Development of culturing methods for *Ureaplasma* spp. and validation of a quantitative multiplex PCR.

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Abstract

Ureaplasma is een van de kleinst zelf-replicerende organismes uit de *Mycoplasma* familie. *Ureaplasma* staat bekend om tijdens zwangerschap infecties te veroorzaken en vroeggeboorte te induceren. Een infectie met *Ureaplasma* tijdens de zwangerschap, kan leiden tot infectie van de neonaat. In de neonaat kan *Ureaplasma* leiden tot luchtweginfecties zoals longontstekingen. Het Erasmus Medisch Centrum voert geen diagnostiek uit voor *Ureaplasma*. Hierdoor zijn er geen diagnostische analyses beschikbaar voor onderzoek. Er is daarom een kweekmethode voor *Ureaplasma* ontwikkeld gebaseerd op de urease activiteit van *Ureaplasma* in medium en op agar. Daarnaast is een kwantitatieve multiplex PCR ontwikkeld voor *Ureaplasma urealyticum*, *Ureaplasma parvum* en *Mycoplasma pneumoniae* middels plasmide standaardreeksen. De multiplex qPCR is gevalideerd door singleplex en multiplex analyses te vergelijken middels lineaire regressie en Wilcoxon Signed Rank test.

De resultaten laten zien dat de vloeibare kweek voor *Ureaplasma* een simpele methode is om *Ureaplasma* groei te detecteren. De kleuromslag van het medium bevestigd urease activiteit en *Ureaplasma* groei. Op *Ureaplasma* agar groeit *Ureaplasma,* facultatief anaeroob, als donkere “zee-egel” kolonies. De *Ureaplasma* kolonies kunnen al na 48 uur incubatie met het blote oog zichtbaar zijn.

De multiplex qPCR voor *Ureaplasma urealyticum*, *Ureaplasma parvum* en *Mycoplasma pneumoniae* heeft een respectievelijke detectie limiet van 13, 15 en 10 genoom kopieën met een respectievelijke efficiëntie van 106,38%, 102,30% en 105,31%. De validatie voldoet aan de gestelde criteria maar de standaardlijnen van de singleplex en multiplex reacties verschillen statistisch voor ieder target (P=<0,05). Het verschil wordt mogelijk veroorzaakt door de 10^4 kopieën standaard die meer cycli afwijkt tussen singleplex en multiplex dan alle andere standaarden. Deze afwijking is voor ieder target vaker geconstateerd waardoor de validatie alsnog is geaccepteerd.

Concluderend, er is een specifieke kweek en detectie methode voor *Ureaplasma* ontwikkeld in vloeibaar medium en op agar. En er is een specifieke en gevoelige kwantitatieve multiplex PCR ontwikkeld voor *Ureaplasma urealyticum*, *Ureaplasma parvum* en *Mycoplasma pneumoniae.*

List of abbreviations

Abbreviation	Definition
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BBA	Brucella Blood Agar
BPD	Bronchopulmonary dysplasia
CCUG	Culture Collection University of Gothenburg
Cq	Quantification Cycle
CTAB	Cetyltrimethylammonium bromide
CV	Coefficient of variance
DDQII	Deep Dark Quencher II (type 2)
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
EMC	Erasmus Medical Center
EtOH	Ethanol
IgA	Immunoglobulin A
ISO	International Organization for Standardization
LOD	Limit of detection
m10B	Modified 10B broth
mA8	Modified A8 agar
MBA	Multiple banded antigen
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
NF-$_k$B	Nuclear factor kappa B
NTC	No template control
OD	Optical density
PBS	Phosphate Buffered Saline
PPLO	Pleuropneumonia-like Organisms
pPROM	Preterm premature rapture of membranes
qPCR	Quantitative Polymerase chain reaction
RFU	Relative Fluorescence Units
RPM	Rounds per minute
rRNA	Ribosomal Ribonucleic acid
TLR	Toll-Like receptor
UNG	Uracil N-Glycosylase

1.0 Introduction

Every year there are between 150.000 and 200.000 births in the Netherlands[1]. It may happen that a newborn is born prematurely (birth before 37 weeks of gestation). In 2017 there were 11.978 (7,2% of total births) premature births and 728 (0,4% of total births) were extremely premature (birth before 27 weeks)[2]. Prematurity can have serious effect on the neonate, which can result in lung diseases, gastrointestinal problems and higher risks of infection[3]. Premature birth can be caused by age, high blood pressure, smoking, drugs and alcohol use or infections[4, 5]. Infections during pregnancy have a serious impact on the neonate. One of the organisms known to cause infections during pregnancy and in neonates is *Ureaplasma*[6]. *Ureaplasma* may infect the respiratory tract of neonates leading to pneumonia[6]. Additionally, the presence of *Ureaplasma* in the respiratory tract is associated with the development of chronic lung diseases. Nowadays researchers are investigating whether there is a causal relationship between *Ureaplasma* infection and lung diseases such as bronchopulmonary dysplasia (BPD) in premature neonates[6].

Ureaplasma is a member of the *Mycoplasma* family and one of the smallest self-replicating organisms. Just like *Mycoplasma* species, *Ureaplasma* does not have a cell wall and is resistant to beta-lactam antibiotics such as ampicillin and penicillin[5].

Ureaplasma is divided into fourteen different serotypes (serovars)[5]. The classification of the serovars are based on genome size, 16s rRNA, manganese growth response and differences in the multiple banded antigen (MBA) genes[7]. Within the fourteen serotypes, two different variations of *Ureaplasma* were found (biovars) and named *Ureaplasma urealyticum* (*U. urealyticum*) and *Ureaplasma parvum* (*U. parvum*)[5]. Nowadays, serovars 1, 3, 6 and 14 are classified as *U. parvum* and serovars 2, 4, 5, 7-13 are classified as *U. urealyticum*[5].

All serovars share the same virulence factors[8]:

1. Urea hydrolysis

Ureaplasma has the ability to hydrolyze urea into ammonium (NH_4^+) and carbamate (NH_2O_2) with urease[9]. The generated ammonium molecules may have a toxic effect on the surrounding tissue causing tissue damage[9]. The synthesis of urease distinguishes *Ureaplasma* from other *Mycoplasma* species, such as *Mycoplasma pneumoniae*, which do not express urease. The reason why *Ureaplasma* express urease is not completely clear but researchers suggest that *Ureaplasma* uses urea for the energy metabolism and adenosine triphosphate (ATP) synthesis[10].

2. Multiple banded antigen

MBA is a surface-exposed lipoprotein that is specifically found on *Ureaplasma* species[11]. Especially the C-terminus of MBA causes the immune system to start an immune response to the MBA[8, 12]. However, *Ureaplasma* has the ability to introduce antigenic variation in the C-terminus of the MBA to evade the immune system[8].

3. IgA protease

IgA protease can degrade IgA antibodies. IgA is a common mucosal antibody, which can be found in the respiratory tract and urogenital tract[13]. Degrading of IgA can result in ineffective first defense, which may cause infection or colonization by *Ureaplasma* during pregnancy or in neonates[14].

Ureaplasma species are commonly found as commensals on the mucous membranes in the urinary tract of 40-80% of, especially, women[5]. However, *Ureaplasma* can also act as a potential pathogen. During pregnancy, *Ureaplasma* can infect the amniotic fluid and placenta, which lead to chorioamnionitis, preterm premature rapture of membranes (pPROM) and/or premature birth[15]. Especially *U. parvum* is found to significantly cause more (subclinical) infections and leading to premature birth, than *U. urealyticum*[16]. The neonate can acquire *Ureaplasma* at birth via vertical transmission or during pregnancy via infected amniotic fluid. *Ureaplasma* may affect the neonate, especially, in the respiratory tract leading to: pneumonia or an invasive infection such as meningitis or sepsis[5]. *Ureaplasma* has been associated with the development of chronic lung diseases such as BPD.
A few studies have found that lipoproteins of *U. urealyticum* and *U. parvum* are responsible for the activation of the Nuclear Factor kappa B (NF-$_k$B) pathway by activating different Toll-like Receptors (TLRs)[17, 18]. The immune system reacts to *Ureaplasma* with an increased influx of neutrophils and macrophages to the infection site but also increasing the levels of pro-inflammatory cytokines like interleukin 1 alpha (IL-1α), interleukin 1 beta (IL-1β) and interleukin 6 (IL-6)[6, 19]. During pregnancy, infection with *Ureaplasma* also causes an increased level of prostaglandin, which may trigger premature birth[6].

Currently, the diagnostic testing for *Ureaplasma* infection is only performed in two laboratories in the Netherlands: Maasstad Hospital in Rotterdam and Radboud University Medical Center Nijmegen by culturing or quantitative Polymerase chain reaction (qPCR)[20]. These diagnostic methods only detect the presence of *Ureaplasma*. Based on the presence of *Ureaplasma* cannot be concluded if *Ureaplasma* is causing the infection. Because that *Ureaplasma* can be found as a commensal in the urinary tract or as non-symptomatic colonizers in the respiratory tract of neonates. This can lead to invalid conclusions and possibly wrong treatment. To diagnose if *Ureaplasma* is causing the infection, new and better diagnostic tools must be developed.

At the Erasmus Medical Center there are no culture methods and PCR assays for *Ureaplasma* directly available for research. Therefore this thesis describes the development of specific and sensitive diagnostic tools for detecting *Ureaplasma* spp. by culture and qPCR. The developed methods will make it possible to perform clinical studies and development of new specific diagnostic tools. Good diagnostics lead to better and specific treatment.

1.1 Aim

The main aim of this research is:

- To develop a culture method for detection of *Ureaplasma* spp.
- To develop a reliable, specific and sensitive quantitative multiplex PCR for detecting and quantifying of *U. urealyticum, U. parvum* and *M. pneumoniae* in DNA samples.

2.0 Experimental strategy

2.1 Development of *Ureaplasma* culture methods

Two culture methods will be developed for culturing and detection of *Ureaplasma*: broth and agar culturing. Broth and agar culture media will be tested with a reference strain and clinical isolates of *Ureaplasma*. For broth culturing, the ability for changing the color of the broth, by raising the pH of the broth, is important for *Ureaplasma* detection (see also 2.1.1 Background). To test the specificity of the *Ureaplasma* media, *Mycoplasma hominis* (*M. hominis*) and an urease positive *Staphylococcus aureus* (*S. aureus*) will be cultured in *Ureaplasma* media to test whether other *Mycoplasma's* and/or urease positive bacteria can grow in *Ureaplasma* media. The *Ureaplasma* cultures will be used for the development of the qPCR. For agar culturing, we will assess colony growth and colony morphology and if *Ureaplasma* colonies can be differentiated from *Mycoplasma* colonies on this agar (see 2.1.1 Background).

2.1.1 Background

The principle of *Ureaplasma* culturing in broth is mainly the detection of urease activity. The broth contains Pleuropneumonia-like Organisms broth (PPLO), nutrients and vitamins that are required for growth, antibiotics and most importantly urea and Phenol red. When *Ureaplasma* is added to the *Ureaplasma* broth, *Ureaplasma* will hydrolyze the urea, which forms bicarbonate and two ammonium molecules (see Figure 1[9]). The formation of ammonium is the key factor for the detection of *Ureaplasma* growth. The broth is pH 6,0 at start of culture but when urea is hydrolyzed and ammonium is formed the pH will start to rise. Phenol red in the broth will change color from clear yellow to clear red. The color change of the medium thus suggests *Ureaplasma* growth.

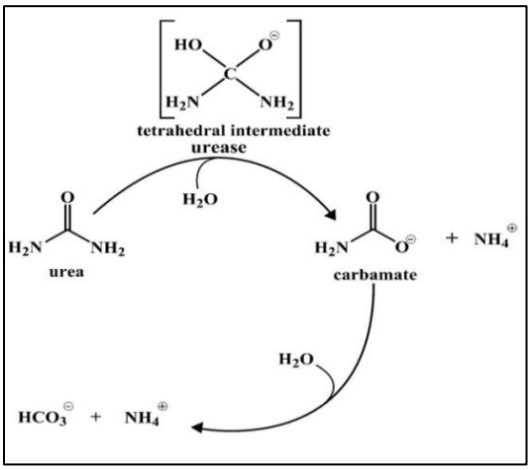


Figure 1: Urea hydrolysis by bacterial urease [9]

Agar culturing

Ureaplasma grows on plain agar under microscopic view as a "fried egg" morphology[21]. However, other *Mycoplasma* spp. colonies also have a "fried egg" morphology (See Figure 2[22]) [21]. To differentiate *Ureaplasma* from *Mycoplasma* colonies, an additional chemical compound can be added like Manganese sulfate ($MnSO_4$) or Calcium chloride ($CaCl_2$) [23]. The addition of $MnSO_4$ or $CaCl_2$ causes a change in the morphology of the *Ureaplasma* colony[23]. At the colony site of *Ureaplasma* a golden brown precipitation reaction will occur. The *Ureaplasma* colonies will then look like golden brown "sea urchin" colonies. (See Figure 2[22]). The precipitation reaction differentiates *Ureaplasma* colonies from other *Mycoplasma* colonies.

Figure 2: *Mycoplasma* colonies with "fried egg" morphology (colonies pointed with black arrows) and *Ureaplasma* colony with "sea urchin" morphology (colony pointed with white arrow)[22]

2.2 Development of multiplex qPCR

To develop the *Ureaplasma* PCR, primers and Dual-labeled TaqMan probes will be designed and selected with following criteria primer length is 16-24 base pairs (bp), GC-content is 45-60%, annealing temperature is ~60°C and primers must be specific to only *Ureaplasma*[24]. The probes must have a maximum length of 30 bp, GC-content is 40-60%, annealing temperature must be higher than the annealing temperature of the primers, and must not contain a guanosine (G) at the 5' of the probe[24].
The analytical specificity will be tested with different bacterial strains and confirmed due to the probe fluorescent signal during PCR and by gel electrophoresis. BLAST (NCBI) will be used for theoretical specificity of the primers and probes.
A plasmid will be constructed with the desired, specific PCR product to make the PCR quantitative and to determine the detection limit and efficiency of each PCR assay.
The multiplex qPCR will contain *U. urealyticum*, *U. parvum* and *M. pneumoniae*. This combination is chosen because *Ureaplasma* and *Mycoplasma* are both potential pathogens for neonates in the respiratory tract. Usually, *M. pneumoniae* causes more infections in children instead of neonates[5], some studies describe that *M. pneumoniae* can cause neonatal pneumonia and that *M. pneumoniae* can be isolated from the urogenital tract. Although it is unusual, *M. pneumoniae* may be vertical transmitted and causing neonatal pneumonia[25-27].
The multiplex qPCR will be validated by linear regression (best fit line) of the standard series and Wilcoxon Singed Rank test to check for significant difference between singleplex PCR and multiplex PCR. Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines will be followed when validating the multiplex qPCR.

3.0 Materials and methods

3.1 Testing of *Ureaplasma* culturing methods

To test whether *Ureaplasma* can grow in our own developed *Ureaplasma* broth (m10B) and agar (mA8), the reference strain *U. urealyticum* 50782T (UU 50782T) of the Culture Collection University of Gothenburg (CCUG) was used. To test if UU 50782T can be cultured in m10B broth (see recipe below), 100µl of UU 50782T was added to 9,9ml m10B in a T25 cell culture flask (CellStar; Frickenhausen, Germany) and incubated until pH change, which may take less than 12 hours till 72 hours, in a humidified environment at 37°C+5% CO_2.
To test our developed *Ureaplasma* agar (mA8, see recipe below), mA8 agar bases with different precipitants and incubation methods were tested to test the optimal agar base to culture *Ureaplasma*. A pre-made 50µl stock aliquot of cultured UU 50782T (CFU/ml =2,52*10^7) was 10-fold diluted till 10^{-7} CFU/ml in filter sterile PBS. 50µl of the dilutions 1*10^{-5} till 1*10^{-7} CFU/ml was plated in duplicate on mA8 plain agar (see recipe below), mA8 with additional 1,1mM $MnSO_4$*H_2O, mA8 with additional 1,1mM $MnSO_4$*H_2O + 15mM $MgCl_2$*$6H_2O$ and mA8 with additional 1,1mM $CaCl_2$*$2H_2O$. One plate of each was incubated anaerobically (in a jar made anaerobically with a candle) and incubated at 37°C for three days. Other plates were incubated aerobically for three days at 37°C+5% CO_2. Colonies were counted under a light reverse microscope (Nikon SMZ 2T). Microscopic pictures of colonies were taken using a Leica microscope with camera (DFC 450C) under 100x and 400x objective.

To test whether clinical isolates of *Ureaplasma* can be cultured in our m10B and on mA8 media, twelve pre-cultured clinical *Ureaplasma* isolates (a kind gift from Dr. Biernat; Jagiellonian University, Krakow, Poland) were thawed and 100µl is added to 900µl m10B broth in a 5ml Falcon tube (Falcon®; Reynosa, Mexico). Uninoculated broth was used as negative control and strain UU 50782T was used as positive control. The tubes were incubated aerobically, with cap slight loose, in a humidified incubator at 37°C+5% CO_2 for maximal 5 days while checking twice per day for color change of the broth. Cultures with pH change were immediately put in the fridge or frozen at -80°C. 50µl of pre-cultured isolates were plated on mA8 with 1,1mM $CaCl_2$. The plates were incubated in a jar (made anaerobically with a candle) at 37°C for at least 3 days.

The m10B broth and mA8 with 1,1mM $CaCl_2$ were tested on specificity by culturing other bacterial strains. We cultured in m10B and mA8 *M. hominis* ATCC 23114, two clinical isolates of *M. hominis* (isolated by the Medical Microbiology and Infectious diseases (MMIZ) department of EMC) and *S. aureus* ATCC 29214 (urease positive bacteria). *S. aureus* was a pre-made 50µl stock (CFU unknown) and was diluted in 1,5ml filter sterile PBS and diluted till an OD_{600} of 0,5 (~$4.0*10^8$ CFU/ml) and then diluted till $1*10^3$ CFU/ml in filter sterile PBS. As viability control of *S. aureus,* 50µl of both dilutions were plated and spread on a blood agar plate (Tryptic Soy Agar II + 5% Sheep blood; BD, Heidelberg, Germany) and incubated overnight at 37°C+5% CO_2. The clinical *M. hominis* isolates were cultured by the MMIZ department of the EMC. We scraped the colonies and dissolved the colonies in 1 ml filter sterile PBS and diluted 100x in filter sterile PBS. 100µl of *M. hominis* suspension (ATCC 23114, undiluted and 100x diluted clinical isolate) and 100µl of $1*10^3$ and $1*10^5$ CFU/ml diluted *S. aureus* suspensions were added to 900µl m10B in 5ml Falcon tubes (Falcon®) in duplicate. Uninoculated broth was used as negative control and UU 50782T was used as positive control. 50µl of the suspensions were plated on mA8+$CaCl_2$ and incubated anaerobically in a jar (made anaerobic with a candle) at 37°C for maximal 6 days.

Recipe of *Ureaplasma* broth (m10B):

Our m10B recipe is a combined recipe of modified ATCC medium 1331 and Shepard's 10B broth[28]

2% PPLO (BD), 2,2% Yeast extract (BD), 20% Heat inactivated (30 min at 56°C) Horse serum (Bio-West; Nuaillé, France), 1,65mM L-Cysteine-HCl (Sigma; Steinheim, Germany), 33,30 mM Urea (VWR; Solon, USA), 9,08mM Putrescine dihydrochloride (Sigma), 0,125 mM phenol red, 0,5% Isovitalex Enrichments (BD) and 10^5U/100 ml Penicillin (Hospital Pharmacy, EMC) were dissolved in MilliQ; pH adjusted to pH 6,0; Sterilized by filtration (Millipore 0,22µM Vacuum filter system). Broth stored at 4°C or -20°C for long-term storage.

Recipe of *Ureaplasma* agar (mA8):

Our mA8 recipe is a combined recipe of ATCC media 1332 and A7 agar of Shepard[29]

1,5% PPLO (BD), 0,5% Tryptic Soy Broth (BD), 0,4% NaCl (Sigma), 0,2% KH_2PO_4 (Merck; Darmstadt, Germany), 1,4% Agar (BD); dissolved in MilliQ, pH adjust to 6,0 and sterilized by autoclave 121°C for 20 minutes. Hereafter, filter sterile 2,5g/L Yeasolate (BD), 0,825mM L-Cysteine*HCl (Sigma), 16,65mM Urea (VWR), 22,7mM Putrescine dihydrochloride (Sigma), 20% Heat inactivated (30 min at 56°C) Horse serum (Bio-West), 0,5% Isovitalex Enrichments (BD), 10^5U/100ml Penicillin (Hospital Pharmacy, EMC) was added. To a petridish (VWR, Ø 9cm), 14ml agar was pipetted. Plates were stored at 4°C. Additional precipitant can be added filter sterile 1,1mM $MnSO_4*H_2O$, 1,1mM $MnSO_4*H_2O$ + 15mM $MgCl_2*$ $6H_2O$ or 1,1mM $CaCl_2*2H_2O$.

Development of culturing methods for *Ureaplasma* spp. and validation of a quantitative multiplex PCR.

3.2 Development and validation of a quantitative multiplex PCR

For the preparation of the PCR validations, DNA of bacterial strains were isolated with the chloroform/isopropanol DNA precipitation method as described below.

Bacterial DNA isolation

Bacterial culture was centrifuged at 13.000 RPM for 10 minutes in a 1,5ml tube (Eppendorf; Hamburg, Germany), supernatant was discarded, 400µl T10E1 (10mM Tris, 1mM EDTA in MilliQ) and 50µg Proteinase K was added. The pellet was suspended by pipetting up and down and 70µl 10% SDS was added, mixed on vortex and incubated for 10 minutes at 65°C. Then 100µl 5M NaCl and 100µl 10% Cetyltrimethylammonium bromide (CTAB) was added and mixed for 10 seconds on a vortex till solution turned white and then incubated for 10 minutes at 65°C. Then 500µl chloroform:isoamylalcohol (24:1) was added, mixed on a vortex for 20 seconds and centrifuged for 3 minutes at 13.000 RPM. The aqueous upper layer was carefully transferred to a clean 1,5ml tube (Eppendorf) and 40µg Glycogen and 360µl isopropanol (Sigma) was added, mixed by gently inverting the tube and incubated for 10 minutes at -20°C. The sample was then centrifuged for 10 minutes at 13.000 RPM, supernatant was discarded and 500µl 70% EtOH was added and centrifuged again for 10 minutes at 13.000 RPM. The supernatant was discarded and pellet was dried in a heatblock at 65°C for 5-10 minutes and resuspended in 50µl T10E1. The purity and concentration of isolated DNA samples were measured with the Nanodrop spectrophotometer Denovix DS-11 FX and DNA samples were stored at -20°C.

Primers and probes information used in multiplex PCR

Table 1: Primers and probe sequences used for multiplex PCR

Name	Sequence 5' – 3'	Target bacteria	Target gene	Gene reference NCBI
UUreCFwd	AAGGTCAAGGTATGGAAGATCCAA	*Ureaplasma*	*Urease C* complex 90 bp PCR product	AF085730.2 1519 – 1608bp AF085724.2 1515 –1604bp
UUreCRev	TTCCTGTTGCCCCTCAGTCT	*Ureaplasma*		
UUreCP1probe	FAM- TCCACAAGCTCCAGCAGCAATTTG-TAMRA	*U. parvum*		
UUreCP2probe	FAM-ACCACAAGCACCTGCTACGATTTGTTC-TAMRA	*U. urealyticum*		
UUreCP2bprobe	HEX-ACCACAAGCACCTGCTACGATTTGTTC-TAMRA	*U. urealyticum*		
MpnP1TaqFwd	AAGCACGAGTGACGGAAACAC	*M. pneumoniae*	P1 adhesin (*MPN141*) 73 bp PCR product	CP017343.1 184844 – 184916bp
MpnP1TaqRev	CACCACATCATTCCCCGTATTAG	*M. pneumoniae*		
MpnP1TaqP2probe	Cy5–CTCCACCAACAACCTCGCGCCTA–DDQII	*M. pneumoniae*		

The primers and probes for *Ureaplasma* were obtained from Jongyoun Yi et al; 2005[30]. The *M. pneumoniae* primers and probes are in-house developed and described in Spuesens E.B, et al; 2010[31]. All primers and probes were synthesized and purified by Eurogentec (40nmol, purified with RP cartridge gold for primers and RP-HPLC for probes.) All stocks solutions were 200µM, dissolved in MilliQ and stored at -20°C. See Table 1 for primer and probe sequences, target genes and target locations.

Constructing *Ureaplasma Urease C* plasmid

To make the PCR quantitative, we want to construct plasmids with the target specific product transformed. The formed plasmids will be used to calculate the copy numbers and to generate a standard curve.

To amplify the 90bp product of *Urease C* of *U. urealyticum* and *U. parvum*, isolated DNA of the reference strains *U. urealyticum* 50782T and *U. parvum* ATCC 27818 was used in a high fidelity PCR. All components (primers, DNA polymerase, buffer, dDNTP's and DNA isolates were thawed on ice, vortex and shortly centrifuged before use. The reaction mix contained 1x High Fidelity buffer (Biolabs; Ipswich, USA), 0,2mM dDNTP's, 1µM UUreCFwd primer, 0,5µM UUreCRev primer, 1U High Fidelity DNA Polymerase (Biolabs), 2µl DNA template and MilliQ was added to obtain a final volume of 25µl. As "no template control (NTC)" MilliQ was used and as negative control *M. hominis* ATCC 23114 was used. The used PCR protocol was 30" 98°C; 40 cycli of 10" 98°C – 15" 60°C – 15" 72°C and single cycli 5' 72°C and 4°C infinite (Applied Biosystems 2720 Thermal Cycler). For gel electrophoresis a 2% agarose gel was made in 0,5x TBE buffer (108,8g/L Trizma base, 55g/L Boric acid and 14,9g/L EDTA) with 0,2µg/ml Ethidium Bromide. 10µl of PCR product was transferred to a clean tube and Orange Loading Dye (Thermo Scientific; Waltham, USA) was added. In total 10µl of PCR sample was pipetted in one of the gel slots. 100bp DNA Plus ladder (Thermo Scientific) was used as a DNA product size reference. The gel ran in 0,5x TBE at 120V for 45-60 minutes. The gel was visualized under UV-light in Gel Doc™ XR+ (Bio-Rad) and analyzed with Image Lab™ Software 6.0.1.

The samples with the expected PCR fragment size (90 bp) were used for further purification with Sodium acetate precipitation. To 15µl PCR product was 1,5µl 3M Sodium acetate pH 5,2, 37,5µl ice cold 100% EtOH and 20µg Glycogen added. The suspension was incubated on dry ice for 30 min, then centrifuged for 15min at 13.000 RPM, supernatant was discarded and 500µl ice cold 70% EtOH was added, centrifuged again for 10min at 13.000 RPM, supernatant was discarded and pellet was air dried and resuspended in 10µl MilliQ.

For ligation of the DNA product in the plasmid pBluescript KS II (+) (Stratagene; Agilent Technologies), was added together in a final volume of 10µl ~60ng of purified DNA, 50ng/µl HincII cut PBluescript II KS (+) plasmid, 1x T4 Ligation Buffer (Fermentas; Waltham, USA), 5% PEG 4000 (Fermentas) and 100U T4 Ligase (Fermentas) and filled up to 10µl with MilliQ. The ligation mix was incubated overnight at 16°C. A no ligase and NTC control were also made to test the ligation reaction.

For cloning of the constructed plasmids, a competent XL-1 Blue *E.coli* strain was thawed on ice. To 50µl of competent cells 10µl ligation suspension was added and mixed by gently ticking. The suspension was incubated on ice for 30 min, heat shocked at 42°C for 2 min and put back on ice for 2 min. Whole suspension was plated on LB agar (10g/L Tryptone (BD), 5g/L Yeast extract (BD), 5g/L NaCl (Sigma) and 15g/L Agar (BD)) with 0,1mM IPTG, 20µg/ml X-gal and 100µg/ml Ampicillin and spread out with spatula. Plates were incubated overnight with agar up at 37°C. Blue colonies were picked from plate and suspended in 20µl MilliQ. Whole bacterial suspension was added to 5ml LB broth with 100µg/ml Ampicillin (10g/L Tryptone (BD), 5g/L Yeast extract (BD), 5g/L NaCl (Sigma)) and incubated overnight while shaking at 37°C. From 2 ml of bacterial suspension the plasmids were purified with the Nucleospin plasmid EasyPure kit (Macherey-Nagel Ref. 740727.250) according to manufactures protocol. The purified plasmids were measured for concentration and purity with the nanodrop spectrophotometer

Denovix DS-11 FX. The plasmids were checked for the right insert by PCR with plasmid specific M13 primer. In reaction mix of total 25µl 1x High Fidelity buffer (Biolabs), 0,2mM dDNTP's, 0,5µM M13 forward primer (5' GTAAAACGACGGCCAG 3'), 0,5µM UUreCRev primer, 1U High Fidelity DNA Polymerase (Biolabs), 2µl DNA template and filled up to 25µl with MilliQ. Used PCR protocol 30" 98°C; 40 cycli of 10" 98°C – 15" 60°C – 15" 72°C and single cycli of 5' 72°C and 4°C infinite (Applied Biosystems 2720 Thermal Cycler). For gel electrophoresis a 2% agarose gel was made in 0,5x TBE buffer with 0,2µg/ml Ethidium Bromide. Orange Loading Dye (Thermo Scientific) was added to the PCR samples. In total 15µl of PCR sample was pipetted in one of the gel slots. 100bp DNA Plus ladder (Thermo Scientific) was used as a product size reference. The gel ran in 0,5x TBE at 120V for 45-60 minutes. The gel was visualized under UV-light in the Gel Doc^{Tm} XR+ (Bio-Rad) and analyzed with Image Lab^{Tm} Software 6.0.1. The constructed plasmids were also analyzed by LIGHTrun sequencing at GATC Biotech (part of Eurofins genomics, Köln, Germany). The constructed plasmids were stored at -20°C. The copy numbers/µl was calculated with the formula: Number of copies = (ng * 6.022x10^{23}) / (total plasmid size with insert (bp) * 1x10^{9} * 650). The plasmid with the insert of *U. urealyticum* was called pBUreCU. The plasmid with the insert of *U. parvum* was called pBUreCP.

Singleplex PCR

First the singleplex PCR to detect *Urease C* of *U. urealyticum* was tested separately using template DNA from reference strain *U. urealyticum* CCUG 50782T (UU 50782T). Specificity was tested with isolated DNA of UU 50782T, *U. parvum* ATCC 27818, *M. pneumoniae* M129, *M. pneumoniae* MAC, *M. genitalium* G37, *S. pneumoniae* D39 and *S. aureus* ATCC 29213. The reaction mix contained 1x TaqMan® Universal PCR Mastermix with UNG (Applied Biosystems; Warrington, UK), 0,8µM of UUreCFwd and UUreCRev, 312,5µM of UUreCP1probe or UUreCP2probe, 1µl DNA template and MilliQ was added to obtain a final volume of 20µl. Reaction mix was pipetted in a 96-wells green hard shell qPCR plate with clear wells (Bio-Rad; Hercules, USA). PCR protocol was 2' 50°C, 10' 95°C and 40 cycli of 15" 95°C and 1' 60°C (plate read). The PCR product was checked with gel electrophoresis on a 2% agarose gel in 0,5x TBE buffer (108,8g/L Trizma base, 55g/L Boric acid and 14,9g/L EDTA) with 0,2µg/ml Ethidium Bromide. Orange Loading Dye (Thermo Scientific) was added to each PCR sample. In total 20µl of PCR sample was pipetted in one of the gel slots. 100bp DNA Plus ladder (Thermo Scientific) was used as a product size reference. The gel ran at 120V for 45-60 minutes. The gel was visualized using a UV-light in Gel Doc^{Tm} XR+ (Bio-Rad) and analyzed with Image Lab^{Tm} Software 6.0.1.

The specificity for *U. parvum* ATCC 27818 and *M. hominis* ATCC 23114 was performed after optimizing the *Urease C* PCR. The reaction mix contained 1x TaqMan® Universal PCR Mastermix with UNG (Applied Biosystems), 1µM of UUreCFwd, 0,5µM UUreCRev, 0,25µM of UUreCP1probe or UUreCP2probe, 1µl DNA template and MilliQ was added to obtain a final volume of 20µl. Reaction mix was pipetted in a 96-wells green hard shell qPCR plate (Bio-Rad). MilliQ was used as NTC. Used PCR protocol 2' 50°C, 10' 95°C and 40 cycli of 15" 95°C and 1' 60°C (plate read).

To validate the *Urease C* plasmid as a standard series it was analyzed in both *U. urealyticum* and *U. parvum Urease C* PCR. The plasmids were thawed on ice, mixed by gently ticking and shortly centrifuged before use. A 10-fold dilution series of the plasmids were made with concentrations of 10^7 till 10^1 copy numbers/µl. Standard series were tested in duplicate. *UU 50782T* and *U. parvum* ATCC 27818 were used as positive controls for the corresponding plasmid and MilliQ was used as NTC. The reaction mix contained 1x TaqMan® Universal PCR Mastermix with UNG (Applied Biosystems), 1µM of UUreCFwd, 0,5µM UUreCRev, 0,25µM of UUreCP1probe or UUreCP2probe, 1µl DNA template and MilliQ was used to obtain a final volume of 20µl. Reactions were performed in duplicate in a green hard shell qPCR plate with clear wells (Bio-Rad). Used PCR protocol 2' 50°C – 10' 95°C – 40 cycli of 15" 95°C and 1' 60°C (plate read).

Validating duplex and triplex qPCR

The duplex PCR (to detect *U. urealyticum* and *U. parvum*) is validated by testing the standard series of the *Urease C* of *U. urealyticum* (pBUreCU) and *U. parvum* (pBUreCP) as singleplex PCR and duplex PCR. The triplex (to detect *U. urealyticum*, *U. parvum* and *M. pneumoniae*) is validated by testing the standard series of pBUreCU, pBUreCP as duplex PCR and standard series of *M. pneumoniae* with P1 fragment (pBmpP1) as singleplex PCR. The standard series contained a concentration of $1{,}52*10^7$ till $1{,}52*10^1$ copy numbers/reaction for *U. parvum*, $1{,}38*10^7$ till $1{,}38*10^1$ copy numbers/reaction for *U. urealyticum* and $1*10^7$ till $1*10^1$ copy numbers/reaction for *M. pneumoniae* and were all diluted in MilliQ. All plasmids were thawed on ice, mixed by gently ticking and shortly centrifuged before use.

<u>*Ureaplasma* duplex qPCR validation</u>

The *Ureaplasma* singleplex PCR reaction mix contained 1x TaqMan® Universal PCR Mastermix with UNG (Applied Biosystems), 1µM of UUreCFwd, 0,5µM UUreCRev, 0,25µM of UUreCP1probe or UUreCP2bprobe, 5µl DNA template and MilliQ was used to obtain a final volume of 20µl.

The *Ureaplasma* duplex PCR reaction mix for *Ureaplasma* duplex PCR contained 1x TaqMan Universal PCR Mastermix with UNG (Applied Biosystems), 1µM of UUreCFwd, 0,5µM UUreCRev, 0,25µM of UUreCP1probe and UUreCP2bprobe, 5µl DNA template and MilliQ was used to obtain a final volume of 20µl. MilliQ was used as NTC. Reactions were performed in triplicate in a Green hard shell qPCR plate with clear wells (Bio-Rad). Used PCR protocol 2' 50°C – 10' 95°C – 40 cycli of 15" 95°C and 1' 60°C (plate read).

Triplex qPCR validation

The *M. pneumoniae* singleplex PCR reaction mix contained 1x IQ Multiplex Powermix (Bio-Rad), 0,4µM of MpnP1TaqFwd, 0,6µM MpnP1TaqRev, 0,20µM of MpnP1TaqP2probe, 5µl DNA template and MilliQ was used to obtain a final volume of 20µl.

The *Ureaplasma* duplex qPCR reaction mix contained 1x IQ Multiplex Powermix (Bio-Rad), 1µM of UUreCFwd, 0,5µM UUreCRev, 0,25µM of UUreCP1probe and UUreCP2bprobe, 5µl DNA template and MilliQ was used to obtain a final volume of 20µl.

The triplex qPCR reaction mix contained 1x IQ Multiplex Powermix (Bio-Rad), 1µM of UUreCFwd, 0,5µM UUreCRev, 0,25µM of UUreCP1probe and UUreCP2bprobe, 0,4µM of MpnP1TaqFwd, 0,6µM MpnP1TaqRev, 0,20µM of MpnP1TaqP2probe, 5µl DNA template and filled with MilliQ till 20µl. MilliQ was used as NTC. Reactions were performed in duplicate in a Green hard shell qPCR plate with clear wells (Bio-Rad). Used PCR protocol 2' 95°C – 15" 95°C (40 cycles) and 1' 60°C (40 cycles + plate read).

All real-time PCR reactions were performed with the CFX96 Real-Time System C1000 touch Thermal Cycler (Bio-Rad). PCR data analysis was performed with Bio-Rad CFX manager program V 3.1.1517.0823. Statistical analysis and making of graphs was performed with Graph Path prism 5. Linear regression of the standard curve and Wilcoxon signed Rank test were used for comparing the singleplex PCR with multiplex PCR reaction. For PCR precision, the coefficient of variation (CV%) was calculated per standard between singleplex PCR and multiplex PCR.

Used criteria for multiplex qPCR validation[32]

- The Cq difference between singleplex and multiplex PCR must be lower than 1 Cq when using a single threshold
- Coefficient of determination (r^2) must be >0,98 with linear regression of the mean Cq.
- Limit of detection (LOD) must be the same when testing singleplex and multiplex. LOD is the lowest detectable concentration that can be detected with reliable results (standard deviation <0,5).
- Efficiency must be within 90-110% and calculated with the slope that is achieved with linear regression line. Efficiency% = $(10^{(-1/\text{The Slope Value})}-1)*100\%$
- CV% between singleplex and multiplex PCR must be <5%
  CV% is calculated with the formula: (standard deviation / mean)*100%

4.0 Results

4.1 Result of *Ureaplasma* culturing

We tested our own developed *Ureaplasma* broth m10B and agar mA8 by culturing reference strain UU 50782T. We assessed whether UU 50782T was able to grow and raise the pH (by the urease activity of *Ureaplasma*) of m10B causing a color change of the media. As expected, the broth with UU 50782T did show a color change from clear yellow to clear red what indicates *Ureaplasma* growth after 48 hours of incubation. (see Figure 3). This result suggests that m10B is a suitable culture media to culture and detect *Ureaplasma*.

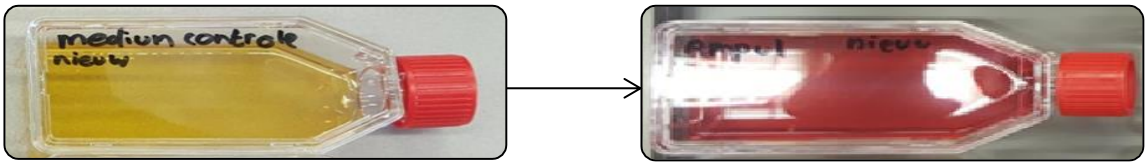


Figure 3: The culturing of UU 50782T in m10B. Left an uninoculated control (clear yellow)=negative. Right an inoculated broth with UU 50782T (clear red)=positive after 48h incubation.

We tested four different types of mA8 agar recipes namely mA8 plain, mA8 with 1,1mM $MnSO_4*H_2O$, mA8 with 1,1mM $MnSO_4*H_2O$ + 15mM $MgCl_2*6H_2O$ and mA8 with 1,1mM $CaCl_2*2H_2O$. We assessed the effect on colony growth and colony morphology of the grown colonies of UU 50782T for each recipe. We also assessed the effect of aerobically and anaerobically incubation on the colony growth and morphology of UU 50782T. We counted the visible precipitation sites. The colony count of the different agars is shown in Figure 4A. Figure 4A shows that *Ureaplasma* can grow aerobically as well as anaerobically, suggesting that *Ureaplasma* is a facultative anaerobic bacteria. The major difference between tested agars is that agar with $MnSO_4$ did not showed any growth but when $MgCl_2$ was added the growth was returned. The agar plate with $CaCl_2$ showed the most colony growth comparing to the plain agar without precipitant. Remarkable was that the colonies on agar with $CaCl_2$ are much larger than those on the basic agar and agar with $MnSO_4$+$MgCl_2$ (see Figure 4B). The *Ureaplasma* colonies on mA8 with $CaCl_2$ were visible with the naked eye, which makes detection of *Ureaplasma* growth easier.

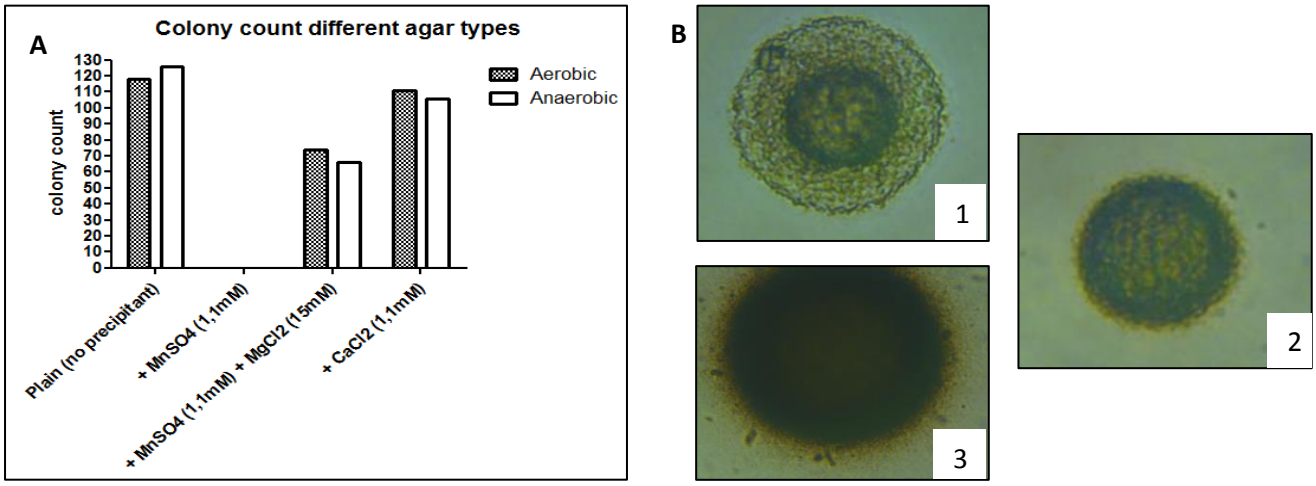


Figure 4: **A)** Result of *Ureaplasma* colony growth on different agar bases (all counted from dilution 10^{-5} CFU/ml) n=1. Incubated aerobically and anaerobically T=3 days. **B)** Microscopic pictures of the colonies from graph 4A. On plain basic agar without precipitant a clear "fried egg" morphology is seen (picture 1). On agar with $MnSO_4$ + $MgCl_2$ (picture 2) and with $CaCl_2$ (picture 3) a "sea urchin" colony can be seen with dark brown pigments. All pictures are from aerobic cultures colonies at objective 400x.

We also cultured twelve pre-cultured clinical isolates of *Ureaplasma*. All strains were cultured in m10B and on mA8 with $CaCl_2$. In total 5 out of 12 strains (3x *U. parvum* and 2x *U. urealyticum*) were viable for growth. Seven other strains seems not viable for growth (See Table 2). The uninoculated broth control was clear yellow (negative) and the positive control of UU 50782T was clear red (positive).

Table 2: Result of culturing pre-cultured clinical isolates of *Ureaplasma* strains

Strain number	Designation*	Broth culture**	Agar culture***
UU 2983	*U. Urealyticum*	Positive	Positive
UU 6050	*U. Urealyticum*	Negative	Negative
UU 2922	*U. Urealyticum*	Negative	Negative
UU 1319	*U. Urealyticum*	Negative	Negative
UU 1427	*U. Urealyticum*	Negative	Negative
UU 2880	*U. Urealyticum*	Positive	Positive
UP 2990	*U. parvum*	Positive	Positive
UP 3763	*U. parvum*	Positive	Positive
UP 3861	*U. parvum*	Negative	Negative
UP 3002	*U. parvum*	Positive	Positive
UP 5918	*U. parvum*	Negative	Negative
UP 6054	*U. parvum*	Negative	Negative

* Designation by Jagiellonian University, Krakow, Poland by PCR

** Broth color changed from clear yellow to clear red = Positive; No color change = Negative (see also Figure 3)

*** Golden brown "sea urchin" colonies were observed as seen in Figure 4 B3= Positive;
No growth or other morphology = Negative

The specificity of our m10B broth and mA8 with $CaCl_2$ is determined by culturing a *M. hominis* ATCC strain and two clinical isolates of *M. hominis* and a *S. aureus* reference strain. We assessed in m10B for color change and turbidity of the broth. On mA8 agar, we assessed for colony growth and morphology. *M. hominis* ATCC 23114 and one clinical *M. hominis* strain did show growth in our *Ureaplasma* broth (seen as light turbidity) when shaking but did not change the color of the broth. On mA8 with $CaCl_2$, *M. hominis* grows as small clear colonies with typical "fried egg" morphology (see Figure 5B). Unexpectedly, one of the two clinical *M. hominis* samples showed *Ureaplasma* growth in broth (color change as seen as in figure 3) and on agar (see Figure 5A and 5B). All uninoculated controls were negative. *S. aureus* did not showed growth. The color of the broth was unchanged (clear yellow) and on agar no colony growth was observed. There was growth on the blood agar control plate what indicates that a viable culture was added to the *Ureaplasma* broth and agar. These results shows that mA8 with $CaCl_2$ can differentiate *Ureaplasma* colonies, by the urease activity of *Ureaplasma*, from other *Mycoplasma* colonies.

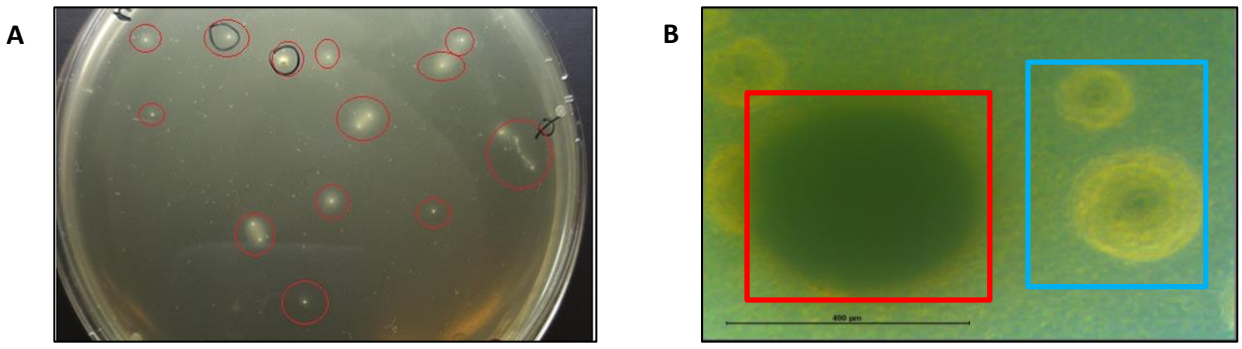


Figure 5: A) *Ureaplasma* agar with $CaCl_2$ with *Ureaplasma* colonies seen with the naked eye (red circled). Cultured from clinical isolate of *M. hominis*. **B)** Microscopic picture of a *Ureaplasma* colony (red "sea urchin") with *M. hominis* colonies (blue "fried egg") from the plate of figure 5A. Picture objective is 100x.

Development of culturing methods for *Ureaplasma* spp. and validation of a quantitative multiplex PCR.

4.2 Multiplex qPCR development

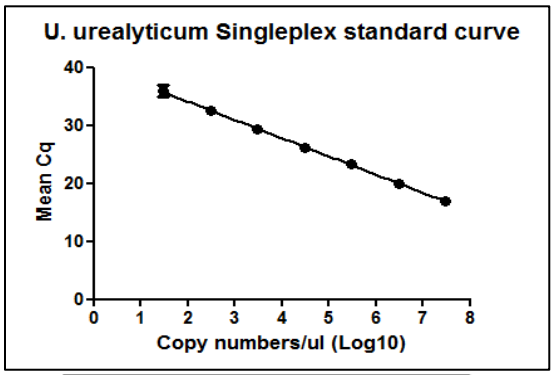
4.2.1 Singleplex PCR *Ureaplasma*

The primers and probes were first tested to check if the expected product of the *Urease C* gene (90bp) will be amplified. The first trial was performed with the reference strain UU 50782T. As expected, there was only an amplification signal to the target for what the probes where designed for. There was no cross-reaction between *U. urealyticum* and *U. parvum* observed. The expected PCR product size was confirmed by gel electrophoresis (result not shown).

The analytical specificity of the *Urease C* PCR was tested with bacterial DNA of UU 50782T, *U. parvum* ATCC 27818, *M. pneumoniae* M129, *M. pneumoniae* MAC, *M. genitalium* G37, *S. pneumoniae* D39 and *S. aureus* ATCC 29213. There was tested for amplification signals of the probes and gel electrophoresis was performed to check for cross-reactions of the primers. There was no amplification and cross-reaction observed to any of the tested bacterial strains only UU 50782T and *U. parvum* ATCC 27818 showed amplification. The designed primers amplify both *U. urealyticum* and *U. parvum*. The probe is specific to only *U. urealyticum* or *U. parvum* (result not shown).

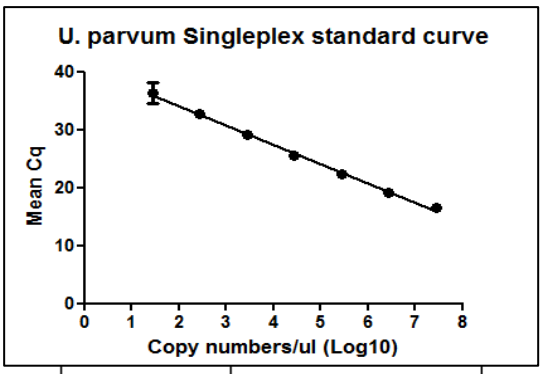
To make the *Urease C* PCR quantitative, plasmids were constructed and cloned with the desired PCR product of the *Urease C* complex of *U. urealyticum* or *U. parvum*. The copy numbers of the purified plasmids were calculated and based on copy numbers, a standard series was made. With the standard series, the efficiency and detection limit of the singleplex PCR was determined with a standard curve.
The *U. urealyticum* singleplex PCR shows a standard curve with a r^2 >0,99 and a efficiency of 108,10%. The lowest standard (10^1 copy number/µl) showed a Cq difference of >1 between the duplicates. The average Cq difference is <1 between all the standards (see Figure 7A and 7C). The PCR targeting *U. parvum* showed a standard curve with a r^2 >0,99 and an efficiency of 99,91%. Also the lowest standard concentration (10^1 copy number/µl) has a Cq difference of >2 between the duplicates (see Figure 7B and 7C). All CV% are <5% (see Figure 7D) and all NTC's were non-reactive (Cq >40). The constructed plasmids can be used to produce a reliable standard curve and quantification values.

A



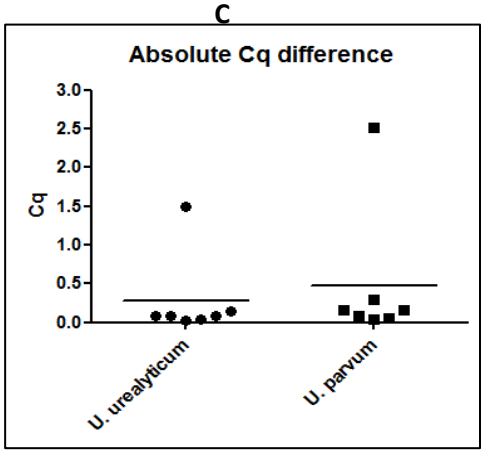
	U. urealyticum
LOD	30 copy numbers/ul
Slope	-3,142
R^2	0,9975
Efficiency%	108,10

B



	U. parvum
LOD	27 copy numbers/ul
Slope	-3,324
R^2	0,9923
Efficiency%	99,91

C



D

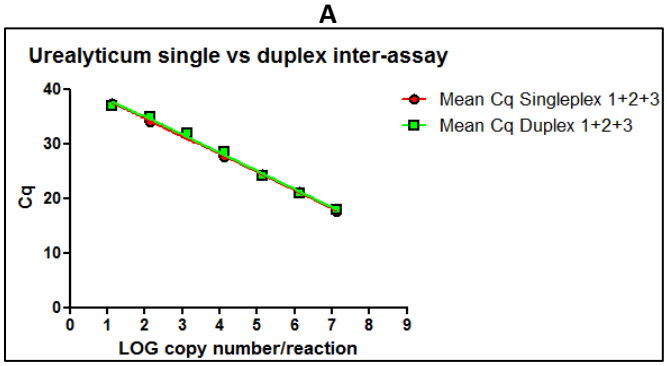
Coefficient of variation (CV%) Intra-assay singleplex PCR				
U. urealyticum			U. parvum	
Copy number/reaction	Intra-assay		Copy number/reaction	Intra-assay
3,06E+07	0,18		2,76E+07	1,25
3,06E+06	0,49		2,76E+06	0,58
3,06E+05	0,26		2,76E+05	0,28
3,06E+04	0,23		2,76E+04	0,17
3,06E+03	0,21		2,76E+03	0,38
3,06E+02	0,06		2,76E+02	0,08
3,06E+01	2,93		2,76E+01	4,91

Figure 6: Result of standard series in singleplex *Ureaplasma* PCR. **A)** Standard curve of *U. urealyticum*. Plotted: mean Cq per standard with standard deviation. Threshold was set at 200 RFU **B)** Standard curve of *U. parvum*. Plotted: mean Cq per standard with standard deviation. Threshold was set at 200 RFU. **C)** Absolute Cq difference between standards replicates. **D)** Coefficient of variation intra-assay singleplex based on mean Cq and standard deviation.

4.2.2 Validation duplex PCR

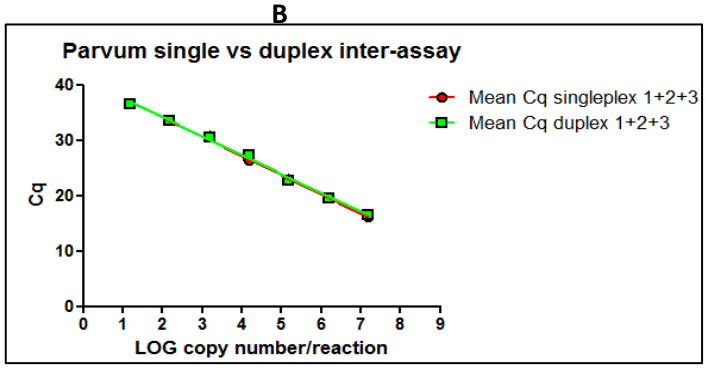
To validate the duplex qPCR for *U. urealyticum* and *U. parvum*, the standard series of both targets were pooled and tested three times (n=3) in triplicate and compared by the singleplex reaction. The data between singleplex and duplex is compared by linear regression and Wilcoxon Signed Rank test to check for significant differences where *P*=<0,05 is significant different. Both targets show no significant difference between the singleplex and duplex reaction (*U. urealyticum P*=0,6766; *U. parvum P*=0,4654). The mean Cq difference of the standards in singleplex and duplex in both targets is <1. The LOD for *U. urealyticum* PCR is 13 copies/reaction with a efficiency of 100,71% in singleplex and 100,88% in duplex with a r^2 of >0,99 (see Figure 8A). The LOD of *U. parvum* is 15 copies/reaction with a efficiency of 94,06% (singleplex) and 96,26% (duplex) with a r^2 of >0,99 (see Figure 8B). All these results are within the validation criteria Cq difference <1, efficiency is 90-110%, LOD is not different between singleplex and duplex, r^2 = >0,98 and all CV% are <5% what makes this a reliable, sensitive duplex qPCR (See Figure 8C and 8D). All NTC's were Cq >40. The threshold value was set at 150 RFU for FAM as well as HEX channel during analyzing. This duplex qPCR can be used for a reliable quantification of *U. urealyticum* and *U. parvum* in a DNA sample.

A



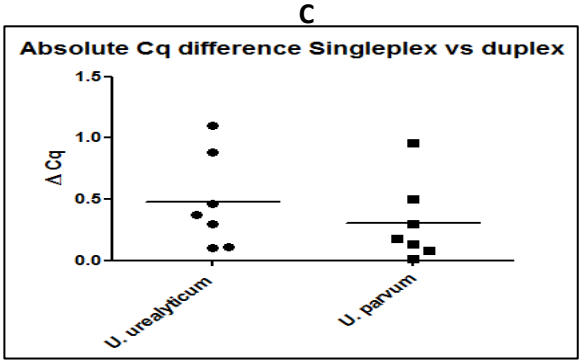
	Singleplex	Duplex
LOD	13 copies/reaction	13 copies/reaction
R2	0,9971	0,9929
Slope	-3,305	-3,301
Efficiency	100,71%	100,88%
Slopes equal?	p=0,9777 (not significantly different)	
Wilcoxon test	p=0,6766 (not significantly different)	

B



	Singleplex	Duplex
LOD	15 copies/reaction	15 copies/reaction
R2	0,9977	0,9968
Slope	-3,473	-3,415
Efficiency	94,06%	96,26%
Slopes equal?	p=0,6209 (not significantly different)	
Wilcoxon test	p=0,4654 (not significantly different)	

C



D

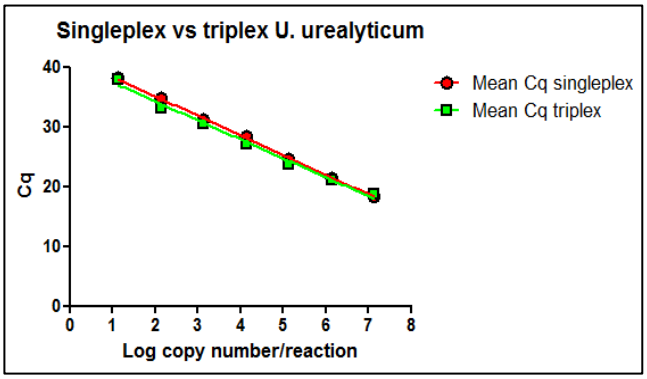
Coefficient of variation (CV%) Singleplex vs Duplex				
U. urealyticum			U. parvum	
Copy number/reaction	Inter-assay		Copy number/reaction	Inter-assay
1,38E+07	1,81		1,53E+07	2,15
1,38E+06	0,35		1,53E+06	0,04
1,38E+05	0,88		1,53E+05	0,55
1,38E+04	2,77		1,53E+04	2,54
1,38E+03	0,23		1,53E+03	0,69
1,38E+02	1,79		1,53E+02	0,28
1,38E+01	0,71		1,53E+01	0,16

Figure 7: Result of standard series in singleplex and duplex *Ureaplasma* PCR. **A)** Standard curve of *U. urealyticum* singleplex vs duplex PCR. Plotted: Linear regression of the mean of the means Cq per standard of three assays. Threshold was set at 150 RFU. **B)** Standard curve of *U. parvum* singleplex vs duplex PCR. Plotted: Linear regression of the mean of the means Cq per standard of three assays. Threshold was set on 150 RFU. **C)** Absolute Cq difference (Δ Cq) between mean of the means of singleplex vs duplex PCR. **D)** Coefficient of variation between singleplex and duplex PCR based on mean Cq and standard deviation.

4.2.3 Validation triplex PCR

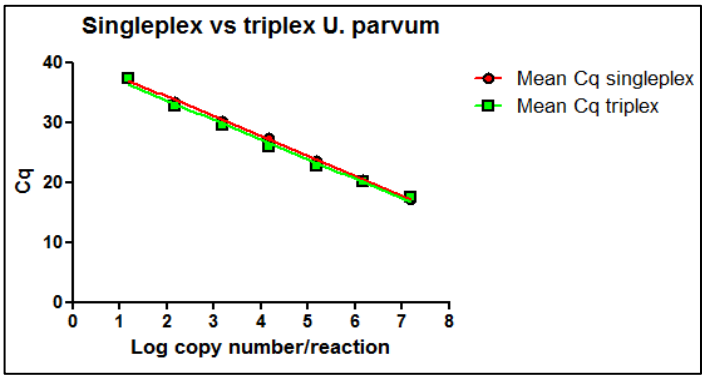
For the validation of the triplex PCR, the standard series of *M. pneumoniae*, *U. urealyticum* and *U. parvum* were pooled. The standard series of *M. pneumoniae* is compared to the singleplex reaction. The standard series of *U. urealyticum* and *U. parvum* were compared to the duplex reaction. The validation has been performed once (n=1) with the standard series in duplicate. The data of each target was compared by linear regression and Wilcoxon Signed Rank test for statistical differences (*P*=<0,05 is significantly different). The results show that all targets were equally well amplified in singleplex and triplex reactions (see Figure 9A, 9B and 9C). However, the Wilcoxon Signed Rank test showed for every target a significant difference of the medians (*P*=<0,05) between singleplex and triplex (see Figure 9A, 9B and 9C). A few samples, especially the standard 10^4, showed a greater Cq difference in all targets (Cq difference >1) while the Cq difference of most of the other standards is <1 and acceptable (see Figure 9D). Looking to all the other data; the LOD is the same, average Cq difference of the whole standard series is <1, all r^2 are >0,99, all efficiencies are acceptable (90-110%) and all CV% are <5% (see figure 9D and 9E). All tested NTC's were Cq >40. For hydrolysis probe with FAM and HEX a threshold of 150 RFU is used for analysis and for Cy5 channel a threshold of 100 RFU is used for analysis. Despite the significant difference of the median of each target, all targets are conform the criteria. Therefore, the triplex qPCR can be used to quantify *U. urealyticum*, *U. parvum* and *M. pneumoniae* in a single reaction.

A



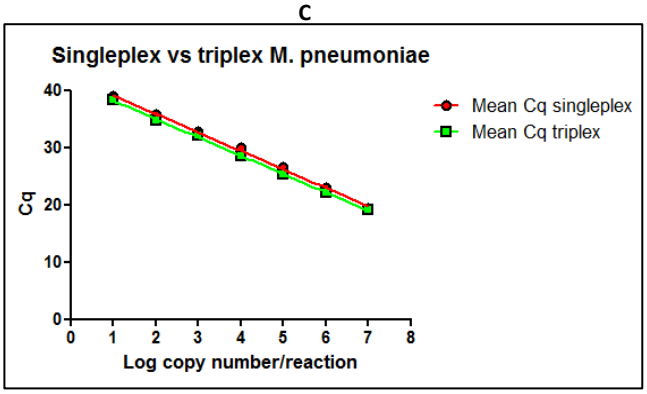
	Singleplex	Triplex
LOD	13 copies/reaction	13 copies/reaction
R2	0,9991	0,9912
Slope	-3,286	-3,178
Efficiency	101,52%	106,38%
Slopes equal?	p=0.4631 (not significantly different)	
Wilcoxon test	p=0,0469 (significantly different)	

B



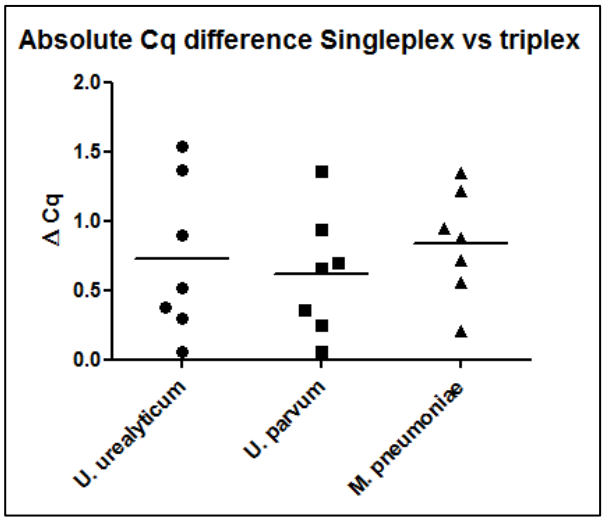
	Singleplex	Triplex
LOD	15 copies/reaction	15 copies/reaction
R2	0,9985	0,992
Slope	-3,314	-3,268
Efficiency	100,33%	102,30%
Slopes equal?	p=0.7551 (not significantly different)	
Wilcoxon test	p=0,0469 (significant different)	

C



	Singleplex	Triplex
LOD	10 copies/reaction	10 copies/reaction
R2	0,9982	0,9992
Slope	-3,226	-3,201
Efficiency	104,17%	105,31%
Slopes equal?	p=0.7459 (not significantly different)	
Wilcoxon test	p=0,0156 (significant different)	

D



E

Coefficient of variation (CV%) Singleplex vs Triplex PCR						
U. urealyticum			U. parvum		M. pneumoniae	
Copy numbers/reaction	Inter-assay		Copy numbers/reaction	Inter-assay	Copy numbers/reaction	Inter-assay
1,38E+07	1,15		1,53E+07	1,04	1,00E+07	0,77
1,38E+06	1,26		1,53E+06	1,26	1,00E+06	2,76
1,38E+05	2,65		1,53E+05	2,87	1,00E+05	3,33
1,38E+04	3,49		1,53E+04	3,60	1,00E+04	3,25
1,38E+03	1,19		1,53E+03	1,65	1,00E+03	1,58
1,38E+02	3,22		1,53E+02	1,41	1,00E+02	1,91
1,38E+01	0,11		1,53E+01	0,13	1,00E+01	1,02

Figure 8: Result of standard series in singleplex and triplex PCR. **A)** Standard curve of *U. urealyticum* singleplex vs triplex PCR. Plotted: Linear regression of the mean Cq per standard. Threshold was set at 150 RFU. **B)** Standard curve of *U. parvum* singleplex vs triplex PCR. Plotted: Linear regression of the mean Cq per standard. Threshold was set on 150 RFU. **C)** Standard curve of *M. pneumoniae* singleplex vs triplex PCR. Plotted: Linear regression of the mean Cq per standard. Threshold was set on 100 RFU. **D)** Absolute Cq difference (Δ Cq) between mean of singleplex vs triplex PCR. **E)** Coefficient of variation between singleplex and triplex PCR based on mean Cq and standard deviation.

5.0 Discussion and conclusion

5.1 Culturing of *Ureaplasma*

The Erasmus Medical Center does not perform routine diagnostic for *Ureaplasma* infections. Therefore there are no culture and molecular assays directly available for research. To make future research and diagnostics possible, we wanted to develop a culture method to culture and detect *Ureaplasma*. Now, we have developed optimal culture methods for the cultivation and detection of *Ureaplasma* by using the urease activity of *Ureaplasma*.

The *Ureaplasma* broth is designed to detect urease activity by the formation of ammonium and the raise of pH. The raise of the pH changes the color of the pH indicator from yellow to red[33]. Our m10B recipe is based on two different recipes. Our m10B has not been compared to other *Ureaplasma* broth recipes. We don't know if our recipe has better growth performance than other recipes.
We did observed, that some pre-cultured strains of *U. urealyticum* and *U. parvum* were not viable to change the pH of the broth. This may indicate that some strains are not viable for growth anymore. A study shows that urea has a great effect on *Ureaplasma* growth but also quickly initiate a death phase after a relative short stationary phase[34]. This may have happened to some of the pre-cultured *Ureaplasma* strains.

The *Ureaplasma* agar mA8 detects urease activity at the colony site of *Ureaplasma* by a precipitation reaction. Based on various recipes we tested different precipitants mA8+$MnSO_4$, mA8+$MnSO_4$+$MgCl_2$ and mA8+$CaCl_2$. We observed a very poor growth on mA8+$MnSO_4$. It is described that manganese inhibits colony growth of *Ureaplasma*[23], although every serotype reacts differently on certain concentrations of $MnSO_4$[23]. In later recipes, $MnSO_4$ was replaced by $CaCl_2$, called A8 agar[35]. Our results show that $CaCl_2$ increases the amount of colony growth but also increased colony size. The colonies on mA8+$CaCl_2$ were easily visible on agar with the naked eye. Normally, *Ureaplasma* colony growth is observed through a microscope but our result shows that they can be easily detected with the naked eye. Therefore, mA8 with $CaCl_2$ was chosen as the best recipe for culturing of *Ureaplasma* on agar.

We tested the specificity of m10B and mA8+$CaCl_2$ by culturing *M. hominis* and *S. aureus* (urease positive) in m10B and on mA8 with $CaCl_2$. We observed that *M. hominis* can grow in m10B. However, *M. hominis* did not changed the color of the *Ureaplasma* broth. *M. hominis* did also grow on mA8+$CaCl_2$ but did not show any precipitation reaction or morphology change (*M. hominis* keeps his typical "fried egg" morphology), making differentiation between *Ureaplasma* and *Mycoplasma* colonies, based on morphology, possible.

These experiments were performed with mostly "clean" strains. When patient material will be tested, drug-resistant bacteria present in the sample may influence the culture and may overgrow *Ureaplasma*. Therefore PCR must always be performed to confirm the presence of *Ureaplasma*.

At this moment we are collecting neonatal respiratory samples of the Neonatology department of EMC. With our culture method, we will test the samples for the presence of viable *Ureaplasma*. This will be the first step to answer the question about the prevalence of *Ureaplasma* in neonatal respiratory diseases. And to answer the question if *Ureaplasma* is associated in developing of BPD.

5.2 Multiplex qPCR development

The Erasmus Medical Center does not perform routine diagnostic for *Ureaplasma* infections. Therefore there are no culture and molecular assays directly available for research. To make future research and diagnostics possible, we want to develop a quantitative multiplex PCR for *U. urealyticum*, *U. parvum* and *M. pneumoniae*.

We validated the triplex according the MIQE guidelines[36]. A validation of a multiplex qPCR has no general specific criteria. The followed MIQE guideline only shows the information that is needed for the publication of a quantitative experiment but does not describe specific criteria. Therefore we used criteria that are based on the MIQE guidelines for the validation of the multiplex qPCR (see also 3.2 Development and validation of a quantitative multiplex PCR)[32].

The singleplex PCR with standard series of both *Ureaplasma* targets shows a good standard curve (r^2 = >0,99). However, the lowest concentration of the standard curve (10^1 copies/µl) shows a high difference between the duplicates with also a relative high CV%. This can be caused due to bad homogenization or pipetting errors. During this method only 1µl of DNA template is used for the qPCR. Because all other results were conform criteria, we think that this PCR is acceptable and continued with multiplex[32].

The duplex PCR with *U. urealyticum* and *U. parvum* showed no significant difference (*P*=>0,05) in performance and result comparing with the singleplex PCR. The limit of detection is different than is shown during the singleplex validation because the amount DNA template was increased to 5µl instead of 1µl. However, the 10^4 copies/reaction standard shows a greater CV% in both targets than all other standards for unknown reason. Although, all results are conform criteria and is therefore validated successfully[32].

The triplex PCR with *U. urealyticum*, *U. parvum* and *M. pneumoniae* showed a significant difference of the medians between singleplex and triplex for all targets (*P*=0,0469 *U. urealyticum*; *P*=0,0469 *U. parvum*; *P*=0,0156 *M. pneumoniae*). Probably, the difference is caused by the 10^4 copy numbers/reactions standard. This standard shows the greatest difference in Cq values and CV% than all other standards. This difference has been observed during all of the other multiplex validation assays and the reason of this difference remains unclear. Despite the significant difference between singleplex and triplex, all other results are conform our used criteria and therefore we concluded this validation reliable and successful[32].

During this triplex validation, we used the mastermix IQ Multiplex Powermix of Bio-Rad instead of the TaqMan® Universal PCR Mastermix by Applied Biosystems. We changed the mastermix due to inadequate amplification results when using the TaqMan® Universal PCR Mastermix in the triplex PCR. The new mastermix is designed for multiplex PCR setups up to five targets and for genomic and plasmid DNA[37].

Our developed triplex qPCR is the first qPCR with the combination *U. urealyticum*, *U. parvum* and *M. pneumoniae* as we know to date. We are the first laboratory, as we know to date, that will research the bacterial load of *Ureaplasma* in neonatal respiratory samples. However, determination of the bacterial load of *Ureaplasma* is not completely new. There are research papers describing bacterial load of *Ureaplasma* in especially urogenital samples of men and woman[38, 39]. there is not much known about *Ureaplasma* load in neonates during infections and development of BPD.

Our triplex may be used for diagnostic perspectives. However, the triplex qPCR is not validated according to the International Organization for Standardization (ISO). Our triplex qPCR can therefore only provide an estimation in the presence and bacterial load of *Ureaplasma* or *M. pneumoniae* in a sample. We cannot conclude by the presence or bacterial load of *Ureaplasma* or *M. pneumoniae* if the infection is caused by *Ureaplasma* or *M. pneumoniae*. Our triplex PCR is validated for research perspectives.

We are developing another multiplex qPCR combining *U. urealyticum*, *U. parvum*, *M. hominis* and *M. genitalium*. Because these are all urogenital bacteria, they can theoretically be vertical transmitted to the neonate during birth. With this multiplex qPCR, the prevalence and bacterial load of *Ureaplasma* in pregnant woman with of chorioamnionitis and preterm birth can be analyzed.

5.3 Overall conclusion

The developed culture method in broth and agar can specifically culture *U. urealyticum and U. parvum* in clinical isolates, and differentiate *Ureaplasma* from *Mycoplasma* by using the urease activity of *Ureaplasma*.
The developed multiplex qPCR is a reliable, sensitive and specific quantification method for the detection and quantification of *U. urealyticum*, *U. parvum* and *M. pneumoniae* DNA in DNA isolates.

With the developed methods, studies on the role of *Ureaplasma* in development of BPD in neonates can be started and makes development of new diagnostic tools possible.

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I especially want to thank Wendy Unger for her great supervision and for giving me the opportunity to perform a research, which was completely new for me but also for the whole group.
I had a great internship where I did learn a lot about *Ureaplasma* and *Mycoplasma* and how challenging it is to develop and validate diagnostic analysis such as qPCR.
It gave me a really good feeling to know that we as Laboratory of Pediatrics of the EMC are the first in the whole EMC that is working on such difficult bacteria and that we succeed to culture and quantitate *Ureaplasma*. I expect that the developed analysis will produce a lot of interesting scientific articles in the near future and I am looking forward to read them!

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