Diplomarbeit

The Role of *Klebsiella oxytoca* in Antibiotic-Associated Colitis

Comparison of Polymerase Chain Reaction as a New Detection Method for *K. oxytoca* to Conventional API 20 E Testing

eingereicht von

Paul Weberhofer

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Univ. - Prof. Dr. Thomas Hinterleitner
und
Univ. - Prof. Dr. Christoph Högenauer

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Zusammenfassung

Einleitung: *Klebsiella oxytoca* wurde kürzlich als neuer Erreger der Antibiotika-assoziierten hämorrhagischen Colitis (AAHC) von Högenauer, C., et al., NEJM, 2006 **355**(23): p. 2418-26. beschrieben. Der Nachweis von *K. oxytoca* mittels Stuhlkultur bei AAHC wird durch das Vorkommen vieler anderer *Enterobacteriaceae* im Darm erschwert. Unser Ziel war es daher, einen neuen spezifischen und sensitiven Test für *K. oxytoca* zu etablieren. Der herkömmliche mikrobiologische Nachweis von *K. oxytoca* erfolgt mittels API 20E Test *(Analytischer-Profil-Index)*. Beim API 20E Test handelt es sich um ein Testverfahren mit einer Keimidentifizierung anhand von Wahrscheinlichkeitsangaben nach biochemischen Farbreaktionen. Eine für *K. oxytoca* hochspezifische neue PCR-Methode wurde 2003 (Kovtunovych et al., Res Microbiol, 2003. **154**(8): p. 587-92) beschrieben. **Methodik:** Ein 344-bp großes *pehX* Gen von *K. oxytoca* kodiert das Enzym Polygalakturonase. Dieses Enzym spaltet eine Polygalakturonkette von demethoxyliertem Pektin. Diese Fähigkeit ist spezifisch für *K. oxytoca* und unterscheidet sich von anderen *Klebsiella spp*. Insgesamt wurden von uns 143 *Klebsiella* Stämme, die zuvor mit dem API 20 E Test identifiziert wurden, mit den Primerpaaren *PEH-C* und *PEH-D* mittels PCR für das *pehX* Gen getestet. **Ergebnisse:** Gemäß API 20E waren von den 143 Stämmen 77 *K. oxytoca*, 64 *K. pneumoniae* und 2 *K. terrigena*. Die PCR konnte hingegen nur 68 Stämme als *K. oxytoca* identifizieren, die restlichen 75 *Klebsiella spp.* waren nicht *K. oxytoca* zuzuordnen. Fünf Proben wurden anhand der PCR Ergebnisse als *K. oxytoca* neu identifiziert. Vierzehn Proben, die nach API 20E Bestimmung vermeintlich *K. oxytoca* waren, konnten mittels PCR nicht als solche bestätigt werden. Insgesamt unterscheiden sich die zwei Nachweismethoden in 19 von 143 Stämmen, das entspricht 13 %. In einer zusätzlich durchgeführten Indol-Reaktion dieser 19 diskrepanten Keime korrelierten die Ergebnisse überwiegend mit den API 20E Ergebnissen. **Diskussion:** Die Aussagekraft dieser PCR ist auf den Nachweis des *K. oxytoca* spezifischen *pehX* Gen beschränkt. Eine Zuordnung zu anderen *Klebsiella spp.* ist mit dieser Methode nicht möglich. Die PCR ist aufgrund der oben beschriebenen Ergebnisse für den Nachweis von *K. oxytoca* möglicherweise nicht so spezifisch wie bisher beschrieben. Nach den derzeitigen Ergebnissen bleibt weiterhin offen, ob die PCR für *K. oxytoca* das spezifischere Nachweisverfahren als der API 20E Test darstellt. Ob eine 16S rRNA-Analyse die Spezifität der Nachweisverfahren weiter verbessert, bleibt abzuwarten.

Abstract

Introduction: *Klebsiella oxytoca* has been recently described as the causative organism of antibiotic-associated hemorrhagic colitis (Högenauer, C., et al., NEJM, 2006 **355**(23): p. 2418-26). Because the identification process of *K. oxytoca* in stool cultures is impaired by other *enterobacteriaceae*, our aim was to establish a new specific and sensitive testing method for *K. oxytoca*. Conventional detection of *K. oxytoca* is carried out by API 20 E testing. API (Analytical Profile Index) is a testing method that identifies bacterial strains due to colour change of substrates after biochemical reactions. The aim of our study referred to the question, if identification of *K. oxytoca* using PCR (Polymerase Chain Reaction) is more specific and more objective compared to routine API 20 E testing. A *K. oxytoca*-specific PCR method was described in 2003 by Kovtunovych et al., Res Microbiol, 2003. **154**(8): p. 587-92 **Methods:** Kovtunochvych et al. demonstrated that specific discrimination of *K. oxytoca* to other *Klebsiella spp*. is performed by detection of a polygalaturonase (*pehX*) gene. An amplicon of *pehX* with 344 bp (base pairs) was obtained in all *K. oxytoca* strains and in none of the other tested *Klebsiella spp.* Polygalacturonase is an enzyme that cleaves a polygalacturonic chain of demethoxylated pectin. This is a capability which is unique for *K. oxytoca* amongst the *Klebsiella spp.* If a sequence of this specific enzyme is amplified with PCR, proper discrimination of *K. oxytoca* to the other *Klebsiella* strains can be achieved. We tested 143 *Klebsiella* strains using primer pairs *PEH-C* and *PEH-D* with PCR. All these 143 strains had been identified with API 20 E before. **Results:** On the basis of the API results, 77 strains were detected as *K. oxytoca*, 64, were identified as *K. pneumoniae* and 2 of these 143 *Klebsiella* strains were identified as *K. terrrigena*. According to PCR, only 68 out of 143 *Klebsiella spp*. were identified as *K. oxytoca*. The remaining 75 *Klebsiella* strains were not classifiable as *K. oxytoca.* Five *K. oxytoca* strains were newly identified with PCR, whereas 14 strains that had previously been identified as *K. oxytoca* according to API results, could not be identified with PCR. In total the results of the testing methods were divergent in 19 out of 143 strains. This equals around 13 %. An additional indole-reaction was carried out on these 19 discrepant strains. These results correlated with API test results in 84%. **Discussion:** PCR does not seem to be as specific as expected for the identification of *K. oxytoca* according to the results described above. It still remains open, if PCR is a more specific testing method in comparison with API. It remains to be seen if a 16S rRNA-analysis of the discrepant strains will resolve this question.

Glossary and Abbreviations

AAHC	antibiotic associated hemorrhagic colitis
API 20 E	analytical profile index using 20 substrate for enterobacteriaceae
BAL	broncho-alveolary lavage
bp	base pairs
C. difficile	Clostridium difficile
CABG	coronary artery bypass graft
CCU	coronary care unit
CDAD	Clostridium difficile associated diarrhea
COPD	chronic obstructive pulmonary disease
CVC	central venous catheter
DANN	desoxyribonucleic acid
gangrene di. III sin.	gangrene digitorum III sinistra
K. o.	Klebsiella oxytoca
K. oxytoca	Klebsiella oxytoca
K. pn.	Klebsiella pneumoniae
K. pneumoniae	Klebsiella pneumoniae
K. terrig.	Klebsiella terrigena
Klebsiella spp.	Klebsiella species
oblit. thrombang.	obliterative thrombangitis
PAD	peripheral arterial disease
PCR	polymerase chain reaction
pehX	polygalaturonase gene
RT-PCR	reverse transcriptase Polymerase Chain Reaction
SBP	spontaneous bacterial peritonitis
st. p.	status post
UC	ulcerative colitis
VAP	ventilator-associated pneumonia
16S rRNA	ribosomal ribonucleic acid

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1 Introduction

1.1 Antibiotic treatment and intestinal microflora

One of the major side-effects of oral antibiotic therapy is the consecutive imbalance and disorder of the enteric microbiological symbiosis. Especially in the large intestine, this can lead to irritations of the bacterial equilibrium. The physiological concentration of bacteria rises from around 10^6/ml in the ileum to the large intestine, where the bacterial concentration is between 10^{11}/ml and 10^{12}/ml. [1]

This is one of the major reasons why the large intestine in particular is endangered by negative consequences resulting from oral administration of antibiotics. The spectrum of symptoms can vary from no symptoms at all up to bloody diarrhea. On the one hand, some patients get along well after treatment with antibiotics and do not develop intestinal side effects. On the other hand, there are many patients who develop intestinal symptoms of varying intensities. In addition to the fact that these side-effects are accompanied by a reduced quality of life, they can potentially be harmful to the patient.

The reasons for these different intestinal disorders following oral antibiotic therapy are diverse. The changes in bacterial flora may result from the level of susceptibility of different bacterial strains to antibiotics amongst other reasons. For example, some bacteria produce *β-lactamase* in order to enzymatically destroy the β - lactam-structure of some antibiotics such as penicillins or cephalosporins. Whilst other bacteria get destroyed, certain resistant bacterial strains can prevail against the physiologic flora and bear the risk of intestinal damage. [2, 3]

1.2 Possible consequences of oral antibiotic administration

1.2.1 *Clostridium difficile*

One major problem in the aftermath of antibiotic treatment is that facultative pathological bacteria such as *Clostridium difficile* proliferate and crowd out physiological bacteria and unfold their virulent potential. *C. difficile* was first described in 1935 by Hall and O'Toole, as a gram-positive anaerobic bacillus and it was named "the difficult clostridium" because it resisted early attempts at isolation and grew very slowly in culture.[4] *Clostridium difficile* is the most common identifiable bacterial cause of diarrhea in the United States.[5] *C. difficile* can be acquired by the oral route - often nosocomially - by an infected person or a healthcare worker who can serve as a vector. Post-antibiotic microfloral changes of the intestine can create an environment that allows *C. difficile* to proliferate.[6] Asymptomatic *C. difficile* carrier have the potential to contribute significantly to disease transmission in long-term care facilities. Clinical factors, such as previous *C. difficile* associated diarrhea (CDAD) and recent treatment with antibiotics, may be predictive of asymptomatic carriage[7]. The prevalence of *C. difficile* amongst the healthy population in the Western world ranges from 4,2 % to 15,3 %.[8] Intake of antibiotics and hospitalization, may trigger the harmful potential of *C. difficile* and its toxins A and B as described above. These toxins can cause severe inflammation of the colonic mucosa, also known as pseudomembranous colitis.

In addition to the well described inflammation of the colonic resulting from *C. difficile* and its toxins, colitis after antibiotic treatment can occur in the absence of *C. difficile* and other known causative organisms of infectious bowel disease. These other causative organisms can be *Yersinia enterocolitica*, diarrheagenic subtypes of *E. coli*, *Shigella dysenteriae* and *Salmonella enterica* to mention the most important bacteria. Recent findings have shown that there is an association between antibiotic-associated hemorrhagic colitis (AAHC) and *Klebsiella spp*. AAHC was first described by Toffler et al. and occurs usually after penicillin therapy.[9] The cause of antibiotic-associated hemorrhagic colitis was still unclear in 1978. In the following years, various etiological reasons have been discussed and proposed in medical literature. They range from local allergic reactions to antibiotic treatment, to microcirculatory and ischemic effects directly or indirectly resulting from antibiotics [10-12]. Finally, the role of *Klebsiella oxytoca* - a gram-negative bacterium - was suggested, which has been demonstrated and proven as an infectious cause of antibiotic-associated hemorrhagic colitis (AAHC) by Högenauer et al. [13]

1.2.2 Antibiotic-associated hemorrhagic colitis and *Klebsiella oxytoca*

Antibiotic-associated hemorrhagic colitis (AAHC) is a colonic inflammation that is often misdiagnosed and underestimated, which can lead to serious illness if the administration of antibiotics is continued [14]. Comparable to *C. difficile*, there are also asymptomatic carriers of *K. oxytoca* (1,6% of healthy subjects) [13]. However, it still remains unclear if AAHC caused by *K. oxytoca* is an infection or if it is caused in asymptomatic carriers under certain conditions.[15] Taking the possible severity and the remaining missing knowledge of antibiotic-associated hemorrhagic colitis into account, AAHC is a disorder that still needs to be studied intensively. In order to discover and reveal as much relevant information and details as possible about the pathophysiology, causality and the prevalence, as well as the clinical presentation of antibiotic-associated hemorrhagic colitis, it is absolutely necessary to work with a highly reliable testing method for *K. oxytoca*. In order to achieve this aim of finding a reliable testing method, the major task of this diploma thesis comprises the comparison between conventional biochemical testing of bacterial strains and PCR (polymerase chain reaction) testing for *Klebsiella oxytoca*. The working hypothesis is that PCR testing furnishes a more specific proof for *K. oxytoca* identification than conventional API testing methods. It might therefore be necessary to reconsider routine testing for *Klebsiella spp*.

2 Background

2.1 *Klebsiella species*

Klebsiella spp. are gram-negative bacteria that are both aerobe and anaerobe. This fact also constitutes the reason why *Klebsiella spp.* on the one hand can cause acute respiratory diseases such as hospital-acquired pneumonia [3]. On the other hand though, *Klebsiella spp.* are also *enterobacteriacae* and, consequently, can survive and proliferate in anaerobe environments such as the gut or the urinary tract. This is also the reason for the infections and inflammations of these organs caused by *Klebsiella spp*. Up to 15 species (including a few subspecies) are described in medical literature [16, 17]:

Table 1: The known subspecies of *Klebsiella*

K. oxytoca	*K. pneumoniae subsp. pneumoniae*
K. pneumoniae	*K. pneumoniae subsp. rhinoscleromatis*
K. terrigena	*K. rhinoscleromatis*
K. ornitholytica	*K. singaporensis*
K. planticola	*K. mobilis*
K. varicola	*K. trevisanii*
K. granulomatis	*K. pneumoniae subsp. ozeanae*
K. ozeanae	

2.2 Differentiation of *K. oxytoca* and *K. pneumoniae*

The most important species in the above group are *K. pneumoniae* and *K. oxytoca.* These two species are facultatively causative organisms of various diseases and nosocomial infections [3, 16]. *Klebsiella spp.* have been first mentioned in 1883 by the German pathologist Karl Friedländer as a causative organism of severe pneumonia. The bacterial genus *Klebsiella* was finally described and designated in honor of the German microbiologist Edwin Klebs by Trevisan two years later in 1885. Trevisan also described the *Klebsiella pneumoniae* (formerly known as Friedländer bacillus) species in 1887 [36].

Klebsiella spp., being a new group of *enterobacteriaceae,* had been first classified in the in 1985, which is one century after the first description of the bacterial species. At that time, the contribution of these new groups of *enterobacteriaceae* to human diseases was in the initial stages of medical discussion [18].

The focus of this diploma thesis will be placed on *K. oxytoca*, which is closely related to *K. pneumoniae*. In biochemical differentiation, *K. oxytoca* and *K. pneumoniae* are only distinguished by a positive indole-reaction for *K. oxytoca* and a negative reaction for *K. pneumonae*. Indole testing is also integrated in the 20 reactions in API 20 E test. Indole reaction is described separately in the following chapter.

2.3 Indole testing

Indole testing aims at the detection of the enzyme tryptophanase. Tryptophanase catalyzes the reaction from tryptophan to indole, pyruvic acid and ammonia (see figure1) [19] . If the examined bacterial species is able to split indole from tryptophan after adding the indole reagent, a change of colour of the bacterial colony can be observed. In simple terms, if the bacterial broth turns red or red-violet after having come in contact with the indole reagent, it is able to split indole from tryptophan and therefore the bacterial strain is indole-positive (e.g. *K. oxytoca*). If this effect fails to appear, the bacterium is indole-negative (e.g. *K. pneumoniae*). Although this method of differentiation is well-established in daily clinical routine, it remains a subjective method of identification. The examiner has to decide, based on his experience, if the change of colour took place or not. Subsequently, there may be uncertainty as to other objective ways of bacterial identification such as PCR – *polymerase chain reaction*. Therefore, regarding the recent findings of the connection between antibiotic-associated colitis and *K. oxytoca*, it is of importance to establish a very sensitive detection method of *K. oxytoca* in order to learn more about this newly described disease (AAHC).

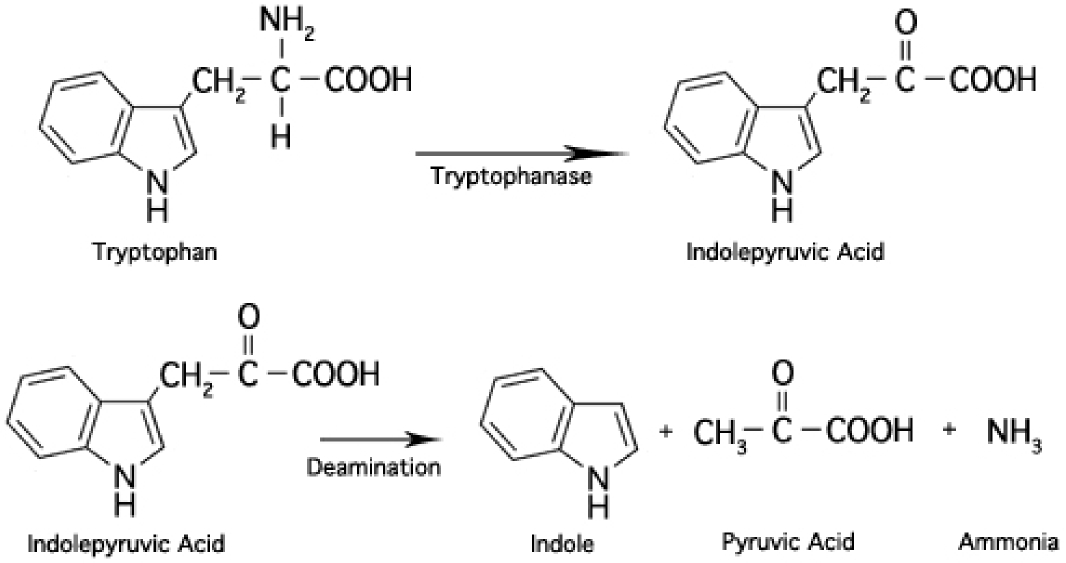
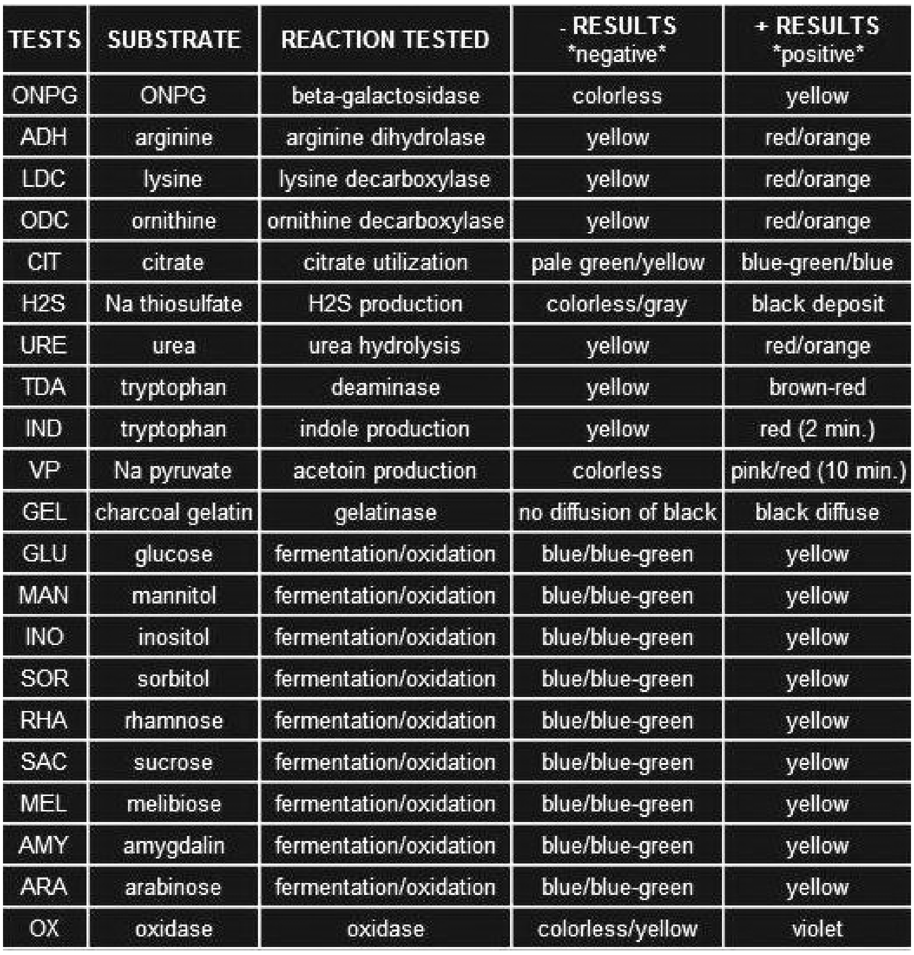


Figure 1: After reaction with the indole reagens, indole, pyruvic acid and ammonia (NH_3) are cleaved from tryptophan.

3 Aims

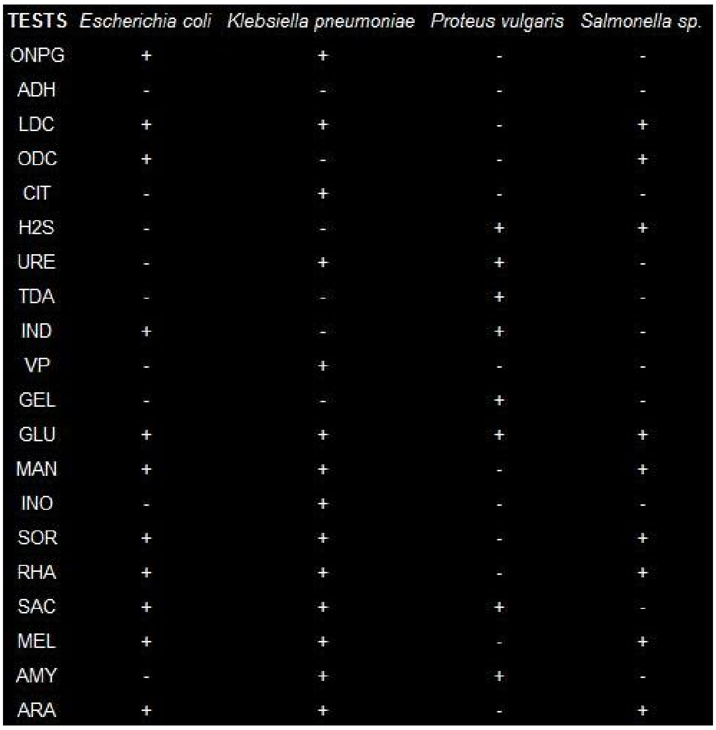
3.1 Conventional identification of bacterial strains

In conventional clinical routine, *enterobacteriaceae* are classified by using API 20E testing methods. API 20E stands for *analytical profile index* for ***e**nterobacteriaceae* and leads to a quick identification of certain *enterobacteriaceae*. API is based on 20 small testing tubes, with a predefined combination of substrates, all following specific physiological reactions (see figure 2) [20]. Reliable testing results can just be achieved with pure bacterial cultures. In order to compare and analyze these results in a reliable way, it is absolutely necessary to carry out the manufacturer´s instructions carefully, as there are API 20E tests offered by various companies.



TESTS	SUBSTRATE	REACTION TESTED	- RESULTS *negative*	+ RESULTS *positive*
ONPG	ONPG	beta-galactosidase	colorless	yellow
ADH	arginine	arginine dihydrolase	yellow	red/orange
LDC	lysine	lysine decarboxylase	yellow	red/orange
ODC	ornithine	ornithine decarboxylase	yellow	red/orange
CIT	citrate	citrate utilization	pale green/yellow	blue-green/blue
H2S	Na thiosulfate	H2S production	colorless/gray	black deposit
URE	urea	urea hydrolysis	yellow	red/orange
TDA	tryptophan	deaminase	yellow	brown-red
IND	tryptophan	indole production	yellow	red (2 min.)
VP	Na pyruvate	acetoin production	colorless	pink/red (10 min.)
GEL	charcoal gelatin	gelatinase	no diffusion of black	black diffuse
GLU	glucose	fermentation/oxidation	blue/blue-green	yellow
MAN	mannitol	fermentation/oxidation	blue/blue-green	yellow
INO	inositol	fermentation/oxidation	blue/blue-green	yellow
SOR	sorbitol	fermentation/oxidation	blue/blue-green	yellow
RHA	rhamnose	fermentation/oxidation	blue/blue-green	yellow
SAC	sucrose	fermentation/oxidation	blue/blue-green	yellow
MEL	melibiose	fermentation/oxidation	blue/blue-green	yellow
AMY	amygdalin	fermentation/oxidation	blue/blue-green	yellow
ARA	arabinose	fermentation/oxidation	blue/blue-green	yellow
OX	oxidase	oxidase	colorless/yellow	violet

Figure 2: 20 Substrates and reactions of the API 20E



TESTS	*Escherichia coli*	*Klebsiella pneumoniae*	*Proteus vulgaris*	*Salmonella sp.*
ONPG	+	+	-	-
ADH	-	-	-	-
LDC	+	+	-	+
ODC	+	-	-	+
CIT	-	+	-	-
H2S	-	-	+	+
URE	-	+	+	-
TDA	-	-	+	-
IND	+	-	+	-
VP	-	+	-	-
GEL	-	-	+	-
GLU	+	+	+	+
MAN	+	+	-	+
INO	-	+	-	-
SOR	+	+	-	+
RHA	+	+	-	+
SAC	+	+	+	-
MEL	+	+	-	+
AMY	-	+	+	-
ARA	+	+	-	+

Figure 3: Characteristic reactions of some *enterobacteriaceae* using API 20E

This type of verification procedure forms an integral part of microbiological testing in daily clinical work. Figure 3 shows characteristic reactions of some enterobacteriaceae (*E.coli, K. pneumoniae, Proteus vulgaris and Salmonella spp.*) [20].

Concerning the discrimination of *K. pneumoniae* and *K. oxytoca*, indole reaction is the main criterion, as mentioned in chapter 2.2.1. One of the main disadvantages of indole reaction and of API testing, although both methods work well, is that the results are strictly dependent on the examiner and they are therefore a subjective identification method. API 20E testing mainly depends on the experience and the skills of the examiner to achieve the right differentiation of *Klebsiella spp*. Beside this, API testing methods can not declare results for 100 percent. This testing method can just make an identification probable (see figure 5).

3.2 Routine identification of *Klebsiella spp.* with API 20 E

Klebsiella strains differ from other *enterobacteriaceae*, as they form shiny and gluey colonies (see figure 4). In order to test this possible *Klebsiella* strain shown in figure 4 on the left using API 20 E, it is necessary to take a single, well-isolated colony of the isolation plate and dilute it with 5ml NaCl 0,85%. Drops of this dilution are now pipetted onto the 20 substrates in the testing tubes followed by addition of reagents into some of the 20 substrates.

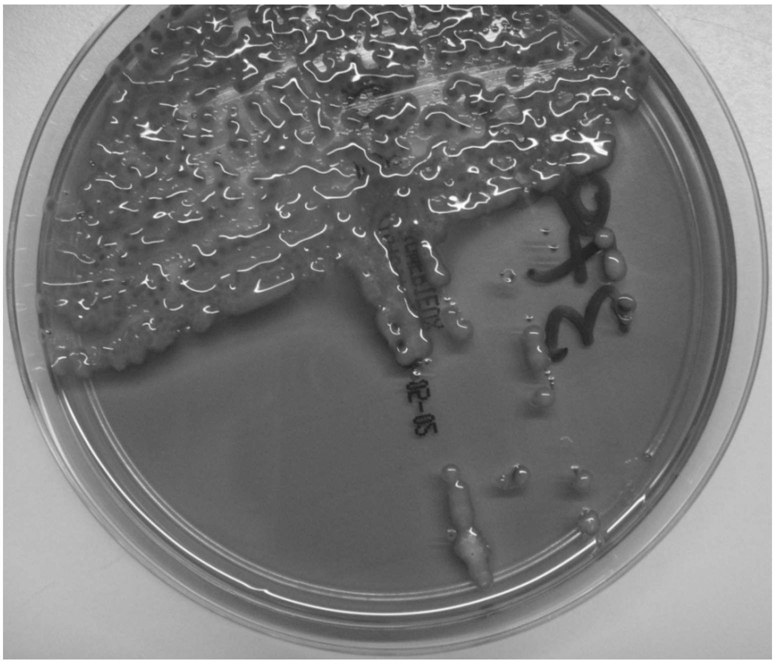
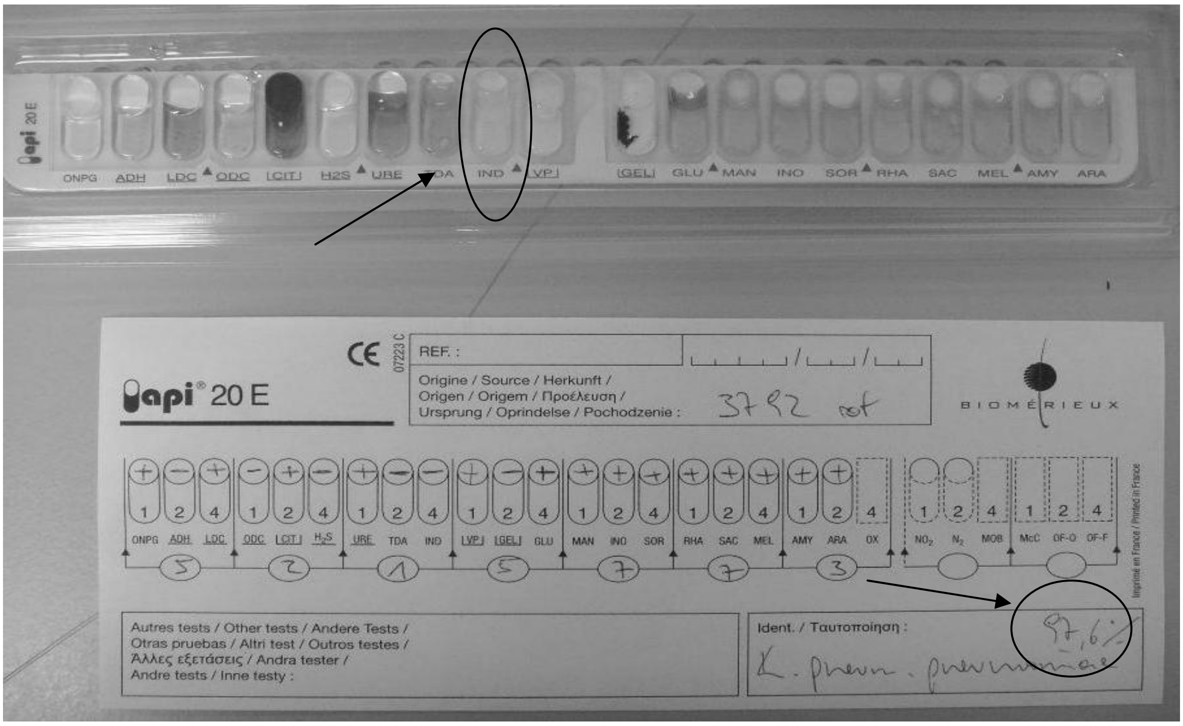


Figure 4: Typical *Klebsiella* colony. Shiny surface and gluey characteristics

After incubation at 36°C for 18-24 hours, the strip containing the 20 substrates is read. Whether the corresponding results are positive or negative, they are written in a given field on the protocol. These results refer to the colour reaction that took place after the bacterial dilution came in contact with the 20 substrates and the reagents that are required for of the API 20 E test (also see figure 5). After having added all the positive and negative results with a computer program, the results are displayed as probabilities. Identifying the bacterial strain of the colony in figure 4 with API 20 E test is shown in figure 5. The twenty testing substrates shown in figure 2, chapter 3.1, are in the upper part of the figure, and the corresponding API protocol with the results is shown underneath. According to this particular API 20 E test (see figure 5) for the bacterial colony shown in figure 4, the tested bacterial strain was identified as *K. pneumoniae pneumoniae* with a probability of 97.6 %. Test results are shown on the right bottom corner of the protocol of figure 5.



api® 20 E

REF. :

Origine / Source / Herkunft /
Origen / Origem / Προέλευση /
Ursprung / Oprindelse / Pochodzenie : 37 92 rot

BIOMÉRIEUX

ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	NO2	N2	MOB	McC	OF-O	OF-F
+	−	+	−	+	−	+	−	−	+	−	+	+	+	+	+	+	+	+	+							
1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
5			2			1			5			7			7			3								

Autres tests / Other tests / Andere Tests /
Otras pruebas / Altri test / Outros testes /
Άλλες εξετάσεις / Andra tester /
Andre tests / Inne testy :

Ident. / Ταυτοποίηση : 97,6%
K. pneum. pneumoniae

Imprimé en France / Printed in France

Figure 5: API 20 E substrates above and the protocol analyzing the different reaction below, indole reaction and identification probability both marked separately.

The main criterion that discriminates *K. pneumoniae* from *K. oxytoca* is the indole reaction. Figures 6 and 7 show this indole reaction of the API 20 E test, once being positive and once being negative, in comparison.

Looking at the key reaction – indole-reaction- in detail, it is depending on the examiner's experience to interpret the change of colour. Figures 6 and 7 demonstrate an obvious difference between a positive indole reaction (figure 6) and a negative indole reaction (figure 7) of two different bacterial strains. But there might be cases, where this apparent discrimination is missing and just a tendency toward a positive or negative reaction is derivable. This demonstrates that API 20 E remains a subjective testing method to a certain degree, whereas PCR is possibly a more objective testing technique in comparison.

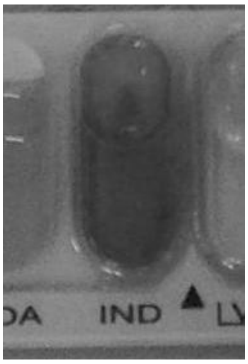


Figure 6: positive indole reaction in API 20 E

Figure 7: negative indole reaction in API 20 E

3.2.1 Alternative detection of *K. oxytoca* using PCR

As described in the previous chapters, API testing remains a subjective testing method. We therefore proposed that polymerase chain reaction (PCR) is a more objective and reproducible detection method for *K. oxytoca* compared to API. PCR is an objective and well-accepted identification method of bacteria and other pathogens [21]. This testing method is able to amplify a special fragment million times in order to provide objective evidence. Beside this, PCR can amplify and detect even smallest amounts of a pathogen, which also improves detection, if the quantity of the tested material is low. Considering these facts, we created a PCR-based identification method for *K. oxytoca*, in order to identify this bacterial strain in a possibly even more sensitive and specific way than API 20 E. The aim of the study was a comparison between conventional microbiological testing using API 20 E and PCR, being a new detection method for *K. oxytoca*. Another aim of the study was to prove or disprove the API results in order to increase the specificity and sensitivity of *K. oxytoca* identification. Maybe it is also possible to detect more strains of this facultative pathogen in hospitalised and ill people, as well as in the healthy population compared to the data known up to now.

The expected aim was to re-test *Klebsiella spp.* with polymerase chain reaction. This new identification method for *K. oxytoca* is the main topic of my diploma thesis. The PCR method was established for a PCR amplicon of 344bp that is typical for *Klebsiella oxytoca* (see chapter 4.3) [22].

The PCR amplicons of the tested *Klebsiella spp*. (n=143) were applied on gel electrophoresis and were subsequently photographed under ultraviolet light.

4 Material and Methods

4.1 Design of the study

This diploma thesis covers a comparative study between conventional API 20E testing of bacteria and PCR testing for the identification of *K. oxytoca*. PCR testing has been accomplished at the 'Institut für molekulare Biowissenschaften' at the Karl-Franzens-University Graz. The *Klebsiella* strains (n=143) were previously analyzed and identified with API 20E in the 'Mikrobiologisches Labor der Universitätsklinik für Innere Medizin' and the Institute of Hygiene, both departments of the Medical University of Graz for the most part. However, some bacterial strains were also obtained from different foreign laboratories.

4.2 Origin of the tested bacterial strains

Strain number MH 43-1 is a strain from a Japanese patient, number 311768/1 and 311768/2 as well as 70763/07 have their origins in Freiburg/Germany (Prof. Kist). The remaining bacteria were either isolated from the inductees of the Austrian Army or from in- and out-patients of the Department of Internal Medicine of the Medical University of Graz.

4.3 Polygalacturonase (*pehX*) gene

Although different approaches to identify the diversity of *Klebsiella spp.* have been described in medical literature [23, 24], it could not be clearly demonstrated until Kovtunovych et al. [22], which testing method is most specific for the differentiation of *Klebsiella spp*. Kovtunovych et al. demonstrated that specific discrimination of *K. oxytoca* to other *Klebsiella spp.* is performed by amplification of a polygalaturonase (*pehX*) gene. Polygalacturonase is an enzyme that cleaves a polygalacturonic chain of demethoxylated pectin. This is a capability which is unique for *K. oxytoca* amongst the *Klebsiella spp.* If a sequence coding for this specific enzyme is amplified with PCR, proper discrimination of *K. oxytoca* to the other *Klebsiella* strains can be obtained. Kovtunovych et al. demonstrated that an amplicon of *pehX* with 344 bp (base pairs) was obtained in all *K. oxytoca* strains and in none of the other tested *Klebsiella spp.*, which were *K. pneumoniae, K. terrigena, K. planticola* and *K. ornithinolytica.* Derived from the above cited publication [22], the aim was to establish a PCR based on the detection of this described *pehX* gene.

4.3.1 DNA preparations

The aim was to reproduce the PCR according to the PCR amplification details from this particular publication mentioned above.

Prior to the PCR, it is necessary to isolate the DNA. In order to achieve this, the different *Klebsiella spp.* were incubated overnight at 37°C on blood agar for the strains to proliferate and to gain adequate material for the DNA preparation.

In order to obtain the template DNA, a loopful of bacteria was heated in distilled sterile water for five minutes, followed by centrifugation at 13000 rpm for another five minutes.

Both suggested primers PEH-C and PEH-D were used for the PCR amplification, which included an initial denaturation step of 2 min at 95°C, followed by 30 amplification cycles (94°C, 20 s; 59°C, 20 s; 72°C, 30 s) and a final elongation step of 10 min at 72°C. PCR products were finally analyzed by electrophoresis in 1,5% agarose gels, stained with ethidium bromide, visualized and photographed under ultraviolet light.

4.3.2 DNA components

In order to run the PCR, it is necessary to prepare a so-called *Master Mix* at first. This *Master Mix* is composed of the following components which are all required for the amplification process during the PCR:

Table 2: Master Mix ingredients

12,5 µl	2x PCR buffer
1,0 µl	*PEH-C* (primer)
1,0 µl	*PEH-D* (primer)
0,5 µl	Taq-polymerase (*fire pol*)
7,5 µl	H_2O
22,5 µl	MM (mastermix)

The 2xPCR buffer provides an adequate chemical environment for optimum activity and stability of the DNA polymerase. The two Primer *PEH-C* and *PEH-D*, which are complementary to the five prime and the three prime ends of the DNA region, are needed for the annealing process. After melting with the single-stranded DNA template, they enable the Taq-polymerase to bind to the primer-template hybrid in order to start the DNA synthesis. Distilled water is also required.

4.3.3 DNA Amplification

Unfortunately, under the conditions described in by Kovtunovych et al., it was not possible to establish a detection of *K. oxytoca* strains.

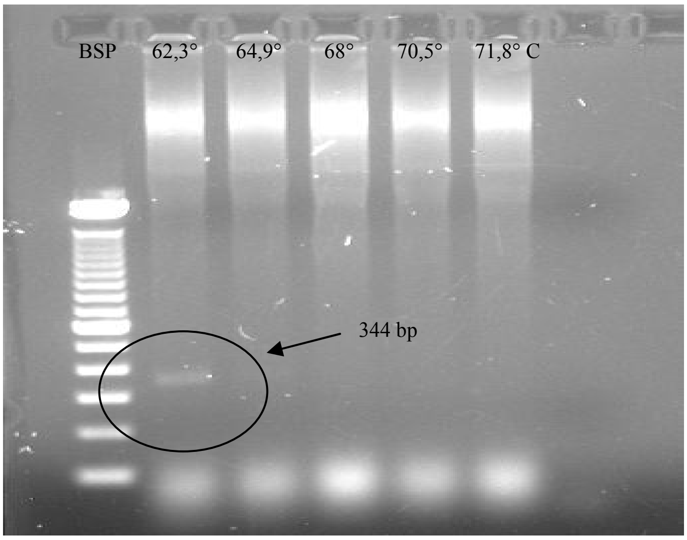


Figure 8: Annealing temperature ranging from 62,3° C to 71,8° C
First visible 344bp band is marked in the figure
BSP = Base pair standard

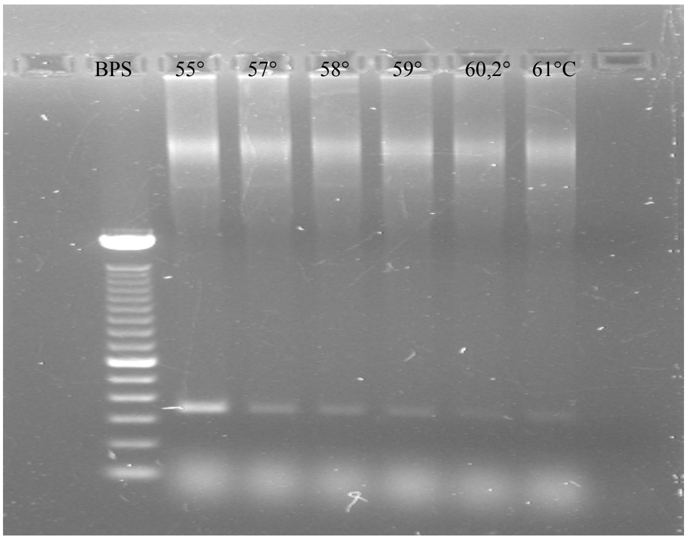


Figure 9: Annealing temperature ranging from 55° C to 61° C
BSP = Base pair standard

Therefore, it was necessary to adapt the ingredients to the settings of our laboratory. After an analysis of the unsuccessful first results, the possible explanation for the failed detection was that the annealing temperature had not been at its optimal level. To find out the best temperature for this step during the DNA amplification, we tried out different annealing temperatures. The annealing temperature ranged at first from 62,3°C to 71,8° (see figure 8). Beside this, we also changed the elongation time from 30 seconds to 1 minute and shortened the final elongation step from the suggested 10 minutes to 2 minutes. Having changed the settings as described above, a slight and modest 344-bp band appeared at 63,2°C. It can be concluded from these results that the

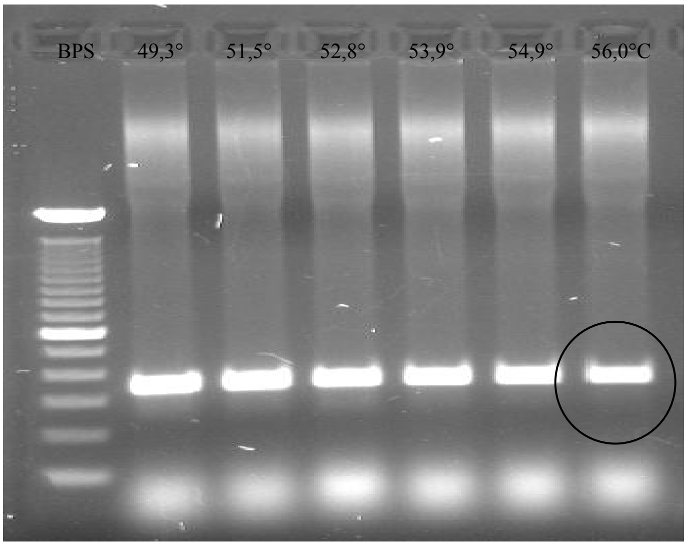


Figure 10: Annealing temperature ranging from 49,3°C to 56°C

BSP = Base pair standard

A 344bp band encircled is at 56,0° C, which was the final annealing temperature we for the experiments

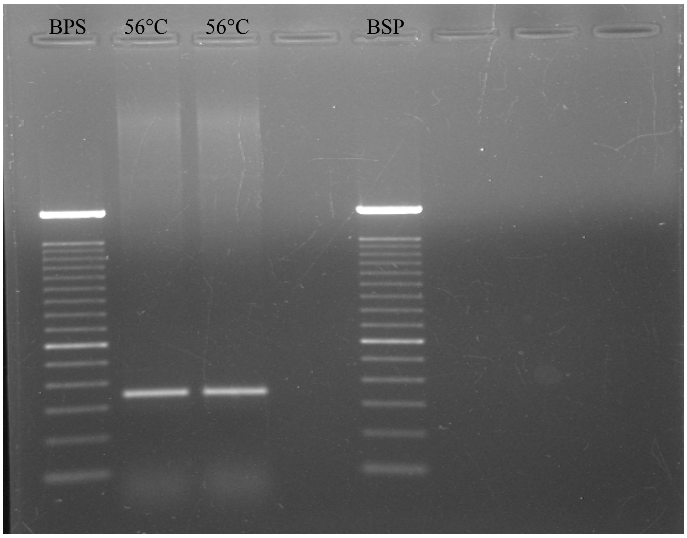


Figure 11: Final annealing temperature of 56°C

Applied volume 3µl per slot

BSP = Base pair standard

perfect temperature might even be below 62,3°C. Due to this, we performed another gradient, this time ranging from 55°C to 61°C (see figure 9). As compared to the first gradient with higher temperatures, there had now been 344-bp bands at every temperature. Interestingly, the intensity of the bands increased towards the lower temperatures, which means that more DNA is annealed and consequently amplified. Concluding from this observation, it was necessary to run another temperature gradient, this time ranging from 49,3° to 56°C (see figure 10). Although there had been bands at the aspired 344-bp, there is a possible problem arising with these low temperatures: If the temperature is lowered too much during the annealing process, one major problem that might happen can be that unspecific amplification might occur [25]. This would mean that false positive results can occur easier and the *K. oxytoca*-specific 344bp bands may not be valid for the specific detection of the *pehX* gene any more. Depending on the amount of the template DNA, the annealing temperature can be lowered, if the amount of template DNA is decreased as well [26]. If this coherence is taken into account, no unspecific amplifications occurs. Concerning the adjustments of our PCR, in order to avoid this possible falsification, an annealing temperature of 56°C was finally agreed on as compared to 59°C in the publication by Kovtunovych (see figure 11).

5 Results

5.1 Tested bacterial strains

Having managed to attach the PCR components to the conditions of the laboratory as described above, I started to test the *Klebsiella* strains. First and foremost, I was aiming to retest the bacterial strains that had already been identified as *K. oxytoca* by API 20E. Secondly, the intention was also to retest different *Klebsiella* strains that were identified as non-*oxytoca Klebsiella spp.* in the API test (mainly *K. pneumoniae* but also two *K. terrigena* strains) to maybe detect even more *K. oxytoca* species. *Klebsiella* strains that were identified with API testing had their origin in different organ systems. These locations are listed in table 3 and table 4.

5.1.1 Origin of the *Klebsiella* strains

Most of the *K. pneumoniae* strains were extracted from stool samples (n=45). Urinary tract (n=5) followed by the respiratory tract (n=3) were two more separate groups to be mentioned. Other locations (n=11) include strains from CVC infections, decubital ulcers and unknown origin. Two *K. terrigena* strains (06/10 T and 07/01 T) are both derived from stool samples from antibiotic-associated colitis (see table 3). All these results refer to API 20 E results.

Table 3: Origin of *K. pneumoniae* and *K. terrigena* strains

Localisation of *K. pneumoniae* strains according to API results (n= 64)	
Stool	45
Urinary tract	5
Respiratory tract	3
Other localisations	11
Localistation of *K. terrigena* strains (n=2)	
Stool/antibiotic-associated colitis	2
K. pneumoniae* and *K. terrigena all identified as non-*oxytoca* by API 20 E	**n=66**

Altogether, 66 strains were identified as different *Klebsiella spp.* than *K. oxytoca* (*K. pneumoniae* [n=64] and *K. terrigena* [n=2]) out of 143 *Klebsiella* strains tested. Also see chapter 5.3, *table 6*.

Compared to the 66 *K. pneumoniae* and *K. terrigena* strains described above, 77 *Klebsiella* strains were identified as *K. oxytoca* with API (see table 4).

Most of these *K. oxytoca* strains were also extracted from stool samples (n=46) whereas 11 out of these 46 *Klebsiella* probes were derived from patients suffering antibiotic-associated hemorrhagic colitis (AAHC). The residual 35 stool samples were extracted from patients suffering antibiotic associated diarrhea, ulcerative colitis, Crohn's disease and other forms of diarrhea. Some of these *Klebsiella* strains that were also extracted from stool samples, were derived from healthy carriers. Remaining localisations include respiratory tract (n=9), cutaneous smear (n=7), urine (n=2), wound smear (n=2), central venous catheter (n=2), blood (n=2) and others (n=7). Origins of the strains are listed in table 3 and table 4. All these results refer to API 20 E test results.

Table 4 Origin of *K. oxytoca* strains

Origin of *K. oxytoca* strains According to API results (n=77)	
Stool (without AAHC)	35
Stool/AAHC	11
Respiratory tract	9
Cutaneous smear	7
Urine	2
Wound	2
Blood	2
Central venous catheter	2
Others	7
***K. oxytoca* positive after API**	**n=77**

5.2 Results of the tested *Klebsiella spp.*

Table 5 on pages 19 to 21, comprises all the characteristics of the 143 tested *Klebsiella spp*. Laboratory numbers are listed on the left. Strain identification including the strain numbers are listed aside. PCR results refer to the detection of the *K. oxytoca*-specific *pehX gene* whether the specific 344bp bands showed up in gel electrophoresis or not. If the detection was positive there is a mark (x) in the table cell belonging to the column named ‘PCR positive for *pehX* gene’. If there was no band at the expected 344bp compared to the 100bp DNA ladder in gel electrophoresis, detection was negative and these *Klebsiella* strain had not been identified as *K. oxytoca* by PCR. These results are inserted (marked with x) in the column titled ‘no bands for *pehX* gene’. Because of the fact that this PCR test, which had been established for this study, is a specific detection method for *K. oxytoca*, it can just detect *K. oxytoca* only and can not furnish the proof of other *Klebsiella spp.* Therefore, further categorization of the remaining bacterial strains that had been *K. oxytoca*-negative in PCR, can not be achieved. API 20 E results and the results of the indole reaction are listed next to the PCR results. Clinical diagnoses and the localisations, where the *Klebsiella strains* are derived from, are also listed in table 5. Abbreviations belonging to table 5 are specified in glossary and abbreviations on page vii.

5.2.1 Characteristics of the *Klebsiella spp.*

Table 5: Characteristics of the tested *Klebsiella* strains with the PCR and API 20E results, clinical diagnosis, localisation and origin and laboratory number.

Laboratory Number	**Strain**	**PCR positive for *pehX* gene**	**No Bands for *pehX* gene**	**API 20E**	**Indole**	**Clinical diagnosis**	**Localisation**
1	KLOX 04 / 1	x		K.o.	pos	bacteriemia	CVC
2	KLOX 04 / 2	x		K.o.	pos	PAD	wound
3	KLOX 04 / 3	x		K.o.	pos	unknown	stool
4	KLOX 04 / 4	x		K.o.		enteritis	stool
5	KLOX 04 / 5	x		K.o.	pos	unknown	wound
6	KLOX 04 / 6	x		K.o.	pos	colitis	stool
7	KLOX 04 / 7	x		K.o.	pos	otitis	ear
8	KLOX 04 / 8	x		K.o.	pos	shunt	stool
9	KLOX 04 / 9	x		K.o.	pos	rhinoconjunctivitis	eye
10	KLOX 04 / 10	x		K.o.	pos	pneumonia	sputum
11	KLOX 04 / 11		x	K.o.	pos	CABG surgery	tracheal secretion
12	KLOX 04 / 12	x		K.o.	pos	enteritis	stool
13	KLOX 04 / 13	x		K.o.		unknown	stool
14	KLOX 04 / 14	x		K.o.		unknown	stool
15	KLOX 04 / 15	x		K.o.		unknown	stool
16	KLOX 04 / 16	x		K.o.		endocarditis	stool
17	KLOX 04 / 17		x	K.o.	pos	Crohn’s disease	stool
18	KLOX 04 / 18	x		K.o.	pos	pneumonia/CCU	BAL
19	KLOX 04 / 19	x		K.o.	Pos	diarrhea	stool
20	KLOX 04 / 20	x		K.o.	pos	recurrent diarrhea	stool
21	KLOX 04 / 21		x	K.o.	pos	antibiotic-associated colitis	stool
22	ACX-1	x		K. pn.	neg	antibiotic-associated colitis	stool
23	ACX-2	x		K.o.	pos	antibiotic-assoc. colitis /UC	stool
24	ACX-3	x		K. pn.	neg	Antibiotic-associated Colitis	stool
25	AHC 1	x		K.o.	pos	AAHC	stool
26	AHC 2	x		K.o.	pos	AAHC	stool
27	AHC 4	x		K.o.	pos	AAHC	stool
28	AHC 6	x		K.o.	pos	AAHC	stool
29	AHC 6.2	x		K.o.	pos	AAHC/follow up	stool
30	AHC 7	x		K.o.	pos	AAHC	stool
31	AHC 8	x		K.o.	pos	AAHC	stool
32	AHC 8.2	x		K.o.	pos	AAHC	stool
33	MH 43 – 1	x		K.o.	pos	AAHC	stool
34	DSM 4798	x		K.o.	pos	laboratory strain	laboratory strain
35	DSM 5175	x		K.o.	pos	laboratory strain	pharyngeal tonsils

Table 5 continued

Laboratory Number	**Strain**	**PCR positive For *pehX* gene**	**No bands for *pehX* gene**	**API 20E**	**Indole**	**Clinical diagnosis**	**Localisation**
36	AHC 9	x		K.o.	pos	laboratory strain	stool
37	73	x		K.o.		healthy carrier	stool
38	141, F. L.		x	K.o.	pos.	healthy carrier	stool
39	149	x		K.o.		healthy carrier	stool
40	235	x		K.o.		healthy carrier	stool
41	328	x		K.o.		healthy carrier	stool
42	281, M. S.		x	K.o.	neg.	healthy carrier	stool
43	M. E.	x		K.o.		sepsis-COPD	blood
44	3		x	K. pn.		healthy carrier	stool
45	8		x	K. pn.		healthy carrier	stool
46	14		x	K. pn.		healthy carrier	stool
47	28		x	K. pn.		healthy carrier	stool
48	40		x	K. pn.		healthy carrier	stool
49	47		x	K. pn.		healthy carrier	stool
50	83		x	K. pn.		healthy carrier	stool
51	103		x	K. pn.		healthy carrier	stool
52	108		x	K. pn.		healthy carrier	stool
53	265		x	K. pn.		healthy carrier	stool
54	277		x	K. pn.		healthy carrier	stool
55	325		x	K. pn.		healthy carrier	stool
56	311768/1		x	K. pn.		unknown	unknown
57	311768/2	x		K.o.		unknown	unknown
58	70763/07	x		K.o.		unknown	unknown
59	70763/07	x		K.o.		unknown	unknown
60	A. K.		x	K. pn.		unknown	unknown
61	03/06 O	x		K.o.	pos	urinary tract infection Crohn’s disease	urine
62	03/11 O		x	K.o.	pos	sepsis-cholangitis	blood
63	03/12 O	x		K.o.	pos	VAP	tracheal secretion
64	03/20 O	x		K.o.	pos	thigh	smear
65	03/23 O		x	K.o.	pos	VAP	tracheal secretion
66	03/27 O	x		K.o.	pos	COPD	sputum
67	03/28 O		x	K.o.	pos	CVC- bacterial culture	CVC
68	04/26 O	x		K.o.	pos	pneumonia	sputum
69	04/35 O	x		K.o.	pos	VAP	sputum
70	04/38 O	x		K.o.	pos	gangrene dig. III sin., oblit. thrombang.	smear
71	05/05 O	x		K.o.		antibiotic-associated colitis	stool
72	06/07 O		x	K.o.	pos	antibiotic-assoc. colitis	stool
73	06/09 O	x		K.o.		AAHC	stool
74	06/10 T	x		K. terrigena	neg	antibiotic-assoc. colitis/UC	stool
75	06/11 O		x	K.o.	pos	antibiotic-assoc. diarrhea	stool
76	06/12 P		x	K. pn.		antibiotic-assoc. colitis/UC	stool

Table 5 continued

Laboratory Number	**Strain**	**PCR positive For *pehX* gene**	**No Bands for *pehX* gene**	**API 20E**	**Indole**	**Clinical diagnosis**	**Localisation**
77	06/13 O		x	K.o.	pos	colonic hemorrhagies	stool
78	06/14 P		x	K. pn.		colonic hemorrhages	stool
79	06/15 P		x	K. pn.		rightsided antibiotic-assoc. colitis	stool
80	06/16 P	x		K. pn.	pos	segmental antibiotic-assoc. colitis	stool
81	06/17 P		x	K. pn.	pos	relapsed AAHC	stool
82	06/19 O	x		K.o.		hämorrhagic colitis	stool
83	06/21 P	x		K.pn	pos	follow up UC	stool
84	06/23 O		x	K.o.	pos	pseudomembranous colitis	stool
85	06/25 O	x		K.o.		purulent bronchitis/ nosocomial	sputum
86	06/28 O	x		K.o.		urinary tract infection	urine
87	06/31-O	x		K.o.		unknown	smear
88	06/32-O	x		K.o.		unknown	smear
89	06/33-O		x	K.o.	pos	unknown	smear
90	06/34 O	x		K.o.		wound, PAD IV	smear
91	06/35-O	x		K.o.		unknown	smear
92	07/01 T		x	K. terrigena		segmental antibioticacolitis	stool
93	07/02 O	x		K.o.		AAHC	stool
94	07/04 O	x		K.o.		pseudomembraneous colitis	stool
95	07/05 P		x	K. pn.		colitis	stool
96	07/06 O	x		K.o.		follow up colitis	stool
97	07/07 O	x		K.o.		follow up colitis	stool
98	07/08 O		x	K.o.	pos	AAHC	stool
99	07/09 P		x	K. pn.		follow up, healthy	stool
100	07/11 O	x		K.o.		Crohn's disease	stool
101	E.B.		x	K. pn.		antibiotic assoc. diarrhoe	stool
102	F. Ko.		x	K. pn.		pancreatitis	stool
103	F. Kö. I		x	K. pn.		urinary tract infections	urine
104	F. Kö. II		x	K. pn.		urinary tract infections	urine
105	F. Kö. III		x	K. pn.		urinary tract infections	urine
106	K. I.		x	K. pn.		cesariean delivery	unknown
107	G. H.		x	K. pn.		CVC	CVC
108	C. G.		x	K. pn.		diarrhea	stool
109	M. R.		x	K. pn.		urinary tract infections	urine
110	E. H.		x	K. pn.		decubital ulcer	decubital ulcer
111	A. G.		x	K. pn.		SBP prophylaxis	unknown
112	H. W.		x	K. pn.		pneumonia	sputum
113	F. P.		x	K. pn.		pneumonia	sputum
114	K. S.		x	K. pn.		burstitis	unknown
115	A. Pi.		x	K. pn.		exacerbated COPD	sputum
116	A. Pr.		x	K. pn.		abdominal pain	unknown
117	H. L.		x	K. pn.		urinary tract infections	urine

Table 5 continued

Laboratory Number	**Strain**	**PCR positive For *pehX* gene**	**No bands for *pehX* gene**	**API 20E**	**Indole**	**Clinical diagnosis**	**Localisation**
118	H. P.		x	K. pn.		infection, unknown origin	unknown
119	R. H.	x		K.o.		healthy carrier	stool
120	T. R.		x	K. pn.		vertigo	unknown
121	A. G.	x		K.o.		gastroenteritis	stool
122	B. D.		x	K. pn.		gastroenteritis	stool
123	S. H.		x	K. pn.		gastroenteritis	stool
124	S.S.	x		K.o.		gastroenteritis	stool
125	A. R.		x	K. pn.		jaw surgery	unknown
126	E. A.		x	K. pn.		healthy carrier	stool
127	G. S.		x	K. pn.		healthy carrier	stool
128	G. H.		x	K. pn.		healthy carrier	stool
129	A. B.		x	K. pn.		healthy carrier	stool
130	S. S.		x	K. pn.		healthy carrier	stool
131	Nr. 3		x	K. pn.		healthy carrier	stool
132	C. T.		x	K. pn.		healthy carrier	stool
133	B. K.		x	K. pn.		healthy carrier	stool
134	I. K.		x	K. pn.		healthy carrier	stool
135	R.		x	K. pn.		healthy carrier	stool
136	T.		x	K. pn.		healthy carrier	stool
137	S. L. I		x	K. pn.		healthy carrier	stool
138	S. L. II		x	K. pn.		healthy carrier	stool
139	K 35		x	K. pn.		healthy carrier	stool
140	K 36		x	K. pn.		healthy carrier	stool
141	K 37		x	K. pn.		healthy carrier	stool
142	K 38		x	K. pn.		healthy carrier	stool
143	K 39		x	K. pn.		healthy carrier	stool

Laboratory numbers 58 and 59 are the same strains (strain number 70763/07), but differently cultured.

░ = PCR verified *K. oxytoca*, API identification *K. pneumoniae* or *K. terrigena*

▓ = According to API 20 E identification *K. oxytoca*, no *K.oxytoca*-specific bands in PCR though, therefore other *Klebsiella spp* or *enterobacteriaceae.*

Klebsiella strains highlighted in light grey boxes (n=5) are *K. oxytoca* strains that were newly identified according to PCR test results and gel electrophoresis. These strains had previously been identified as K. *pneumoniae* (n=4) and *K. terrigena* (n=1). Results are separately described in chapter 5.3.1. Boxes that are highlighted in dark grey (n=14) could not be identified as *K. oxytoca* strains with PCR. According to API results these fourteen strains were identified as *K. oxytoca*. The results of the remaining tested strains were concordant between PCR and API 20 E.

Healthy carriers were mainly inductees from the Austrian army and volunteers participating in previous studies [13]. Their names are abbreviated, intitials are listed in table 5. Supplementary indole testing was also done in many cases. For the interpretation of our comparison between PCR and API, indole testing also plays an important role because this additional testing method may substantiate one testing method more than the other. In detail, indole testing is especially of interest concerning the 19 strains with discordant test results. Although indole testing is also integrated in API 20 E (see chapter 3.1.1), it is the key substrate in differentiating between *K. pneumoniae* and *K. oxytoca* as mentioned before. Performing the test alone, without other substrates influencing the final result of the testing method, as in API, a better discrimination between *K. pneumoniae* and *K. oxytoca* may be achieved. Nineteen strains were discordantly identified between API and PCR. Additional indole testing was performed on these *Klebsiella* strains. The results of these 19 indole reactions are described in chapter 5.4.1 and chapter 5.4.2.

5.3 PCR results

First of all, it needs to be stressed that the bacteria that were identified via PCR have been pre-selected by other testing methods. The PCR results are guided by the presumption that *Klebsiella spp*. had already been sorted out from other *enterobacteriacae*. For this reason, the results of this study are not valid for any detection at random of *enterobacteria*, just for *K. oxytoca.* This is also the reason why the results refer to *K. oxytoca* only and if its detection was positive or negative. The results of this study do not consider other *Klebsiella spp.* such as *K. pneumoniae, K. terrigena* and the test results can therefore not detect these aforementioned *Klebsiella* strains.

All together I have tested 143 different bacterial strains with PCR. Laboratory number 58 and 59 are the same *K. oxytoca* strain, but they were cultured at different occasions. In gel electrophoresis there were 344bp (base pair) bands detectable from 68 strains, which stands for a positive result as described in the previous chapters. There were no bands detectable from the other 75 strains tested which in turn stands for a negative result.

5.4 Discrepant results between API 20E and PCR

The first incongruity that attracts attention is that there were more positive *K. oxytoca* strains detected with API 20E than with PCR (n=77 with API as compared to n=68 with PCR, see tables 4 and 5 below). PCR could only identify 43% of all tested *Klebsiella* strains as *K. oxytoca* (68 out of 143), whereas API results detected 54% (77 out of 143) bacterial strains as *K. oxytoca*.

Table 6: PCR results

PCR	n = 143
PehX gene *(K. oxytoca)* positive	68
PehX gene *(K. oxytoca)* negative	75

Table 7: API 20E results

API 20E	n = 143
K. oxytoca positive	77
K. oxytoca negative (*K. pneumoniae n= 64, K. terrigena n=2*)	66

Looking at the results in detail, in comparison to API testing, five newly identified *K. oxytoca* strains were discovered with PCR, whereas 14 strains that were originally identified as *K. oxytoca* by API 20E-testing could not be verified and detected by PCR testing. Altogether, the two different types of tests divers in 19 out of 143 strains, which equals around 13% of all the tested *Klebsiella* strains. This would mean that within eight tested bacterial strains there is at least one differently identified strain comparing API 20E and PCR.

5.4.1 Newly identified *K. oxytoca* strains with PCR

Five *Klebsiella* strains were newly identified as *K. oxytoca* according to PCR results. These five strains were incorrectly identified as different *Klebsiella spp.* by API 20 E. This applies to laboratory number 22 (strain ACX-1), laboratory number 24 (strain ACX-3), laboratory number 80 (strain 06/16 P) and laboratory number 83 (strain 06/21 P). All these four strains have been formerly detected as *K. pneumoniae*. The fifth strain that was not detected with API 20 E was laboratory number 74; strain 06/10 T, which was originally identified as *K. terrigena*. With this conventional routine test, 5 strains of *K. oxytoca* would have been missing (see table 8).

Table 8: *Klebsiella oxytoca* strains that were not detected with API 20E testing methods and that were newly identified with PCR

Laboratory Number	**Strain Nr.**	**PCR positive for *K. oxytoca***	**No Bands for pehX gene**	**API 20E**	**Indole**	**Clinical diagnosis**	**Localisation**
22	ACX-1	x		K. pn.	neg	Antibiotic-associated Colitis	stool
24	ACX-3	x		K. pn.	neg	Antibiotic-associated Colitis	stool
74	06/10 T	x		K. terrigena	neg	Antibiotic-assoc. Colitis/UC	stool
80	06/16 P	x		K. pn.	pos	segmental antibiotic-assoc. colitis	stool
83	06/21 P	x		K.pn	pos	follow up UC	stool

Regarding the five strains that turned out to be *K. oxytoca* after PCR (listed in table 8 above), indole reaction that was performed additionally was non-uniform. It was positive in two cases (strain number 06/16 P and 06/21 P, both *K. pneumoniae* according to API results) but negative in three strains (strain number ACX-1, ACX-3 both *K. pneumoniae* and strain number 06/10 T, formerly *K. terrigena* by API 20E). A positive indole reaction stands for *K. oxytoca*. As compared to these five recently detected *K. oxytoca* strains, there have been 14 *K. oxytoca* strains that could not be detected with PCR.

5.4.2 Fourteen *K. oxytoca* strains that could not be identified with PCR

On the one hand, five strains were newly identified as *K. oxytoca*. On the other hand, 14 *Klebsiella* strains that had previously been detected as *K. oxytoca* with API 20 E, were not verified with PCR. No *K. oxytoca*-specific 344bp bands in gel electrophoresis showed up for these 14 strains. Looking at the clinical diagnoses and the localisations of these 14 discrepant *Klebsiella* strains in detail, most of these strains were extracted from patients suffering intestinal diseases. Two strains were derived from healthy carriers.

Table 9: According to API 20E these strains are identified as *K. oxytoca*. These strains could not be identified as *K. oxytoca* with PCR.

Laboratory Number	**Strain No.**	**PCR positive For *K. oxytoca***	**No Bands**	**API 20E**	**Indole**	**Clinical diagnosis**	**Localisation**
11	KLOX 04 / 11		x	KO	pos	CABG surgery	tracheal secretion
17	KLOX 04 / 17		x	KO	pos	Crohn's disease	stool
21	KLOX 04 / 21		x	KO	pos	recurrent diarrhea	stool
38	141, L. F.		x	KO	pos	healthy carrier	stool
42	281, S. M.		x	KO	neg	healthy carrier	stool
62	03/11 O		x	KO	pos	sepsis-cholangitis	blood
65	03/23 O		x	KO	pos	VAP	tracheal secretion
67	03/28 O		x	KO	pos	CVC	CVC
72	06/07 O		x	KO	pos	antibiotic-assoc. colitis	stool
75	06/11 O		x	KO	pos	antibiotic-assoc. diarrhea	stool
77	06/13 O		x	KO	pos	colonic hemorrhagies	stool
84	06/23 O		x	KO	pos	pseudomembranous colitis	stool
89	06/33 O		x	KO	pos	unknown	smear
98	07/08 O, A. R.		x	KO	pos	AAHC	stool

Concerning strain number 07/08 O, A. R., which was extracted from stool from a patient diagnosed with AAHC, PCR was performed twice and the *K. oxytoca*-negative result was verified. API 20 E was also performed twice and it was both times positive for *K. oxytoca.* Examining the supplementary indole reaction in detail, indole testing was positive 13 times out of these 14 *Klebsiella* strains. These indole results are all discordant with PCR results, because a positive indole reaction stands for *K. oxytoca*. Just in one case, the result of indole reaction was congruent with the PCR result. This is strain number 281 (S.M.), isolated from stool sample derived from a healthy carrier. Summarizing indole reaction and API test results concerning these 14 cases listed above in table 9, both test results point towards *K. oxytoca*, whereas PCR was not able to detect *K. oxytoca*-specific 344bp bands for these 14 bacterial strains. Summing up the 5 newly identified *Klebsiella* strains (chapter 5.4.1) and the 14 other discrepant results between API and PCR described above, indole reaction confirmed API 20 E results in 16 of the 19 cases, which equals 84%. PCR and indole reaction was only concordant in the remaining 3 out of 19 cases, which equals 16%.

5.4.3 Origin of the strains from the discrepant results

Examining the clinical diagnoses of the patients, wherefrom the five newly identified *Klebsiella spp.* were derived (table 8), it is noticeable that all these five strains were isolated from patients suffering of infections or inflammations of the large intestine. Four of the five strains were extracted from patients diagnosed with antibiotic-associated colitis and one strain was isolated from a patient with ulcerative colitis. Compared to the intestinal localisations of these five newly identified *K. oxytoca* strains according to PCR results, the 14 *Klebsiella* strains that could not be proven as *K. oxytoca* by polymerase chain reaction have their origin from various locations. In most cases, the clinical diagnosis was antibiotic-associated colitis. However some isolates were derived from non-intestinal diagnoses as endocarditis or sepsis. Two isolates were obtained from healthy carriers. The localisations, wherefrom the strains were extracted, are also non-uniform as compared to the five new *K. oxytoca* strains described. Although being extracted from stool samples in most cases, these strains were also derived from blood, tracheal secretion and infection of the central venous catheter (see table 9).

Concluding from the PCR results concerning the five newly identified *K. oxytoca* strains and regarding the fact that they were derived from patients diagnosed with antibiotic-associated colitis, these cases of colonic infections would have been misdiagnosed and a possible causality between non-hemorrhagic antibiotic associated colitis caused by *K. oxytoca* would have been overseen.

5.4.4 Gel electrophoresis of the incongruent results

Although the PCR results are already mentioned in tables 5 - 10, this chapter demonstrates the described results with the corresponding pictures of gel electrophoresis. The PCR results on gel electrophoresis are shown in figures 12 - 14. Positive results appear as white bands at 344-bp, whereas missing bands at 344-bp stand for a negative result. The bands are compared to the two 100-bp DNA ladders each delimiting the applied probes on the left and on the right. The left 100-bp DNA ladder is applied into the first slot of the agarose gel; the right DNA ladder is pipetted in the slot following the blank on the right side. In figure 10 there is only one 100-bp DNA ladder on the left side, because there were just 11 probes pipetted on the gel and one DNA ladder is sufficient to analyze the results. The 'blank' represents the negative control and indicates a contamination in case there is a band at the same position as the remaining examined probes (the so called 'blank' is named '*neg.*' on the gel electrophoresis figures). The blank should always be negative in order to obtain proper results. Each band on the ladder stands for 100-bp. The amplified sequence of the *K. oxytoca*-specific *pehX* gene at 344-bp can therefore be found between the third and the fourth 100-bp band of the DNA ladder.

Figure 12 shows that there are characteristic 344bp bands at all strains except for 'Ko' (this is laboratory number 60), strain numbers 11, 17, 21 and the negative control. Number 11, 17, and 21 were all identified as *K. oxytoca* according to the results of API 20E testing. Figure 12 also detects two more *K. oxytoca* strains (as mentioned in chapter 5.1.1, table 6 and shown in chapter 4.4, table 3), which are number 22 and 24 that were previously identified as *K. pneumoniae* by API 20 E testing.

All discordant results are marked with columns. Striated caskets emphasize *Klebsiella* strains that could not be detected with PCR. Newly identified *K. oxytoca* strains are marked with caskets without filling.

▨ = striated caskets bring out *Klebsiella* strains that were formerly identified as *K. oxytoca* according to API 20 E but could not be verified with PCR testing

☐ = caskets without filling point out *Klebsiella* strains that were newly identified as *K. oxytoca* with PCR

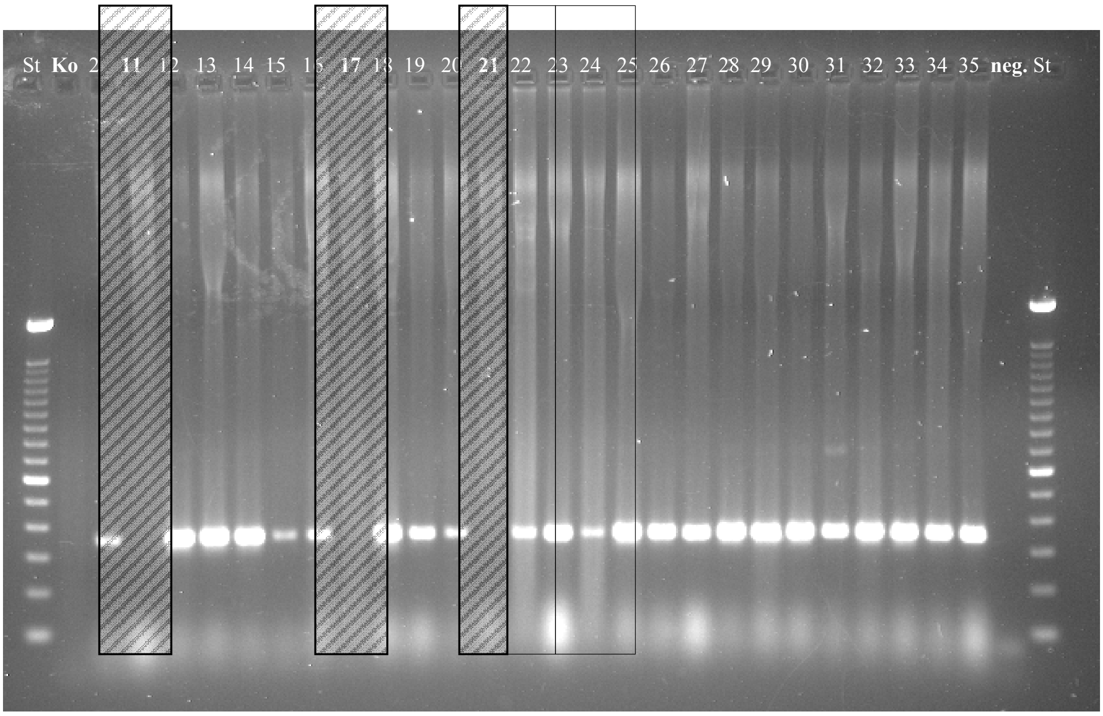


Figure 12: Missing 344bp bands for strain Ko, probe number 11, 17, 21 and the negative control

Probes number: Ko (=laboratory number 60), 2, 11 - 35, neg.

St.: 100bp DNA ladder

Applied volume: probes 10µl DNA ladder: 3µl

Reaction batch: 25µl

Gel electrophoresis in figure 13 demonstrates that the formerly API-identified *K. oxytoca* strains number 38 and 42 could not be confirmed with PCR (also see chapter 5.1.1). No 344bp bands appear for these strains. The remaining results in figure 12 and figure 13 are concordant between API 20 E and PCR results.

Figure 14 shows results from six different *Klebsiella* strains, this was still during the starting phase of our *K. oxytoca*-specific PCR detection with gel electrophoresis. Referring to number *36 aqua* the bacterial strain in this case was boiled with distilled aqua only instead of Cheelex. Due to the fact that this method also worked well, we agreed on using aqua instead of Cheelex (an Insta/Gene matrix) for the boiling procedure during the DNA extraction (also see chapter 4.3.1). The suffix *old* indicates that these strains were cultured at different occasions. Results were the same. PCR was therefore repeated for these strains (laboratory numbers 7, 36, 56 and 57).

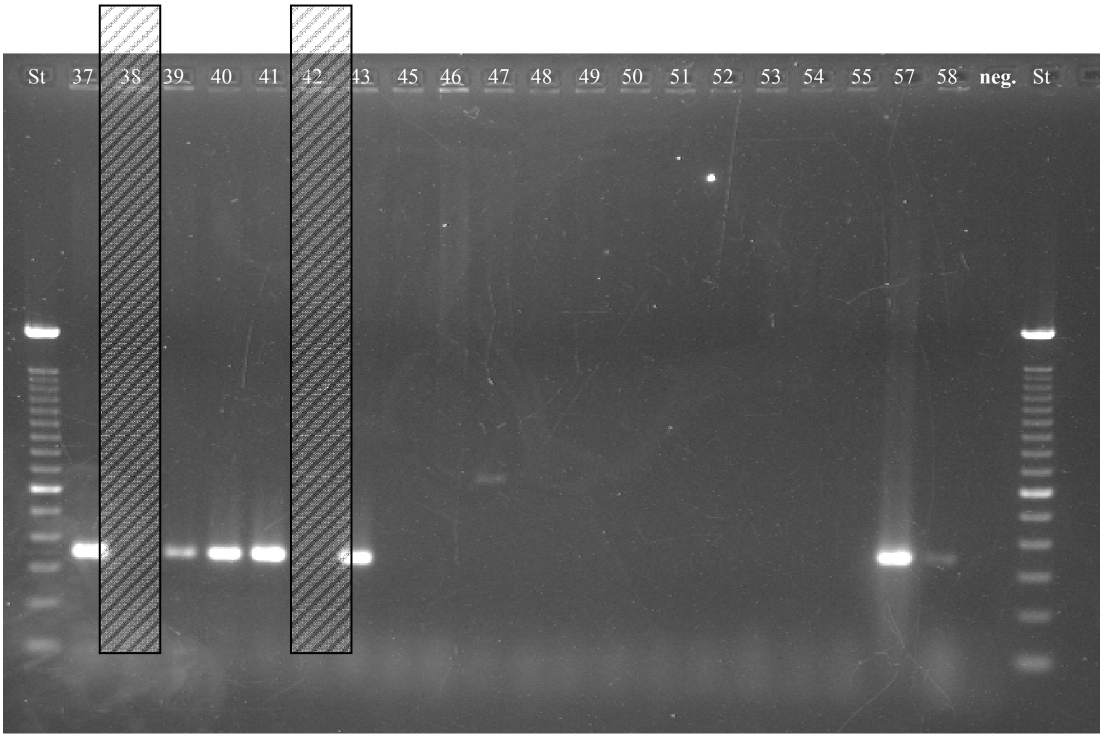


Figure 13: Missing 344bp bands for probe numbers 38, 42, 45-55 and the negative control

Probes number: 37-43, 45-55, 57, 58, neg.

St.: 100bp DNA ladder

Applied volume: Probes 10µl DNA ladder: 3µl

Reaction batch: 25µl

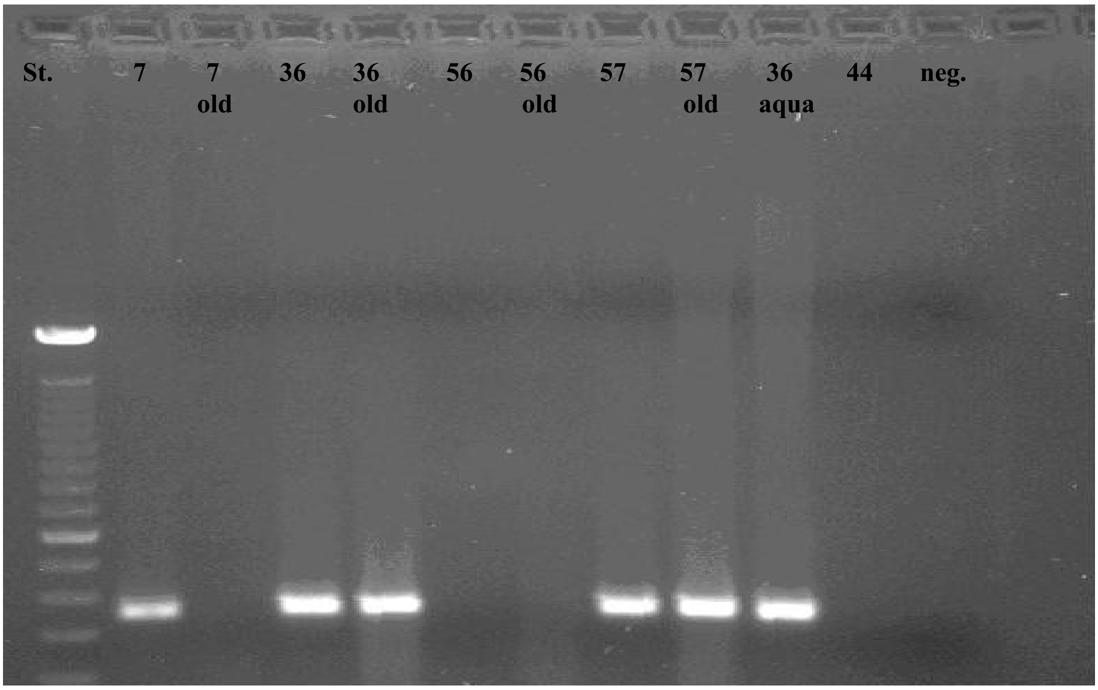


Figure 14: Missing 344bp bands for probe numbers 7old, 56 + 56 old, 44 and the negative control

Probes number: 7 + 7 old, 36 + 36 old, 56 + 56 old, 57 + 57 old, 36 aqua, 44 and neg. control

St.: 100bp DNA ladder

Applied volume: Probes 10µl DNA ladder: 3µl

Annotation: strain number *36 aqua* was boiled with distilled water whereas the other strains have been boiled with cheelex during the DNA isolation.

Old indicates that these strains were bred again on new agar

Figure 15 demonstrates that strain numbers 62, 67, 72, 75 and 77 don't show bands at 344bp. Hence, these strains are not identifiable as *K. oxytoca.* They were possible false positive API 20E results. Gel electrophoresis of figure 15 also newly detects two more *K. oxytoca* strains previously identified as *K. terrigena* and *K. pneumoniae*. These are number 74 and number 80. Unfortunately the quality of this figure is not as good as the original photograph, which brings out a 344bp band of number 74 more clearly. Nevertheless, there is a slight band visible for number 74 in figure 15. The last photograph of gel electrophoresis that shows discrepant results, as compared to API 20E, is figure 16. It shows that number 84, 89 and 98 are no *K. oxytoca* whereas number 83 is newly identified as *K. oxytoca*.

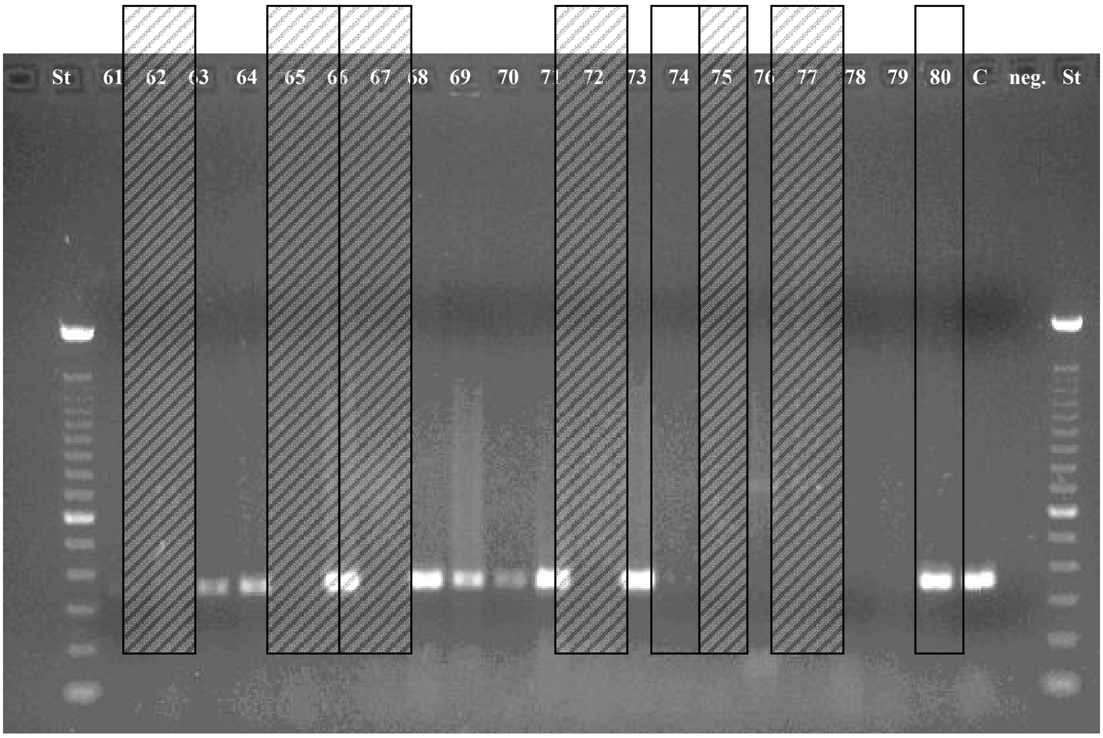


Figure 15: Missing 344bp bands for number 62, 65, 67, 72, 75 – 79 and the negative control

Probes number: 61 – 80, C (=control strain) , neg.

St.: 100bp DNA ladder

Applied volume: Probes 10µl DNA ladder: 3µl

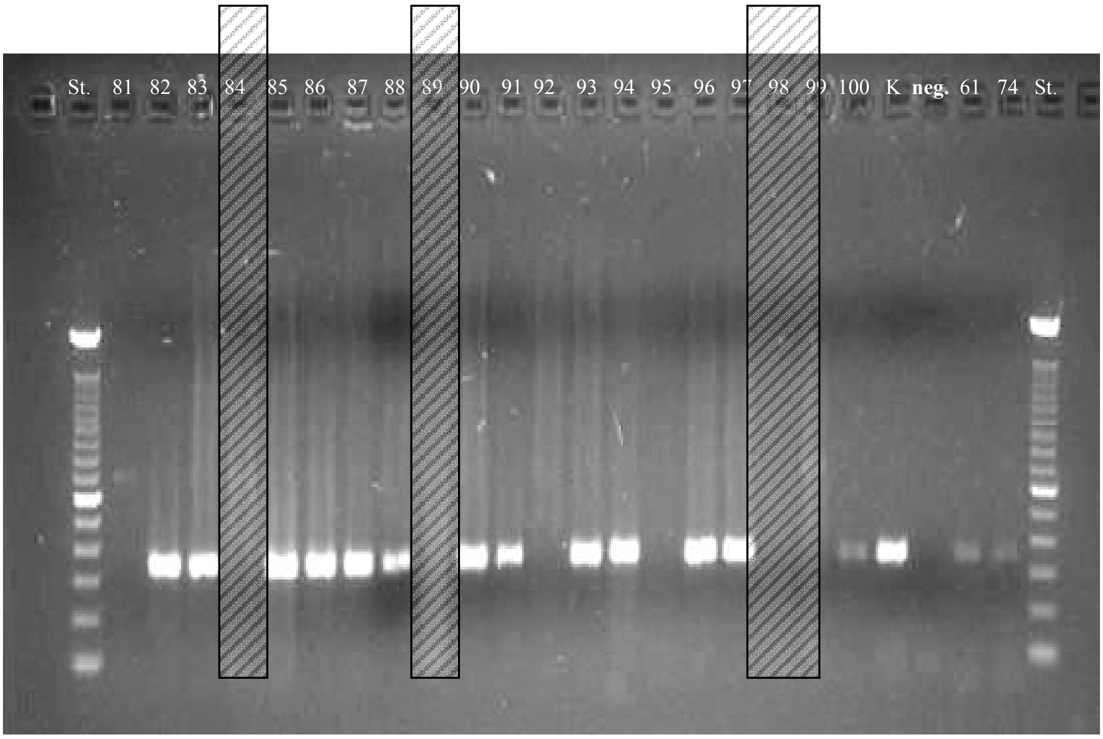


Figure 16: Missing 344bp bands for strains number 81, 84, 89, 92, 95, 98, 99 and the negative control

Probes number: 81-100, K, neg., 61, 74

St.: 100bp DNA ladder

Applied volume: Probes 10µl DNA ladder: 3µl

5.5 Statistic analysis of the results

Routine identification of *Klebsiella spp.* is performed with API 20 E test, as mentioned in the chapters above. Concerning our aim of evaluating PCR as a new testing method for *Klebsiella oxytoca*, it is also necessary to statistically compare these two methods. Because API is well established in conventional bacterial identification, PCR, being the new test is compared to up-to-now gold standard API 20E. Data for the comparison using four-fold test is shown in Table 10.

Table 10: Data for four-fold test

		bacterial strains n= 143
API +	PCR +	63
API -	PCR +	5
API +	PCR -	14
API -	PCR -	61
		n=143

\+ …………positive detection of *K. oxytoca*
\- ………… no detection of *K. oxytoca*

Summarizing the results that were described in previous chapters, table 10 comprises these results in a four fold test. Both testing methods, API and PCR, detected 63 *K. oxytoca* strains (out of 143 tested). Five *K. oxytoca* strains were only found by means of PCR, 14 *K. oxytoca* strains were just identified using API 20 E detection methods and 61 strains could not be identified as *K. oxytoca* neither using PCR nor API. The comparison of the two different testing methods shown in table 11, refers to API as gold standard of detection, because it had been the only established technique used in routine enterobacterial diagnosis up to now. Although we are aiming to proof PCR being a possibly more specific testing method than API 20 E for *K. oxytoca*, this new identification technique must be compared to a well working routine testing method at first.

5.5.1 PCR compared to API 20 E

As described, first of all, it is necessary to compare PCR, being a new detection method for *K. oxytoca*, to routine *Klebsiella* testing accomplished by API 20 E.

Table 11: Comparison between PCR and API 20 E in a four-fold test, considering API 20 E as gold standard of detection

	API 20 E *K. oxytoca* negative	API 20 E *K. oxytoca* positive	total amount
PCR *K. oxytoca* negative	61	14	75
PCR *K. oxytoca* positive	5	63	66
total amount	66	77	n=143

The sensitivity, which equals true positive results (n=63) divided by true positive (n=63) plus false negative results (n=14) for PCR-testing in comparison with the API results is 82%. The specificity of PCR compared to API is 92%. Specificity is calculated by dividing true negative results (n=61) by false positive (n=5) plus true negative results (n=61). There is a significant coherence (confidence interval for $\kappa = 0.95$, $P < 0.0001$) Observers agreement is 87 %, expected agreement 50 %. For this case shown in table 11, API is regarded as gold-standard of detection. As mentioned repeatedly, we are aiming to test PCR with API 20 E, a well working routine testing methods of *enterobacteriaceae* and gold standard in clinical identification up to now. Making allowances to this situation, also shown in table 11, PCR can deliver false-positive results.

Although false positive PCR results are mainly described for reverse-transcriptase PCR [27, 28], there are also case reports describing standard PCR (as used for our study, for detection of a specific amplicon) possibly false positive. This aspect is separately discussed in chapter 6.

As described in the previous chapters, API remains a subjective testing method and it is therefore possibly more error-prone than other, more objective testing techniques. PCR, being a more objective and specific detection method, should therefore furnish proof of all the *K. oxytoca* strains. If the *K. oxytoca*-specific *pehX* gene is present in the tested probe for PCR, amplification must be possible. False-negative PCR results are described for real-time PCR results detecting viral pathogens. Rapidly evolving RNA viruses present a challenge for diagnosis because they accumulate mutations that may render them undetectable. Detection may therefore be false negative [29]. Others describe no false-negative results at all using PCR diagnostic for viral detection [30]. Negative results can also occur due to the lack of an appropriate gene sequence or faulty reagents and/or faulty thermal cycler [31].

Based on these descriptions in literature in order to exclude false negative results, false negative results are not supposed to occur in the settings of our laboratory. Neither had we been detecting viral pathogens, nor had the reagents and the thermal cycler been defective. Gene sequences are therefore - if existing in the examined bacterial strain - appropriate and should be detected [29-31]. Beside eliminating false negative results, it is also important to exclude false positive results, which are mainly described using reverse-transcriptase PCR, as mentioned previously [27, 28]. These facts back up our hypothesis that the PCR we use is a more specific detection method for *K. oxytoca* compared to API 20 E. Changing the angle of vision now towards PCR being gold standard of detection, API might be false positive and false negative in some cases. Referring to the 19 discrepant detection results of the *Klebsiella* strains, API results being either false positive or false negative are compared to PCR in table 12 below.

Table 12: Regarding PCR results as gold standard of detection, the discordant API 20E results are either false positive or false negative

API 20E positive (for *K. oxytoca*)		**API 20E negative (for *K. oxytoca*)**	
68	all *K. oxytoca positive* (after PCR)	75	*K. oxytoca* negative (after PCR*)*
-5	false negative (1)	5	false positive (3)
63		80	
63		80	
14	false positive (2)	-14	false negative (4)
API 20E positive: n=77		API 20E negative: n=66	

(1)........false negative: these five strains were not detected with API

(2)........false positive: these 14 strains were wrongly identified as *K. oxytoca* with API

(3)........false positive: these five strains would contribute to the negative *K. oxytoca* results

(4)........false negative: these 14 strains are to be deducted from the interim 80 negative *K. oxytoca* results

5.5.2 API 20 E compared to PCR

Analogue to table 11, which compared PCR identification to API results, table 13 compares the two methods vice versa, PCR being gold standard of detection this time. API is evaluated compared to PCR. Sensitivity, which is true positive strains (n=63) divided by true positive (n=63) and false negative strains (n =5), equals 93 % for API 20 E (as compared to 82 % comparing PCR to API). Specificity is calculated by dividing true negative results (n=61) by false positive (n=14) plus true negative results (n=61). Specificity for API compared to gold-standard PCR is 81 %.

Table 13 Comparison between API 20 E and PCR in a four-fold test, considering PCR as gold standard of detection

	PCR *K. oxytoca* negative	PCR *K. oxytoca* positive	total amount
API *K. oxytoca* negative	61	5	66
API *K. oxytoca* positive	14	63	77
total amount	75	68	n=143

On the one hand, summarizing the statistical results comparing the two testing methods, sensitivity for API is higher regarding PCR as gold-standard and comparing API to PCR (93% as compared to 82% for PCR being compared to routine testing method API, chapter 5.5.1). One can deduct from these results that API is a possibly more effective testing method of verifying *K. oxytoca* strains than PCR. On the other hand, analyzing the specificity of the two testing techniques, regarding PCR as gold-standard, specificity of API is lower (81% as to 92%) compared to that of PCR in the precious chapter. This would confirm that PCR has almost no wrong negative results and can rather assure if detection was negative for *K. oxytoca* than API 20 E does.

6 Discussion

The main conclusion after investigating 143 different strains of *Klebsiella spp*. is that routine API 20E testing can currently be considered as a sufficient testing method for identification of the bacterial subspecies. Although there were 19 results that were not concordant comparing API 20E and PCR testing, the less bothersome and more established way of the detection of *Klebsiella oxytoca* remains the API test. Regarding the five newly identified *K. oxytoca* strains using PCR, they were predominately derived from patients experiencing antibiotic-associated colitis. This would raise the question, if *K. oxytoca* was also the causative organism of antibiotic associated colitis in these cases that would have been missed by API testing alone. It should therefore be considered to retest *Klebsiella* strains, which are derived from patients with colitis using PCR, because PCR seems to be more specific than API. This should be done to get even more certainty about the origin of the infection and subsequently gain more information about the virulence of *K. oxytoca*.

As described previously, API is a subjective test that might be fault prone. Aldridge and Hodges describe that API 20 E test had more discrepant results placing bacteria into the correct genus but in the wrong subspecies, compared to other testing methods, which were Entero-Set and conventional tube media [32]. Beside the statistical results described in the previous chapters, this would also suggest that PCR is a more specific testing method than API. There is not much information about false-positive PCR in medical literature. If false positive PCR results occur, they concern reverse-transcriptase polymerase chain reaction (RT-PCR) in most of the cases. RT-PCR, which is commonly used for HIV detection for example, proves the evidence of gene expression by amplifying a defined piece of RNA (ribonucleic acid). False-positive results can occur, when the discrimination of mRNA (messenger RNA) and contaminating genomic DNA is not possible. This is a common problem in RT-PCR [27, 28, 33]. Regarding the PCR used for our aim, there are some case reports described, depicting PCR not infallible and to evaluate results cautious [34, 35].

Although PCR is having a higher specificity than API, API seems to be more sensitive in the identification of *K. oxytoca*. Beside this, additional indole reaction tests rather confirm API results, as the concordance between test results of API and indole reaction is 84%, whereas the concordance of PCR and indole reaction is only 16%. A possible explanation is that the *pehX* gene coding for the polygalacturonase is not as specific for *K. oxytoca* strains as previously suggested and described by Kovtunovych et al. [22]. If this is the case, some *K. pneumoniae* strain might also carry this gene, while some *K. oxytoca* strains are lacking the *pehX* gene. It therefore still remains open, if PCR is a more specific testing method in comparison with API for the identification of *K. oxytoca*. Additional genetic testing by 16S rRNA-analysis for subspecies identification, especially in strains with discrepant results between API and PCR testing, might help resolving this question.

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8 Appendage

8.1 Anhang –Projektplan

(Arbeits-)Titel	The Role of *Klebsiella oxytoca* in Antibiotic-Associated Colitis
Untertitel (optional)	Comparison of Polymerase Chain Reaction as a new detection method for *K. oxytoca* to conventional API 20 E testing Rolle von
Betreuer/in: Institut/Klinik: Zweite/r Betreuer/in:	Univ. Prof. Dr. Thomas A. Hinterleitner Klinische Abteilung für Gastroenterologie und Hepatologie der Universitätsklinik Graz Univ. Prof. Dr. Christoph Högenauer Klinische Abteilung für Gastroenterologie und Hepatologie der Universitätsklinik Graz
Kernfrage und Zielsetzung *1. Wie lautet die Fragestellung?* *2. Warum ist diese Frage von Bedeutung?* *3. Welche Ergebnisse sind im Wesentlichen zu erwarten?* *4. Worin besteht der theoretische Kern der Arbeit?*	1. PCR als spezifischerer Nachweis von *Klebsiella oxytoca* aus Stuhlkulturen im Vergleich zu konventioneller mikrobiologischer Diagnostik. Herkömmliche biochemische Nachweismethoden sind möglicherweise nicht absolut zuverlässig in der oftmals schwierigen Unterscheidung zwischen *Klebsiella spp*. Daher ist der Versuch, den Nachweis von *K. oxytoca* mittels hochspezifischer PCR zu liefern, angezeigt. 2. Unter Antibiotikatherapie ist ein neuer Krankheitsauslöser im Darm beschrieben worden – *Klebsiella oxytoca* (Högenauer et al N Engl J Med 2006;355:2418-26). Antibiotika-assoziierter Diarrhoen durch *Clostridium difficile* und *K. oxytoca* sind schwere klinische Krankheitsbilder. Durch das Etablieren eines spezifischen und sensitiven PCR-Nachweises von *K. oxytoca* besteht die Möglichkeit, Risikogruppen sowie gesunde Träger frühzeitig zu erkennen und eventuell neue Aufschlüsse über das Krankheitsgeschehen zu gewinnen. 3. PCR von *K. oxytoca* aus Stuhlkulturen liefert möglicherweise eine exaktere Diagnostik als etablierte Nachweisverfahren bezüglich der Unterscheidung zu anderen *Klebsiella spp*. und Neuerkenntnisse über die Prävalenz von *Klebsiella oxytoca*. 4. Hintergrund: Definition und Ätiologien von Antibiotika-assoziierten Durchfallserkrankungen. Erregerspektrum, fakultativ und obligat pathogene Erreger, *Clostridium difficile*, *K. oxytoca,* Krankheitsbilder.

8.2 Lebenslauf

Paul WEBERHOFER
(Geboren am: 26/12/1983 in Graz)

Ausbildung:

seit 10/2002 **Medizinische Universität Graz -** Studium der Humanmedizin

3. Abschnitt seit 10/2007
2. Abschnitt am 20. 09. 2007 beendet
1. Abschnitt am 10. 09. 2003 beendet

09/1994 – 06/2002 **Graz International Bilingual School (GIBS)**.

Unterrichtssprache: Englisch.
Reifeprüfung 2002 mit gutem Erfolg bestanden.

Vertiefte Ausbildung:

- **Klinisch-topografische Anatomie der Eingeweide**
- **Klinische Endokrinologie – Eine multidisziplinäre Erfahrung**
- **Medizinische Parasitologie**
- **Neuro - und Gastrointestinale - Pharmakologie**
- **Medizinische Ernährungsmedizin**

Famulaturen

- 08/2007 **Krankenhaus der Elisabethinen**
  Abteilung für Innere Medizin
- 07/2007 **Krankenhaus der Barmherzigen Brüder Graz-Eggenberg**
  Abteilung Neurologie und Psychiatrie
- 08/2006 **Hospital de São João in Porto, Portugal**
  Auslandsfamulatur, Pädiatrie
- 02/2006 **LKH Graz-West**
  Medizinische Abteilung
- 07/2005 **LKH Bruck/Mur**
  Medizinische Abteilung
- 07/2004 **Unfallkrankenhaus Graz**
  Fachabteilung Unfallchirurgie

Graz, Mai 2008