the WASPLab, usually including a blood agar plate and a Uriselect plate (incubated in ambient air) and a chocolate agar plate (incubated in CO_2). An anaerobic plate is incubated in a separate incubator. In addition, plates are inoculated for antibiotic susceptibility testing. In 2013, a new MALDI-TOF MS application was employed, which made species identification directly on blood cultures bottles possible. In 2015, blood culture systems were distributed to the clinical chemistry laboratories located in five hospitals in Skåne, which made it possible to load blood cultures bottles into the incubators around the clock. Positive bottles are transported to the clinical microbiology laboratory in Lund.

4. Species identification of aerococci

As mentioned, aerococcal colonies on a blood agar plate are in most cases indistinguishable from alpha-hemolytic streptococci. At the time our studies on aerococci began, all urinary cultures that grew bacteria with the appearance of alpha-hemolytic streptococci at the clinical microbiology laboratories in Malmö and Lund were routinely considered to be of no clinical importance and no further species identification or antimicrobial susceptibility testing were carried out. The message from the laboratory to the referring clinician was short and concluded that the finding was of no clinical importance. Thus, no data was available on how many of these alleged alpha-hemolytic streptococci were in fact aerococci or other bacteria. A correct identification of aerococci in urinary cultures was of potential importance, since several reports had suggested aerococci as a cause of UTI (16, 17, 27, 28, 52). As mentioned, microscopy after Gram staining of colonies would have been able to separate aerococci from streptococci, but this was not part of the diagnostic routine in Malmö and Lund, mostly due to practical reasons.

During a 2-month period in 2010, we screened all urinary cultures in Skåne for the presence of aerococci. Colonies that resembled alpha-hemolytic streptococci were Gram stained and tested for catalase activity. Most of these bacteria grew in clusters and were catalase negative. Thus, they were neither streptococci nor staphylococci. This work is more comprehensively presented in Paper I (3).

The workflow for blood cultures in the clinical microbiology laboratory was thus different from the above mentioned for urine cultures at the time we started investigating aerococci in 2010. Microscopy after Gram staining was performed on all positive blood culture bottles as the initial diagnostic step. Preliminary identification of aerococci would thus have been "a staphylococcal species" based on the microscopy finding. All positive blood cultures were cultivated on agar plates over-night, of which some were incubated in 5 % CO_2. If staphylococci were expected from the microscopy, the appearance of colonies resembling alpha-hemolytic streptococci was a cause of puzzlement when colonies on the agar

plates were inspected the next morning. In the years before the introduction of MALDI-TOF MS, species identification in general relied on phenotypic tests, which were based on biochemistry, or genetic tests, such as PCR and sequencing of the 16S rRNA gene. The antibiogram could also contribute with information about species identity. This has been suggested in several studies regarding aerococci (16, 17, 53). As an example, *A. urinae* are resistant to sulfonamides, but in general susceptible to penicillin, which could help discriminate *A. urinae* from streptococci and enterococci.

For aerococci, secure identification relied on sequencing of the 16S rRNA gene (19, 30, 31) prior to the implementation of MALDI-TOF MS. This procedure is however costly, time-consuming and not well suited for blood culture diagnostics, where prompt species identification is of great importance.

Phenotypic properties of aerococcal species, including fermentation of different carbohydrates, enzyme tests and growth characteristics have been relatively comprehensively described (16, 17, 31, 32, 34, 54) and a combination of these tests can theoretically be used for species identification of aerococci. As Christensen *et al.* suggested, testing the combination of LAP, PYR and sucrose would be sufficient to discriminate aerococcal species from each other (54). The use of such a scheme would however be rather impractical for routine diagnostic work, since most Gram-positive cocci growing in clusters are not aerococci. Commercially available phenotypic systems, such as the VITEK 2 (bioMérieux, Marcy l'Etoile, France) are also based on biochemical reactions but have the advantage of being easier to implement in a clinical setting, since they can be used more universally. The same procedure is used for a large group of bacteria, such as "Gram-positive cocci". The VITEK 2 systems generate a computer-based interpretation of the biochemical reaction pattern and suggest a species name. This implicates that a valid database is required - broad enough to include all relevant species in a group. Also, it is necessary that such systems clearly report failures of identification when it occurs. As an example, rather than misidentifying an *Aerococcus* as *Granulicatella*, it should report something similar to "no reliable identification was possible". Several of these commercially systems have been evaluated for aerococci (29, 32, 55) with varying, but mostly poor, results. In conclusion, none of the tested systems in the studies referred to in this chapter could reliably identify all aerococcal species. In fact, species identification methods used in several publications on aerococcal infections has been unreliable and misclassification has probably occurred (56-59).

MALDI-TOF MS and aerococci.

In a study by Christensen *et al.* the authors evaluated MALDI-TOF MS on a collection of catalase-negative, Gram-positive cocci, including 35 strains of different aerococcal species (60). All of the *A. urinae* and *A. sanguinicola* strains were identified correctly to the species level, but MALDI-TOF MS failed to safely identify two *A. viridans* strains. It was also shown that the score values were improved when an extension of the database was generated. In this way, the sensitivity of the method to correctly identify well-characterized aerococcal strains was evaluated.

We investigated the usefulness of MALDI-TOF MS as an identification method for aerococci in a clinical setting. This is described in Paper I of this thesis (3).

5. Clinical presentation of aerococcal bacteriuria.

UTI classification.

The topic of UTI classification will not be fully covered in this thesis. But worth mentioning, the definition of UTI is dependent on what criteria are used.

In this paragraph, some widely used classification systems of UTI will be briefly summarized (61-63). UTI can be defined either as symptomatic or as asymptomatic (ASB). Sometimes ASB is referred to as colonization. Typical symptoms and signs that support the presence of a symptomatic UTI are fever (>38^{o} C), urgency, frequency, dysuria and suprapubic tenderness. The clinical presentation can determine whether a patient suffers from cystitis (involving the bladder), pyelonephritis (involving the kidneys) or urosepsis (systematic effects). Predisposing risk factors can be used to classify a UTI as uncomplicated or complicated. For instance, male gender is in most cases is considered to be a complicated UTI. The result from a urine culture, such as the type of microorganism isolated, the number of CFU/L of the organism, and if the microorganism is isolated in pure culture, may also influence the UTI classification. An elevated level of leukocytes in urine supports the diagnosis of UTI and the lack of leukocytes supports ABS.

To conclude, the classification of UTI relies both on laboratory findings and clinical symptoms and signs.

Previous studies on aerococcal bacteruria.

For many years, aerococci were unappreciated as uropathogens. This may be explained by the fact that *A. viridans* very rarely causes UTI and remained the only known aerococcal species for decades, and perhaps by the fact that aerococci in urine cultures have been misclassified as streptococcal species. Observations made by Christensen *et al.* in the 1980s led to the discovery of a new aerococcal species, which was isolated from urine specimens.

Initially these aerococci were referred to as *Aerococcus*-like organisms (ALO), but were later designated as *A. urinae* (19).

In the publication from 1989 (16), the presence of UTI caused by ALO was investigated at a hospital in Denmark during a 4-month period. Patient charts were reviewed and signs and symptoms of UTI were recorded (rectal temperature $\geq 38^{o}$ C, dysuric complaints and/or pollakisuria). Significant bacteruria was defined as $\geq 10^{5}$ CFU/ml. Pyuria was determined with microscopy. During the study period, 29 patients were identified of whom 11 had ALO in pure culture. In samples with mixed culture, aerococci were most commonly isolated with *E. coli* (n=17). Twenty patients were female and 9 were male and the median age was 75 years (range 49-88). Forty-one patients had predisposing conditions for UTI. This was the first study that published the finding of this novel aerococcal species and, perhaps as important, suggested that it should not be considered to be just a contaminant in urine samples. The authors reported that 72 % of the patients had symptomatic UTI. ALO was isolated in about 0.8 % of all urine samples during the study period.

A second study was conducted in 1991 by Christensen *et al* (17). Sixty-three patients with urine cultures that grew ALO were described. The patient characteristics were consistent with previous findings, even though the fraction of men was slightly higher (45 %). A majority (55 %) of the patients presented with symptoms of UTI according to the authors.

In a study by Schuur *et al.*, the presence of *A. urinae* in urine cultures was investigated in the south of the Netherlands in two different laboratories (27). *A. urinae* was found in 0.31 % and 0.44 % of all urine samples during one year. Species identification was based on biochemical tests. *A. urinae* was found in pure culture in 83 % of the cases. Clinical data was retrieved by a questionnaire sent to the treating physician. The patients were of old age and predisposing conditions for UTI were common. The authors reported that 98 % of the patients had symptoms indicating a UTI. However, only 40 of 54 physicians returned the questionnaire, which may have influenced the results. And also, criteria for UTI used in this study included vague symptoms such as abdominal pain and general discomfort, which is not specific for UTI.

In 2005, Sierra-Hoffman *et al.* performed a retrospective chart review of all patients that had been admitted to a hospital during one year with a urine culture positive for *A. urinae* (52). *A. urinae* was found in 0.25 % of all urine samples. Aerococcal species identification was based on biochemical tests. Sixty-four cases were identified, but only 54 medical records were available. Ninety-two percent of the patients were female. Patients were classified as being either colonized or having a UTI based on clinical data and laboratory findings. Thirty-two patients (59 %) were considered to have a UTI. In the UTI group, the proportion of patients

having a urinary catheter was significantly higher (41 % vs. 5 %). Thirty-one percent of the patients in the UTI group had *A. urinae* in pure culture, as compared to 45 % in the colonized group. In mixed cultures, aerococci were most commonly isolated with *E. coli* (n=22).

A few years later, Shelton-Dodge *et al.* performed a study with a similar approach (28). In this study, medical charts from patients with *A. urinae* (n=66) and *A. sanguinicola* (n=52) in urine were reviewed. All samples were analyzed at the same clinical microbiology laboratory. The species identification was based on biochemical tests in this study as well (including the PYR and LAP tests). *A. urinae* was isolated from 0.19 % and *A. sanguinicola* in 0.15 % of all urine samples. To differentiate between UTI and colonization, clinical diagnosis was used. In the *A. urinae* group, 79 % of the patients were diagnosed with a UTI and 67 % of the *A. sanguinicola* patients were diagnosed with a UTI. The mean age was 82 years (range 24-101 years). The majority of the patients were female, and a majority of the male patients had underlying prostate disease.

Cattoir *et al.* retrospectively analyzed clinical and microbiological data of 29 cases of aerococcal UTI from a hospital in France (29). Three different aerococcal species were detected: *A. urinae* (n=20), *A. sanguinicola* (n=8) and *A. viridans* (n=2). Species determination was performed through sequencing of the 16S rRNA gene. Patients were categorized as either being infected or colonized based on the classification described in (52). The demographics and clinical characteristics of the 29 patients were in line with results from previous studies; 76 % were female, median age was 73 years (range 19-96 years), and 31 % had predisposing urological disease.

Based on these studies, patients suffering from aerococcal UTI have been found to be of old age (mean or median age was >65 years in all studies), predominately female and many patients have underlying conditions predisposing for UTI. Aerococci were isolated in pure culture in 38-83 % of the cases and 55-98% of the patients were considered to have a symptomatic UTI (Table 1). Several factors may have influenced the range of these numbers. Different definitions of UTI were used in the studies, and also, the clinical guidelines for sampling urine for culture may have varied (such data is not presented in any study). Most studies have included only in-hospital patients whereas one study received urine samples from nursing homes and general practitioners as well. The population base is not clearly defined in any of the studies and it is hard to draw conclusions about incidence of aerococcal UTI in the general population based on them.

The role of aerococci as colonizers of the urinary tract and their role in UTI are thus not fully explained by these studies, although they together strongly suggest that *A. urinae* and *A. sanguinicola* should not be disregarded as just contaminants in urine samples. *A. viridans* seems to be of little or no clinical importance as a

urinary tract pathogen, even though a few case reports of *A. viridans* UTI have been published (64, 65).

Table 1. Studies on aerococcal UTI.
Adapted from Rasmussen (66).

Study and year	Species	No of patients	% of cultures	% pure culture	Mean or median age in years	% male	% with UTI
Christensen, 1989	ALO	29	0.8	38	75	31	72
Christensen, 1991	ALO	63	-	57	74	46	55
Schuur, 1997	*A. urinae*	40	0.4	83	80	50	98
Sierrra-Hoffman, 2005	*A. urinae*	54	0.25	40	>65	7	60
Cattoir, 2010	*A. urinae* *A.sanguinicola* *A. viridans*	29	-	38	73	30	93
Shelton-Dodge, 2011	*A.urinae* *A.sanguinicola*	46+57	0.15-0.19	-	82	13-24	67-79

Although most studies on aerococcal UTI describe older patients, there are reports on aerococcal UTI in children. Murray *et al.* reported a case of pyelonephritis in a 12-year old boy (67) and three publications describe young boys, aged 5 ,7 and 12 years, with extremely foul-smelling urine caused by *A. urinae* (68-70).

In Paper IV, we presented population-based data on the prevalence of aerococci in clinical urine specimens (1). In paper V, we performed a prospective observational treatment study of aerococcal UTI (4). MALDI-TOF MS was used as the primary species identification method in these studies.

6. Clinical presentation of invasive aerococcal infections.

ALO/ *Aerococcus urinae*

Christensen *et al*. described two cases of ALO bacteremia in 1991 (18). One of the cases was a fatal endocarditis. A few years later, the Danish ALO study group performed a nation wide survey and reported seventeen cases of ALO bacteremia, corresponding to an incidence of 0.5 cases per million inhabitants per year according to the authors (20). All but two patients were male and the median age was 78 years (range 37-90 years). Most patients were considered to have predisposing conditions, such as prostatic or cardiac disease. Sixteen patients had ALO in pure culture and six patients were diagnosed with IE, confirmed by echocardiography. Five of the IE patients died from the infection. Ten patients were diagnosed with urosepticemia of whom nine grew ALO in urine.

Kristensen *et al.* presented one case of *A. urinae* IE, where the primary focus was the urinary tract, confirmed by growth of *A. urinae* in urine (23). Several other studies have reported cases of severe IE caused by *A. urinae*, often with rather spectacular outcome (21, 25, 26, 71-75).

In a study by Senneby *et al.* (76), we retrospectively identified 16 cases of *A. urinae* bacteremia during a 6-year period in Skåne, which corresponded to an incidence of 3 cases per million inhabitants per year. Fifteen patients were male and 12 had urological conditions. However, *A. urinae* was rarely found in urine cultures, even though a UTI was suspected in many cases. Three patients were diagnosed with IE of whom one patient also suffered from spondylodiscitis and one patient developed septic embolization to the brain. One fatality was recorded.

Sunnerhagen *et al*. published a registry-based study on aerococcal IE in Sweden where 14 cases of *A. urinae* IE were reported (77). Ten of the patients were male, and the median age was 79 years. The patients were older than those with streptococcal or staphylococcal IE and in most cases a urinary tract focus was suspected. In contrast to previous case reports on aerococcal IE, no fatalities were recorded and the outcome seemed to be relatively favourable.

Other cases of spondylodiscitis have been reported but this seems to be a rare manifestation of *A. urinae* bacteremia (78-82). Other uncommon *A. urinae* invasive infections include peritonitis associated with peritoneal dialysis (83, 84), spontaneous bacterial peritonitis (85), postpartum infection (86), infections in the genital area (24, 76, 87) and hip abscess (88).

Aerococcus sanguinicola

Ibler *et al.* described *A. sanguinicola* bacteremia in a case series of six patients (33). Most patients were of old age (median 70 years, range 40-92 years) and suffered from underlying neurological conditions. Four patients were male and two patients had an indwelling urinary catheter. The origin of infection was considered to be the urinary tract in three cases and the gallbladder in one case, (*Proteus mirabilis* also was isolated from blood in that case). Two patients had IE, confirmed with echocardiography, with *A. sanguinicola* in pure culture. Two additional cases of *A. sanguinicola* IE were described in Sunnerhagen et al. (77).

In Paper II we described eleven cases of *A. sanguinicola* bacteremia that were identified retrospectively for the years 2006-2012. (2).

In Paper III, we performed a population-based study of aerococcal bacteremia for the years 2012-2014, using MALDI-TOF MS as the primary identification method. (5).

Aerococcus christensenii

Carlstein *et al.* described a case of polymicrobial chorioamnionitis where *A. christensenii*, among other pathogens, was isolated in foul-smelling amniotic fluid (89). The patient was a healthy woman that developed fever, tachycardia and vomiting after labour had been induced with an intracervical balloon. The patient recovered after intravenous antibiotic therapy. The child showed no signs of infection.

Jose *et al.* described a case of subacute bacterial endocarditis in a 60-year-old male with a B-cell lymphoproliferative disease (90). Echocardiography revealed a vegetation on the aortic valve and blood cultures grew *A. christensenii.* The patients underwent aortic valve replacement and received six weeks of treatment with ceftriaxone and recovered.

Aerococcus viridans

As mentioned, *A. viridans* is foremost a lobster pathogen, not a human pathogen. Publications describing invasive disease with *A. viridans* must be interpreted with caution. Numerous studies reporting invasive *A. viridans* infections have used identification methods, based on biochemistry, that are unable to safely identify the aerococcal isolates to the species level.

- Three cases of meningitis reported by Nathavitharana *et al.* (91).
- Cases of infective endocarditis reported in several publications (92-95).
- A case of post-operative *A. viridans* bacteremia reported by Tekin *et al.* (96).

Other studies have failed to report which methods were used for species identification of the alleged *A. viridans* isolates.

- Case reports on *A. viridans* endocarditis reported by Park *et al.* and Zhou *et al.* (97, 98).
- A case of penicillin-resistant *A. viridans* bacteremia in a patient with sickle-cell disease reported by Swanson *et al.* (99) .
- A case where a heart-transplantation patient suffered from a paraaortic abscess due to *A. viridans* reported by Park *et al.* (100).
- A case of *A. viridans* aortic pseudoanerysm and endocarditis reported by Yadav *et al.* (101)

Aerococcal isolates that are resistant to penicillin are however likely to be *A. viridans*, since penicillin-resistance is very uncommon in other aerococcal species (this is further presented in Chapter 7).

In the following studies the isolates were more reliably identified as *A. viridans*:

- A case of *A. viridans* bacteremia in a HIV-positive patient reported by Razeq *et al.* (102).
- A case of penicillin-resistant *A. viridans* bacteremia reported by Uh *et al.* (103).
- A case of odontogenic *A. viridans* infection reported by Jiang *et al.* (104)

7. Aerococcal antibiotic susceptibility.

In our study from 2012 on *A. urinae* bacteremia (76), a case of ciprofloxacin resistant *A. urinae* infection is described. A patient with *A. urinae* bacteremia was discharged with ciprofloxacin as follow-up treatment, since a UTI was suspected as the origin of the infection. The patient returned to hospital shortly thereafter due to fever and new blood cultures yielded again growth of *A. urinae*. A transoesophageal echocardiography (TEE) revealed a vegetation on the mitralis valve. The *A. urinae* isolate was resistant against Ciprofloxacin (MIC > 32 mg/L).

Antibiotic resistance is not a major issue regarding aerococci, since most clinically relevant species are susceptible against beta-lactam antibiotics, such as penicillin, cephalosporins and carbapenems. Some strains are however resistant to antibiotics commonly used in UTI treatment, as in the example above, which can be problematic. In this chapter, aspects of antibiotics and aerococci will be covered.

EUCAST guidelines for antibiotic susceptibility testing of aerococci.

Aerococci are rather demanding to cultivate and have special requirements. The European committee on antibiotic susceptibility testing (EUCAST) specify which media and growing conditions should be used when performing susceptibility testing on *A. urinae* and *A. sanguinicola* (105). MH-F agar or broth is recommended, which is MH agar or broth supplemented with 5 % defibrinated horse blood and 20 mg/L β-NAD. The isolates should be incubated in 5 % CO_2 in 34-36º C. The results should be recorded after 16-20 hours, or if poor growth, after 40-44 hours of incubation.

In the EUCAST document for aerococci, two methods for AST determination are used; the disk diffusion method and broth microdilution (BMD) according to ISO-standard 20776-1 (106). An alternative method for MIC determination is Etest (bioMérieux, France), which is often used in clinical routine due to practical reasons. Carkaci *et al.* compared MIC values obtained by BMD and Etests for six

antibiotics, tested on 120 aerococcal isolates, and the results from the two methods were in agreement within ± one dilution (107).

Some studies have reported that the choice of culturing medium can influence the results of AST for aerococci (108, 109). This will be discussed further in the Trimethoprim-sulphamethoxazole paragraph.

Penicillin (benzylpenicillin, penicillin G)

In general, *A. urinae* and *A. sanguinicola* display low MIC-values against penicillin. If the newly introduced EUCAST clinical breakpoints for *A. urinae* and *A. sanguinicola* were applied (110), more than 99 % of reported isolates would be sensitive (Table 2 for references). In some publications, MIC 0.25 mg/l have been reported for a small number of isolates (5, 28, 111, 112). This is equal to one MIC step above the MIC breakpoint, which would be interpreted as penicillin resistance according to EUCAST. The number of resistant isolates corresponds to less than one percent of all tested isolates. Thus, for non-allergic patients, penicillin could be considered as the drug of choice for treatment of invasive infections with *A. urinae* and *A. sanguinicola*.

A. viridans isolates seem to display higher MIC-values for penicillin than other aerococcal species (5, 103), but no EUCAST clinical breakpoints have been established for *A. viridans*.

Ampicillin and Amoxicillin

The EUCAST clinical breakpoint for ampicillin is S ≤ 0.25 mg/l. Amoxicillin can be interpreted from the ampicillin MIC. More than 98 % of the tested isolates are sensitive to ampicillin/amoxicillin (Table 2 for references). Amoxicillin could be considered as a reasonable treatment option for aerococcal UTI.

Cefotaxime, Ceftriaxone and Ceftibuten

If MIC breakpoints for viridans group streptococci were used, 95% of the tested *A. urinae* and 84% of the tested *A. sanguinicola* isolates would be sensitive to cefotaxime, and 76% of *A. urinae* and 95% of *A. sanguinicola* isolates would be sensitive to ceftriaxone (Table 2 for references). All isolates tested in Oskoii *et al.*

displayed MIC-values >32 mg/l for ceftibuten (4), which suggests that it should be avoided in the treatment of aerococcal UTI.

Carbapenems

Aerococci are in general sensitive to carbapenems. When applying EUCAST breakpoints for meropenem (S ≤ 0.25 mg/L), 99% of *A. urinae* and 96% of *A. sanguinicola* isolates would be sensitive (Table 2 for references). All tested aerococcal isolates in Senneby *et al*. (5) had MIC-values ≤0.125 mg/l for imipenem, which support the assumption that aerococci are sensitive to imipenem as well.

Vancomycin

Ninety-seven percent of tested aerococcal isolates are sensitive to vancomycin according to the EUCAST MIC breakpoint (S ≤ 1 mg/l) (Table 2 for references). Vancomycin could thus be a rational treatment option for penicillin-allergic patients with invasive aerococcal infections.

Gentamicin

No clinical MIC breakpoints have been established for gentamicin and aerococci. The MIC-values presented in studies range from 0.125 - >256 mg/l for *A. urinae* and 2-32 mg/l for *A. sanguinicola* (2, 5, 29, 76, 112). Thus, some aerococcal isolates are highly gentamicin resistant. Several studies have investigated if synergism is achieved when combining gentamicin with other antibiotics (77, 112, 113). Synergism will be discussed in a separate paragraph below.

Ciprofloxacin and Levofloxacin

As previously mentioned, most patients with aerococcal bacteremia, and many of the patients with aerococcal UTI, are elderly men, often with urological underlying conditions. A flouroquinolone is an attractive treatment option for infections in this type of patient. However, ciprofloxacin and levofloxacin resistance is not uncommon in aerococci. When EUCAST breakpoints for aerococci are applied,

92% of *A. urinae* and 39% of *A. sanguinicola* isolates are sensitive to ciprofloxacin (Table 2 for references). For levofloxacin, 84% of *A. urinae* and 39% of *A. sanguinicola* isolates are sensitive. Resistance is thus an issue regarding flouroquinolone treatment of aerococcal infections, especially concerning *A. sanguinicola*.

The underlying genetic causes for flouroquinolone resistance in aerococci has been presented in two publications (114, 115), which will be further discussed below.

Nitrofurantoin

The nitrofurantoin MIC breakpoint for treatment of uncomplicated aerococcal UTI is set to 16 mg/l. All tested isolates in the studies referred to in Table 2 are considered to be sensitive. Nitrofurantoin is, alongside mecillinam, recommended as a first-choice treatment for cystitis according to Swedish guidelines. The results in Oskoii *et al.* (Paper V of this thesis) suggest that nitrofurantoin is an appropriate treatment for aerococcal cystitis (4).

Mecillinam

No clinical breakpoints have been established for mecillinam and aerococci. As mentioned, mecillinam is a first-choice drug for cystitis in Sweden and thus many patients with aerococcal UTI will receive mecillinam empirically. The MIC values presented in our studies do not rule out the possibility that mecillinam could have an *in vivo* effect against aerococci (1, 4). This is supported by the fact that mecillinam achieved clinical success in all patients in Oskooi *et al.*, although the number of patients treated was low (4).

Trimethoprim (TR) and Trimethoprim-sulphamethoxazole (SXT)

Several studies have reported that aerococci are resistant to TR or SXT (1, 4, 27, 29, 55). Most aerococcal isolates in these studies have MIC values >32 mg/l, but a few isolates display MIC-values as low as 0.125 mg/l. However, Humphries *et al.* argued in a publication from 2011 that the choice of culturing media influences the AST results of SXT on aerococci (109). In their study, all tested *A. urinae* isolates

(n=27) were susceptible to SXT in MH broth with lysed horse blood (LHB). According to Humphries *et al.*, conventional media contains thymidine, which is able to inhibit the SXT effect. The authors argued that lysed erythrocytes release an enzyme, called thymidine phosphorylase, which converts thymidine to thymine and thus restores the STX effect on the bacteria. Hizel *et al.* investigated this further and unexpectedly found that identical *A. urinae* isolates were susceptible to SXT in broth supplemented with LHB but resistant to SXT when tested with Etests on agar supplemented with LHB (108). It is still unclear what is the cause of these results and how it should be interpreted.

Synergistic effects of antibiotic combinations

In a study by Zbinden *et al.*, the killing kinetics of penicillin with and without an aminoglycoside were investigated (26). Two *A. urinae* isolates were tested and a synergistic effect with the antibiotic combination was noted in both cases. In a study by Skov *et al*., the authors investigated the effect of combining penicillin or vancomycin with gentamicin on two *A. urinae* isolates (112). The authors concluded that there was a synergistic effect of both antibiotic combinations. In Sunnerhagen *et al.*, two methods were used to evaluate the effect of combining penicillin with gentamicin on thirteen *A. urinae* and two *A. sanguinicola* isolates (77). The results from the two methods combined suggested synergy only in a minority of cases (n=7), of which all were *A. urinae*. Hirzel *et al.* described time-kill results on three *A. urinae* isolates for penicillin, ceftriaxone, gentamicin and daptomycin alone and in combination (113). Synergism was noted for penicillin or ceftriaxone combined with gentamicin in two of the isolates. The authors also concluded that daptomycin is bactericidal against *A. urinae* and could constitute a treatment option in certain cases.

Antibiotic resistance mechanisms in aerococci

Resistance mechanisms in aerococci have not been thoroughly studied, since only three publications have investigated possible resistance mechanisms.

Cattoir *et al.* investigated point mutations in the quinolone-resistance determining regions (QRDRs) of DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) and the presence of efflux pumps in nineteen *A. urinae* and eight *A. sanguinicola* isolates (114). Two isolates of each species that displayed MIC-values > 32 mg/l for one or more of the tested quinolones had amino acid substitutions in the QRDRs of *gyrA* and *parC*. The MICs for ciprofloxacin were

similar in the presence or absence of reserpin (an efflux pump inhibitor), indicating that this mechanism is not of great importance in aerococcal flouroquinolone resistance.

Guilarte *et al.* made a similar approach and when they investigated flouroquinolone resistance in 80 *A. urinae* isolates (115). Ten isolates were non-susceptible to flouroquinolone and all of them had mutations in the QRDR of *parC*, whereas two of the isolates also had mutations in the QRDR of *gyrA*. In line with previous results, the authors found no evidence of efflux pumps as the cause of flouroquinolone resistance.

Lupo *et al.* used the cefinase disk assay (Oxoid) to detect betalactamase production in *A. urinae* isolates (n=80) (111). Almost all isolates were susceptible to penicillin but a few were resistant to ceftriaxone. Betalactamase production was however not detected in any of the isolates and the authors proposed that the ceftriaxone resistance could be explained by alterations in the penicillin-binding proteins of the aerococcal cell wall.

Table 2.
Antibiotic sensitivity of *A. urinae* and *A. sanguinicola*.

	EUCAST breakpoints for *A. urinae* and *A. sanguinicola* (MIC mg/l)	**Proportion of sensitive isolates in % and number (*n*) of isolates tested (references)**	
Antibiotic	S ≤/R >	*A. urinae*	*A. sanguinicola*
Benzylpenicillin	0.125/ 0.125	99 %, n=389 (28, 76, 107, 111, 112, 116)	99 %, n=104 (2, 28, 32, 107)
Ampicillin and Amoxicillin [a]	0.25/0.25	98 %, n=342 (1, 29, 107, 111, 112, 116)	98 %, n=63 (2, 29, 32)
Meropenem	0.25/0.25	99 %, n=209 (107, 116)	96 %, n=54 (32, 107)
Ciprofloxacin[b]	2/2	92%, n=172 (1, 4, 112)	39%, n=77 (1, 4, 32)
Levofloxacin[b]	2/2	84 %, n=262 (28, 52, 108, 116)	32 %, n=56 (28, 32)
Vancomycin	1/1	97%, n=388 (5, 28, 29, 76, 107, 111, 116)	97 %, n=115 (5, 28, 29, 32, 107)
Nitrofurantoin[b]	16/16	100 %, n=180 (1, 4, 108)	100%, n=65 (1, 4)
Rifampicin	0.125/0.125	100 %, n=250 (5, 107, 116)	100 %, n=50 (5, 107) 0%, n=15 (32)[c]
	EUCAST breakpoints for viridans group streptococci		
Cefotaxime	0.5/0.5	95%, n=266 (5, 76, 107, 116)	84 %, n= 74 (2, 5, 32, 107)
Ceftriaxone	0.5/0.5	76%, n=294 (28, 111, 112, 116)	95%, n=41 (28)

[a]Amoxicillin can be interpreted from ampicillin MIC. [b]Only uncomplicated UTI. [c]All isolates in this study had MIC 2 mg/L.

8. Virulence mechanisms and aerococci

Aerococcal virulence has not been thoroughly studied and very little is known about how aerococci cause infection.

It has been shown that *A. urinae* can produce biofilm and activate platelets (117). In the study by Shannon *et al.,* five *A. urinae* isolates from clinical blood cultures were subjected to analysis. All isolates produced biofilm and the presence of 10% plasma had a stimulatory effect on biofilm formation. Four isolates induced platelet aggregation in platelet-rich plasma, retrieved from five donors. One isolate induced aggregation in all five donors. This isolates was further investigated for the ability to activate platelets. It was demonstrated that the isolate activated platelets and that the complement system, fibrinogen and immunoglobulin G (IgG) were involved in the process. The authors concluded that both biofilm formation and platelet aggregation is of importance in the pathogenesis of IE caused by *A. urinae*.

In a study by Carkaci *et al.,* genes coding for potential virulence factors were searched for in aerococcal isolates using whole genome sequencing (118). Forty *A. urinae* and eight *A. sanguinicola* isolates from clinical urine and blood cultures were sequenced. The authors searched for similarities between aerococcal genes and known virulence genes in a virulence factor database (VFDB) and they concluded that gene homologues associated with antiphagocytosis and bacterial adherence were identified in the aerococcal isolates.

Bacterial induced platelet aggregation

Many Gram-positive bacteria, including staphylococci and enterococci, that cause IE have the ability to activate platelets and to induce aggregation (117, 119-121). This has been proposed to be important for bacterial virulence. On the other hand, platelet activation can also contribute to host defense, since activated platelets release antibacterial peptides (122-124). Resistance against such peptides in *Staphylococcus aureus* isolates has been showed to correlate with an endovascular

infectious source (125). Thus, the exact role of bacterial induced platelet activation in the pathogenesis of IE is complex. There are three basic principles through which bacteria can affect platelets (126). Firstly, platelets can be activated by the immune response to the infection - an effect that is mediated by inflammatory cytokines. The other two principles involve secreted bacterial products that activate platelets or binding of bacteria to platelets. The binding can be either direct, which means that a bacterial surface protein binds to a platelet receptor, or indirect, which involves a mediator (often a plasma protein such as fibrinogen) that binds both to the bacterium and the platelet. This indirect activation mechanism requires presence of plasma IgG, which mediates activation through the FcγRIIa receptor on the platelet surface (119-121). When bacteria activate platelets, a signal-transduction cascade is evoked (127). The platelet integrin GPIIb/IIIa becomes activated and platelet expression of surface molecules is increased. The GPIIb/IIIa binds to plasma fibrinogen, which lead to platelet aggregation. The bacterial induced aggregation have been described to be concentration dependent (126). This means that a threshold exists, below which no aggregation occurs and above which maximum aggregation occurs. Another feature of bacterial induced aggregation is the presence of a lag time before aggregation is initiated (117, 120, 126). This lag time can be shortened if the bacterial concentration is increased, but it can never be eradicated. The time-to-aggregation have been reported to range between 2-25 minutes for different bacterial species.

Biofilm formation

The process of biofilm formation can be described as microorganisms that attach to and grow on surfaces where they produce an extracellular matrix, which mainly consists of polysaccharides (128). This matrix is often referred to as extracellular polymeric substance (EPS) matrix. The phenotypic properties of microorganisms in a biofilm differ from microorganisms in a planktonic (floating) state. For instance, when microorganisms are embedded in the biofilm, the metabolism slow down, probably due to limited access to nutrients or oxygen (129). The organisms also become more resistant to host defences and antibiotics (129-134), probably due to the inhibited penetration of antimicrobial compounds in the biofilm and to the altered metabolism of the bacteria.

Bacterial biofilms have wide clinical implications, since modern health care involves the use of many synthetically products with surfaces suitable for biofilm formation, such as urine and venous catheters and prosthetic joints and other implantable devices. Many Gram-positive cocci can produce biofilms, such as *Enterococcus faecalis* and *Enterococcus faecium* (134, 135), and *S. aureus* and

coagulase-negative staphylococci (130, 136). Biofilm-associated bacteria that are commonly isolated from indwelling urine catheters are *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis* and *Enterococcus faecalis* (128). As mentioned, it has been demonstrated that *A. urinae* form biofilms, and this might be of clinical importance in patients with indwelling urine catheters or in the process of aerococcal IE. In a recent publication, a case of *A. urinae* pacemaker endocarditis was described in which the ability of the bacteria to form biofilm could have had importance (137).

In Paper II, we demonstrated that clinical *A. sanguinicola* isolates can produce biofilms and induce platelet aggregation (2).

Cell wall-anchored proteins with an LPXTG-motif

Proteins with a so-called LPXTG-motif (where the letters LPTG represent specific amino acids and X means any amino acid) are widely spread among Gram-positive bacteria where they play a central role for survival and virulence. Proteins containing this motif are involved in fundamental bacterial processes such as adhesion, immune evasion, internalization and iron acquisition (138). For example, the M-protein of *Streptococcus pyogenes* displays a wide spectrum of functions, such as binding to plasma proteins and inhibition of phagocytosis (139, 140). In *S. aureus*, around 20 different proteins with the LPXTG-motif have been identified, including protein A (Spa) and two clumping factors (ClfA and ClfB) (141).

The LPXTG-proteins share common structural characteristics. There is a signal sequence in the NH_2-terminal end, which directs the protein for secretion through an export apparatus called the Sec pathway (142, 143). In the COOH-terminal end, there is a cell wall sorting region containing the LPXTG-motif, a hydrophobic membrane-spanning domain and a tail with positively charged amino acids (144). The membrane bound enzyme sortase recognizes the LPXTG-motif and cleaves and attaches the protein to the peptidoglycan of the cell wall (142, 145, 146). The attachment of LPXTG-proteins will be disrupted if the sortase enzyme function is impaired. Several studies have described attenuation of virulence when sortase knockout mutants of streptococcal, staphylococcal and *Listeria* strains are used to infect animals (147-149). For example, *S. aureus* sortase mutants did not cause renal abscesses and acute infections in mice in a study by Mazmanian *et al.* (145). Inhibition of sortase also caused reduced binding to IgG, fibrinogen and reduced biofilm production in staphylococcal strains (150, 151). Chemical compounds that

inhibit sortase activity have been suggested as ideal anti-virulence drugs, since the growth of bacteria is not affected (141, 152). The bacterial virulence would be impaired but with a reduced risk of resistance development.

In paper VI of this thesis, we described the proteome of two *A. urinae* isolates through the use of masspectrometry-based proteomics and identified two quantitatively dominating LPXTG-proteins. Also, we described genes encoding surface proteins with an LPXTG-motif in 25 *A. urinae* isolates (*manuscript in preparation)*.

9. Present investigations.

Paper I

Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry Is a Sensitive and Specific Method for Identification of Aerococci

MALDI-TOF MS had recently been introduced in Malmö and Lund, but was not fully implemented yet when we started our investigations on aerococcal bacteriuria. MALDI-TOF MS was a very promising method for species identification, but was not validated in a clinical setting for identification of aerococci. In this study, we defined the sensitivity of the method by prospectively collecting suspected aerococci from urine cultures during two months. Urine cultures with growth of $>10^7$ CFU/L of colonies resembling alpha-hemolytic streptococci were selected and the colonies were tested for catalase activity and Gram stained. Catalase negative bacteria that grew in clusters in Gram stain were subjected to sequencing of the 16S rRNA gene. In total, we sequenced 48 isolates of which 22 were *A. urinae* and 19 were *A. sanguinicola*. These aerococcal isolates were then analyzed with MALDI-TOF MS, and all isolates were correctly identified to the species level.

After MALDI-TOF MS was implemented in the routine diagnostic work, we defined the specificity of the method in regard of identifying aerococci. During a 2-month period, all isolates that were identified as aerococci by MALDI-TOF MS (with a score ≥ 1.8) were subjected to sequencing of the 16S rRNA gene. There was a total agreement between the two methods for all identified *A. urinae* isolates (n=25) and *A. sanguinicola* isolates (n=19).

In conclusion, we demonstrated that MALDI-TOF MS was a reliable identification method for aerococci, with high sensitivity and specificity.

Paper II

Bacteremia with Aerococcus sanguinicola: Case Series with Characterization of Virulence Properties

In this work, we focused on the clinical presentation of *A. sanguinicola* bacteremia and described potential virulence properties of the isolates.

We retrospectively identified blood cultures in Skåne with growth of suspected or confirmed *A. sanguinicola* between 2006-2012. We obtained clinical information from the patients' medical charts. MIC values were determined for commonly used intra-venous antibiotic and we investigated the isolates ability to form biofilm and induce platelet aggregation.

Results and conclusion

We found 11 patients with *A. sanguinicola* bacteremia. All patients were of male gender and the median age was 82 years (range 67-93). Five patients had *A. sanguinicola* in pure culture in their blood cultures and six patients had additional bacterial species isolated. Eight of the patients had an indwelling urinary catheter and six had underlying urological conditions, such as prostatic disease. A majority had dementia or memory impairment, but none received immunosuppressive treatment. Nine of the patients had fever and five patients presented with hematuria. All but one fulfilled the criteria for sepsis and nine of them had organ dysfunction. Two patients, terminally ill in cancer, died at hospital, both with *A. sanguinicola* in pure culture. Two patients had IE according to Duke´s criteria, one with *A. sanguinicola* in pure culture, and one with additional growth of coagulase-negative staphylococci. Both of the IE patients recovered. In six patients, a urine culture was performed but none yielded growth of aerococci. The MIC values were in line with previous reports, with low MICs for penicillin, cefotaxime and vancomycin and high MICs for ciprofloxacin.

All eleven isolates produced biofilms, tightly attached to the plastic surface, after 24, 48 and 72 hours of incubation. For a majority of the isolates, the biofilm produced after 72 hours were significantly increased in the presence of human plasma. Platelet aggregation was determined through aggregometry in platelet-rich plasma from three human donors. Three isolates were unsuccessful in inducing platelet aggregation in plasma from any of the donors. The other isolates induced aggregation in plasma from one or more donors.

To conclude, we demonstrated that *A. sanguinicola* can cause invasive infections with severe outcome in elderly men, of whom many had underlying urological

conditions, and that *A. sanguinicola* produce biofilm and induce platelet aggregation, virulence mechanism that could be of significance in IE. The presence of plasma strongly stimulated biofilm formation, a fact that supports the possibility that biofilm formation could have a part in aerococcal IE.

Paper III

A population-based study of aerococcal bacteraemia in the MALDI-TOF MS-era

The purpose of this study was to describe the incidence and the clinical presentation of aerococcal bacteremia in a setting with a well-defined population-base in the MALDI-TOF MS-era. No such investigation had been undertaken before. Aerococci in blood cultures were identified retrospectively for the years 2012-2014 in Skåne. The patients' medical charts were systematically reviewed.

Results and conclusion

We identified 77 cases of aerococcal bacteremia (*A. urinae* n=49, *A. sanguinicola* n=13, *A. viridans* n=14 and *A. christensenii* n=1). The correlating incidences were 14 cases per 1000 000 inhabitants per year for *A. urinae* and 3.5 cases per 1000 000 inhabitants for *A. sanguinicola* and *A. viridans*.

Thirty patients (61%) had *A. urinae* in pure culture, 6 patients (46 %) had *A. sanguinicola* and 5 patients (36 %) had *A. viridans* in pure culture. Only seven patients with *A. urinae* bacteremia and one patient with *A. sanguinicola* bacteremia had aerococci in urine cultures.

Patients with *A. urinae* and *A. sanguinicola* bacteremia had a median age of 82 and 86 years, respectively, whilst patients with *A. viridans* bacteremia had a median age of 64 years ($p<0.0001$ for difference, Kruskal-Wallis test).

There was a male dominance, especially in the *A. urinae* group (80%).

Underlying conditions were common. Sixty-five percent of the *A. urinae* patients, 23 % of the *A. sanguinicola* patients and 21 % of the *A. viridans* patients had urinary tract disorders ($p=0.002$ for difference, chi-squared test). Forty-one percent of the *A. urinae* patients, 38 % of the *A. sanguinicola* patients and none of the *A. viridans* patients had indwelling urinary catheters ($p=0.02$ for difference, chi-squared test).

Fever was the most common symptom. Fifty-one percent of the *A. urinae* patients, 69 % of the *A. sanguinicola* patients and 21 % of the *A. viridans* patients fulfilled criteria for sever sepsis (p=0.04 for difference, chi-squared test). Cefotaxime was the most common initial antibiotic treatment. In total, four patients were admitted to the intensive care unit and 6 patients died within 30-days (*A. urinae* n=5, *A. sanguinicola* n=2 and *A. viridans* n=1). Five patients with *A. urinae* in pure culture were diagnosed with IE of whom three fulfilled the Duke´s criteria (153). None of them had *A. urinae* in urine.

MIC-values for ten antibiotics were determined on all isolates with Etests (bioMérieux, France). The *A. urinae* and *A. sanguinicola* isolates displayed low MICs for penicillin, cefotaxime, imipenem and vancomycin. The *A. viridans* isolates had in general slightly higher MIC-values for the beta-lactam antibiotics, except for imipenem. Several isolates of all species had high MICs for ciprofloxacin and clindamycin.

In conclusion, the incidence of aerococcal bacteremia was several times higher in our study than previously reported, probably due to the implementation of MALDI-TOF MS. *A. urinae* was the most common cause of aerococcal bacteremia and the only species causing IE, and predisposing factors seem to be old age, male gender and urological conditions. *A. viridans* seems to be a contaminant in most cases, since it was isolated with other low-grade pathogens in a majority of cases (such as coagulase-negative staphylococci), underlying urinary tract diseases were rare and the focus of bacteremia was unknown in 71 % of the patients. Interestingly, only a few patients had aerococci isolated in urine cultures. A careful clinical assessment should be performed for patients with aerococcal bacteremia and the risk of IE or the possibility of contamination should be considered.

Paper IV

Epidemiology and antibiotic susceptibility of aerococci in urinary cultures

In this study we described the prevalence of aerococci in urine cultures. The population-base was the inhabitants of Skåne. All aerococcal isolates from the clinical microbiology laboratories in Malmö and Lund was collected during a 3-month period. Species identification was performed with MALDI-TOF MS. Only cultures with $\geq 10^7$ CFU/L of aerococci and with no more than one additional species were included. Antibiotic susceptibility testing was performed with Etests and disk diffusion tests.

Results and conclusion

The most commonly isolated pathogen during the study period was *E. coli* (n=9204). *A. urinae* was isolated in 64 cultures (73 % were monocultures) and *A. sanguinicola* in 40 cultures (98 % were monocultures). The other species isolates in these cultures were *E. coli* (n=14), *S. aureus* (n=1), *Citrobacter koseri* (n=1) and *Klebsiella oxytoca* (n=1). Aerococci were isolated in 0.62 % of all positive urine cultures or in 0.27 % of all cultures sent to the laboratories. The estimated incidence of aerococcal bacteruria was 33 cases per 100 000 inhabitants, based on these results.

The median age of patients with aerococcal bacteruria was 83 years, which was higher compared to the median age of 69 years for patients with *E. coli* ($p<0.0001$, Mann-Whitney test) and to the median age of 66 years for patients with *Enterococcus faecalis* ($p<0.0001$, Mann-Whitney test).

Female gender dominated in the *E. coli* group (84 %), which was significantly different from the almost even gender distribution in the aerococcal group ($p<0.0001$, chi-squared test). The proportion of samples collected from primary care and hospital was recorded. Aerococci were more often isolated from hospital patients compared to *E. coli* ($p=0.02$, chi-squared test).

In general, the MIC-values for ampicillin and cefalotin were low for all aerococcal isolates. Most isolates were resistant to trimethoprim. A variation between the aerococcal species was noted for ciprofloxacin - most *A. urinae* isolates had low MIC-values (MIC_{90} 4 mg/l) and most *A. sanguinicola* isolates had high MIC-values (MIC_{90} >32 mg/l). The MIC_{90} for mecillinam was 8 mg/l for *A.* urinae and 16 mg/l for *A. sanguinicola*. The MIC_{90} for nitrofurantoin was 4 mg/l for both species.

In conclusion, we demonstrated that aerococcal bacteruria were more common than previously thought and that several antibiotics could be considered as attractive treatment options based on the MIC-values. However, to establish the optimal treatment, clinical treatment studies were needed.

Paper V

A prospective observational treatment study of aerococcal urinary tract infection

In this study, we aimed to evaluate different treatment options for aerococcal UTI by performing a prospective observational study during a two-month period. Urine cultures positive for aerococci were identified with MALDI-TOF MS in the clinical microbiology laboratory in Lund. The inclusion criteria were the same as in Paper IV, except for exclusion of urine cultures that also grew a primary pathogen (*E. coli* or *Staphylococcus saprophyticus*).

A standardized interview with the referring physician was performed within a few days after culture positivity to obtain clinical information. Information from the medical charts was also collected. The patients were interviewed a couple of days after the treatment was completed. Clinical success was defined as disappearance of all symptoms. A control culture was collected 1 week after therapy completion. Microbiological success was defined as no growth of the aerococcal species that grew in the first culture. MIC-values were determined for mecillinam, nitrofurantoin, ciprofloxacin, trimethoprim, cephalotin and ceftibuten with Etests.

Results and conclusion

Totally, 31 629 urine cultures were sent to the laboratory during the study period of which 144 were eligible for inclusion (311 cultures grew aerococci, corresponding to 1 % of cultures). Finally, 91 patients were enrolled in the study. Sixty-six patients had urine cultures with *A. urinae* (51 in pure culture) and 25 patients had urine cultures with *A. sanguinicola* (18 in pure culture).

Seventy-two patients (79 %) met the criteria for UTI of whom 58 had cystitis and 14 had pyelonephritis.

The features of these patients are summarized in Table 1 of the original article. The clinical and microbiological outcomes after treatment for the patients are summarized in Table 2 and 3 in the original article.

Nitrofurantoin achieved clinical success in 71 % and microbiological success in 76 % of the *A. urinae* UTI cases (n=21). It seemed to be harder to treat *A. sanguinicola* UTI effectively with nitrofurantoin, since only six of twelve patients reported clinical success. All patients with *A. urinae* UTI reported clinical success with ciprofloxacin (n=9) and pivmecillinam (n=6). Amoxicillin was given to three patients with *A. urinae* and one patient with *A. sanguinicola,* with 100 % clinical

and microbiological success. Three patients with *A. sanguinicola* and one patient with *A. urinae* received cefadroxil with successful outcome as well.

Nineteen patients did not meet the UTI criteria. Five patients received no antibiotic treatment and had growth of aerococci in their control urine culture, which indicates that asymptomatic aerococcal bacteruria occurs.

These results should be interpreted with caution, since the study had several limitations, such as the observational study design, the relatively low number of patients and loss to follow-up. Possibly, some of the patients with *A. sanguinicola* that were unsuccessfully treated with nitrofurantoin could have had urogenital conditions other than UTI explaining their symptoms. However, several antibiotics could serve as treatment options for aerococcal UTI according to the results presented in this study.

Paper VI

Proteomic and genomic profiling of Aerococcus urinae surface proteins identifies two quantitatively dominating cell wall-anchored proteins

In the final study of the thesis we aimed to describe the surface proteome and genes encoding surface proteins of *A. urinae*. In order to do so, we utilized mass spectrometry (MS)-based proteomics and next-generation sequencing. Two *A. urinae* strains, denoted Col10a and AU3, with available whole genome sequences were selected for MS-analysis. One surface and one cell protein fraction were prepared for MS analysis through trypsin digestion steps.

Twenty-three *A. urinae* isolates, recovered from clinical blood cultures, were whole genome sequenced with Illumina HiSeq technology.

Results and conclusion

In total, 1118 and 1168 proteins were identified for Col10a and AU3, of which 765 and 683 were detected in the surface fractions from the two isolates. Basically all surface fraction proteins were also detected in the cellular fractions, as expected. The relative abundance of proteins in the surface fraction was determined.

In both *A. urinae* strains, the most abundant surface-located protein had a predicted LPXTG-sequence and a signal peptide protein domain. The relative

abundance for this protein was approximately 73 % and 26 % of the total proteins on the surface (for Col10a and AU3 respectively).

Another pair with identical domain structures was also detected among the most abundant surface proteins on both strains. We designated these proteins as Aerococcal surface proteins (Asp) 1 and 2 and the corresponding genes as *asp1* and *asp2*. No similarities in domains were found between the Asp proteins and other described proteins in databases (except for the LPXTG-motif and the signal peptide) and no function could be predicted either.

The genomes of Col10a and AU3 and the genomes of the 23 additional *A. urinae* isolates were analyzed in regard of *asp* genes. Variants of these genes were found in all isolates and they were all identically organized in a chromosomal locus denoted by us as the Locus encoding Aerococcal Surface Proteins (LASP). According to the annotations of the genomes, this locus was, in a 5' – 3' order, constituted by a pyruvate carboxylase gene, a gene designated as a "hypothetical cytosolic protein", the asp-gene(s) and a 16S rRNA methyltransferase gene.

A phylogenetic analysis was performed and the genes were denoted *asp1-6*. Some isolates had only one *asp* gene (*asp1*), but most isolates had two *asp* genes in their genomes. Four LASP-variants were found ($LASP_{1-4}$). Our analysis of the 25 genomes revealed that these isolates had in median 13 genes encoding LPXTG-proteins (range 6-24). The function of the Asp proteins is unknown, but they could be involved in adhesion or biofilm formation, since LPXTG-proteins in other species are involved in such processes.

In Paper VI, we demonstrated for the first time that *A. urinae* has genes encoding surface proteins with the LPXTG-motif and that two of these proteins quantitatively dominate the surface. Further studies on the function of the Asp proteins could improve our understanding of how aerococci cause infection.

9. Conclusion and future perspectives.

When we started our studies on aerococcal infections, the general awareness of aerococci was low. Basically nothing was known about the incidence of aerococcal UTI in Sweden and little was known about the clinical importance of such infections. Even if the work by Christensen and others had significantly increased the knowledge about aerococcal infections, it had only a moderate impact on the clinical microbiology field until MALDI-TOF MS was introduced. Before that, aerococci were easily misclassified and secure species identification was complicated and time-consuming.

MALDI-TOF MS has become the primary method for species identification in clinical bacteriology, due to its speed and accuracy. It has undoubtedly contributed to a significant improvement in diagnosing bacterial infections in general and has led to a paradigm shift in regard of diagnosing aerococcal infections. Thus, aerococci have risen from being a curiosity in the outskirts of bacteriology, studied mostly by a group of Danish experts, to become well known bacteria for clinical microbiologists and physicians treating infections.

The incidence increase

In the introduction of this thesis, the prevalence of aerococci in blood and urine cultures in Skåne for the years 2009-2017 was presented (Figure 1 and 2). The increase of cases is striking, especially concerning *A. urinae* in urine cultures, even when adjustments are made for the increase of the total number of cultures. Based on numbers presented in Paper V, 2,3 % of all positive urine cultures and 1 % of all urine samples sent to the laboratory grew aerococci. These numbers are much higher than in previous reports (Table 1).

The most obvious explanation for the initial increase, around the years 2011-2012, is the employment of MALDI-TOF MS. However, the steady increase of cases during the years that followed raises questions whether other factors or events also played a role. One such event that took place in 2015 was the implementation of

the WASPLab. The handling of urine cultures became more standardized than before in regard of streaking, incubation time, imaging and perhaps other unknown factors. All urine samples are in principle treated the same by WASPLab and the human factor is erased. This may have influenced the recovery rate of aerococci in urine specimens. Another factor that could have had a role is the presumed increase of awareness in the laboratory. This is of course harder to measure. But when a research group (such as ours) picks up interest in a certain pathogen it is not inconceivable that it will have effects on the number of cases identified in the laboratory. A final possibility is of course that an actual increase of cases has occurred, maybe due to an aging population. It is perhaps most likely that the increase in cases could be attributed to a combination of all of the things mentioned.

Clinical significance

When a urine or blood culture yields growth of aerococci, it is of course of importance to assess the clinical significance of such a finding. The risk of contamination should always be considered as a possibility, especially regarding urine cultures. In the case of aerococcal bacteremia, the risk of serious complications like endocarditis or spondylodiscitis must be carefully assessed. In the following sections, the clinical significance or aerococcal bacteruria and bacteremia is reviewed.

Urinary tract infections

The most dominating aerococcal species in clinical samples is *A. urinae*. When isolated in urine, a majority of the patients will most likely have a symptomatic UTI (in Paper V, 80 % of the patients met the UTI criteria and in previous reports the number has varied between 55-98% (Table 1)). Some patients will thus have ASB. Regardless of which pathogen is isolated in a urine culture, the possibility of colonization or contamination should of course always be considered before initializing treatment. *A. sanguinicola* is less frequently isolated in urine than *A. urinae*, but is however seemingly clinically relevant in a majority of cases also (in Paper V, 76 % of the patients met the criteria for UTI).

On the other hand, *A. viridans* is almost never isolated in urine and its clinical relevance in UTI must be considered to be very limited.

Invasive infections

A. urinae and *A. sanguinicola* can cause severe disease, most often in the elderly male population, although their exact contribution to disease in cases with mixed cultures is difficult to assess. However, the severity of invasive aerococcal infections has most likely been exaggerated in the past, since knowledge on clinical presentation relied almost entirely on case-reports. In general, it is more likely that spectacular cases are reported than less serious cases. According to a review by Rasmussen, the incidence of IE in patients with aerococcal bacteremia was 50 % in published reports and the case-fatality rate of the IE cases was 46 % (66). In Paper III, five patients (10 %) with *A. urinae* bacteremia were diagnosed with IE, with one in-hospital fatality. Three patients with *A. urinae* bacteremia (6 %) died within 30 days. Thus, the outcome of *A. urinae* bacteremia in our study was more favourable than previously reported. However, further studies are needed to establish risk factors for aerococcal IE and to determine which patients should undergo IE investigation.

An interesting observation is the absence of aerococci in urine cultures from many patients with aerococcal bacteremia. It is unclear why, but several potential explanations could be thought of. The conditions used for urine cultures are not optimal for aerococci and could possibly affect the recovery rate. In some cases, antibiotics have been given to a patient before urine is collected. A third possible explanation is that the bacteremia is not always originating from the urinary tract, but rather from another source, such as the intestines.

When a blood culture yields growth of *A. viridans*, it is in most cases of low clinical significance. Possibly, *A. viridans* temporary dwell on the skin when a blood cultures is drawn, and thereby contaminating the culture.

Cases with *A. christensenii* invasive infections are rare, but all seem to originate from the female genital tract and be of clinical importance.

Male dominance

An interesting feature of aerococcal bacteremia is the male dominance. The cause of this skewed gender distribution is not understood. The prostate is perhaps involved since this organ is exclusively found in males and many patients have predisposing urological conditions, such as benign prostate hyperplasia, which may facilitate the spread of bacteria from the urinary tract to the blood.

Treatment of aerococcal infections

When *A. urinae* is encountered in a urine culture and the patient has a symptomatology in concordance with an UTI, several treatment options are at hand. Nitrofurantoin, amoxicillin and cefadroxil should probably be recommended in the case of cystitis, and ciprofloxacin in the case of pyelonephritis, unless the isolate is resistant. For *A. sanguinicola* UTI, the options are a bit more restricted due to higher prevalence of flouroquinolone resistance. Also, nitrofurantoin did achieve clinical success in only six of twelve cases in Paper V. However, the number of patients was low in this group and the results should be interpreted with caution. Amoxicillin or cefadroxil are presumably also reasonable treatment options for *A. sanguinicola* UTI.

The use of pivmecillinam is not ruled out by the MIC-values obtained in our studies for either of the two species and could serve as an alternative, even though no breakpoints have been established and the experience from pivmecillinam treatment of aerococcal UTI is limited. Trimethoprim and trimethoprim-sulphamethoxazole should probably be avoided until further notice regarding how to interpret the varying AST results in different studies.

For invasive aerococcal infections, betalactam antibiotics seem to be safe treatment options. Penicillin should be considered as the drug-of-choice for non-allergic patients. Vancomycin is the obvious option for penicillin-allergic patients. If an aminoglycoside should be added to a betalactam antibiotic or vancomycin in the treatment of IE is not entirely clear. A synergistic effect is present *in vitro* in about half of the tested isolates according in the literature. The potential benefits from a combination treatment of IE must be reviewed in the light of potential side effects, such as renal failure, and the treating physician should do a careful clinical assessment before initiating an aminoglycoside treatment.

Aerococcal virulence mechanisms

As mentioned, bacterial biofilms is a major health issue and is involved in infections on foreign materials, such as urinary catheters. For the year 2017 in Skåne, 15 % (n=248) of all urine cultures that grew aerococci were performed on urine collected from indwelling urinary catheters and nephrostomies. Presumably, aerococci grew in biofilms on many of those catheters. It is also possible to conceive that aerococci form biofilms on the uroepithelium or on prostate tissue *in vivo*, although no such evidence has been presented. Plasma strongly stimulates aerococcal biofilm formation, and this could be of importance not only in the blood stream, but also in a situation of local inflammation where plasma exudates

to the tissue. In the cases where aerococci cause IE, biofilm formation is likely to be involved. No evidence is however present that aerococcal isolates that cause IE have an enhanced ability to produce biofilm compared to other, non-IE or non-invasive, aerococcal isolates. Such investigations are thus called upon to increase the understanding of aerococcal pathogenesis.

Whether the capability to induce platelet activation and aggregation is favourable for bacteria has been debated, since thrombocytes release anti-bacterial peptides when activated. Also, the aggregation around bacteria could serve as a host defence, since the bacterial infection is encapsulated and thus further spread of bacteria in the body is inhibited. On the other hand, it is obviously not beneficial for the host when bacteria, thrombocytes and fibrinogen are deposited on a damaged heart valve, though bacteria in a vegetation are likely to be protected from effective clearance of the immune system. The effect of antibiotics is also likely impaired when bacteria reside in such environments.

We have demonstrated that *A. urinae* have surface proteins with the LPXTG-motif and that the Asp-proteins are quantitatively dominating the surface. All 25 isolates had genomes containing the LASP, with one or two *asp* genes. The arrangement of genes in the LASP has a high degree of similarity to the arrangement of genes in the *mga* regulon of *S. pyogenes*, which encode important streptococcal virulence factors. Altogether, these results suggest that the Asp-proteins are of importance for aerococcal virulence.

It would be of interest to screen aerococcal isolates from different sources, such as urine and blood, and compare the variants of the *asp* genes found in these genomes. In analogy with the M-protein, where certain types are more prone to cause severe disease, it is not unlikely that more or less "virulent" Asp-proteins exist.

Further studies are however needed to establish the functions of the Asp-proteins. Possibly, they could act as adhesion molecules, perhaps to urothelium, or be involved in the biofilm formation process. Other thinkable functions for the Asp-proteins are binding of plasma proteins or involvement in platelet-bacteria interaction or immune evasion.

Finally, is there a human aerococcal habitat?

Some publications suggest that *A. urinae* could be part of the urinary tract flora. Pearce *et al.* compared the urine microbiome of females with urgency urinary incontinence (UUI) with the urine microbiome of non-UUI females, using both sequencing and culture methods (154). *A. urinae* was isolated in both groups, but more frequently in urine from UUI patients. In two other studies, *A. urinae* was isolated in urine samples from patients suffering from overactive bladder (155, 156). On the other hand, when Coorevits *et al.* examined urine from 101 healthy men and women, *A. urinae* was not detected (157).

Some publications indicate that *A. christensenii* could be a part of the female genital flora. When Collins *et al.* proposed *A. christensenii* as a novel species, the bacteria were isolated from vaginal samples (30). Di Pietro *et al.* found an association between *Chlamydia trachomatis* infection and *A. christensenii* in women (158). Doyle *et al.* reported a structural alteration in the microbial communities in the placenta from preterm births, where *A. christensenii* was found in great abundance (159). Carlstein *et al.* reported on a case of chorioamnionitis in a pregnant woman caused by a polymicrobial infection, including *A. christensenii* (89). Another study suggested an association between presence of specific genera in the vaginal microbiota, including *Aerococcus,* and bacterial vaginosis (160). However, the aerococci were not identified to the species level.

Thus, some studies indicate that *A. urinae* and *A. christensenii* could be part of the human bacterial flora, although further investigations are needed to confirm this.

Populärvetenskaplig sammanfattning på svenska.

Bakteriesläktet *Aerococcus* består av åtta arter av s.k. Grampositiva kocker. Den första arten, *A. viridans*, beskrevs 1953 och kopplingar gjordes mellan den och en allvarlig sjukdom hos hummer. Det skulle dröja åtskilliga decennier innan nästa aerokockart, *A. urinae*, upptäcktes. Denna kopplades till urinvägsinfektioner (UVI) och bakteriemi, dvs. växt av bakterier i blodet, hos människa. Därefter har två andra arter vilka kan orsaka sjukdom hos människa, *A. sanguinicola* och *A. christensenii*, identifierats. Övriga aerokockarter förekommer enbart hos djur eller har ej visats orsaka sjukdom hos människa.

Det var tidigare okänt hur vanliga aerokocker var i urin- och blodprover från svenska patienter och vilken betydelse de eventuellt hade som sjukdomsframkallande bakterier. Det fanns svårigheter med att artbestämma aerokocker. Deras koloniutseende på odlingsplattor liknade dessutom en annan grupp av bakterier, alfa-streptokocker, och misstogs därför ofta för att utgöras av dessa. Alfa-streptokocker förorenar ofta urinprov vid provtagning.

Under den första delen av 2010-talet introducerades en ny analysmetod inom bakteriediagnostiken som var baserad på s.k. masspektrometri (MALDI-TOF MS). Analysinstrumentet undersöker proteininnehållet i bakterier, vilket genererar ett masspektra eller ”fingeravtryck” för varje art. Därefter sker en jämförelse mellan det erhållna masspektrat med masspektra i en databas. På så sätt bestäms en arttillhörighet med stor säkerhet. MALDI-TOF MS visades sig vara en väldigt tillförlitlig metod för att artbestämma många av våra vanligt förekommande bakterier.

I denna avhandlings första delarbete (**Paper I**) utvärderade vi MALDI-TOS MS och dess tillförlitlighet i att identifiera aerokocker. Vi fann att MALDI-TOF MS var en metod med god sensitivitet och specificitet i avseende att identifiera *A. urinae* och *A. sanguinicola* jämfört med en referensmetod (sekvensering av 16S rRNA-genen).

I det andra delarbetet (**Paper II)** beskrev vi den kliniska presentationen av bakteriemi med *A. sanguinicola*. Vi identifierade retrospektivt alla fall i Skåne mellan åren 2006-2012 och tog del av patientjournalerna samt undersökte bakterieisolatens förmåga att bilda biofilm och inducera trombocytaggregation (dvs hopklumpning av blodplättar). Elva patienter identifierades och alla var män (medianålder var 82 år). Många patienter hade urinvägssjukdomar och urinvägskateter. Nio patienter uppfyllde kriterierna för svår sepsis (systemisk påverkan med organsvikt) och två patienter dog på sjukhuset. Två patienter fick hjärtklaffinfektion (endokardit), vilket är en allvarlig komplikation som oftast kräver långvarig antibiotikabehandling och ibland kirurgi. Alla bakterieisolat från

de elva patienterna bildade biofilm på plastytor men endast en del fick trombocyter att aggregera. Dessa två egenskaper kan vara viktiga för bakteriers sjukdomsframkallande förmåga, speciellt i avseende på att infektera främmande material och att etablera en infektionshärd på hjärtklaffar.

I nästa delarbete (**Paper III**), undersökte vi retrospektivt förekomsten av aerokocker i blododlingar i Skåne under en treårsperiod. Vi identifierade 77 fall av aerokockbakteriemi, vara *A. urinae* stod för 49 fall och *A. sanguinicola* för 13 fall. Merparten av dessa patienter var gamla (medianåldern var > 80 år) och de flesta var män (80 % respektive 54 % för *A. urinae* och *A. sanguinicola*). Många patienter hade sjukdomar i urinvägarna sedan tidigare och många hade även inneliggande urinvägskatetrar. En majoritet av patienterna med någon av dessa två arter i blodet hade ett misstänkt urinvägsfokus (dvs. ursprunget för infektionen förmodades vara urinvägarna), även om få patienter hade aerokocker i urinodlingar. En majoritet av dessa patienter drabbades av organsvikt som en orsak av infektionen. Vanligast var att njurarna drabbades. Fem patienter med *A. urinae* i blodet fick diagnosen hjärtklaffinfektion (endokardit). En patient med endokardit avled. Totalt avled tre patienter med *A. urinae* och två patienter med *A. sanguinicola* inom 30 dagar efter inläggning på sjukhus.

Fjorton fall av bakteriemi orsakat av *A. viridans* identifierades också, men merparten av dem bedömdes vara orsakade av föroreningar av blododlingarna. Således verkar *A. viridans* spela en jämförelsevis mindre betydande roll beträffande allvarliga infektioner än *A. urinae* och *A. sanguinicola*.

Vi undersökte känsligheten hos bakterieisolaten från patienterna för tio antibiotika genom att bestämma det s.k. MIC-värdet, dvs. den minsta koncentration uttryckt i mg/l som hämmar bakteriernas tillväxt. Aerokockerna var i nästan alla fall känsliga för betalaktamantibiotika (en grupp antibiotika där penicillin ingår) och vancomycin.

I nästa delarbete (**Paper IV**) undersökte vi hur vanliga aerokocker var i urinodlingar under en tremånadersperiod i Skåne och beskrev bl.a. ålder och kön på patienterna. Vi testade även aerokockernas känslighet för olika antibiotika. I ett uppföljande arbete (**Paper V**), observerade vi behandlingseffekten av olika antibiotika mot UVI orsakat av aerokocker genom intervjuer med läkare och patient (under och efter behandling), samt genom att undersöka urinprov efter avslutad behandling för att notera om aerokockerna var eradikerade. Sammanfattningsvis fann vi i dessa två studier att aerokocker var betydligt vanligare än vad man tidigare trott. *A. urinae* och *A. sanguinicola* tillsammans återfanns i nästan 1 % av alla urinprov som togs i Skåne under studieperioden på två månader i delarbete V. Patienterna var gamla (medianålder var 83 respektive 79 år i de två studierna) och könsfördelningen hos patienterna med UVI orsakat av aerokocker var ganska jämnt fördelad mellan män och kvinnor. Som jämförelse

kan nämnas att patienter med UVI orsakat av *E. coli*, den vanligaste bakterien i urinodlingar, oftast är kvinnor (84 % i vår studie) och att de dessutom är yngre (medianålder 69 år i vår studie). Vi utvärderade behandlingseffekten av de antibiotika som ordinerats till 50 patienter med *A. urinae* och 19 patienter med *A. sanguinicola* och nitrofurantoin uppnådde bra effekt vid behandling av *A. urinae*, men något sämre effekt mot *A. sanguinicola*. Nitrofurantoin är ett av två förstahandsmedel för UVI-behandling i Sverige. Det andra medlet, pivmecillinam, gavs endast till sex patienter med *A. urinae* men med bra effekt. Ett annat antibiotikum, ciprofloxacin, som vanligtvis ges till patienter med mer komplicerade urinvägsinfektioner (t.ex. njurbäckeninflammation), hade bra effekt i nio fall av UVI orsakat av *A. urinae*. Dock noterades en ganska utbredd resistens mot ciprofloxacin hos *A. sanguinicola*-isolaten.

I denna avhandlings sista delarbete (**Paper VI**) använde vi masspektrometri och helgenomssekvensering för att kartlägga ytproteiner och gener kodande för sådana hos *A. urinae*. Vi fann att två proteiner dominerade ytan kvantitativt, dvs. utgjorde en stor andel av den totala mängden protein på ytan. Dessa proteiner hade strukturella likheter med proteiner från andra bakterier (t.ex. streptokocker och stafylokocker), som man vet har stor betydelse för dessa bakteriers sjukdomsframkallande förmåga. De är framför allt i den del av proteinet som förankras i bakteriens cellvägg som likheterna finns (det s.k. LPXTG-motivet). Dessa två LPXTG-proteiner hos *A. urinae* fick namnet Asp-proteiner. Generna som kodade för dessa proteiner fick namnet *asp*-gener och de fanns organiserade på ett liknande sätt i genomet hos alla undersökta bakterieisolat. Asp-proteinerna har än så länge okända funktioner, men med tanke på deras rikliga förekomst på ytan, och att *asp*-generna förekommer hos alla undersökta bakterieisolat, tror vi att de har en viktig betydelse för *A. urinae*. Möjligtvis är Asp-proteinerna involverade när aerokocker bildar biofilm eller fäster till celler i urinvägarna.

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