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Acinetobacter baumannii: An evaluation of five susceptibility test methods to detect tobramycin resistance in an epidemiologically related cluster.

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MBChB *summa cum laude*, DTM&H, FCPath(Med. Micro)SA

Submitted in fulfilment of the requirements for the degree of

Master of Medicine (MMed)

in the Department of Clinical Laboratory Science, Division of Medical Microbiology, Faculty of Health Sciences, University of Cape Town.

August, 2011

Dedicated to my parents.

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DECLARATION

I, Vineshree Mischka Moodley, hereby declare that the work on this dissertation is based on independent work performed by myself (except where acknowledgements indicate otherwise), and that neither the whole work or any part of it has been, is being, or is to be submitted for another degree in this or any other university.

This work has also not been published prior to registration for the abovementioned degree.

Signature: Signed by candidate

Date: 27 July 2011.

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ABSTRACT

***ACINETOBACTER BAUMANNII*: AN EVALUATION OF FIVE SUSCEPTIBILITY TEST METHODS TO DETECT TOBRAMYCIN RESISTANCE IN AN EPIDEMIOLOGICALLY RELATED CLUSTER.**

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Abstract

BACKGROUND
Acinetobacter baumannii is a major pathogen causing nosocomial infections, particularly in critically ill patients. This organism has acquired the propensity to rapidly develop resistance to most antibiotics. At several hospitals within Cape Town, tobramycin and colistin remain frequently the only therapeutic options. The Vitek2 automated susceptibility testing (AST) is used in the clinical laboratory to determine selected susceptibility profiles. The suspicion of a possible AST-related technical error when testing for susceptibility to tobramycin in *A. baumannii* precipitated this study.

METHODOLOGY
Forty *A. baumannii* strains isolated from clinical specimens (June-December 2006) which exhibited MICs close to the tobramycin breakpoints were included in this prospective study. AST was compared to disk diffusion, Epsilometer test and agar dilution using broth microdilution (BMD) as the reference method. Additionally, PCR was performed to detect the *aac*(3)-II' gene which encodes an aminoglycoside modifying enzyme with activity against tobramycin.

RESULTS
The tobramycin susceptibility results revealed errors in 25/39 isolates (10 very major and 15 minor errors) when AST was compared to BMD ($p<0.001$), 12/39 (1 very major and 11 minor errors) when Etest was compared to BMD, and 15 errors (3 very major and 12 minor errors) when disk diffusion was compared to BMD. Additionally, the tobramycin resistance gene, *aac*(3)-II,' was detected in 21/25 of the discrepant isolates, confirming the resistant phenotype detected by the reference method. Molecular typing showed that these isolates were genetically related.

CONCLUSION
Clinical laboratories using the Vitek2 system for routine use should consider an alternative susceptibility testing method to determine susceptibility to tobramycin.

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ABBREVIATIONS

%	percentage
°C	degrees Celcius
aac(3)-IIa	aminoglycoside acetyltransferase(3)-*IIa* gene
AAC(3)-IIa	aminoglycoside acetyltransferase(3)-IIa enzyme
AFLP	amplified fragment length polymorphism
AME	aminoglycoside modifying enzyme
AST	automated susceptibility testing
ATCC	American Type Culture Collection
BMD	broth microdilution
CLSI	Clinical Laboratory Sciences Institute
DNA	deoxyribonucleic acid
ESBL	extended spectrum beta-lactamase
ESI-MS	electrospray ionisation mass spectrophotometry
Etest	epsilometric test
GSH	Groote Schuur hospital
hrs	hours
ICU	intensive Care Unit
kb	kilobase
kDa	kiloDaltons
NHLS	National Health Laboratory Services
mcF	McFarland
MDR	multidrug-resistant
µg	microgram

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µl	microliter
mg	milligram
ml	millilitre
mM	millimoles
mm	millimetre
MIC	minimal inhibitory concentration
ng	nanogram
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PFGE	pulse field gel electrophoresis
pmole	picomole
OXA	oxacillinase
rRNA	ribosomal RNA
SANAS	South African National Accreditation Society
SOP	standard operating procedure
Taq	*Thermus aquaticus*
TLR	Toll-like receptor
U	unit
UK	United Kingdom
US	United States of America
UV	ultraviolet
V	volts
VAP	ventilator-associated pneumonia
VME(s)	very major error(s)

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The Protocol

PART A

SUPERVISORS

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Gilles Zambardi, Research and Development Division, bioMérieux, La Balme, France.

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RESEARCH PROPOSAL

OBJECTIVE and AIMS:

The overall objective of this study is to evaluate the Vitek®2 automated susceptibility testing system (bioMérieux, Durham, North Carolina) for tobramycin susceptibility testing of *Acinetobacter baumannii* using broth microdilution as the reference standard.

***Aims*:**

1. To compare the Vitek®2 automated susceptibility testing with disc diffusion, Epsilometric test (E-test), agar dilution, and broth microdilution (reference standard) methodology for aminoglycoside susceptibility testing of *A. baumannii* in the diagnostic microbiology laboratory at Groote Schuur Hospital.

2. To determine whether the choice of nutrient agar and bacterial cell inoculum used in the manual method and in the Vitek®2, respectively, impacts on the outcome of susceptibility testing.

3. Determination of genetic relatedness of *A. baumannii* strains with discrepant Vitek®2/ broth microdilution tobramycin susceptibility results.

4. Identification of the mechanism of tobramycin resistance in selected *A. baumannii* strains.

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BACKGROUND

Acinetobacter baumannii has emerged as a major nosocomial pathogen in many intensive care units throughout the world (1, 2, 5, 21, 24). These non-fastidious, strictly aerobic Gram-negative organisms present several problems. Firstly, *Acinetobacter* species are opportunistic organisms with the capacity for long-term survival in the hospital environment. The organism has the ability to colonize human skin, thereby contributing to cross-transmission in the hospital.

Secondly, these organisms have been associated with fatal infections in critically ill patients. Acinetobacter has been implicated in bacteraemia, respiratory tract and surgical site infections. Factors predisposing to infection include prolonged hospital stay, admission to an intensive care unit, mechanical ventilation, previous treatment with antimicrobials, invasive procedures or instrumentations, burns, immunosuppression, and previous sepsis (1, 2, 5, 12, 24). *A. baumannii* bacteraemia is associated with a high crude mortality rate (varying between 17 and 52%) and prolonged hospital stay (2, 13, 15). Patient characteristics that play a major role in outcome include age, immunosuppression, recent surgery, acute respiratory failure, acute renal failure, septic shock, and appropriate choice of initial antibiotic therapy (6, 11).

Thirdly, its intrinsic resistance to many antimicrobial agents as well as the propensity to develop resistance to newly developed antibiotics has made *A. baumannii* a formidable adversary. It is of great concern that the incidence of carbapenem-resistant acinetobacter isolates is increasing. Measures to limit the spread include enforcement of hand washing, sterilization of ventilator equipment, dedication of equipment to individual patients, use of isolation facilities, restriction of antibiotic usage, and ward closure (5, 12). The choice of available antibiotics, however, grows more limited. In multi-resistant acinetobacter infections, some remaining options include polymyxins (colistin), sulbactam and tigecycline (1). Known resistance mechanisms of *A. baumannii* to antimicrobials are the production of broad-spectrum β–lactamases, carbapenemases, aminoglycoside-modifying enzymes, mutations in outer membrane porins, and alterations in penicillin-binding proteins. As therapeutic possibilities decrease, patient mortality increases due to inappropriate empiric antimicrobial treatment.

Accurate identification of Gram-negative non-fermenters in the clinical laboratory relies mainly on automated or semi-automated systems. These systems have the advantages of decreasing laboratory turn-around time, perceived cost-effectiveness, and are able to be interfaced to laboratory and hospital information systems. Unfortunately, they have limitations. Numerous studies have reported on errors in the accuracy of various automated systems in identification and sensitivity testing especially amongst the non-fermenters (14, 16).

A. baumannii is endemic in some hospitals' intensive care units within the Cape Town metropole. The majority of these isolates are multi-resistant organisms, only remaining susceptible to tobramycin and colistin. Surveillance data collected by the National Antibiotic Surveillance Forum in 2006 revealed that of the 161 blood culture isolates of *A. baumannii* cultured at Groote Schuur Hospital (a tertiary academic institution), 57%

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were susceptible to piperacillin-tazobactam and ciprofloxacin, while 42% were susceptible to ceftazidime. Importantly, only 55% and 57% were susceptible to the carbapenems, imipenem and meropenem, respectively (17). Similar rates of resistance are seen at other hospitals in South Africa (see Table 1).

TABLE 1: ***Susceptibility rates for Acinetobacter species isolated from blood from clinical diagnostic laboratories in different provinces of South Africa in 2006 (17).***

LABORATORY	TOTAL NUMBER OF ISOLATES	PITZ	CIPRO	CTAZ	IMI	MERO
Gauteng						
Chris Hani Baragwaneth	132	85%	50%	51%	69%	71%
Charlotte Maxeke Johannesburg Academic	70	56%	51%	53%	51%	53%
Dr George Mukhari	61	62%	56%	75%	49%	51%
Tshwane						
Pretoria Academic	40	80%	63%	65%	68%	68%
Western Cape						
Groote Schuur	**161**	**57%**	**57%**	**42%**	**55%**	**57%**
Tygerberg	124	80%	79%	74%	77%	77%

Note: PITZ-piperacillin-tazobactam, CIPRO-ciprofloxacin, CTAZ-ceftazidime, IMI-imipenem, MERO-meropenem

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With respect to aminoglycoside sensitivity, statistics show that of the *A. baumannii* isolated in the diagnostic laboratory at Groote Schuur Hospital, from all clinical specimens during the period 1/10/2005-30/09/2006, 82.75% of 1125 acinetobacter isolates were resistant to gentamicin, 71.63% of 1195 isolates were resistant to amikacin, and 42.29 % of 1206 isolates were resistant to tobramycin (NHLS Laboratory Information System). An increasing number of Vitek®2 reports were being sent out from the laboratory noting *A. baumannii* isolates with MICs close to the tobramycin breakpoints (CLSI breakpoints). Further, an inconsistency between the antibiograms, as detected by disc diffusion and Etest strips, and the Vitek®2 automated susceptibility testing was noted. These discrepancies drew our attention to possible technical

problems with the current Vitek®2 system. In this context, an extensive literature search and discussions with bioMérieux indicated that problems with tobramycin susceptibility testing of *A. baumannii* had not been reported previously.

The following study, to evaluate the accuracy of the Vitek®2 automated susceptibility testing for aminoglycoside antibiotics, was predicated by the limited therapeutic options and the consequences of initiating septic patients on inappropriate therapy.

METHODOLOGY

Seventy-eight isolates were collected from specimens sent to the clinical diagnostic microbiology laboratory during a seven month period (June 2006 – December 2006). Technologists at our laboratory were requested to make single colony plates of *A. baumannii* from clinical isolates with MICs/disc zone sizes close to the CLSI breakpoints for tobramycin (4). These isolates were specifically chosen as we had noted discrepancies between the Vitek®2 and Etests in this group previously. A few fully sensitive and resistant isolates were also chosen to evaluate the complete range of isolates analysed by the Vitek®2 in the clinical diagnostic laboratory. Duplicate specimens from the same patient were avoided except where the sensitivity pattern to aminoglycosides changed.

The majority of the isolates were obtained from sputum, blood culture, pus swabs, tissue culture and urine specimens of ICU patients (medical, surgical and paediatric ICUs from four hospitals), including three pus swabs taken during a surveillance exercise of the ICUs (See Table 3, Part D). The remainder of the isolates were obtained from patients in the general wards. Groote Schuur Hospital and Red Cross Children's Hospital represent tertiary academic institutions, with Mowbray Maternity Hospital, G.F. Jooste and Victoria Hospital being secondary level institutions.

All isolates will be inoculated on to fresh MacConkey agar to obtain single colonies that will be utilized for identification and susceptibility testing.

Storage

The agar plates will be stored at 4 ºC. Single colony plates will be sub-cultured if necessary (maximum twice) to ensure viability of the organisms. Following susceptibility testing, all isolates will be stored on beads at -70 ºC (Viabank VIM tubes, ABTEK Biologicals Ltd, Liverpool). Recovery of organisms from beads will be performed according to the manufacturer's instructions.

IDENTIFICATION OF BACTERIA

The identification of the isolates will be confirmed as per standard laboratory protocol and manufacturer's instruction using the Vitek®2 system (See SOP 1, Part D).

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Introduction

The Vitek®2 Gram-Negative Identification Card (GN-ID) intended for the automatic identification of significant fermenting and non-fermenting gram-negative bacteria, utilises the results of 41 biochemical tests to identify an isolate. The results of the biochemical tests are compared to the expected corresponding set of reactions for each organism. A qualitative value (percentage probability) is calculated based on the observed test reactions versus the typical reactions for each organism. Thus, a perfect match would provide a percentage probability of 99%. A percentage probability of 85-97% indicates that a test reaction pattern is sufficiently close to the biochemical profile of a particular organism or organism group. Results are usually available within 3 hours.

Methodology

Fresh isolates (18-24 hours old) will be identified using the Vitek®2 system (See Standard Operating Procedure 1, Part D). An isolated colony from the single colony plate will be selected and emulsified in sterile saline in a clear plastic tube to obtain a density of 0.6 and 1.0 McFarland, respectively. Cassettes with tubes and cards will be loaded into the Vitek®2 according to the manufacturer's instructions.

The identification of each isolate will be confirmed twice, at 0.6 McFarland (manufacturer's recommendation) and 1.0 McFarland, respectively. Only Vitek®2 results with a percentage probability above 94 % (Very Good, Excellent Identification categories) will be considered as acceptable identifications.

The identification of each isolate will also be confirmed independently by the bioMérieux Research and Development Division using API phenotypic testing panels.

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ANTIBIOTIC SUSCEPTIBILITY

Antibiotic susceptibility testing will be performed on all isolates. The following methods will be employed: disc diffusion; MIC determination by Etests, agar dilution and broth microdilution; and automated susceptibility testing by Vitek®2.

Disc diffusion

Introduction

Disc diffusion is one of the most popular methods of antimicrobial susceptibility testing due to its ease of use and low cost. This method is based on the diffusion through agar of an antimicrobial drug released from an impregnated disc. When a disc is applied to the agar surface, the antibiotic begins to diffuse immediately. This results in a

concentration gradient that diminishes with distance from the edge of the disc. The zone edge is formed by the growth of the organism at the point in the agar where the antibiotic concentration is at the critical concentration, which is defined as the concentration that is just capable of inhibiting bacterial growth. Unlike other dilution methods, an MIC value is not generated. Instead, a zone diameter is used to predict an MIC value, and which category of susceptibility the strain belongs to.

Several factors can influence the result of disc diffusion testing. These include the disc content (amount of drug per disc), the diffusion characteristics of the drug, the disc size, the depth of the agar, the density of the inoculum, and incubation time (10, 23). To ensure standardization, commercial media obtained from three different manufacturers will be used in the study. In addition, antibiotic discs with the same lot numbers will be used, and a mechanical rotary device will be used to distribute the bacterial inoculum evenly across the agar surface. Quality control will be performed for each batch of media used.

Methodology

Disc diffusion testing (modified Bauer-Kirby method) will be performed in accordance with CLSI standards (3, 4). Cation-adjusted Mueller-Hinton media will be utilised for the disc diffusion testing. The disc diffusion will be performed in triplicate on three different commercial media [bioMérieux (Randburg, South Africa), Bio-Rad Laboratories (Johannesburg, South Africa), and Greenpoint NHLS Media Laboratory (Cape Town, South Africa)] to exclude any discrepancies as the cation levels in the media could influence the aminoglycoside zone diameters (10). The aminoglycosides that will be tested by disc diffusion are gentamicin (10 µg), amikacin (30 µg), and tobramycin (10 µg) [Oxoid, Basingstoke]. These antibiotics were chosen as they are used at local hospitals for treatment of acinetobacter infections.

Isolated colonies from the single colony plate will be emulsified in normal saline to achieve an organism suspension with a density equivalent to 0.5 McFarland. The suspension will then be evenly applied onto the three different commercial Mueller-Hinton media using a mechanical rotary device. The antibiotic discs are applied by tabbing them onto the agar using a disc dispensing device. Each plate must be checked to ensure that the discs are making complete contact with the agar as well as to ensure that they are >24 mm apart (centre-to-centre). The plates will be inverted and incubated within 15 minutes in an aerobic incubator at 35 ºC for 24 hours.

After 24 hours, the susceptibilities will be determined using the methodology outlined in CLSI standards (See SOP 2, Part D). The diameters of the zone of inhibition will be measured to the nearest millimetre using a sliding calliper. The zone diameter will be interpreted using CLSI interpretative standards (4). The mean of the zone diameters obtained from all three media will be used in the analysis.

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Epsilometric Tests (Etest)

Introduction

The Etest (AB Biodisk, Solna, Sweden) is a quantitative method for determining the antimicrobial susceptibility of a bacterial species. The system utilises a predefined antibiotic gradient which is used to determine the MIC (in µg/ml) of different antibiotics against organisms as tested on agar media using overnight incubation. The MIC generated is the minimal concentration of a drug, in µg/ml, which will inhibit the growth of a particular bacterium under defined experimental conditions.

The Etest is a thin, inert, non-porous plastic strip that is 5 mm wide and 60 mm long. One side of the strip carries the MIC reading scale (in µg/ml) and a two letter code that represents the antibiotic being tested. A predefined exponential gradient of the antibiotic is present on the other surface of the strip. The gradient covers a continuous concentration range across 15 two-fold dilutions of a conventional MIC method.

When an Etest is applied to the inoculated agar surface, there is immediate transfer of the antibiotic into the agar matrix. A stable, continuous and exponential gradient is formed under the strip. A symmetrical ellipse of inhibition becomes visible after the recommended incubation period. The Etest has been shown to be reproducible and equivalent to CLSI reference dilution methods. However, even under the best controlled conditions, a dilution test may not give the same end point each time it is performed. The reproducibility of the conventional dilution test is within +/- 1 two-fold dilution of the end point.

Methodology

MIC determination by Etest will be performed as per manufacturer's instructions. Once again, a single Etest will be placed on three different commercially available Mueller-Hinton agars to exclude the influence of variation of cation concentration on susceptibility testing.

Individual bacterial colonies are homogenised in saline to 0.5 McFarland. A swab is then immersed into the suspension, excess fluid is removed, and the surface of the agar is swabbed evenly in three directions. The Etest will then be placed on the surface of the agar using a sterile forceps. Thereafter, the plate is placed into an aerobic incubator and incubated at 35 ºC for 24 hours (See SOP 3, Part D).

The plates will be read only if sufficient growth is seen after the recommended incubation period. The MIC of an antibiotic is the point at which the ellipse intersects the scale. The end point is defined as complete inhibition of all growth including hazes and isolated colonies.

CLSI interpretative standards will be utilized to interpret the MIC values obtained (4). Since the Etest is a continuous gradient, values in between two-fold dilutions may be obtained. These values will be rounded up to the next two-fold dilution before

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interpretation. The Etest results will be compared to the reference broth microdilution results, and the results obtained from the medium which most closely agrees with the results from the reference method will be used for analysis purposes.

Vitek®2 Susceptibility Testing

Introduction

The Vitek®2 system is an automated susceptibility test method that utilises plastic reagent cards containing antibiotics in a 64-well format. The system employs repetitive turbimetric monitoring of bacterial growth during an abbreviated incubation period of 4-10 hrs. Using a unique algorithm, an MIC is calculated. The Vitek®2 uses an "expert system" computer software programme to interpret susceptibility results and analyses the results for atypical patterns or unusual resistance phenotypes.

Methodology

Susceptibility testing will be performed using the same standard operating procedure as for Vitek®2 identification. A Vitek®2 Gram Negative Susceptibility card (NO-22) will be used to determine the susceptibility of each isolate at 0.6 McFarland and 1.0 McFarland, respectively.

The concentrations of the aminoglycoside antibiotics in the card are as indicated in Table 2.

TABLE 2: ***Concentrations of antibiotics in NO-22 card.***

Antibiotic	Code	Concentration	Calling Range
Amikacin	AN	8, 16, 64	≤ 2; ≥ 64
Gentamicin	GM	4, 16, 32	≤ 1; ≥ 16
Tobramycin	TM	8, 16, 64	≤ 1; ≥ 16
Netilmicin	NET	4, 16, 32	≤ 1; ≥ 32

Table obtained from AST NO-22 Card, Package Insert, bioMérieux, 2006.

Agar dilution

Agar dilution, the second reference method, is a truncated method that incorporates one or two selected concentrations of antimicrobials, usually at breakpoint values (10, 22).

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This technique involves the incorporation of different concentrations of the antimicrobial substance into a nutrient agar medium followed by the application of a standardised inoculum of bacteria to the surface of the agar plate. The plates are then incubated at 35 ºC for 18 hrs. Sensitive organisms are inhibited by the concentration of antibiotic in the agar, and no growth is evident at the points of inoculation. Resistant organisms appear as distinct colonies of bacterial growth (23).

The agar dilution method will be performed by the Research and Development Division of bioMérieux in LaBalme, France. The methodology used for testing will follow CLSI standards. Only tobramycin susceptibility will be performed for all isolates as tobramycin is the antibiotic that we have experienced the most problems with. The agar dilution technique will be performed in duplicate by two independent technologists. The results obtained will be compared to the reference method, and the lab worker whose results most closely match the results of reference method will be used for analysis.

Broth microdilution

The broth microdilution test serves as the reference method for susceptibility testing. Serial dilutions of antimicrobial agent to be tested are added to broth in a 96 well plate. A standardized bacterial suspension is then added. Growth is assessed after incubation for a defined period of time (16–20 h). At the end of the incubation period, the wells are visually inspected. Cloudiness indicates that the bacterial growth has not been inhibited by the concentration of antibiotic in the well, whereas a clear well indicates that the bacteria have been inhibited by the concentration of antibiotic in the well (23).

The broth microdilution will be performed by the Research and Development Division of bioMérieux in LaBalme, France, due to lack of local resources and expertise. Testing will follow CLSI standards. The broth microdilution will be performed on three aminoglycoside antibiotics, i.e. gentamicin, amikacin, and tobramycin. The method will be performed in duplicate by two technologists working independently. CLSI interpretative criteria will be utilized for interpretation of the MIC values obtained (4). Discrepant results will be reconciled by choosing the result with the higher MIC value. This accords with standard laboratory practice as theoretically these results would be used to influence choice of antibiotic in critically ill patients.

Quality control

To ensure standardization of results, all susceptibility testing performed in Cape Town will be performed by a single investigator working under standardised conditions using standard operating procedures in a SANAS accredited laboratory. The investigator will be observed by two independent observers (from bioMérieux) to ensure that no deviation from the standard operating procedures occurs.

Quality control procedures as outlined in CLSI guidelines as well as the Vitek®2 manufacturer's guidelines will be followed (3, 4). Quality control using the recommended

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American Type Culture Collection (ATCC) organisms will be performed for each lot number of media, antibiotic discs, Etests, and Vitek®2 cards utilised in the study.

MOLECULAR TESTING

DETECTION OF MECHANISM OF RESISTANCE

The mechanisms of aminoglycoside resistance amongst *A. baumannii* isolated at the clinical diagnostic laboratory have previously been researched by the Division of Medical Microbiology, University of Cape Town (8). The prevalent mechanism of resistance to tobramycin was an aminoglycoside modifying enzyme encoded by the *aac(3)-IIa* gene (8).

Based on this observation, a PCR will be performed on all isolates to confirm the presence or absence of the *aac(3)-IIa* gene.

Methodology

The NucliSens® easyMag® platform (bioMérieux, Durham, North Carolina) will be used for DNA extraction. This is an automated system for extraction of nucleic acids based upon silica extraction technology. The samples are incubated with lysis buffer overnight. The target nucleic acids are captured by silica particles. The NucliSens® easyMag® magnetic device attracts all the magnetic silica, enabling the system to purify the nucleic acids through several wash steps. The heating step releases the nucleic acids from the silica, and the magnetic beads are then separated from the eluate by the magnetic device.

The quantity and quality of DNA extracted will be confirmed using the Nanodrop® ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA).

The PCR assay utilised has been optimised and validated by previous work performed by medical scientists at the Institute of Infectious Diseases and Molecular Medicine, University of Cape Town (8). The sequences of the primers that will be used are shown in Appendix 1.

The mastermix consists of magnesium chloride(25 mM), 2.5 mM of each dNTP, forward and reverse primers (20 pmoles each), and 2.5U *Taq* polymerase in buffer made up to a final volume of 50 µL per reaction (8). Genomic DNA (8-80 ng/µL) will be added to the mastermix. The mixture will be placed in a thermocycler using the following cycling conditions: an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of 95 °C for 1 minute, 51 °C for 45 seconds, 72 °C for 1 minute, and a final extension at 72 °C for 5 minutes (9). MOS-1, a known *Acinetobacter baumannii* strain with the *aac(3-)IIa* gene will be used as a positive control, while MOS-2 will be used as a negative control (8).

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The PCR product will be separated in an agarose gel and visualised by transillumination. The expected product size is 786 bp (8). The assay will be repeated on all samples with an initial negative result. To confirm the integrity of DNA extracted, a 16s rRNA PCR will also be performed on all isolates (See SOP 5, Part D).

Selected isolates will be sequenced by the DNA sequencing facility at the University of Stellenbosch, South Africa. The DNA sequences will be analysed using Chromas Lite v2.01 (Technelysium Pty Ltd) and BioEdit Version 7.0.9 software. The sequences will be blasted on Genbank for nucleic acid similarity with existing sequences.

Quality control

The molecular tests will be performed in designated molecular areas within a SANAS accredited laboratory. Positive and negative controls will be included for all stages of molecular testing. Standard operating procedures will be strictly adhered to.

EPIDEMIOLOGY OF ISOLATES

REP-PCR

The isolates will be typed by rep-PCR using the Diversilab System by the bioMérieux Research and Development team in France. This will be performed to determine if there is a predominant clone that is responsible for the disparity in tobramycin susceptibility testing. The Diversilab System is an automated DNA fingerprinting and analysis tool based on rep-PCR technology. The technique consists of three parts: isolation of DNA from bacteria with a Diversilab™ *Acinetobacter* kit, amplification of the isolated DNA using rep-PCR, and detection of the amplified material by electrophoresis. Rep-PCR primers bind to many specific repetitive sequences interspersed throughout the bacterial genome. Multiple fragments of differing lengths are amplified. These fragments are then separated by mass and charge via electrophoresis. A unique fingerprint is created with multiple bands of varying intensity. Software analysis of the fingerprints generated may be used to create dendrograms, scatterplots, etc. This will then be used to evaluate the degree of similarity between the isolates utilised in this study. See Part D for detailed methodology.

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Pulsed field gel electrophoresis (PFGE)

Introduction

PFGE is considered the gold standard for typing of *A. baumannii* (18, 19). This technique has the highest discriminatory power, and interlaboratory reproducibility is possible through the use of standardised protocols (18, 19). PFGE will be performed to further investigate the relatedness of *A. baumannii* strains included in this study to a previously characterised tobramycin resistant strain isolated at Groote Schuur Hospital. The methodology for the PFGE has already been optimized by medical scientists at the Institute of Infectious Diseases and Molecular Medicine, University of Cape Town (8).

Methodology

Briefly, agarose plugs containing a bacterial suspension and sodium dodecyl sulphate ($C_{12}H_{25}NaO_4S$), to lyse the bacteria, will be prepared. After lysis, the plugs will be washed and the DNA will be digested with Apa1 (Roche). Following digestion, the plugs will be loaded into an agarose gel in preparation for electrophoresis. The electrophoresis will be carried out in a Gene Navigator® PFGE machine (Amersham Biosciences AB, Uppsala, Sweden) for 23 hours, after which it will be stained with ethidium bromide, destained, and photographed (See SOP 7, Part D). The patterns will be visually inspected and interpreted using Tenover criteria (20). An analysis using GelCompare II Version 4.6 (Applied Maths, Sint-Martens-Latem, Belgium) software will be used to further analyse the profiles obtained (7).

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DISCUSSION AND IMPACT

This study has been proposed to inform the diagnostic laboratory on whether the Vitek®2 automated system is an accurate means of testing for tobramycin susceptibility in *A. baumannii*. In addition, an assessment of the accuracy of the various methods in determining sensitivity is critical as inappropriate antimicrobial therapy is associated with increased morbidity and mortality.

ENVISAGED OUTCOMES

The results of this study may lead to changes in standard operating procedure with respect to aminoglycoside susceptibility testing at Groote Schuur Hospital, as well as other NHLS laboratories in South Africa. In addition, a revision of the product or package insert may have to be made by the manufacturer.

This study will form the basis of research toward a MMed dissertation. The findings of this project are expected to be published in an international peer reviewed journal, and the data is expected to be presented at an international microbiology congress either as an oral presentation or as a poster submission.

ETHICAL CONSIDERATIONS

Ethical approval was sought from the University of Cape Town Human Research Ethics Committee (REF REC 458/2006). See Appendix 1, Part D.

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APPENDIX 1:

Sequence of *aac(3)-IIa* gene which encodes the AAC(3)-IIa enzyme.

ATGCATACGCGGAAGGCAATAACGGAGGCAATTCGAAAACTCGGAGTCCAAACCGGTGACCTGTTGATGGTGCA

TGCCTCACTTAAAGCGATTGGTCCGGTCGAAGGAGGAGCGGAGACGGTCGTTGCCGCGTTACGCTCCGCGGTTGG

GCCGACTGGCACTGTGATGGATACGCGTCGTGGGACCGATCACCCTAACGAGGAGACTCTGAATGGCGCTCGGTT

GGATGACAAAGCCCGCCGTACCTGGCCGCCGTTCGATCCCGCAACGGCCGGGACTTACCGTGGGTTCGGCCTGCT

GAATCAATTTCTGGTTCAAGCCCCCGGCGCGCGGCGCAGCGCGCACCCCGATGCATCGATGTCGCGGTTGGTCCG

CTAGCTGAAACGCTGACGGAGCCTCACGAACTCGGTCACGCCTTGGGGAAAGGGTCGCCCGTCGAGCGGTTCGTC

CGCCTTGGCGGGAAGGCCCTGCTGTTGGGTGCGCCGCTAAACTCCGTTACCGCATTGCACTACGCCGAGGCGGTT

GCGGATATCCCCAACAAACGATGGGTGACGTATGAGATGCCGATGCTTGGAAGAAACGGTGAAGTCCGCCTGGA

AAACGGCATCAGAATACGATTCAAACGGCATTCTCGATTGCTTTGCTATCGAAGGAAAGCCGGATGCGGTCGAAA

CTATAGCAAATGCTTACGTGAAGCTCGGTCGCCATCGAGAAGGTGTCGTGGGCTTTGCTCAGTGCTACCTGTTCGA

CGCGCAGGACATCGTGACGTTCGGCGTCACCTATCTTGAGAAGCACTTCGGAGCCACTCCGATCGTGCCAGCACAC

GAAGCCGCCCAGCGCTCTTGGCGAGCCTTCCGG**TTA**

The start and stop codon for the *aac(3)-IIa* gene are indicated in bold type. The forward primer is indicated in green and the reverse primer is indicated in blue. The arrows indicate the direction of transcription.

(Adapted from: Jacobson, R. K. 2007. M. Sc thesis. Association of *IS1133* with an aminoglycoside resistance gene, *aacC2a*, in *Acinetobacter baumannii* isolates. University of Cape Town).

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Literature Review

Part B

LITERATURE REVIEW

Acinetobacter baumannii has emerged as a major nosocomial pathogen world-wide (1, 20, 21, 115, 124). The organism has gained increased notoriety with its classification as a high priority pathogen by the Antimicrobial Availability Task Force of the Infectious Diseases Society of America (109). This bacterium presents several problems to the microbiologist and clinician. *A. baumannii* has the capacity for long-term survival in the hospital environment, rapidly filling the ecological niche left vacant by the elimination of competing bacteria by broad-spectrum antibiotics (111). Infections due to *A. baumannii* are associated with increased morbidity and mortality in critically ill patients in ICUs. Genetic interchange with other bacterial species is possible with significant implications for the rapid acquisition of resistance determinants, and subsequent spread of multi-drug resistant strains (82). Considerable information has been obtained about the mechanisms of antibiotic resistance, but we are only beginning to understand the virulence factors and pathogenicity potential of this complex bacterium.

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1. ***MICROBIOLOGY***

1.1 TAXONOMY OF ACINETOBACTER

The taxonomy of *Acinetobacter* spp. has a convoluted history. The organism was first described in 1911 by the Dutch microbiologist, Beijerinck, who isolated the organism from the soil in a calcium-acetate-containing minimal medium (82, 111). In 1954, Brisou and Prevot proposed the current genus designation to separate the non-motile from the motile organisms within the genus *Achromobacter* (82). This designation was accepted in 1968. In 1991, *Acinetobacter* spp. were designated as belonging to the family *Moraxacellaceae,* within the γ-subclass of the Proteobacteria (94).

Acinetobacter was initially classified as a single species genus in 1968, but at present there are 33 species (111). Twenty-three of these have been named; the remaining 10 are classed as genomic species. Genospecies 1(*Acinetobacter calcoaceticus),* genospecies 2 (*A. baumannii),* genospecies 3, and genospecies 13 compose the *A. calcoaceticus- A. baumannii* complex*.* These four species are closely related genotypically, and are phenotypically very difficult to distinguish. The difficulty of accurate speciation has created problems in establishing the epidemiology and true clinical importance of *A. baumannii*. However, accurate speciation is important as clinical significance and antimicrobial susceptibility varies between species (111). In addition, the epidemiology and propensity for spread is also species dependent. Species identification is, however, often difficult for both clinical diagnostic and research purposes. Whilst the majority of acinetobacters are environmental organisms which are not associated with human disease, *A. baumannii* has emerged as a successful pathogen in the 21st century.

1.2 NATURAL HABITATS

Acinetobacter spp. are ubiquitous organisms. They have been isolated from numerous sites, including the soil and water, from which *A. calcoaceticus*, and *Acinetobacter johnsonii* are common isolates (26, 111). Acinetobacter have been found colonising animals (including arthropods) and have even been isolated from food (9, 60). *Acinetobacter lwoffii, Acinetobacter radioresistens* and *A. johnsonii* are commonly found colonising human skin (111). In addition to colonising skin, *A. baumannii* has the ability to colonise indwelling devices including urinary catheters, neurosurgical shunts, and central venous lines (26, 111). In the hospital environment, *A. baumannii* are usually associated with moist environments such as ventilators, humidifiers, and sinks. The natural habitat of *A. baumannii* outside the hospital, however, is yet to be determined (111).

Recovery of acinetobacter from various clinical specimens, for example sputum, urine, or faeces, can represent colonization of the body site, contamination when obtaining the specimen through a colonised opening, or true infection of the site.

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1.3 SPECIES IDENTIFICATION

Acinetobacter spp. are short gram-negative bacilli, often appearing cocco-bacillary on Gram stain (122).They may appear gram-positive when stains are made directly from blood culture bottles (122).

Acinetobacter may be cultivated on several types of media commonly utilised in the diagnostic laboratory. *A. baumannii* is saccharolytic, and may therefore appear as a lactose-fermenter on MacConkey agar (122). The colonies are approximately 0.5-2.0mm in diameter, domed, mucoid, and non-pigmented (122).

The identification of acinetobacter to genus level depends on the following phenotypic traits: a strictly aerobic, non-motile, non spore-forming, non-fermenting gram-negative bacillus, which is catalase-positive, oxidase-negative, with a G+C content of 39 to 47% (82, 122). *A. baumannii* has the following differentiating phenotypic reactions from other non-fermenting gram-negative bacilli: It is saccharolytic (utilises glucose, lactose, and xylose, but not maltose), non-haemolytic, and grows at 42ºC (12, 57). Special chromogenic medium is available for rapid identification of *A. baumannii* within 24 hours (43).

Several automated and semi-automated identification tests have been developed for commercial use. These include the API20E, Vitek2, MicroScan WalkAway, and BD Phoenix. These tests have made identification of bacterial species much easier, quicker and more cost-efficient. However, they have been shown to have a limited database, and tend to group *A. baumannii*, genomic group 3 and genomic group 13TU into one group, identified as *Acinetobacter baumannii* (26, 82).

The problems associated with phenotypic testing led to the development of other tests for identification. The DNA transformation assay of Juni utilises the ability of a strain to

transform *Acinetobacter* strain *BD413 trypE27*, a tryptophan auxotroph, to a wild-type phenotype, as a pre-requisite to belong to the genus *Acinetobacter* (52). Molecular tests include16s rDNA sequence analysis, DNA-DNA hybridization, protein SDS PAGE fingerprinting, amplified fragment length polymorphism (AFLP) fingerprinting, amplified rDNA restriction analysis (ARDRA), and ribotyping (26, 40, 82). Although they are more accurate, they are laborious, require considerable skill and, therefore tend to be restricted to reference or research laboratories.

2. ***PATHOGENESIS AND VIRULENCE FACTORS***

Acinetobacter first began to be recognized as a nosocomial pathogen in the 1970s. Today, *A. baumannii* accounts for 2-10% of gram-negative bacterial infections in Europe, and 2.5% of these infections in the United States (45, 50).

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2.1 INFECTIONS

A. baumannii was previously thought to be a low virulence organism causing infections in predominantly immunocompromised and debilitated patients, but this view is changing. This organism is capable of causing a spectrum of diseases.

Acinetobacter has been implicated in clinical infections in immunosuppressed and critically ill patients in intensive care units worldwide, particularly patients requiring mechanical ventilation, and those with wounds or burn injuries (111). It has also been implicated in community-acquired infections (32, 49, 75). *A. baumannii* was identified as a significant cause of skin and soft-tissue infections in US soldiers during the wars in Iraq and Afghanistan (16). Following the tsunami in South-East Asia in December 2004, 17% of 17 people evacuated to Germany with severe trauma had multidrug-resistant *A. baumannii* isolated from wound swabs, sputum and blood cultures (65).

This organism has been implicated in several types of nosocomial infections. In the Surveillance and Control of Pathogens of Epidemiologic Importance (SCOPE) study conducted in the US in 1995-2002, acinetobacter ranked as the 10th leading cause of bloodstream infections (0.6 bloodstream infections per 10,000 admissions) (123). In the same study, acinetobacter had the third highest crude mortality rate (34%) in ICU patients after *Pseudomonas*(39.2%) and *Candida species* infections(38.7%) (123).

This organism is frequently isolated from patients with ventilator-associated pneumonia, and is associated with a high mortality rate. *Acinetobacter* spp. ranked fourth (6.9%) in prevalence amongst gram-negatives causing pneumonia in ICUs as reported to the National Nosocomial Infection Surveillance system in 2003 (39).

Acinetobacter has also been implicated in secondary meningitis; ventriculitis; urinary tract infections; surgical site infections; chronic ambulatory peritoneal dialysis related-peritonitis, and even endocarditis (10, 14, 48, 54, 73, 80, 82, 91).

Risk factors for nosocomial infection include host factors such as a high APACHE II score, prematurity, malignancy and length of hospitalization (26, 74). Prior antimicrobial

therapy (the use of cephalosporins, carbapenems, and quinolones) are additional risk factors for acquisition of nosocomial infection particularly with multidrug-resistant phenotypes (74). The type of medical intervention e.g. surgery, catheterisation, or mechanical ventilation, as well as environmental factors such as insufficient adherence to infection control guidelines by healthcare workers, also plays a role in acquisition of infection.

Risk factors for community-acquired infection include chronic obstructive airways disease, renal failure, diabetes mellitus, smoking and alcohol abuse (26, 32). Community-acquired pneumonia caused by *A. baumannii* is associated with a with an acute onset of respiratory failure and shock, and a high mortality rate (40-64%) (18, 32).

2.2 VIRULENCE FACTORS

The organism possesses several factors that may contribute to its pathogenicity. These include fimbriae or pili for attachment to cell structures, outer membrane proteins (that may function as a resistance mechanism), and lipopolysaccharide (19, 42). Outer membrane protein A (OmpA) is thought to be responsible for inducing apoptosis in the cells that the bacterium invades (19). In addition, acinetobacter is capable of capsule and biofilm production. In one study, more than 60% of *A. baumannii* from clinical isolates formed biofilm (93). This allows acinetobacter to produce infections associated with indwelling devices, such as urinary and intravenous catheters. Biofilms contribute to drug resistance and provide a means for acinetobacter to evade the immune defences. Further, exopolysaccharide from biofilms has been shown to suppress neutrophil activity and contribute to serum resistance (42).

Siderophores for iron acquisition, which is essential for bacterial growth, have been found in acinetobacter (126). Several extracellular enzymes are known to be produced by this bacterium. These include proteases, gelatinases, esterases, leucine arylamidase and phospholipases (17). A Type IV secretion system and quorum sensing machinery have also been detected among the organism's armamentarium (113). Lipopolysaccharide from the bacterium has been shown to be a potent inducer of the pro-inflammatory cytokine cascade in human monocytes, via Toll-like receptor (TLR)-2 and TLR-4 pathways (30). Acinetobacter is capable of uptake of DNA released by other bacteria in its environment. This transformation may result in the acquisition of drug resistance genes and pathogenicity islands. In addition, some strains of *A. baumannii* have been shown to be resistant to serum killing by complement (55).

3. <u>EPIDEMIOLOGY OF ACINETOBACTER BAUMANNII</u>

The incidence of acinetobacter infections has been increasing, both locally and worldwide. Several outbreaks have been reported in the literature. These have been reported in England, France, Portugal, Spain, Australia, Asia, the US and South Africa (21, 22, 24, 46, 69, 70, 77, 82, 115, 116). Eight worldwide clonal lineages that are associated with epidemic spread throughout the world have been described (46). Strains belonging to European Clone I and II have been implicated in outbreaks since the 1980s (26, 79). A study on the dissemination of the southeast (SE) clone of *A.*

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baumannii containing OXA-23 has revealed that it was the cause of outbreaks in several ICU's in the United Kingdom resulting in closures (22). This clone is usually susceptible only to tigecycline and the polymyxins (64).

Acinetobacter may be transmitted from patient to patient through the hands of healthcare workers, and possibly by airborne transmission (26, 111). Colonized patients are thus the primary reservoir for acinetobacter in the hospital environment. Other possible sources are contamination of surfaces close to the patient or medical equipment (e.g. ventilators) (15, 41). Acinetobacter has been shown to survive on dry surfaces from 3 days up to 5 months (56). The simplicity of this organism's nutritional requirements combined with its ability to survive at different temperatures and pH values, explains its remarkable ability to thrive in a variety of environments (56).

Most nosocomial outbreaks are usually related to a single hospital ward, such as the ICU, and caused by a single or a few clones with a common environmental source usually implicated. These outbreaks are usually resolved once the reservoir is identified and eliminated. Infection control measures to inhibit *A. baumannii* transmission include the use of closed tracheal suction, improved hand decontamination using alcohol gels, strategies to clean equipment and the environment, and the use of nebulised colistin for patients with mild-moderate VAP (33, 92, 112).

Several methods have been developed to determine the genetic relatedness of *A. baumannii* implicated in outbreaks. These include phenotypic testing such as biotyping, resistance phenotyping, serotyping, and phage typing (8). The problems with these methods are that they are not discriminatory enough, and up to 20% of bacteria are not typable by phage typing. DNA-based epidemiologic tools (Table 1) include plasmid profile analysis, ribotyping, random amplified polymorphic DNA (RAPD), repetitive extragenic polymerase chain reaction (REP-PCR), amplified fragment length polymorphism (AFLP), integrase gene PCR, multilocus sequence typing (MLST), electrospray ionisation mass spectrometry(ESI-MS), and pulsed-field gel electrophoresis (11, 28, 82, 101-103, 105, 114). It should be noted that many of these techniques are no longer in the domain of a research laboratory.

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TABLE 1: ***Molecular techniques for epidemiological studies.***

Technique	Advantages	Disadvantages
Plasmid profile analysis	Nil.	Plasmids may be lost/ gained. Cumbersome technique.
Ribotyping	Automated systems available.	Labour intensive, poor discriminatory power.
PFGE	Gold standard, highest discriminatory power.	Labour intensive.
RAPD-PCR	Easy, rapid, low cost.	Reproducibility poor; discrimination inferior to PFGE.
AFLP	High discriminatory power, robust.	Expensive, requires experienced technologist.
MLST	Reproducible, portable.	Expensive, time consuming.
ESI-MS	Rapid, high-throughput, easy.	Expensive, not for routine use.

4. ***TREATMENT OF ACINETOBACTER INFECTIONS***

The treatment of acinetobacter infections has become increasingly difficult as therapeutic options diminish. This has prompted the use of off-label drugs, such as colistin.

Antibiotics that are utilised for the treatment of acinetobacter infections include the anti-pseudomonal penicillins, the anti-pseudomonal cephalosporins, sulbactam, monobactams, aminoglycosides, tetracyclines, quinolones, trimethoprim-sulphonamide, and the carbapenems. Options available for drug resistant isolates include the polymixin, colistin, and the glycylcycline, tigecycline (37). Clinical trials of the use of combination therapy, such as colistin and rifampicin, or a carbapenem and an aminoglycoside, for the treatment of MDR acinetobacter infections have yielded somewhat mixed results (53). However, there are numerous case reports of successful treatment of patients with MDR acinetobacter infections with these combination therapies (6, 37). Aminoglycosides in combination with meropenem or polymixin B are recommended for empirical treatment of acinetobacter meningitis (54). Novel agents such as antimicrobial peptides, enzyme inhibitors, and efflux pump inhibitors are currently under investigation for the treatment of pan drug-resistant isolates.

The administration of an appropriate antibiotic in treating sepsis is a strong predictor of mortality (29, 38, 47, 59). In most cases, antibiotic therapy is initiated prior to identification of the causative organism. Empirical therapy is often inappropriate when treating MDR acinetobacter infections, thus leading to an unfavourable outcome. In a study in Turkey where 51% of the acinetobacters isolated from bloodstream infections were resistant to imipenem, only 14 of 41 patients with *A. baumannii* bacteraemia received appropriate antimicrobial therapy (4). A Canadian study involving 14 ICUs documented that the administration of an appropriate antibiotic in the 1st hour of hypotension was associated with a survival rate of 79.9% (59). However, each hour of delay in administration of an antibiotic over the next 6 hours was associated with a 7.6% decrease in survival (59). In a study conducted in Turkey, inappropriate empirical antibiotic therapy in acinetobacter sepsis was associated with a 65% mortality versus 39.5% for patients who received appropriate therapy (29). Another study in Thailand showed that patients with a pan drug- resistant acinetobacter infection had an 80% mortality versus 14% of controls infected with a drug-sensitive acinetobacter (5). Interestingly, only 75 % (42/56) of these patients received appropriate empirical therapy (5). However, the literature is conflicting as some studies have found a poor correlation between patient mortality and inappropriate empirical antibiotic choice (23, 72). The lack of consensus on the contribution of acinetobacter to patient mortality may be due to multiple confounders in these studies. These include study population, study design, the distinction between infection and colonisation in critically ill patients, and lack of information about virulence factors (31). Although the evidence for the impact on mortality has not been established, several studies have shown an increase in patient hospital stay, a requirement for mechanical ventilation and increased treatment costs (31). In general, the outcome for patients infected and colonised with *A. baumannii* seems to be poor.

5. ***MECHANISMS OF ANTIBIOTIC RESISTANCE***

In 1946, Alexander Fleming stated, "*There is probably no chemotherapeutic drug to which in suitable circumstances, the bacteria cannot react by in some way acquiring 'fastness' (resistance)*" (3). This statement still holds true today, particularly with reference to *A. baumannii*. This organism was susceptible to most antibiotics in the 1970s, but has now acquired a remarkable propensity to rapidly gain resistance to most antibiotics (111).

Susceptibility studies carried out in the early 1970s revealed that acinetobacter was susceptible to most commonly used antimicrobials, including ampicillin (60-70% isolates), gentamicin (92.5%), chloramphenicol (57%), and nalidixic acid (97.8%) (7). Thirty years later, *A. baumannii* has emerged as a multidrug resistant organism.

Antibiotic resistance in *A. baumannii* is diverse, and includes target gene mutation, enzymatic modification of the antibiotic, altered membrane permeability, and unregulated efflux pump activity (Figure 1). The efflux systems involve protein transporters that function to reduce the concentration of an antibiotic by transporting them across the bacterial cell membrane into the external environment.

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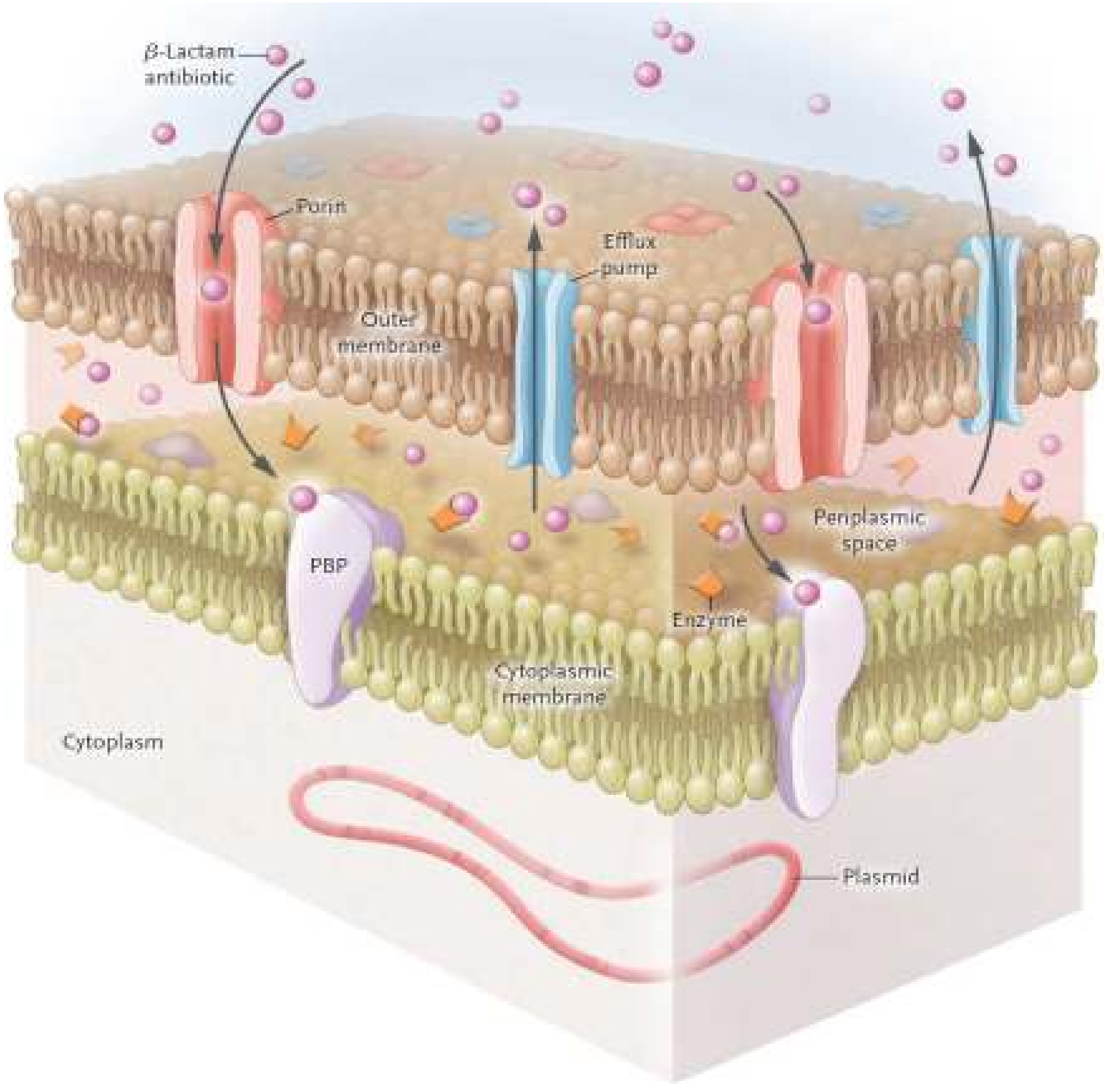

FIGURE 1*: Diagram of the cell wall of A. baumannii.*
Adapted from: Munoz-Price, LS, Weinstein, RA. *Acinetobacter* Infection. NEJM. 2008; 358: 1271-1281.

In a survey by the Centers for Disease Control (CDC) involving 300 hospitals in the US, carbapenem resistance in *A. baumannii* increased from 9% in 1995 to 40% in 2004 (75). In the UK, resistance to carbapenems rose from <0.5% in the 1990s to 24% in 2007 (111). Statistics from South Africa revealed that at Groote Schuur Hospital, carbapenem resistance increased from 43-45% in 2006 to 75-78% in 2008 for *A. baumannii* isolates cultured from blood (*unpublished, National Antibiotic Surveillance Forum, NHLS public sector susceptibility data, personal communication with Dr Colleen Bamford*).

5.1 INTRINSIC RESISTANCE

Acinetobacter has a cell membrane that is significantly less permeable than other Gram-negative bacteria. Sato and Nakae demonstrated that the permeability co-efficients of zwitterionic cephalosporins in the intact cell outer membrane of *A. calcoaceticus* was two to seven times lower than the permeability coefficients of *Pseudomonas aeruginosa* (97). They also demonstrated that the diffusion rates of carbapenems and zwitterionic cephalosporins appeared to be 1-3% that of *Escherichia coli* membrane (97). These authors postulated that this was due to the small number of small-sized porins in the acinetobacter cell membrane (97).

Other mechanisms of intrinsic resistance include outer membrane proteins, such as loss of CarO which is associated with carbapenem resistance, and the intrinsic production of oxacillinase, OXA-51 (76).

5.2 ACQUIRED RESISTANCE

The most common mechanism to explain the rapid gain in resistance of *A. baumannii* is the acquisition of plasmids, transposons and integrons, which carry clusters of resistance genes resulting in simultaneous resistance to several antibacterials (3). Large genomic islands containing multiple antibiotic resistance genes have been identified in acinetobacter; for example, *A. baumannii* AYE, a strain epidemic in France, contains a 86kb resistance island comprising 45 resistance genes inserted in its chromosome (34). Some genes isolated in this resistance island mediate resistance to beta-lactams, streptomycin, aminoglycosides, trimethoprim-sulphonamide, chloramphenicol, tetracyclines, and rifampicin (34). A recent study revealed that MDR *A. baumannii* can possess at least eight resistance determinants that give rise to its MDR phenotype (68).

5.2.1 β-LACTAMS

Resistance to the penicillins is mediated by chromosomal beta-lactamases (ampC). Cephalosporin resistance may be mediated by ampC overexpression through introduction of the insertion sequence IS*Aba1* upstream of bla_{AmpC}, oxacillinases, changes in penicillin binding proteins (PBPs), porin loss and efflux pumps (82, 85, 95, 100). Extended spectrum beta-lactamases (ESBLs) such as PER-1, PER-2, VEB, and CTX-M, have also been isolated in *A. baumannii* (81, 83, 86). Carbapenemases which afford protection against the broad-spectrum carbapenems are also part of *A. baumannii's* armamentarium. OXA-23, an oxacillinase with carbapenemase activity, has now been identified in more than 50 different centres in the UK (Table 2) (63). Other carbapenemases isolated from *A.baumannii* include OXA-24, OXA-58, and IMP/VIM (85). The expression of these carbapenemases are dependent on promoter sequences within the insertion sequence, IS*aba1* (98).

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TABLE 2: ***Oxacillinases isolated from A. baumannii.***

Enzyme	**Geographical Distribution**	**Location**	**Associated IS elements**
OXA-23 Cluster	Europe, Australia,China, Korea, United States, Vietnam, Brazil, Pakistan	Plasmid or chromosomal	IS*Aba1, ISAba4*
OXA-24 Cluster	Spain, Belgium, France, Portugal, United States	Chromosomal or plasmid	None.
OXA-58 Cluster	France, Spain, UK, Australia, Italy, United States, Greece Pakistan, Romania	Plasmid or chromosomal	IS*Aba1*, IS*Aba2*, IS*Aba3*, IS *18*
OXA-51 Cluster	Naturally occurring, therefore found worldwide.	Chromosomal	IS*Aba1*

Adapted from: Peleg AY, Seifert, H, Paterson, DL. *Acinetobacter baumannii*: Emergence of a Successful Pathogen. Clinical Microbiology Reviews. 2008 (July); 21(3): 538-582.

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Oxacillinase-23, OXA-64 and OXA-71 have been isolated from *A. baumannii* strains from South Africa (84, 99).

Resistance to carbapenems may also be mediated by other mechanisms such as porin losses or modifications, changes in outer membrane proteins, or rarely changes in PBPs (25, 87).

5.2.2 QUINOLONES

Quinolone resistance is mediated mainly by mutations in *GyrA* and/or *ParC* which result in alteration of the antibiotic target, the topoisomerases (44, 117, 118). Efflux pumps and porin losses are additional mechanisms that *A. baumannii* employs to defend itself against the quinolones. The AdeM efflux pump, a member of the MATE family, confers resistance to norfloxacin, ofloxacin, and ciprofloxacin (107).

5.2.3 TETRACYCLINES AND GLYCYLCYCLINES

Tetracycline resistance is mediated through several efflux pumps. These include TetA, TetB, TetC, TetD and TetE which afford resistance to the tetracyclines and CmlA which affords resistance to chloramphenicol (3, 89). These efflux pumps are part of the major facilitator superfamily (MFS) group. The AdeABC efflux pump, which is a member of the Resistance Nodulation Division (RND) group, confers resistance to the tetracyclines, chloramphenicol, β-lactams, aminoglycosides, erythromycin, and reduced susceptibility to fluoroquinolones (96). Ribosomal protection by proteins TetM and TetO are another resistance mechanism to tetracyclines (90).

Although tigecycline has only been recently introduced as a novel antibiotic, resistance to it has been documented amongst *A. baumannii* strains (78, 88). The major mechanism of resistance is an efflux pump, the AdeABC efflux pump (96).

5.2.4 POLYMIXINS

Postulated mechanisms of resistance to colistin include mutations that modify the lipopolysaccharide that make the cell membrane more resistant to the detergent action of this group of antibiotics, proteolytic cleavage of the drug, and activation of a broad-spectrum efflux pump (37).

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5.2.5 AMINOGLYCOSIDES

The aminoglycoside group of antibiotics include gentamicin, amikacin, tobramycin (Figure 2), netilmicin, kanamycin and streptomycin. These antibiotics are hydrophilic molecules, consisting of a central aminocyclitol ring linked to one or more amino sugars by pseudoglycosidic bond(s) (66). This group of antibiotics bind specifically to 16S RNA in the 30S ribosomal subunit of the bacterium and inhibit protein synthesis (66). This results in mistranslated proteins that have a fatal secondary effect on the bacterium (66).

FIGURE 2: ***Chemical structure of tobramycin sulphate.***

Downloaded from: www.chemicalbook,com/ChemicalPropertyProduct_EN_CB6678801.htm.

The aminoglycosides have been used as monotherapy or in combination with other antibiotics for the treatment of acinetobacter infections. Many MDR *A. baumannii* retain susceptibility to amikacin or tobramycin. In a global survey (2001-2004), 60% of *Acinetobacter* spp. remained susceptible to amikacin (36). In a study performed in 2007 in private healthcare facilities in South Africa, tobramycin was the most active aminoglycoside against *A. baumannii (*81% susceptible) (13).

Resistance to the aminoglycosides is usually due to decreased affinity of the drug for its target, the bacterial ribosome, either by modification of the drug or the ribosome. The most common mechanism amongst these mechanisms is the enzymatic inactivation of the drug, usually by the production of aminoglycoside-modifying enzymes (AMEs) encoded by genes frequently located on transferable elements (66). These enzymes are proteins that N-acetylate(acetyltransferases), phosphorylate (phosphotransferases), or adenylate (nucleotidyltransferases) the aminoglycoside, thus rendering the antibiotic inactive (Figure 3) (66). Some strains of acinetobacter contain several aminoglycoside resistance genes. In addition, many of these AMEs are found on genetic elements that are associated with additional resistance determinants. Previous research on isolated strains of *A. baumannii* from Cape Town have shown the presence of the AAC(3')-I and ANT(3'')-I enzymes(104).

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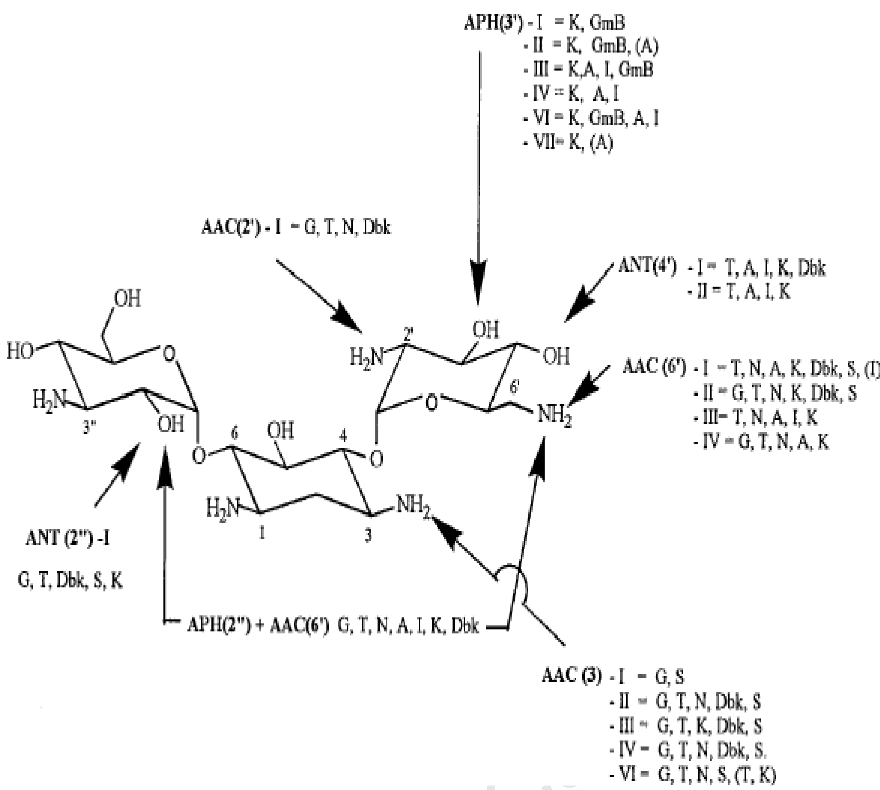

FIGURE 3: ***Major aminoglycoside-modifying enzymes target sites on kanamycin B.***

Kanamycin B is susceptible to the largest number of enzymes. Each group of enzymes inactivates specific sites, but each of these sites is susceptible to distinct isoenzymes (Roman numerals) with different substrate specificities. The main clinically used aminoglycosides on which these enzymes act are as follows: amikacin (A), dibekacin (Dbk), gentamicin (G), gentamicin B (GmB), kanamycin A (K), isepamicin (I), netilmicin (N), sisomicin (S),and tobramycin (T).

Adapted from Mingeot-Leclercq, M-P, Glupczynski, Y, Tulkens, PM. Aminoglycosides: Activity and Resistance. Antimicrobial Agents and Chemotherapy. April 1999, 43(4): 727-737.

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TABLE 3: ***Important aminoglycoside-modifying enzymes and the antibiotics modified.***

ENZYME	AMINOGLYCOSIDE MODIFIED	TYPE OF ENZYME/ LOCATION	REFERENCE
AAC(3)-Ia	Gentamicin	Acetyltransferase Class 1 integron	(104)
AAC(3)-IIa	Gentamicin, tobramycin	Acetyltransferase	(104)
AAC(6')-Ib	Tobramycin, amikacin	Acetyltransferase Class 1 Integron	(104)
AAC(6')-Ih, AAC(6')-Iad	Tobramycin, amikacin	Acetyltransferase Plasmid	(61)
APH(3')-Ia	Kanamycin	Phosphotransferase	(104)
APH(3')-VI	Amikacin, kanamycin	Phosphotransferase Mostly plasmid	(62)
ANT(2")-Ia	Gentamicin, tobramycin	Nucleotidyltransferase	(104)
ANT(3')-Ia	Streptomycin	Nucleotidyltransferase	(104)

The site of modification is indicated in parentheses. A Roman numeral and a letter are used to indicate the pattern of resistance that they confer and to their primary sequence, respectively.

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Other mechanisms of resistance to aminoglycosides include efflux pumps (AdeABC and AbeM) (67, 71). More recently, the production of 16S rRNA methylases which are capable of modifying the 16S RNA molecule have been described as a mechanism of high level resistance to the clinically important aminoglycosides (27, 66, 125).

6. ANTIBIOTIC SUSCEPTIBILITY TESTING IN THE CLINICAL MICROBIOLOGY LABORATORY

Drug susceptibility testing is performed because it serves to guide choice of antimicrobial therapy. In addition, it serves as a predictor of patient outcome, as well as detection of some resistance markers. Susceptibility testing may be performed using a variety of validated methods: disk diffusion testing (Kirby-Bauer method), Epsilometer test, agar dilution and broth microdilution. Often diagnostic laboratories are under pressure from clinicians and hospital administrators to provide rapid and accurate bacterial identification and susceptibility results. To accomplish this, many laboratories have adopted the use of semi-automated and automated testing that have been designed to reduce laboratory turn-around time, increase efficiency and improve cost-effectiveness (106).

Problems have, however, been detected in susceptibility testing of *A. baumannii*. A high level of major errors were detected in tetracycline testing when disc diffusion was compared to broth microdilution (108). In the same study, beta-lactam drugs posed a problem when interpreted by broth microdilution testing (108). Problems have also been reported with susceptibility testing of colistin (35).

Several studies have shown drawbacks of automated systems, particularly with the identification and susceptibility testing of nonfermenting gram-negatives (58, 110, 119-121). The testing of aminoglycosides using automated susceptibility tests has revealed varying results. In a study comparing the Phoenix System with the MicroScan Walkabout in terms of automated susceptibility of nonfermenting gram-negatives, there were 8.6% minor errors associated with amikacin, 14.2% minor errors associated with gentamicin, and 5.7% minor errors associated with tobramycin testing (106). However, a problem with this study was that disc diffusion, not broth microdilution, was used to resolve discrepant results (106). A recent study revealed susceptibility testing using the Vitek®2 showed false susceptibility to amikacin in *A. baumannii* harbouring the *armA* gene, which confers resistance to amikacin (51). Another recent study found that the Vitek®2 incorrectly reported a third of the isolates as falsely susceptible to amikacin (2).

Errors reported by these systems can have serious consequences for the clinical outcome of patients. The accuracy of these tests becomes paramount as available treatments diminish. The results may encourage treatment with inactive agents, or falsely restrict therapeutic options.

CONCLUSION

This study was proposed to inform the diagnostic laboratory on whether the Vitek®2 automated system is an accurate means of testing for tobramycin susceptibility in *A. baumannii,* particularly due to the paucity of published information about tobramycin susceptibility testing, and as tobramycin is frequently used to treat patients at our institution. In addition, an assessment of the accuracy of the various methods in determining sensitivity is critical as inappropriate antimicrobial therapy is associated with increased morbidity and mortality.

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Manuscript

Part C

***Acinetobacter baumannii*: An evaluation of five susceptibility test methods to detect tobramycin resistance in an epidemiologically related cluster.**

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Abstract

***Acinetobacter baumannii* is a major pathogen causing nosocomial infections, particularly in critically ill patients. This organism has acquired the propensity to rapidly develop resistance to most antibiotics. At several hospitals within Cape Town, tobramycin and colistin remain frequently the only therapeutic options. The Vitek®2 automated susceptibility testing (AST) is used in the clinical laboratory to determine selected susceptibility profiles. The suspicion of a possible AST-related technical error when**

testing for susceptibility to tobramycin in *A. baumannii* precipitated this study. Thirty-nine *A. baumannii* strains isolated from clinical specimens (June-December 2006) which exhibited MICs close to the tobramycin breakpoints were included in this prospective study. Tobramycin susceptibility testing by AST, disc diffusion, Epsilometer test and agar dilution was compared to broth microdilution (BMD), the reference method. Additionally, PCR was performed to detect the *aac(3)-IIa* gene which encodes an aminoglycoside modifying enzyme with activity against tobramycin. The tobramycin susceptibility results revealed errors in 25/39 isolates (10 very major and 15 minor errors) when AST was compared to BMD ($p<0.001$), 12/39 (1 very major and 11 minor errors) when Etest was compared to BMD, and 15 errors (3 very major and 12 minor errors) when disc diffusion was compared to BMD. Additionally, the tobramycin resistance gene, *aac(3)-IIa* was detected in 21/25 of the discrepant isolates, confirming the resistant phenotype detected by the reference method. Molecular typing showed that these isolates were genetically related. Clinical laboratories using the Vitek®2 system for routine use should consider an alternative susceptibility testing method to determine susceptibility to tobramycin.

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Acinetobacter baumannii has gained increased notoriety as a highly-resistant nosocomial pathogen globally. This organism has been associated with infections in immunocompromised and debilitated patients, particularly in the intensive care (ICU) setting (2, 14).

This organism has proven to be a formidable adversary. Acinetobacter has the capacity for long-term survival in the hospital environment (4, 9). In addition, its remarkable capacity to acquire resistance has prompted its classification as a high priority pathogen by the Antimicrobial Availability Task Force of the Infectious Diseases Society of America (13). Pan drug-resistant phenotypes have been isolated in many settings (4, 5, 25). *A. baumannii* is endemic in some hospitals' ICUs in the Western Cape, South Africa. The majority of these are multidrug-resistant organisms, retaining susceptibility only to tobramycin and colistin. Susceptibility rates for *A. baumannii* isolated from blood cultures in 2006 at our diagnostic laboratory revealed that only 55% and 57% remained susceptible to imipenem and meropenem, respectively.

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The pressure on clinical diagnostic laboratories to produce rapid identification and susceptibility profiles has resulted in increasing use of automated microbiology systems, such as the Vitek®2 (bioMérieux). Although there are many advantages to the use of this technology, several studies have indicated inaccurate results especially when testing non-fermenting gram-negatives, such as *A. baumannii* (12, 24, 26-28). These inaccuracies have a major impact on patient management as they may encourage the use of inactive antimicrobials in critically ill patients. In addition, they promote the use of broader spectrum antibiotics if narrow spectrum drugs are falsely reported as resistant. Inaccuracies with AST have been reported in the literature regarding aminoglycoside testing (1, 7). A recent study found that up to 1/3rd of *A.*

baumannii isolates (n=107) tested were incorrectly reported as susceptible to amikacin by the Vitek®2 instrument (1).

A discrepancy between tobramycin susceptibility testing using manual methods and the Vitek®2 automated susceptibility test method alerted the clinical diagnostic laboratory to a possible technical error, thus precipitating this study. A prospective study of *A. baumannii* to investigate the accuracy of tobramycin susceptibility testing in comparison to validated susceptibility test methods was conducted. In addition, we hypothesised that the resistance was due to the aminoglycoside modifying enzyme, AAC(3)-IIa (aminoglycoside acetyltransferase), as the gene encoding this enzyme was previously isolated from clinical isolates of *A. baumannii* at our institution (3). Pulse-field gel electrophoresis and rep-PCR using the Diversilab was also performed to compare the clonal relatedness of these isolates.

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MATERIALS AND METHODS

The study was conducted in 4 phases. In phases I and II, the identification was performed, and AST results were compared to the reference broth microdilution. In phase III, the tobramycin resistance genotype was investigated. Finally, in phase IV, the molecular epidemiology of the strains was determined.

Bacterial strains. A total of 39 nonrecurring randomly collected clinical isolates of *A. baumannii* were tested. These isolates were obtained from 5 hospitals in Cape Town, South Africa (Groote Schuur Hospital, Red Cross Children's Hospital, Victoria Hospital, Mowbray Maternity Hospital, and G.F. Jooste Hospital) over a 7 month period (June 2006-December 2006). Most of the isolates were obtained from various clinical specimens from patients in intensive care units, the majority being tracheal aspirates. The isolates selected had zone diameters or MICs close to the tobramycin breakpoints, as defined by the Clinical and Laboratory Standards Institute (CLSI). Appropriate quality control organisms (ATCC strains) were used for all susceptibility testing as per CLSI or manufacturer's recommendations. The isolates were stored on beads (Viabank VIM tubes, Abtek Biologicals Ltd), and were passaged twice on 2% blood agar plates (Greenpoint Media Laboratory, NHLS).

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Identification. All strains were identified twice by the Vitek®2 Gram-Negative Identification card (bioMérieux, LaBalme, France) according to manufacturer's instructions at 0.6, and 1.0 McFarland. A percentage probability above 90% was considered an acceptable identification.

Susceptibility test methods. The susceptibilities of all isolates were tested by disc diffusion (Oxoid, Basingstoke) and Etests (AB Biodisk, Solna, Sweden) using cation-adjusted Mueller-Hinton medium from three commercial manufacturers [bioMérieux (Randburg, South Africa), Bio-Rad Laboratories (Johannesburg, South Africa), and Greenpoint NHLS Media Laboratory (Cape Town, South Africa)] according to CLSI and manufacturer's guidelines, respectively. These tests were performed with inocula from the same subculture. In addition, automated susceptibility using the Vitek®2 NO-22 susceptibility card (bioMérieux) was performed twice, at 0.6 and 1.0 McFarland, respectively. The raw MICs obtained using the Vitek®2 was used for

analysis. Agar dilution and broth microdilution were performed on the isolates in duplicate by two different scientists at the Research and Development Division of bioMérieux (LaBalme, France) using cation-adjusted Mueller-Hinton medium. Results were interpreted using CLSI criteria. Discordant results with the reference method (BMD) were classified as very major errors, major errors, or minor errors.

Quality control organisms included *Pseudomonas aeruginosa* ATCC27853 and *Escherichia coli* ATCC 25922 as recommended by CLSI and the manufacturer.

PCR for detection of *aac(3)-IIa*. Genomic DNA from each isolate was extracted using the EasyMag (bioMérieux, Durham, North Carolina) as per manufacturer's instructions. The quantity and quality of DNA extracted was confirmed using the Nanodrop® ND-1000 spectrophotometer (ranged between 8-78ng/μL). Primers (F: cgc gga agg caa taa c, R: gct tct caa gat agg tg) from previously published sequences were used (3). *A. baumannii* strains, previously isolated at our laboratory and designated MOS-1 and MOS-2, were used as positive and negative controls, respectively.(3)

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The mastermix consisted of magnesium chloride (25 mM), 2.5 mM of each dNTP, forward and reverse primers (20 pmoles each), and 2.5 U *Taq* polymerase in buffer made up to a final volume of 50 μL per reaction. An initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of 95 °C for 1 minute, 51 °C for 45 seconds, 72 °C for 1 minute, and a final extension at 72 °C for 5 minutes was carried out. The final reaction products were separated on a 2 % agarose gel by gel electrophoresis, stained with ethidium bromide, and visualised under UV

light. The amplicon sizes were estimated using a 1 kb Plus DNA molecular weight marker (Invitrogen Life Science). A positive, negative, extraction control and water blank were included for each gel. PCR was repeated for all negative isolates.

The amplicons from two isolates were sequenced by the DNA Sequencing Facility at the University of Stellenbosch, Cape Town, South Africa (ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit using ABi genetic analysers) and blasted on Genbank to determine their nucleic acid similarity.

Pulsed Field Gel Electrophoresis. The relatedness of the isolates was compared by pulsed-field gel electrophoresis (PFGE) by an in-house protocol with minor modifications (3). Following plug preparation and cell lysis, the plugs were digested with Apa-1 (Roche) for two hours. The plugs were then separated on a 1.5 % PFGE agarose gel (BioRad) in 0.5X Tris buffer. The electrophoresis was carried out in a Gene Navigator® PFGE machine (Amersham Biosciences AB, Uppsala, Sweden) for 23 hours with the pulse time increasing from 5-45 s, after which it was stained with ethidium bromide, destained, and photographed using a Fotodyne Inc. UV light box and a Kodak EDAS 290 camera . The fingerprint images were analysed by Gel Compare II software Version 4.6 (Applied Maths, Sint-Martens-Latem, Belgium) using dice similarity index for cluster analysis and the unweighted pair group average (UPGMA) for tree building. All isolates with PFGE banding patterns with >87% similarity were grouped within the same cluster (11). Banding patterns were compared with 1.5% optimisation and 1.5% band position tolerance (11).

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In addition, rep-PCR using the Diversilab (bioMérieux) was performed by the R&D Division, bioMérieux, La Balme, France.

Statistical methods. The χ^2 test was used to examine the association of the various susceptibility testing methods and the reference method. In addition, the degree of correlation between the test method and the reference method was determined using unweighted kappa. Statistical tests were performed using an online statistical calculator (http://faculty.vassar.edu/lowry/vassarstats.html). A p-value <0.05 was considered statistically significant.

Ethics. This research received approval from the University of Cape Town, Health Sciences Faculty, Research Ethics Committee.

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RESULTS and DISCUSSION

Characterisation of clinical isolates. All 39 isolates were identified twice by the Vitek® 2 instrument as *A. baumannii* with a high percentage probability(99%). The identifications were also subsequently confirmed by API phenotype testing performed by the R&D Division, bioMérieux, La Balme, France.

Tobramycin susceptibility and error rates. The susceptibilities of the isolates were determined by multiple manual and automated methods. The interpretative category errors that were used are defined as follows: very major error ((isolate susceptible by reference method is resistant by test method), and minor error (difference between reference method and test method differs by 1 interpretative category). An overall category agreement error rate of less than 10 % was considered an acceptable performance of a susceptibility test method, which included ≤ 1.5 % very major errors and ≤ 3.0 % major errors (10).

The level of categorical agreement between the different methods and the reference method (BMD) varied widely and is shown in Table 1. A statistically significant number of very major (10 isolates) and minor errors (15 isolates) were observed when Vitek®2 AST was compared to BMD ($p<0.001$). Irrespective of the testing method, no major errors were detected (Figure 1). A recent study at the San Antonio Military Medical Centre, Texas, United States, revealed errors with manual and automated susceptibility testing of tobramycin (1). Very major errors were detected in 13.1% of isolates tested by Vitek®2, as compared to 2.8 % VMEs when disc diffusion and E-tests were compared to BMD. The authors also noted VMEs with tobramycin susceptibility testing using other automated systems as well. Our study found a higher level of VMES, with 25.6 % detected in isolates

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tested by the Vitek®2. The use of a higher inoculum when performing AST did not have an effect on the error rates. Although errors occurred with manual testing methods as well, we showed that the Etest correlated the best with the reference method (k 0.5169) (Table 2).

Several studies have been conducted to evaluate automated methods for the detection of aminoglycoside resistance amongst non-fermenters, but few of these have looked at *A. baumannii* in particular. Most studies have detected problems with other aminoglycosides, such as amikacin (1, 5). The manufacturers of the Vitek®2 and other automated systems either suggest non-reporting or confirmation of the susceptibility result by manual methods in these cases.

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Detection of *aac(3)-IIa*. Previous research at our institution had established that the prevalent mechanism of tobramycin resistance amongst *A. baumannii* isolated at the clinical diagnostic laboratory was an aminoglycoside modifying enzyme encoded by the *aac(3-)IIa* gene (8). Based on this information, PCR for the *aac(3-)IIa* gene was performed on the 39 isolates (Figure 2). Presence of the AAC(3)-IIa enzyme was inferred by demonstration of the gene. All isolates with a negative PCR result had a repeat PCR performed. In addition, a 16sRNA PCR was performed on these isolates to confirm the integrity of bacterial DNA.

Twenty-one of the twenty-five isolates that demonstrated discordant results (i.e. either sensitive or intermediate by Vitek, but resistant by BMD) were found to contain the *aac(3)-IIa* gene (Table 1).This confirmed a possible mechanism of resistance to tobramycin. This is not surprising as a worldwide study (which included isolates from South Africa) has shown that the AAC(3)-II enzyme was the commonest aminoglycoside modifying enzyme (AME) present, accounting for resistance to aminoglycosides in 50% of isolates tested(8).

Numerous aminoglycoside-modifying enzymes have been isolated from *A. baumannii.* The *aac(3)-IIa* gene confers resistance to gentamicin, tobramycin, dibekacin, netilmicin, and sisomicin. This resistance profile is common amongst the *Enterobacteriaceae.* The AAC(3)-IIa enzyme is the commonest resistance mechanism in the group exhibiting this resistance profile, accounting for 84.8% of isolates (12). The *aac(3)-IIa* gene has been detected in 21.3% of *Acinetobacter* spp. (12).

Another study concluded that AME genotype was an inadequate predictor of the aminoglycoside phenotype, suggesting that multiple resistance mechanisms were operating simultaneously (1). Although this may be true, our study showed a good correlation between the presence of the *aac(3)-IIa* gene and tobramycin resistance, with the gene being present in 12/19 (63 %) isolates with tobramycin MICs ≥16.

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Four isolates that were resistant to tobramycin by BMD lacked the *aac(3)-IIa* gene. These isolates likely have other mechanisms of resistance to tobramycin, such as AAC-6' or ANT-2", combinations of AMEs, efflux pumps or other resistance mechanisms that were not explored further as this was not the focus of this study. Importantly, though, the failure of the Vitek2 to detect these resistant isolates suggests a wider failure of the system to detect tobramycin resistance.

Alarmingly, another study showed that susceptibility to tobramycin was retained in the presence of a potentially inactivating AME gene in 8 (21.6 %) of isolates (1). We found that just a single isolate that was susceptible by all test methods harboured the *aac(3)-IIa* gene.

Amplicons from two isolates (17 and 28) were sequenced and blasted. A comparison of the sequencing data showed that they were 100% identical to the corresponding sequence of the *aac(3)-IIa* gene. In addition, all the AAC(3) enzymes that confer resistance to tobramycin have a conserved threonine residue at position 74 (12). Conversion of the nucleotide sequences to amino acid residues confirmed that the isolates selected had a threonine residue at position 74.

Our hypothesis to explain the discrepant Vitek® 2 results is that the catalytic activity of the AAC(3)-IIa enzyme is too slow to be detected by rapid automated susceptibility test methods, particularly with a poor substrate like tobramycin (6). Whether this has implications on the clinical outcome of patients treated with tobramycin has yet to be determined.

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Relatedness of isolates. The majority of clinical strains were isolated from various intensive care units from 4 different hospitals. The isolates were recovered from patients aged 4 days to 63 years (mean 29.37 years), with an equal male to female ratio (1.06:1). The isolates were recovered from a variety of clinical specimens, which included blood cultures (*n*=4), tracheal aspirates /sputum (*n*=21), pus swabs (*n*=7), urine (*n*=5), and tissue/fluid cultures (*n*=3).

The relatedness of the isolates was determined by rep-PCR using the Diversilab (bioMérieux) system. The isolates clustered into 4 clones (Figure 3), with the majority of isolates clustering in Cluster C. The results of the Diversilab confirmed the presence of a predominant clone that was not confined to a single hospital (Table 3). In addition, isolates 3, 4 and 11 (which lacked the AAC(3')-IIa enzyme but were resistant to tobramycin), were

scattered across clusters C and D, confirming that tobramycin resistance was not linked to a single clone at our institution.

Pulsed field gel electrophoresis was performed to compare a representative of the two commonest clones (C and A) with the dominant strains that had previously been isolated at our institution (Figure 4). The MOS-1 and MOS-2 strains were isolated from the same patient during the same period of hospitalisation at Groote Schuur Hospital (3). Isolate C2 was highly similar to MOS-1, a tobramycin resistant strain with the *aac(3)-IIa* gene, that had been present in Cape Town since the early 1980s (3). Although it might have been expected that the tobramycin resistant strains had recently emerged due to the increased use of tobramycin at our hospitals, PFGE suggests that is unlikely.

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CONCLUSION

According to our knowledge, this is the first study in South Africa that has assessed the ability of the VITEK®2 to detect *A. baumannii* isolates with reduced susceptibility to tobramycin. Tobramycin, tigecycline and colistin remain the only antibiotics available in the public health sector that are effective against the multidrug resistant strains of *A. baumannii* in the Western Cape, South Africa. Thus, accurate susceptibility testing remains critical.

The data from our study confirms the limitations of both automated and manual tobramycin susceptibility test methods. In conclusion, the VITEK® 2 appears to be unreliable for the detection of tobramycin resistance in *A. baumannii*. It appears that manual methods, such as E-tests, may be more reliable for susceptibility testing when tobramycin is considered as a potential therapeutic agent.

Acknowledgements. This work was supported by BioMerieux and Omnimed. The authors wish to acknowledge Gilles Zambardi and the Research and Development Division, bioMérieux, LaBalme, France for assistance with performing the agar and broth microdilution testing, and the Diversilab rep-PCR.

Conflict of interest. The authors declare no conflict of interest.

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TABLE 1. Tobramycin susceptibility testing results of the 39 isolates using different methods.

Isolate	Disc Diffusion (mm)	Etest (MIC in μg/ml)	Agar dilution (MIC μg/ml)	Vitek 0.6McF (MIC μg/ml)	Broth Microdilution (MIC μg/ml)	*aac(3)-IIa* gene
7	0mm	256	128	>=16	>256	Absent
40	4mm	8	4	4	>256	Present
3	3mm	32	16	8	256	Absent
4	10mm	16	8	8	256	Absent
27	0mm	64	32	>=16	256	Absent
31	12mm	16	16	4	256	Present
18	13mm	16	4	8	128	Present
36	13mm	8	16	4	128	Present
38	0mm	32	16	>=16	128	Absent
10	13mm	16	4	8	64	Present
5	11mm	8	8	8	32	Present
6	14mm	4	2	2	32	Present
11	13mm	16	4	2	32	Absent
12	9mm	32	8	8	32	Present
13	11mm	32	4	4	32	Present
17	13mm	16	2	4	16	Present
22	16mm	8	2	<=1	16	Present
23	21mm	2	<=0.5	<=1	16	Absent
39	15mm	8	2	2	16	Present
8	13mm	8	4	4	8	Present
9	15mm	8	2	2	8	Present
14	14mm	8	4	2	8	Present
15	15mm	8	4	4	8	Present
20	15mm	4	2	<=1	8	Present
26	0mm	64	2	>=16	8	Present
32	17mm	4	2	<=1	8	Present
33	10mm	16	8	4	8	Present
42	14mm	8	4	2	8	Present
29	14mm	8	2	2	4	Absent
24	22mm	2	<=0.5	<=1	2	Absent
35	21mm	2	<=0.5	<=1	2	Absent
2	19mm	2	<=0.5	<=1	1	Absent
16	23mm	2	<=0.5	<=1	1	Absent
21	15mm	2	<=0.5	<=1	1	Absent
28	23mm	1	<=0.5	<=1	1	Present
30	20mm	2	<=0.5	<=1	1	Absent
34	23mm	1	<=0.5	<=1	1	Absent
37	21mm	2	<=0.5	<=1	1	Absent
41	21mm	2	<=0.5	<=1	1	Absent

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KEY:

Red-indicates resistant susceptibility results , blue-intermediate, green-susceptible.

The blocks highlighted in yellow indicate isolates with discrepant results with confirmed presence of *aac(3)-11a* gene.

FIGURE 1. Graph of absolute number of errors detected when each susceptibility test method was compared to BMD.

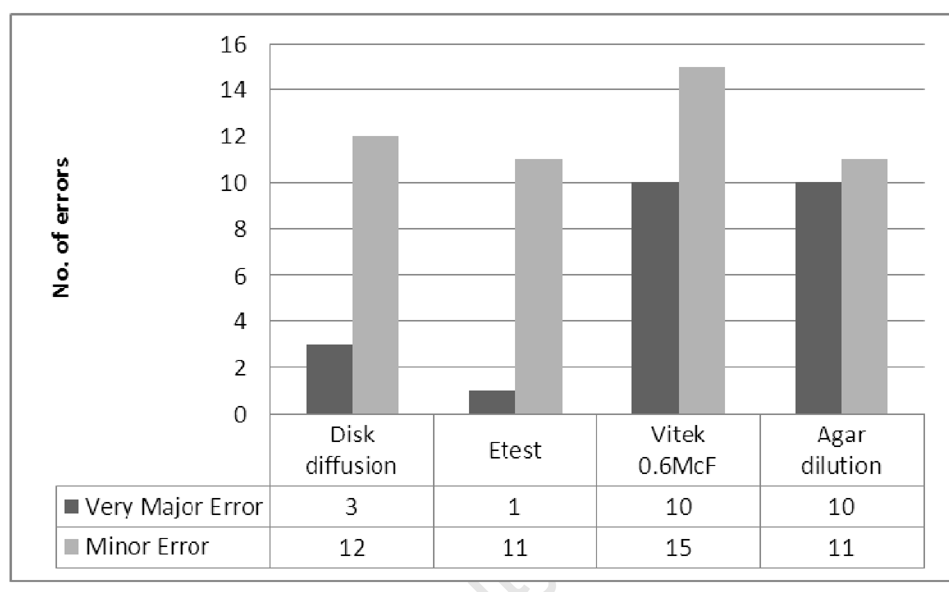

TABLE 2. Statistical analysis of the susceptibility test results.

	Chi-squared	P-value	Unweighted Kappa	Confidence level(k)
Vitek vs BMD	18.48	<0.0001	0.0905	0-0.3041
Agar vs BMD	16.78	0.0002	0.2215	0-0.4477
DD vs BMD	2.92	0.2322	0.3858	0.1547-0.6169
Etests vs BMD	1.32	0.5169	0.5329	0.313-0.7528

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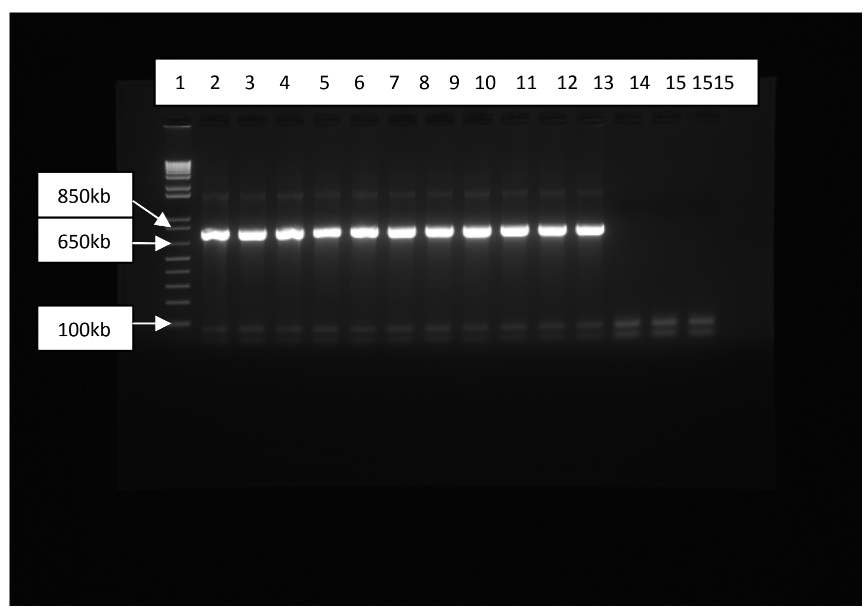

FIGURE 2. Gel electrophoresis for the detection of the presence of amplicons for specimens 21-30.

Lane 1: Molecular weight mark, Lanes 1-11: Specimens 21-30, Lane 12: Positive control, Lane 13: Negative control, Lane 14: Extraction control, Lane 15: Blank.

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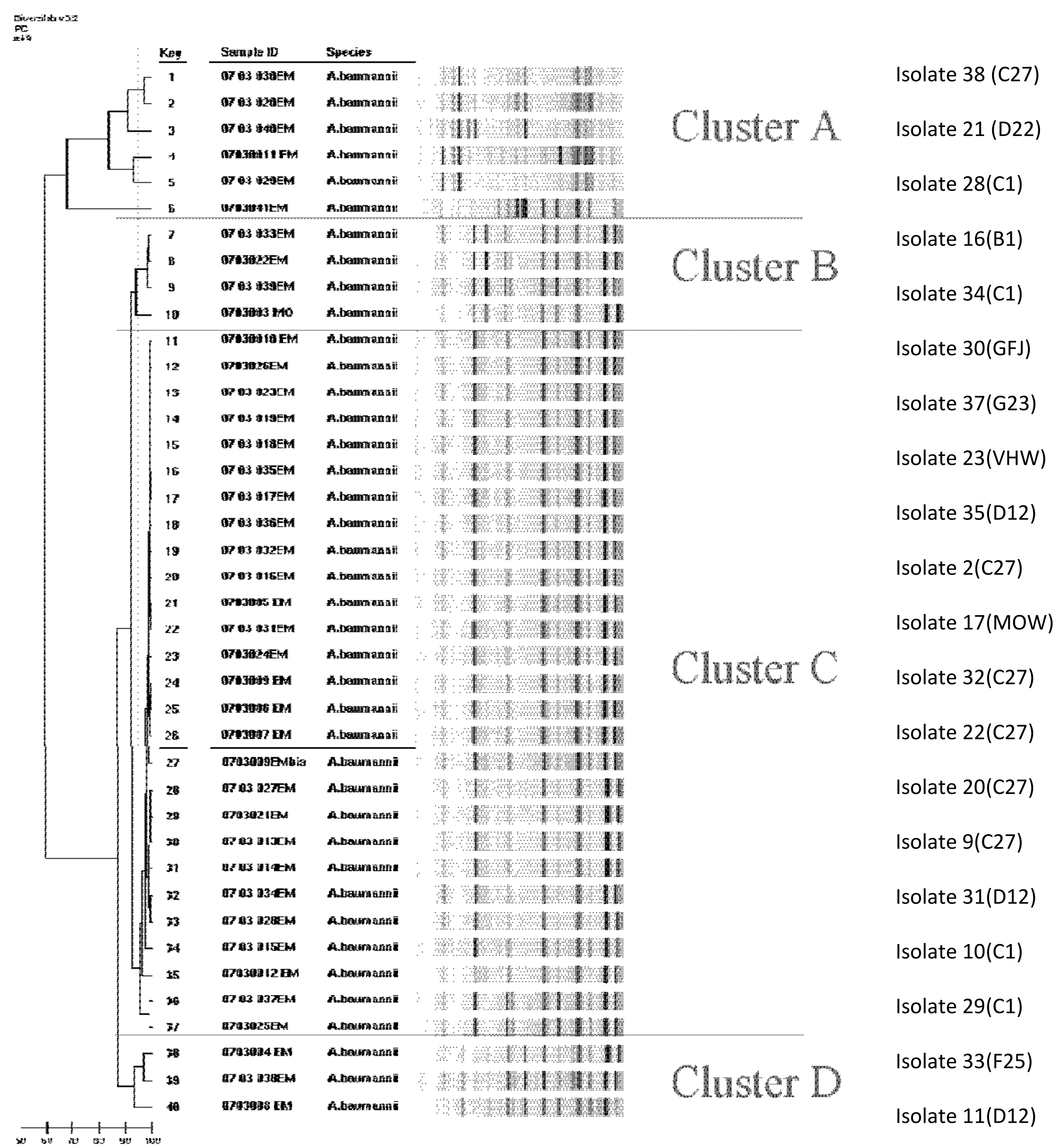

FIGURE 3: Results of Diversilab typing of the 39 isolates as provided by the R&D Division, bioMérieux.

TABLE 3. Stratification of Diversilab clusters by hospital and ward.

	Distribution of strains across hospitals [no. (%)]										
	Groote Schuur Hospital						Red Cross		Mow	GFJ	Vict
Diversilab cluster (no.)	C27	D12	E26	D22	D13	Others	C1	Others			
A (6)	1(16.67)			1(16.67)			2(33.33)	1(16.67)		1(16.67)	
B(4)	1(25)	1(25)				1(25)					1(25)
C(26)	6(23.08)	4(15.38)		2(7.69)	1(3.84)	4(15.38)	8(30.77)		1(3.84)		
D(3)		1(33.33)	1(33.33)				1(33.33)				

Key: C27- Adult Medical ICU, D12- Adult Surgical ICU, E26- Adult Isolation ICU, D22- Adult Cardiothoracic ICU, D13- Adult Neurosurgical ICU, C1- Paediatric ICU, Mow- Mowbray Maternity Hospital, GFJ- G. F. Jooste Hospital (secondary level hospital), Vict- Victoria ICU (secondary level hospital).

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FIGURE 4. Results of PFGE comparing 2 representative isolates of the commonest Diversilab clones, A and C; and MOS-1.

A2: Isolate 16, RUH3: control strain, MOS1: clinical strain with *aac(3)-IIa*, C2: Isolate 32, MOS-2: clinical strain without *aac(3)-IIa.*

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Technical Appendices

Part D

TECHNICAL APPENDICES

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APPENDIX 1: ***Ethics Approval obtained from University of Cape Town, Health Sciences Faculty, Research Ethics Committee.***

UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
e-mail [illegible]

18 December 2006

REC REF: 458/2006

Dr VM Moodley
C/o Dr SP Oliver
Medical Microbiology

Dear Dr Moodley

PROJECT TITLE: AMINOGLYCOSIDE RESISTANCE DETECTION BY AUTOMATED SUSCEPTIBILITY TESTING FOR ACINETOBACTER BAUMANNI

Thank you submitting your study to the Research Ethics Committee for review.

I have pleasure in informing you that the Ethics committee has **formally approved** the above mentioned study (lab-based and no human subjects' involvement. Specimens are being tested anonymously).

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

This serves to confirm that the University of Cape Town Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

The Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROF. M. BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

APPENDIX 2: ***Budget***

Reagents and susceptibility testing material will be sponsored by BioMerieux and Omnimed.

TABLE 1: *Estimated cost of consumables*

	Cost(unit)	Total cost
ANTIBIOTIC DISKS		
Gentamicin	R0.23	R35.05
Amikacin	R0.23	R35.05
Tobramycin	R0.23	R35.05
ETESTs		
Gentamicin	R29.01	R4351.50
Amikacin	R29.01	R4351.50
Tobramycin	R29.01	R4351.50
Vitek®2 CARDS		
GN Identification cards	R40	R2000
No22 Susceptibility cards	R40	R2000
TOTAL		R17 159.65

The reagents and equipment utilised for the molecular aspects of the study will be obtained from the National Health Laboratory Services and the Department of Medical Microbiology, Institute of Infectious Diseases and Molecular Medicine, University of Cape Town.

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APPENDIX 3: ***Data collation***

3.1 ***Selection of isolates***

TABLE 2: ***Isolates collected in diagnostic laboratory from June – December 2006.***

AGE/GENDER	INCLUDED	VIABILITY OF ISOLATE	REASON FOR EXCLUSION	CODE
68/F	Yes	Viable		15
38/F	Yes	Viable		12
55/M	Yes	Viable		24
66/F	Yes	Viable		8
44/F	Yes	Viable		4
5m/M	No	Non-viable	Non-viable	
18m/M	No	Non-viable	Non-viable	
18m/M	No	Non-viable	Non-viable	
50/M	Yes	Viable		23
50/M	No	Viable	Duplicate	
44/M	No	Non-viable	Non-viable	
4d/F	Yes	Viable		28
59/F	Yes	Viable		27
28/F	No	Non-viable	Non-viable	
6m/M	Yes	Viable		5
34/M	No	Viable	*P. putida*	
26/M	No	Viable	Duplicate	
26/M	Yes	Viable		9
37/M	Yes	Viable		42
17/F	No	Non-viable	Non-viable	
24/M	Yes	Viable		31
28/M	No	Non-viable		
22/F	Yes	Viable		25
60/M	No	Non-viable	Non-viable	
66/M	No	Non-viable	Non-viable	
63/M	Yes	Viable		22
25/M	No	Non-viable	Non-viable	
15/F	No	Non-viable	Non-viable	
51/M	No	Non-viable	Non-viable	
4m/F	No	Non-viable	Non-viable	
46d/M	Yes	Viable		7
55/M	Yes	Viable		21
7m/F	Yes	Viable		10
31/F	Yes	Viable		26
44/F	Yes	Viable		38
10m/M	No	Non-viable	Non-viable	
50/F	No	Non-viable	Non-viable	
45/M	Yes	Viable		11
37/F	Yes	Viable		2
19/M	No	Non-viable	Non-viable	
43/F	No	Non-viable	Non-viable	
49/M	Yes	Viable		39
25/M	No	Non-viable	Non-viable	
13/F	Yes	Viable		16
15/M	Yes	Viable		30
35/M	No	Non-viable	Non-viable	

45/F	No	Non-viable	Non-viable	
7d/F	No	Non-viable	Non-viable	
15d/F	Yes	Viable		18
29d/F	No	Non-viable	Non-viable	
65d/F	No	Non-viable	Non-viable	
75d/F	No	Non-viable	Non-viable	
14/F	Yes	Viable		13
14/F	No	Viable	Duplicate	
26/M	No	Non-viable	Non-viable	
84d/M	Yes	Viable		34
Outbreak	Yes	Viable	Duplicate	
Outbreak	Yes	Viable		36
Outbreak	Yes	Viable		40
Outbreak	Yes	Viable		35
24d/F	Yes	Viable		17
27/F	Yes	Viable		33
11m/M	Yes	Viable		29
46/M	Yes	Viable		41
22/M	Yes	Viable		20
56d/F	Yes	Viable		14
33/F	No	Non-viable	Non-viable	
33/F	No	Non-viable	Non-viable	
34/F	Yes	Viable		37
4m/F	No	Non-viable	Non-viable	
19/F	No	Viable	*A.haemolyticus*	
12/M	No	Non-viable	Non-viable	
19/M	Yes	Viable		32
37/M	Yes	Viable		6
69/F	No	Non-viable	Non-viable	
69/F	No	Non-viable	Non-viable	
45/M	Yes	Viable		3

N.B. Rows highlighted in yellow indicate isolates used in the study.

3.2 *Clinical data of isolates selected*

TABLE 3: ***Clinical data of patients from whom strains were isolated***

NO.	AGE/GENDER	WARD	DIAGNOSIS	SPEC TYPE	DATE OF COLLECTION	PURE/ MIXED culture	VITEK/ MANUAL susceptibility testing
02	37/F	C27	Resp failure	culba	17/08	Pure	Manual
03	45/M	E26	Polytrauma	culpu	10/07	Pure	Vitek
04	44/F	C27	Pneumonia	cultr	24/07	Pure	Vitek
05	6month/M	C1 ICU	AVSD	cultr	23/07	Pure	Vitek
06	37/M	G5	Pyrexia	culsp	2/10	Pure	Vitek
07	46 day/M	C1 ICU	Pneumonia	cultr	2/10	Pure	Vitek
08	66/F	D13	Subarachnoid haemorrhage	cultr	23/10	Pure	Vitek
09	26/M	C27	Stab	cultr	16/10	Pure	Vitek
10	7 month/F	C1 ICU	Pneumonia	culsp	06/09	Pure	Vitek
11	45/M	D12	Head injury	cultr	09/10	Mixed with *K.pneumo*	Vitek
12	38/F	D22	Post surgery	cultr	16/10	Mixed; NF	Vitek
13	14/F	C1 ICU	Liver transplant	culsp	09/10	Mixed with *K.pneumo*	Vitek
14	56 day/F	C1 ICU	Pneumonia	culsp	17/10	Mixed; NF	Vitek
15	68/F	D24	Post surgery	culpu	21/07	Mixed with Entero-coccus	Vitek
16	13/F	B1	Bronchiolitis	culsp	22/09	Pure	Vitek
17	24 day/F	Mow NICU	Sepsis	cultr	17/10	Pure	Vitek
18	15 day/F	C1 ICU	Pneumonia	culur	04/07	Mixed with yeast, CNS	Vitek
20	22/M	C27	Pneumonia	cultr	16/10	Pure	Vitek
21	55/M	D22	Post surgery	cultr	09/10	Mixed, NF	Vitek
22	63/M	C27	Renal failure	cultr	09/10	Pure	Vitek
23	50/M	Vict ICU	GSW	culba	27/08	Pure	Manual
24	55/M	D22	Post cardiac surgery	culur	04/12	Pure	Vitek
25	22/F	D12	Polytrauma	culur	04/12	Pure	Vitek
26	31/F	D12	Polytrauma	culur	04/12	Pure	Vitek
27	59/F	D12	Post surgery	culpu	04/12	Mixed; *E.cloacae*	Vitek
28	4 day/F	C1 ICU	Post surgery	cultr	05/12	Pure	Vitek
29	11 day/M	C1 ICU	Pneumonia	culba	05/12	Pure	Manual
30	15/M	GJF	Septic wound	culpu	21/08	Mixed; *S.aureus*	Manual
31	24/M	D12	Polytrauma	culti	06/11	Pure	Vitek
32	19/M	C27	Post surgery	cultr	23/10	Mixed; NF	Vitek

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33	27/F	F25	Myeloprolifer-ative disorder	culfl	21/10	Mixed; *S.aureus*	Vitek
34	84 day/M	C1 ICU	Necrotising fasciitis	culsp	17/07	Mixed; NF	Vitek
35	Outbreak investigation	D12		culpu	18/08	Pure	Manual
36	Outbreak investigation	C1 ICU		culpu	18/08	Pure	Manual
37	34/F	G23	TENS	culsp	05/11	Mixed; NF	Vitek
38	44/F	C27	Pneumonia	cultr	02/11	Mixed; NF	Vitek
39	49/M	F7	Abscess	culti	27/10	Pure	Vitek
40	Outbreak	C1 ICU		culpu	18/08	Pure	Manual
41	46/M	C27	COPD	culba	19/08	Pure	Manual
42	37/M	D12	Polytrauma	culur	23/10	Pure	Vitek

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Key: This data was obtained from the NHLS Laboratory Information System.
M – male, F- female.
Wards: C27- adult medical intensive care unit (ICU) (Groote Schuur Hospital),E26- adult isolation ICU(Groote Schuur Hospital), C1 ICU- paediatric ICU(Red Cross Children's Hospital), G5- adult medical ward(GSH), D13- adult neurosurgical ICU(GSH), D22- adult cardiothoracic ICU(GSH), D24- adult cardiothoracic ward(GSH),B1- paediatric medical ward(RXH), MOW NICU- neonatal ICU (Mowbray Maternity Hospital),VICT ICU- adult ICU(Victoria Hospital), GFJ- general male ward, GF Jooste Hospital, F25- adult vascular surgery ward, G23- adult dermatology ward,F7- adult neurosurgical ward.
Specimens: Culba- blood culture, Culur- urine culture, Culti- tissue culture, Culsp- sputum culture, Culpu- Pus swab culture,Cultr- Tracheal aspirate culture, Culfl- Sterile fluid culture.
Organisms: CNS – coagulase-negative staphylococcus, NF- normal flora.

ANALYSIS OF SAMPLE INFORMATION :

Patient profiles:

Ages ranged from 4day-63 years (Mean age 29.37)

Gender profile Male: Female = 1.06:1 (19:18)

Wards
Majority ICUs 33(including 3 outbreak specimens) 82.5%
Respiratory ICU GSH 8 (20%)
Surgical ICU GSH 7 (17.5%)
Isolation ward GSH 1 (2.5%)
Cardiothoracic ICU GSH 3 (7.5%)
Neurosurgical ICU GSH 1 (2.5%)
Paediatric ICU RXH 11 (27.5%)
NICU Mowbray 1 (2.5%)

ICU Victoria 1 (2.5%)
General wards 7 (17.5%)
General medical ward 1 (2.5%)
Cardiothoracic ward 1 (2.5%)
Paediatric high care 1
Vascular surgery ward 1
Dermatology ward 1
ENT ward 1
Surgical ward GFJ 1

Medical conditions

Respiratory (eg pneumonia) 11 (27.5%)
Trauma-related 8 (20%)
Surgery 8 (20%)
Cardiac 1 (2.5%)
Sepsis 5 (12.5%)
Vascular 1 (2.5%)
Renal 1 (2.5%)
Myeloproliferative disorder 1 (2.5%)
TENS 1 (2.5%)

Specimen types
Blood culture 4 (10%)
Pus swabs 7 (17.5%)
Tracheal aspirates/sputum 21 (14 tracheal apirates) (52.5%)
Urine 5 (12.5%)
Tissue culture 2 (5%)
Fluid culture 1 (2.5%)

Mixed/Pure cultures
Pure 26 (65%)
Mixed 14 [with other organisms(pathogens and normal flora)]

ANALYSIS OF DATA:

TABLE 4: ***Source of isolates***

TYPE OF ICU	NUMBER OF ISOLATES	PERCENTAGE OF ICU ISOLATES
Respiratory	**8**	**20**
Surgical	**7**	**17.5**
Isolation	**1**	**2.5**
Cardiothoracic	**3**	**7.5**
Neurosurgical	**1**	**2.5**
Paediatric	**11**	**27.5**
Neonatal ICU Mowbray	**1**	**2.5**
ICU Victoria	**1**	**2.5**
TOTAL	**33**	**82.5**

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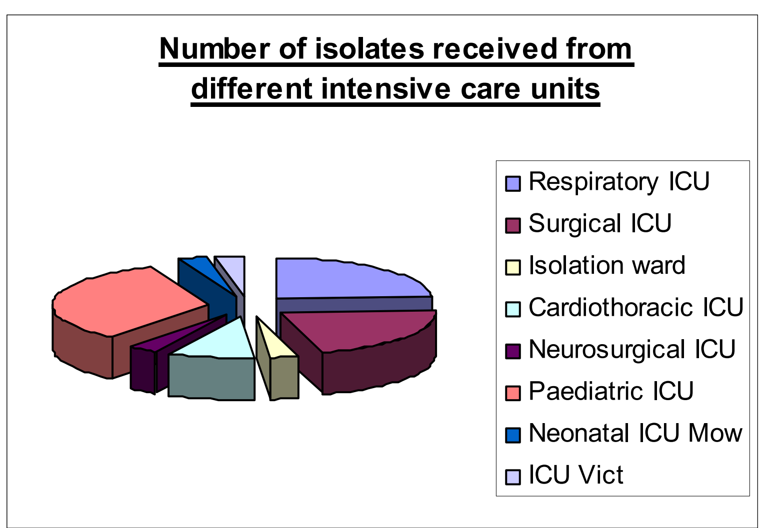

FIGURE 1: ***Graphic representation of isolates and the ICUs they were obtained from.***

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3.3 ***Original antibiotic susceptibility testing as performed by the clinical diagnostic laboratory***

TABLE 5: *Antibiotic susceptibility results of isolates selected.*

ISOLATE	PITZ	CTAZ	CPIME	GENT	AMIK	IMI	MERO	CIP	TS	COL
2	R	R	R	R	R	R	R	R	R	S
3	R	R	R	R	R	R	R	R	R	S
4	R	I	R	R	R	R	R	R	R	S
5	R	I	R	R	R	R	R	R	R	S
6	R	I	R	R	R	R	R	R	R	S
7	R	I	R	R	R	R	R	R	R	S
8	R	I	R	R	R	R	R	R	R	S
9	R	I	R	R	R	R	R	R	R	S
10	R	R	R	R	R	R	R	R	R	S
11	R	R	R	R	R	R	R	R	R	S
12	R	R	R	R	R	R	R	R	R	S
13	R	R	R	R	R	R	R	R	R	S
14	R	I	R	R	R	R	R	R	R	S
15	R	R	S	R	R	S	S	R	R	S
16	R	S	R	S	R	R	R	S	S	NT
17	R	R	R	R	R	R	R	R	R	S
18	R	I	R	R	R	R	R	R	R	S
20	R	R	R	R	R	R	R	R	R	S
21	S	S	S	S	R	S	S	S	S	NT
22	R	I	R	R	R	R	R	R	R	S
23	R	R	R	R	R	R	R	R	R	S
24	R	R	R	R	S	R	R	R	R	NT
25	R	R	R	R	R	R	R	R	R	S
26	R	R	R	R	R	R	R	R	R	S
27	R	R	R	R	R	R	R	R	R	S
28	R	I	R	R	R	R	R	R	R	S
29	R	R	R	R	R	R	R	R	R	S
30	S	R	S	R	R	S	S	R	R	NT
31	R	I	R	R	R	R	R	R	R	S
32	R	R	R	R	R	R	R	R	R	S
33	R	R	R	R	R	R	R	R	R	S
34	R	I	R	R	R	R	R	R	R	S
35	R	R	R	R	R	R	R	R	R	NT
36	R	I	R	R	R	R	R	R	R	NT
37	R	R	R	R	R	R	R	R	R	S
38	R	S	R	R	R	R	R	S	S	S
39	R	R	R	R	R	R	R	R	R	S
40	R	I	R	R	R	R	R	R	R	NT
41	R	R	R	R	S	R	R	R	R	S
42	R	R	R	R	R	R	R	R	R	S

KEY: NT- not tested, R- resistant, I- intermediate, S-susceptible.
ANTIBIOTICS: PITZ-piperacillin-tazobactam, CTAZ- ceftazidime, CPIME- cefepime, GENT-gentamicin, AMIK- amikacin, IMI- imipenem, MERO- meropenem, CIP- ciprofloxacin, TS-trimethoprim-sulphamethoxazole, COL- colistin.

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IDENTIFICATION AND SUSCEPTIBILITY TESTING

Please note that although isolate 25 had some susceptibility tests performed, the remaining tests were not performed as the isolate was not stored on beads in error. The isolate was removed from further analysis.

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APPENDIX 4.1: ***Identification of isolates and susceptibility test results***

SOP 1: ***Identification and susceptibility testing using the Vitek 2***

Day 1
Single colony plates (2% blood agar) are streaked out to obtain fresh single colonies and to exclude mixed cultures. The plates are incubated at 35ºC for 18-24 hours.

Day 2

VITEK:

MATERIALS: VITEK2 DENSICHEK Kit
VITEK 2 Cassette
0.45 % sterile saline, pre-warmed
12 x 75 mm clear plastic disposable test tubes
sterile sticks
Gram-negative ID cards
No 22 susceptibility cards
Purity plates – MacConkey agar

METHOD:

1. Aseptically transfer 3.0 ml of sterile saline into a clear plastic test tube.
2. Select isolated colonies from the single colony plate.
3. Use a sterile stick to transfer a sufficient number of morphologically similar colonies to the saline tube. Prepare the organism suspension with a density equivalent to 0.6McFarland using the VITEK2 DENSICHEK (bioMérieux, Durham, NC).
4. Place the suspension tube, ID card and No22 susceptibility card in the cassette.
5. Enter specimen data and load cassette into Vitek2 machine within 30 minutes.
6. Repeat procedure using organism suspension of 1.0 McFarland.

QUALITY CONTROL
Vitek GN ID cards utilised: BioMerieux, Lot 241019940, Expiry date 01/07/2007.
Results of QC strains (ATCC 27853 and ATCC 25922 are shown in the results table on the following page.

REFERENCES:
Biomerieux, Vitek®2 ID-GNB Product Information Manual

OTHER INFORMATION:

Vitek®2 AST- NO22 Gram Negative Susceptibility Card, package insert, REF 22031.

Vitek AST-NO22 Card Antibiotic Concentrations
Gentamicin 4, 16, 32 µg/ml
Amikacin 8, 16, 64 µg/ml
Tobramycin 8, 16, 64 µg/ml

QUALITY CONTROL
Performed as per manufacturer's recommendation.

TABLE 6: ***MICs for quality control organisms using the Vitek 2***

	Amikacin MIC	*Gentamicin MIC*	*Tobramycin MIC*
***E.coli* ATCC 25922**	< 2 – 4	< 1	< 1
***P.aeruginosa* ATCC 27853**	< 2 – 4	< 1 - 2	< 1

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TABLE 7: ***Results of identification of the 40 isolates using Vitek2 at 0.6 and 1.0 McF, respectively.***

SPECIMEN NUMBER	IDENTIFICATION	CONFIDENCE INTERVAL
2 (0.6 McF)	*Acinetobacter baumannii*	Excellent (99.0)
(1.0 McF)	*Acinetobacter baumannii*	Excellent (99.0)
3	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
4	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
5	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
6	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
7	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
8	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Very Good (94.70)
CONTROL ATCC 27853	*Pseudomonas aeruginosa*	Excellent(98.24)
	Pseudomonas aeruginosa	Excellent(98.24)
ATCC 25922	*Escherichia coli*	Very Good(95.0)
	Escherichia coli	Very Good(95.0)
9	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
10	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
11	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
12	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
13	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
14	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
15	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
16	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
17	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
18	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
20	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
21	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
22	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
23	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
24	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
25	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
26	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
27	*Acinetobacter baumannii*	Excellent (99.0)

	Acinetobacter baumannii	Excellent (99.0)
28	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
29	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
30	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
31	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
32	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
33	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
34	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
35	*Acinetobacter baumanii*	Excellent (98.79)
	Acinetobacter baumannii	Excellent (99.0)
36	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
37	*Acinetobacter baumanii*	Excellent (98.79)
	Acinetobacter baumanii	Excellent (98.79)
38	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
39	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
40	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
41	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)
42	*Acinetobacter baumannii*	Excellent (99.0)
	Acinetobacter baumannii	Excellent (99.0)

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<u>TABLE 8:</u> ***Results of antibiotic susceptibility testing using the Vitek2 AST at 0.6 and 1.0 McF, respectively.***

RESULTS OF MICs OBTAINED FROM VITEK2 AUTOMATED SUSCEPTIBILITY TESTING														
	VITEK 0.6 McFARLAND								VITEK 1.0 McFARLAND					
ISOLATE	GENTAMICIN	AMIKACIN	TOBRAMYCIN	ISEPAMICIN	NETILMICIN		MECHANISM	*aacC2a*	GENTAMICIN	AMIKACIN	TOBRAMYCIN	ISEPAMICIN	NETILMICIN	
2	>=16	32	<=1	>=64	2	Inconsistent	G,I	Absent	>=16	>=64	<=1	>=64	4	Consistent
3	>=16	32	8	>=64	2	Consistent	G,I	Absent	>=16	>=64	>=16	>=64	4	Consistent
4	>=16	>=64	8	>=64	>=32	Consistent	G,A,I,N	Absent	>=16	>=64	8	>=64	>=32	Consistent
5	>=16	>=64	8	>=64	>=32	Consistent	G,A,I,N	Present	>=16	>=64	8	>=64	>=32	Consistent
6	>=16	>=64	2	>=64	16	Consistent	G,A,I	Present	>=16	>=64	2	>=64	16	Consistent
7	>=16	>=64	>=16	>=64	16	Consistent	G,A,T,I	Absent	>=16	>=64	>=16	>=64	16	Consistent
8	>=16	>=64	4	>=64	>=32	Consistent	G,A,I,N	Present	>=16	>=64	4	>=64	>=32	Consistent
9	>=16	>=64	2	>=64	16	Consistent	G,A,I	Present	>=16	>=64	<=1	>=64	16	Consistent
10	>=16	>=64	8	>=64	>=32	Consistent	G,A,I,N	Present	>=16	32	4	>=64	>=32	Consistent
11	>=16	>=64	2	>=64	16	Consistent	G,A,I	Absent	>=16	>=64	4	>=64	16	Consistent
12	>=16	>=64	8	>=64	>=32	Consistent	G,A,I,N	Present	>=16	>=64	8	>=64	>=32	Consistent
13	>=16	32	4	>=64	>=32	Consistent	G,I,N	Present	>=16	32	2	>=64	>=32	Consistent
14	>=16	>=64	2	>=64	>=32	Consistent	G,A,I,N	Present	>=16	>=64	2	>=64	>=32	Consistent
15	>=16	32	4	>=64	>=32	Consistent	G,I,N	Present	>=16	>=64	2	>=64	>=32	Consistent
16	<=1	>=64	<=1	>=64	2	Inconsistent	A,I	Absent	<=1	>=64	<=1	>=64	2	Inconsistent
17	>=16	32	4	>=64	>=32	Consistent	G,I,N	Present	>=16	32	4	>=64	>=32	Consistent
18	>=16	>=64	8	>=64	>=32	Consistent	G,A,I,N	Present	>=16	>=64	4	>=64	>=32	Consistent
20	>=16	>=64	<=1	>=64	16	Consistent	G,A,I	Present	>=16	>=64	<=1	>=64	16	Consistent
21	<=1	>=64	<=1	>=64	2	Inconsistent	A,I	Absent	<=1	>=64	<=1	>=64	2	Inconsistent
22	>=16	>=64	<=1	>=64	16	Consistent	G,A,I	Present	>=16	>=64	<=1	>=64	16	Consistent
23	>=16	16	<=1	>=64	2	Consistent	G,I	Absent	>=16	16	<=1	>=64	2	Consistent
24	>=16	<=2	<=1	2	4	Consistent	G	Absent	>=16	<=2	<=1	2	4	Consistent
25	>=16	32	<=1	>=64	4	Consistent	G,I	NT	>=16	32	<=1	>=64	4	Consistent
26	>=16	>=64	>=16	32	16	Consistent	G,A,T,I	Present	>=16	>=64	>=16	>=64	16	Consistent
27	>=16	>=64	>=16	>=64	4	Consistent	G,A,T,I	Absent	>=16	>=64	>=16	>=64	4	Consistent
28	8	>=64	<=1	>=64	2	Inconsistent	A,I	Present	<=1	>=64	<=1	>=64	2	Inconsistent
29	>=16	>=64	2	>=64	>=32	Consistent	G,A,I,N	Absent	>=16	>=64	2	>=64	>=32	Consistent
30	>=16	32	<=1	>=64	2	Inconsistent	G,I	Absent	>=16	32	<=1	>=64	2	Inconsistent
31	>=16	>=64	4	>=64	>=32	Consistent	G,A,I,N	Present	>=16	>=64	4	>=64	>=32	Consistent
32	>=16	>=64	<=1	>=64	>=32	Consistent	G,A,I,N	Present	>=16	>=64	<=1	>=64	>=32	Consistent
33	>=16	>=64	4	>=64	>=32	Consistent	G,A,I,N	Present	>=16	>=64	2	>=64	>=32	Consistent
34	<=1	<=2	<=1	<=1	<=1	Consistent		Absent	<=1	<=2	<=1	<=1	<=1	Consistent
35	>=16	16	<=1	>=64	4	Consistent	G,I	Absent	>=16	16	<=1	>=64	2	Consistent
36	>=16	>=64	4	>=64	>=32	Consistent	G,A,I,N	Present	>=16	>=64	4	>=64	>=32	Consistent
37	>=16	16	<=1	>=64	4	Consistent	G,I	Absent	>=16	16	<=1	>=64	2	Consistent
38	>=16	>=64	>=16	>=64	2	Consistent	G,A,T,I	Absent	>=16	>=64	>=16	>=64	2	Consistent
39	>=16	>=64	2	>=64	8	Consistent	G,A,I,N	Present	>=16	>=64	2	>=64	16	Consistent
40	>=16	>=64	4	>=64	>=32	Consistent	G,A,I,N	Present	>=16	>=64	4	>=64	>=32	Consistent
41	>=16	<=2	<=1	<=1	2	Consistent	G	Absent	>=16	<=2	<=1	2	4	Consistent
42	>=16	>=64	2	>=64	>=32	Consistent	G,A,I,N	Present	>=16	>=64	2	>=64	16	Consistent
QC: *P. aeruginosa* ATCC 27853	<=1	<=2	<=1	<=1	<=1	Consistent			<=1	<=2	<=1	<=1	<=1	Consistent
QC: *E.coli* ATCC 25922	<=1	<=2	<=1	<=1	<=1	Consistent			<=1	<=2	<=1	<=1	<=1	Consistent
		S	30											
		I	6											
		R	4											
		TOTAL	40											

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SOP 2: ***Disc Diffusion Testing***

MATERIAL: Mueller-Hinton agar (3 different brands: BioRad, BioMerieux,Greenpoint)
Antibiotic discs(Oxoid)- Gentamicin (10 µg)
Amikacin (30 µg)
Tobramycin(10µg)

METHOD:

1. Select isolated colonies from single colony plate.
2. Using a sterile stick to transfer a sufficient number of morphologically similar colonies to the saline tube. Prepare the organism suspension with a density equivalent to 0.5McFarland.
3. Whirl out suspension on to Muller Hinton agar(MHA) (x 3 different brands of MHA).
4. Leave for 3-5 minutes to allow for any excess surface moisture to be absorbed.
5. Apply aminoglycoside-impregnated discs using disc tabbing device. Each disc must be pressed down to ensure complete contact with the agar surface. The discs must be evenly distributed (>24mm from centre to centre).
6. Invert plates and incubate within 15 minutes at 35 degrees Celsius under aerobic conditions for 24 hrs.

READING PLATES AND INTERPRETING RESULTS: See Performance Standards for Antimicrobial Susceptibility Testing; 16th Informational Supplement, M100-S16, Vol. 26 No.3, January 2006, CLSI.

1. After 18-24 hrs of incubation, examine each plate. The zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies are visible, the inoculum was too light and the test must be repeated.

2. Measure the diameters of the zones of complete inhibition, including the diameter of the disc. Measure the zones to the nearest millimetre, using a sliding caliper, which is held on the back of the inverted petri plate.

3. The zone margin is considered the area showing no obvious, visible growth that can be detected with the unaided eye. If any discrete colonies appear within the zone of inhibition, the test will be repeated with a pure culture. If colonies continue to grow within the zone of inhibition, measure the colony-free inner zone.

4. The sizes of the zones must be interpreted according to Table 2B2 (Performance Standards for Antimicrobial Susceptibility Testing; 16th Informational Supplement, M100-S16, Vol. 26 No.3, January 2006, CLSI.)

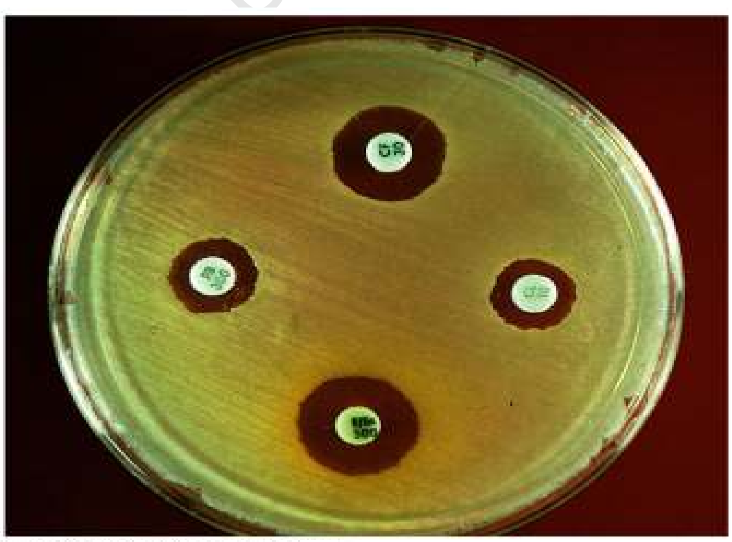

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FIGURE 2: *Example of disc diffusion testing on Mueller-Hinton agar*

Image downloaded from: diverge.hunter.cuny.edu/~weigang/Images/20-17_diskdiffusion.jpg

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QUALITY CONTROL

Performed for each new lot number. See results on next page.
Control organisms – *E.coli* ATCC 25922 and *P. aeruginosa* ATCC 27853.

TABLE 9: ***Lot numbers and expiry dates of reagents used for disk diffusion testing***.

	LOT NUMBER	**EXPIRY DATE**
MUELLER HINTON AGAR:		
Greenpoint	Nil	**21-02-07**
Bio-Rad	2418D	**13-04-07**
BioMerieux	807673101	**16-01-07**
ANTIBIOTIC DISCS:		
Amikacin		**05-2009**
Gentamicin	461298	**07-2009**
Tobramycin	**450224**	**05-2009**

REFERENCES:

Performance Standards for Antimicrobial Disk Susceptibility Tests, Approved Standard- 9th edition, M2-A9 Vol. 26 No. 1, January 2006, Clinical and Laboratory Standards Institute.

Performance Standards for Antimicrobial Susceptibility Testing; 16th Informational Supplement, M100-S16, Vol. 26 No.3, January 2006, CLSI.

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TABLE 9: ***Results of disk diffusion testing***

DATA COLLECTION SHEET: DISC DIFFUSION TESTING												
	GENTAMICIN			OVERALL RESULT	AMIKACIN			OVERALL RESULT	TOBRAMYCIN			OVERALL RESULT
ISOLATE	BIOMERIEUX	BIO-RAD	GREENPOINT		BIOMERIEUX	BIO-RAD	GREENPOINT		BIOMERIEUX	BIO-RAD	GREENPOINT	
2	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	19mm	20mm	18mm	SENSITIVE (19mm)
3	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	10mm	NZ	NZ	RESISTANT (3mm)
4	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	10mm	10mm	11mm	RESISTANT (10mm)
5	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	11mm	11mm	11mm	RESISTANT (11mm)
6	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	14mm	14mm	15mm	INTERMEDIATE (14mm)
7	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)
8	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	12mm	13mm	14mm	INTERMEDIATE (13mm)
9	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	15mm	15mm	16mm	SENSITIVE (15mm)
10	NZ	NZ	NZ	RESISTANT (NZ)	9mm	9mm	8mm	RESISTANT (9mm)	13mm	13mm	13mm	INTERMEDIATE (13mm)
11	NZ	NZ	NZ	RESISTANT (NZ)	8mm	7mm	8mm	RESISTANT (8mm)	13mm	13mm	13mm	INTERMEDIATE (13mm)
12	NZ	NZ	NZ	RESISTANT (NZ)	7mm	NZ	7mm	RESISTANT (5mm)	9mm	8mm	9mm	RESISTANT (9mm)
13	NZ	NZ	NZ	RESISTANT (NZ)	8mm	8mm	9mm	RESISTANT (8mm)	11mm	11mm	13mm	RESISTANT (11mm)
14	NZ	NZ	NZ	RESISTANT (NZ)	8mm	7mm	7mm	RESISTANT (7mm)	14mm	15mm	14mm	INTERMEDIATE (14mm)
15	NZ	NZ	NZ	RESISTANT (NZ)	9mm	8mm	12mm	RESISTANT (10mm)	15mm	15mm	15mm	SENSITIVE (15mm)
16	21mm	22mm	22mm	SENSITIVE (22mm)	8mm	NZ	NZ	RESISTANT (3mm)	22mm	25mm	21mm	SENSITIVE (23mm)
17	NZ	NZ	NZ	RESISTANT (NZ)	9mm	8mm	9mm	RESISTANT (9mm)	13mm	13mm	13mm	INTERMEDIATE (13mm)
18	NZ	NZ	NZ	RESISTANT (NZ)	8mm	NZ	9mm	RESISTANT (6mm)	13mm	12mm	13mm	INTERMEDIATE (13mm)
20	NZ	NZ	NZ	RESISTANT (NZ)	8mm	NZ	8mm	RESISTANT (5mm)	15mm	15mm	15mm	SENSITIVE (15mm)
21	21mm	22mm	21mm	SENSITIVE (21mm)	10mm	9mm	9mm	RESISTANT (9mm)	23mm	25mm	24mm	SENSITIVE (15mm)
22	NZ	NZ	11mm	RESISTANT (4mm)	8mm	NZ	8mm	RESISTANT (5mm)	15mm	16mm	16mm	SENSITIVE (16mm)
23	8mm	8mm	9mm	RESISTANT (8mm)	8mm	8mm	9mm	RESISTANT (8mm)	19mm	22mm	21mm	SENSITIVE (21mm)
24	NZ	NZ	NZ	RESISTANT (NZ)	20mm	22mm	24mm	SENSITIVE (22mm)	20mm	22mm	23mm	SENSITIVE (22mm)
25	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	20mm	22mm	21mm	SENSITIVE (21mm)
26	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (0mm)
27	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (0mm)
28	22mm	22mm	21mm	SENSITIVE (22mm)	NZ	NZ	NZ	RESISTANT (NZ)	23mm	24mm	21mm	SENSITIVE (23mm)
29	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	14mm	15mm	14mm	INTERMEDIATE (14mm)
30	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	20mm	20mm	21mm	SENSITIVE (20mm)
31	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	12mm	11mm	12mm	RESISTANT (12mm)
32	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	15mm	18mm	18mm	SENSITIVE (17mm)
33	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	10mm	10mm	10mm	RESISTANT (10mm)
34	21mm	22mm	21mm	SENSITIVE (22mm)	20mm	23mm	23mm	SENSITIVE (22mm)	22mm	24mm	22mm	SENSITIVE (23mm)
35	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	20mm	22mm	20mm	SENSITIVE (21mm)
36	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	13mm	13mm	13mm	INTERMEDIATE (13mm)
37	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	20mm	22mm	20mm	SENSITIVE (21mm)
38	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (0mm)
39	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	14mm	15mm	16mm	SENSITIVE (15mm)
40	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	NZ	13mm	NZ	RESISTANT (4mm)
41	NZ	NZ	NZ	RESISTANT (NZ)	20mm	21mm	22mm	SENSITIVE (21mm)	20mm	22mm	21mm	SENSITIVE (21mm)
42	NZ	NZ	NZ	RESISTANT (NZ)	NZ	NZ	NZ	RESISTANT (NZ)	14mm	14mm	14mm	INTERMEDIATE (14mm)
QC: *E.coli*	21mm	22mm	20mm	IN RANGE	22mm	22mm	21mm	SENSITIVE (22mm)	21mm	22mm	20mm	SENSITIVE (21mm)
QC: *P.aeruginosa*	19mm	18mm	20mm	IN RANGE	21mm	22mm	24mm	SENSITIVE (22mm)	24mm	24mm	25mm	SENSITIVE (24mm)

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SOP 3: ***Epsilometric tests (E-Tests)***

MATERIALS: 0.85 % saline
Sterile swabs
Etest strips – gentamicin, amikacin, tobramycin
Mueller-Hinton agar (depth 4mm) from 3 different suppliers

METHOD:

1. Remove Etest package from freezer (-20 degrees Celsius) and allow the strips to reach room temperature for approximately 30 minutes.
2. Homogenize individual viable colonies from a 24hr agar plate in saline.
3. Adjust the turbidity to 0.5 McFarland.
4. Ensure agar surface is dry before inoculation. Dip a swab in the inoculum, remove excess fluid and swab the entire agar surface evenly in 3 directions.
5. Allow the agar surface to dry for 10-15 minutes on the bench.
6. Place the Etest strips to the agar surface with a sterile forceps. Once the strip is on the agar, do not move it.
7. Incubate the plate at 35 degrees Celsius under aerobic conditions for 24 hrs.

READING AND INTERPRETATION:

1. Read plates after 24hrs incubation only if sufficient growth is seen and the inhibition ellipse is visible.
2. Read the MIC where the ellipse intersects the scale.
3. Read the endpoint at complete inhibition of all growth including hazes and isolated colonies.

FIGURE 3: ***Example of an Etest.***

Downloaded from: www.uniklinik-ulm.de/uploads/pics/etest11.jpg

QUALITY CONTROL

Performed once (as per manufacturer's instructions) for each lot number of Etests used.

Control organisms – *E.coli* ATCC 25922 and *P. aeruginosa* ATCC 27853.

Gentamicin Etest Lot No BG 2559 Expiry 2011/09
Amikacin Etests Lot BG 1765 Expiry 2011/03
Tobramycin Etest Lot BG 2671 Expiry 2011/03

REFERENCES

Etest Technical Guide 3B, AB BIODISK

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Etest Application Sheet, EAS 004, AB BIODISK, 2004

TABLE 10: ***Epsilometric susceptibility test results***

DATA COLLECTION SHEET: EPSILOMETRIC TESTING (ETESTS)												
	GENTAMICIN			OVERALL RESULT	**AMIKACIN**			OVERALL RESULT	**TOBRAMYCIN**			OVERALL RESULT
ISOLATE	BIOMERIEUX	BIO-RAD	GREENPOINT		BIOMERIEUX	BIO-RAD	GREENPOINT		BIOMERIEUX	BIO-RAD	GREENPOINT	
2	32	32	32	RESISTANT (32)	128	>256	128	RESISTANT	2	16(12)	2 (1.5)	SENSITIVE (2)
3	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	24	32	64 (48)	RESISTANT
4	>256	>256	>256	RESISTANT (>256)	256	>256	256 (192)	RESISTANT (>256)	16(12)	16	16(12)	RESISTANT (16)
5	>256	>256	>256	RESISTANT (>256)	128	256 (192)	128	RESISTANT	8	16(12)	8	INTERMEDIATE (8)
6	>256	>256	>256	RESISTANT (>256)	256 (192)	256	128	RESISTANT	4	4	4	SENSITIVE (4)
7	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	256(192)	256	256	RESISTANT (256)
8	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	8 (6)	8	8 (6)	INTERMEDIATE (8)
9	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	8 (6)	8	8 (6)	INTERMEDIATE (8)
10	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	16	16(12)	16	RESISTANT (16)
11	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	16 (12)	16(12)	16(12)	RESISTANT (16)
12	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	32	32	32	RESISTANT (32)
13	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	32	16	64 (48)	RESISTANT
14	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	8 (6)	8	8	INTERMEDIATE (8)
15	>256	>256	>256	RESISTANT (>256)	64	128 (96)	64	RESISTANT	8 (6)	8	8	INTERMEDIATE (8)
16	2	2	4 (3)	SENSITIVE (3)	>256	>256	>256	RESISTANT (>256)	2 (1.5)	2 (1.5)	2 (1.5)	SENSITIVE (2)
17	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	16	16	16 (12)	RESISTANT (16)
18	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	16 (12)	16	16	RESISTANT (16)
20	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	4	8 (6)	4	SENSITIVE (4)
21	2	2	2	SENSITIVE (2)	64	128 (96)	64	RESISTANT	2 (1.5)	2 (1.5)	2 (1.5)	SENSITIVE (2)
22	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	8 (6)	8 (6)	4	INTERMEDIATE (8)
23	32	32	32	RESISTANT (32)	>256	>256	>256	RESISTANT (>256)	2	2	2	SENSITIVE (2)
24	64	128 (96)	16	RESISTANT	4	4	2 (1.5)	SENSITIVE	2	2	1	SENSITIVE
25	32	32	32	RESISTANT (32)	>256	256	256	RESISTANT (>256)	2	2	2	SENSITIVE (2)
26	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	32	64	64 (48)	RESISTANT
27	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	64	64	64	RESISTANT (64)
28	2 (1.5)	2	2 (1.5)	SENSITIVE (2)	128	128	64	RESISTANT	1	1 (0.75)	1	SENSITIVE
29	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	8 (6)	8	8	INTERMEDIATE (8)
30	64 (48)	64	32	RESISTANT	128	128	128	RESISTANT (128)	2	2	2	SENSITIVE (2)
31	>256	>256	>256	RESISTANT (>256)	128	128	128	RESISTANT (128)	16	16	16 (12)	RESISTANT (16)
32	256	256	256	RESISTANT (256)	>256	>256	>256	RESISTANT (>256)	4	4	4	SENSITIVE
33	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	16	16	16	RESISTANT (16)
34	2 (1.5)	1	1	SENSITIVE	4(3)	2	4(3)	SENSITIVE	2 (1.5)	1	1	SENSITIVE
35	32	32	16 (12)	RESISTANT	128	128	64	RESISTANT	2 (1.5)	2 (1.5)	2 (1.5)	SENSITIVE (2)
36	>256	>256	>256	RESISTANT (>256)	128	128	128	RESISTANT (128)	8	8	8	INTERMEDIATE (8)
37	32	32	32	RESISTANT (32)	128 (96)	128	64	RESISTANT	2 (1.5)	2 (1.5)	2 (1.5)	SENSITIVE (2)
38	256	>256	>256	RESISTANT (>256)	128	128	128	RESISTANT (128)	32	64 (48)	32	RESISTANT
39	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	4	8 (6)	8 (6)	INTERMEDIATE
40	>256	>256	>256	RESISTANT (>256)	128	128 (96)	128	RESISTANT (128)	8 (6)	8	8 (6)	INTERMEDIATE (8)
41	32	32	32	RESISTANT (32)	4 (3)	4	4(3)	SENSITIVE	2	2	2	SENSITIVE
42	>256	>256	>256	RESISTANT (>256)	>256	>256	>256	RESISTANT (>256)	8 (6)	8	8 (6)	INTERMEDIATE (8)
QC: *E.coli*	1	1	1	IN RANGE	2	2	1	IN RANGE	1	1	0.5	IN RANGE
QC: *P.aeruginosa*	2	4	2	IN RANGE	4	4	2	IN RANGE	1	1	0.5	IN RANGE

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4.4 AGAR DILUTION RESULTS

Agar dilution susceptibility testing was performed and analysed used CLSI criteria by the R&D Division, Biomerieux, France. The testing was done in duplicate by 2 different scientists.

TABLE 11: ***Agar dilution susceptibility test results***

ISOLATE	TECH 1	TECH 2	RESULT
2	<=0.5	<=0.5	S
3	16	16	R
4	8	8	I
5	8	8	I
6	2	2	S
7	128	128	R
8	4	4	S
9	2	2	S
10	4	4	S
11	4	4	S
12	8	8	I
13	4	4	S
14	4	4	S
15	4	4	S
16	<=0.5	<=0.5	S
17	2	2	S
18	4	4	S
20	2	2	S
21	<=0.5	<=0.5	S
22	2	2	S
23	<=0.5	<=0.5	S
24	<=0.5	<=0.5	S
25	NT	NT	NT
26	2	2	S
27	32	32	R
28	<=0.5	<=0.5	S
29	2	2	S
30	<=0.5	<=0.5	S
31	16	16	R
32	2	2	S
33	4	8	I
34	<=0.5	<=0.5	S
35	<=0.5	<=0.5	S
36	16	16	R
37	<=0.5	<=0.5	S
38	16	16	R
39	2	2	S
40	4	4	S
41	<=0.5	<=0.5	S
42	4	4	S
ATCC 25922	<=0.5	<=0.5	
ATCC 27853	<=0.5	<=0.5	
ATCC 25923	<=0.5	<=0.5	
ATCC 29213	<=0.5	<=0.5	
ATCC 35218	<=0.5	<=0.5	
ATCC 29212	4	4	

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4.5 BROTH MICRODILUTION RESULTS

Broth microdilution was performed according to CLSI guidelines by the R&D Division, Biomerieux, France. The testing was done in duplicate by 2 different scientists.

TABLE 12 : ***Results of Broth Microdilution Testing***

	BROTH MICRODILUTION									
	GENTAMICIN			AMIKACIN			TOBRAMYCIN			*aacC2a*
ISOLATE	CCH	IC	RESULT	CCH	CD	RESULT	CCH	VM	RESULT	
2	64	64	R	256	256	R	1	1	S	Absent
3	>256	>256	R	256	256	R	128	256	R	Absent
4	>256	>256	R	>256	>256	R	64	256	R	Absent
5	>256	>256	R	128	256	R	32	32	R	Present
6	>256	>256	R	>256	>256	R	8	32	R	Present
7	>256	>256	R	>256	>256	R	>256	>256	R	Absent
8	>256	>256	R	256	>256	R	8	8	I	Present
9	>256	>256	R	128	>256	R	4	8	I	Present
10	>256	>256	R	256	256	R	32	64	R	Present
11	>256	>256	R	>256	>256	R	32	32	R	Absent
12	>256	>256	R	>256	>256	R	32	32	R	Present
13	>256	>256	R	128	128	R	32	32	R	Present
14	>256	>256	R	256	256	R	8	8	I	Present
15	>256	>256	R	128	256	R	8	8	I	Present
16	1	1	S	>256	>256	R	1	1	S	Absent
17	>256	>256	R	128	256	R	16	16	R	Present
18	>256	>256	R	128	>256	R	16	128	R	Present
20	>256	>256	R	128	256	R	4	8	I	Present
21	1	1	S	64	128	R	0.5	1	S	Absent
22	>256	>256	R	>256	>256	R	8	16	R	Present
23	>256	>256	R	256	256	R	8	16	R	Absent
24	>256	>256	R	2	8	S	2	2	S	Absent
25	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
26	>256	>256	R	2	4	S	4	8	I	Present
27	>256	>256	R	>256	>256	R	128	256	R	Absent
28	1	1	S	256	256	R	1	1	S	Present
29	>256	>256	R	256	>256	R	4	4	S	Absent
30	>256	>256	R	256	256	R	1	1	S	Absent
31	>256	>256	R	128	>256	R	32	256	R	Present
32	>256	>256	R	128	256	R	4	8	I	Present
33	>256	>256	R	256	>256	R	8	8	I	Present
34	1	1	S	2	2	S	1	0.5	S	Absent
35	256	256	R	256	256	R	2	2	S	Absent
36	>256	>256	R	256	256	R	64	128	R	Present
37	32	64	R	256	256	R	1	1	S	Absent
38	256	256	R	128	128	R	64	128	R	Absent
39	>256	>256	R	>256	>256	R	4	16	R	Present
40	>256	>256	R	256	>256	R	16	>256	R	Present
41	64	64	R	2	4	S	1	1	S	Absent
42	>256	>256	R	>256	>256	R	8	8	I	Present
ATCC 25922	<=0.5	?	0.25-1	2	2	0.5-4	<=0.5	<=0.5	0.25-1	
ATCC 27853	1	1	0.5-2	2	2	1.0-4.0	<=0.5	<=0.5	0.25-1	
ATCC 25923	<=0.5	<=0.5		NT	1		4	4		
ATCC 25213	<=0.5	<=0.5	0.12-1	1	1	1.0-4.0	<=0.5	<=0.5	0.12-1	
ATCC 35218	<=0.5	1		2	2		1	1		
ATCC 29212	4	4	4.0-16.0	64	64	64-256	4	4	8.0-32.0	

KEY: NT-Not tested, ATCC- American Type Culture Collection, CCH/ IC/CD/VM-different scientists who performed testing, R-resistant, I-intermediate, S-susceptible.

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TABLE 13: ***Summary of tobramycin susceptibility testing***

DATA COLLATION : TOBRAMYCIN								
SPECIMEN NUMBER	DISK DIFFUSION	ETEST	VITEK 0.6	VITEK 1.0	AGAR DILUTION	BROTH MICRODILUTION	ERRORS	CLONES
2	S	S	S	S	S	S		B
3	R	R	I	R	R	R	MINOR	D
4	R	I	I	I	I	R	MINOR	C
5	R	I	I	I	I	R	MINOR	C
6	I	S	S	S	S	R	VERY MAJOR	C
7	R	R	R	R	R	R		D
8	R	I	S	S	S	I	MINOR	C
9	I	I	S	S	S	I	MINOR	C
10	R	R	I	S	S	R	MINOR	C
11	I	I	S	S	S	R	VERY MAJOR	C
12	R	R	I	I	I	R	MINOR	C
13	R	R	S	S	S	R	VERY MAJOR	C
14	I	I	S	S	S	I	MINOR	C
15	I	I	S	S	S	I	MINOR	C
16	S	S	S	S	S	S		A
17	R	R	S	S	S	R	VERY MAJOR	C
18	I	R	I	S	S	R	MINOR	C
20	I	S	S	S	S	I	MINOR	C
21	S	S	S	S	S	S		A
22	S	I	S	S	S	R	VERY MAJOR	C
23	S	S	S	S	S	R	VERY MAJOR	B
24	S	S	S	S	S	S		C
25	S	S	S	S	NT	NT		NT
26	R	R	R	R	S	I	MINOR	C
27	R	R	R	R	R	R		D
28	S	S	S	S	S	S		A
29	I	I	S	S	S	S		C
30	S	S	S	S	S	S		A
31	R	R	S	S	R	R	VERY MAJOR	C
32	S	S	S	S	S	I	MINOR	C
33	R	I	S	S	I	I	MINOR	C
34	S	S	S	S	S	S		A
35	S	S	S	S	S	S		B
36	I	I	S	S	R	R	VERY MAJOR	C
37	S	S	S	S	S	S		B
38	R	R	R	R	R	R		A
39	S	I	S	S	S	R	VERY MAJOR	C
40	R	I	S	S	S	R	VERY MAJOR	C
41	S	S	S	S	S	S		C
42	I	I	S	S	S	I	MINOR	C

KEY: R- resistant, I- intermediate, S- susceptible.

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GENETIC ANALYSIS OF STRAINS

University of Cape Town

PCR for aac(3)-IIa gene:

DNA extraction was performed using the NucliSens Lysis Buffer (bioMerieux), NucliSens Magnetic Extraction Reagents (bioMerieux) and EasyMag (bioMerieux) as per manufacturer's instructions.

Example of nucleic acid extraction worksheet used in the study

EXTRACTION WORKSHEET NUMBER :

METHOD OF EXTRACTION : EasyMag® 2.0 (bioMerieux) **DATE :** 05/11/2009

BSL CABINET NO : TB Cabinet 2 **OPERATOR** : Mischka

INCUBATOR: N/A

INSTRUMENT NUMBER : 00897

PCR TYPE	STORAGE	NO	SAMPLE TYPE	VOLUME EXTRACTED	ELUTION VOLUMES	COMMENTS
				0.010ml	50µl	

TABLE 14: ***Reagents used for DNA extraction***

	LOT Number	Expiry date	Manufacturer
NucliSens® Lysis Buffer (2ml)	09020501	2011-01	bioMerieux
NucliSens® Magnetic Extraction Reagents	Z011AAIMS	2011-01	bioMerieux
Extraction Buffer 1	Z011EA	2011-05	bioMerieux
Extraction Buffer 2	Z010LA	2010-10	bioMerieux
Elution Buffer	Z010GC	2010-07	bioMerieux

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Following extraction, the quantity and quality of the DNA extracted was assessed using the Nanodrop.

ACINETOBACTER SPECTROPHOTOMETRY:

Absorbance measurements were used to assess the quantity and purity of DNA extracted. A ratio of >1.8 was accepted as “pure” for DNA.

The Nanodrop® ND-1000 (Nanodrop Technologies, Inc.) spectrophotometer was utilised. See SOP on next page.

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SOP 4:

STANDARD OPERATING PROCEDURE

Title: NANODROP INSTRUMENT

Document number: CHE0607

Version number: 2

(Changes from previous version highlighted)

Written by: S. Meldau

Checked by: N/A

Approved by: Dr E.P Owen

Active date: 26-10-2009

Date of next review	*Date reviewed*	*Reviewed by*	*Action*
26-10-2010			

Date withdrawn: ....

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NANODROP INSTRUMENT

OBJECTIVE Instructions on how to use the nanodrop instrument for measurement of DNA / PCR concentration.

RESPONSIBILITY
Perform test: Technician / Technologist who is trained and deemed competent in molecular biology techniques.

PRINCIPLE
Spectrophotometric measurements

SPECIMEN
Extracted DNA/RNA in solution or cleaned PCR product

PROCEDURE

1. **Start up:**

- Switch unit on at wall.
- Start computer and double click on the Nanodrop icon on screen.
- Select USER and click on module – "Nucleic Acid" for the measurement of DNA concentration.

Initialization:

- When software starts up a pop up screen will appear – load water sample onto the lower measurement pedestal, lower the sampling arm, and then click "OK". After clicking "OK" the message "Intializing Spectrometer – please wait" will appear.
- When the message disappears the instrument is ready to use. (All data will be logged in the appropriate archive file)

2. **Blank measurement:** Solvents often absorb in the UV range and therefore it is critical to blank the instrument on exactly the same material that the sample is suspended in.

- Ensure measurement pedestal surfaces are clean.
- Load a blank sample (1,5ul) of DNA hydration solution (or other carrier solution used for samples) onto the lower pedestal and lower the sampling arm. Use a 2µl pipette.
- Click the "Blank" button on screen.
- Once blanking complete – wipe blanking solution from both pedestals.
- Analyze an aliquot blank solution as if it was a sample. Use the "Measure" button on screen. The result should be a spectrum with a relatively flat baseline. Wipe the blank from the pedestals and repeat until the spectrum is flat.

3. **Sample measurement:**

- For increased accuracy (optional): Heat DNA samples to 55°C and vortex before measurement. To prevent DNA sample contaminations transfer an aliquot of the sample to be measured (5ul) to a clean tube and use this for quantitation.
- PCR product must be cleaned of dNTP's and primers.
- Type in sample ID in the space provided on screen.
- Place 1,5ul sample on lower pedestal, lower arm and click on "Measure" button on screen.
- Data will automatically be stored in the Nanodrop data archive file on "C" drive.
- Lift arm and wipe the pedestal well before applying next sample. To prevent carry over an aliquot of water can be applied to the pedestals and wiped well.

4. **Shut down**

- Ensure the pedestals are cleaned after last sample. Close program on computer and switch off Nanodrop at wall.

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INTERPRETATION OF RESULTS

The Nanodrop will accurately measure dsDNA / PCR samples up to 3600ng/ml without dilution

Validation of the tesT

- Samples can be compared with results from a spectrophometer using a larger volume. Results compare with 10% from instrument to instrument.
- Absolute accuracy of DNA concentration for PCR is not necessary. PCR can amplify any amount of DNA from one copy to millions. Purity of DNA is more important as contaminants such as hemoglobin inhibit the PCR reaction.

ENTERING OF RESULTS

Record results on work sheet.

Proceedure for abnormal results

- If an abnormal result is obtained repeat the measurement.
- If the ratio is poor repeat the DNA extraction
- If no DNA is recorded repeat the DNA extraction.
- Concentrated samples (greater than 3600ng/ml) dilute the sample and repeat the measurement.

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TABLE 15: *Results of photospectrometric measurements using the Nanodrop*

Sample	ng/µL	A260	A280	260/280
1	39.92	0.658	0.327	2.01
2	19.21	0.384	0.199	1.93
3	28.06	0.561	0.280	2.00
4	38.20	0.764	0.379	2.02
5	28.60	0.572	0.285	2.01
6	29.80	0.596	0.310	1.92
7	26.61	0.532	0.258	2.06
8	24.35	0.487	0.207	2.36
9	43.05	0.861	0.473	1.82
10	32.65	0.653	0.268	2.43
11	38.87	0.777	0.381	2.04
12	45.55	0.911	0.439	2.08
13	18.38	0.368	0.195	1.89
14	55.65	1.113	0.541	2.06
15	22.12	0.442	0.218	2.03
16	31.21	0.624	0.320	1.95
17	17.51	0.350	0.200	1.75
18	35.23	0.705	0.366	1.92
19	49.42	0.988	0.485	2.04
NEG1(Extraction Control)	2.07	0.041	0.056	0.74
20	39.49	0.790	0.381	2.07
21	50.27	1.005	0,504	1.99
22	47.85	0.957	0.455	2.10
23	29.97	0.599	0.282	2.12
24	35.01	0.700	0.345	2.03
25	42.20	0.844	0.414	2.04
26	32.77	0.655	0.330	1.98
27	28.91	0.578	0.288	2.01
28	51.39	1.028	0.478	2.15
29	30.64	0.613	0.286	2.14
30	8.33	0.167	0.089	1.87
31	51.60	1.032	0.493	2.09
32	50.29	1.006	0.474	2.12
33	51.98	1.040	0.496	2.10
34	13.26	0.265	0.131	2.02
35	64.14	1.283	0.618	2.07
36	67.88	1.358	0.633	1.95
37	78.78	1.576	0.742	2.12
38	55.69	1.114	0.532	2.10
39	21.10	0.422	0.203	2.08
NEG2(Extraction Control)	2.40	0.048	0.038	1.26
MOS-1(Positive Control)	1140.58	22.812	10.773	2.12
Positive control 1:100 dilution	31.71	0.634	0.350	1.81
MOS-2(Negative Control)	85.25	1.705	0.845	2.02

For a PCR product between 100-200bp long, a minimum 1-3ng of DNA is required. Therefore to detect a PCR product of 786bp, a minimum of 4-12ng of DNA will be required. The nanodrop confirmed the quantity of DNA extracted which ranged from 8.33 to 78.78 (average 62.2ng).

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POLYMERASE CHAIN REACTION FOR aac(3)-IIa

MASTERMIX:

TABLE 16: ***Contents of mastermix***

Per reaction	
H_2O	23.5 µL
Buffer	10 µL
$MgCl_2$ (25mM)	6 µL
dNTPs (2.5 mM of each dNTP)	4 µL
Forward Primer (10picomoles/ µL)	2 µL
Reverse Primer (10picomoles/ µL)	2 µL
Taq polymerase (5U/ µL)	0.5 µL
Template	2 µL
Total	50 µL

TABLE 17: ***Details of reagents used***

REAGENTS	LOT NO	EXPIRY DATE	MANUFACTURER
GoTaq® Flexi DNA Polymerase	#25803910	17/06/2010	Promega Corporation
Magnesium chloride	#25803910	17/06/2010	Promega Corporation
Molecular weight marker(1kb Plus DNA Ladder) 1µg/µL	Cat No 10787-018		e-invitrogen
Forward and Reverse Primers			Obtained from Tsungai Jongwe, Division of Medical Microbiology.

TABLE 18: ***Details of equipment used***

EQUIPMENT	MANUFACTURER
Nanodrop ND 1000 spectrophotometer	NanoDrop Technologies, Inc.
Thermocycler 4 GeneAmp PCR System 9700	AB Applied Biosystems

Cycling conditions:
95(5’); 35x: 95(1’), 51(45”), 72(1’); 72(5’)

A 2% agarose gel was run at 110V for 1 hour.

Expected product size is 786kb.

Controls:
MOS-1 POSITIVE CONTROL
MOS-2 NEGATIVE CONTROL

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TABLE 19: ***Results of PCR for aac(3)-IIa***

LANE NO	SPECIMEN NO.	RESULT
1	MWM	
2	Isolate 38	Absent
3	Isolate 21	Absent
4	Isolate 28	Present
5	Isolate 16	Absent
6	Isolate 34	Absent
7	Isolate 30	Absent
8	Isolate 37	Absent
9	Isolate 23	Absent
10	Isolate 35	Absent
11	Isolate 2	Absent
12	POSITIVE CONTROL	Present
13	NEGATIVE CONTROL	Absent
14	EXTRACTION CONTROL	Absent
15	BLANK (Water)	Absent
1	MWM	
2	Isolate 17	Present
3	Isolate 32	Present
4	Isolate 22	Present
5	Isolate 20	Present
6	Isolate 9	Present
7	Isolate 31	Present
8	Isolate 10	Present
9	Isolate 29	Absent
10	Isolate 33	Present
11	Isolate 11	Absent
12	POSITIVE CONTROL	Present
13	NEGATIVE CONTROL	Absent
14	EXTRACTION CONTROL	Absent
15	BLANK (Water)	Absent
1	MWM	
2	Isolate 4	Absent
3	Isolate 39	Present
4	Isolate 36	Present
5	Isolate 8	Present
6	Isolate 5	Present
7	Isolate 6	Present
8	Isolate 42	Present
9	Isolate 18	Present
10	Isolate 14	Present
11	Isolate 13	Present
12	POSITIVE CONTROL	Present
13	NEGATIVE CONTROL	Absent
14	EXTRACTION CONTROL	Absent
15	BLANK (Water)	Absent
1	MWM	
2	Isolate 40	Present
3	Isolate 26	Present
4	Isolate 12	Present
5	Isolate 15	Present

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6	Isolate 41	Absent
7	Isolate 24	Absent
8	Isolate 3	Absent
9	Isolate 27	Absent
10	Isolate 7	Absent
11		
12	POSITIVE CONTROL	Present
13	NEGATIVE CONTROL	Absent
14	WATER	Absent
15		

REPEAT PCR OF NEGATIVES

1	MWM	
2		
3	1	
4	2	
5	3	
6	4	
7	5	
8	6	
9	7	
10	8	
11	9	
12	10	
13	POSITIVE CONTROL	
14	NEGATIVE CONTROL	
15	WATER	
1	MWM	
2		
3	18	
4	20	
5	35	
6	36	
7	37	
8	38	
9	39	
10	POSITIVE CONTROL	
11	NEGATIVE CONTROL	
12	WATER	
13		
14		
15		

2% gel run at 110V for 1 hour, 1kb ladder used.

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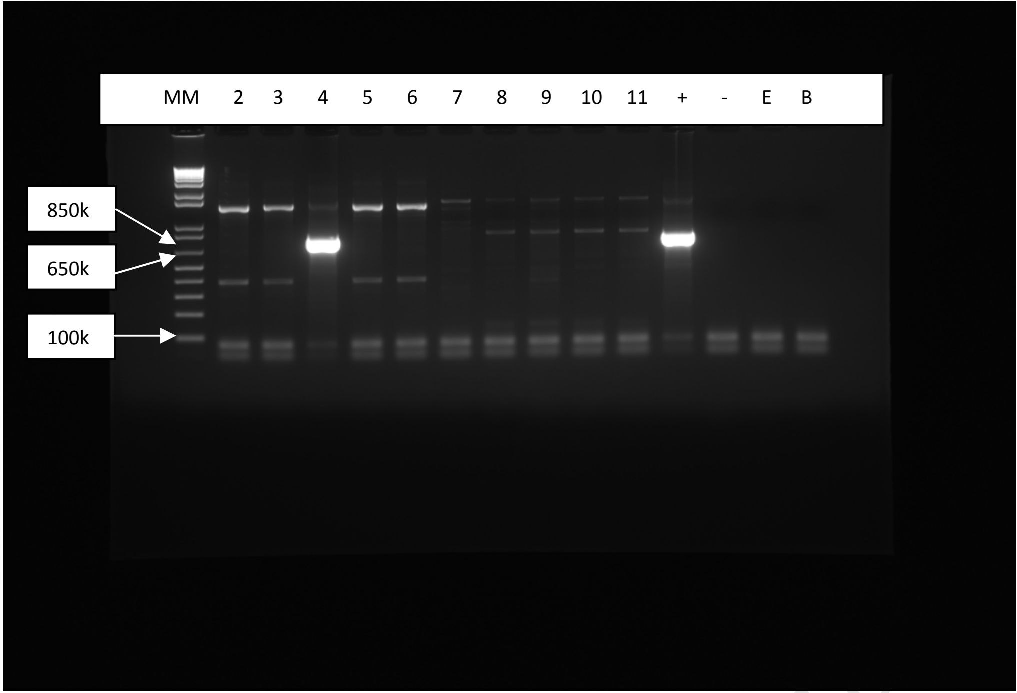


FIGURE 4: GEL 1
Lane 1: Molecular weight mark, Lanes 2-11: Specimens 1-10, Lane 12: Positive control, Lane 13: Negative control, Lane 14: Extraction control, Lane 15: Blank (water).

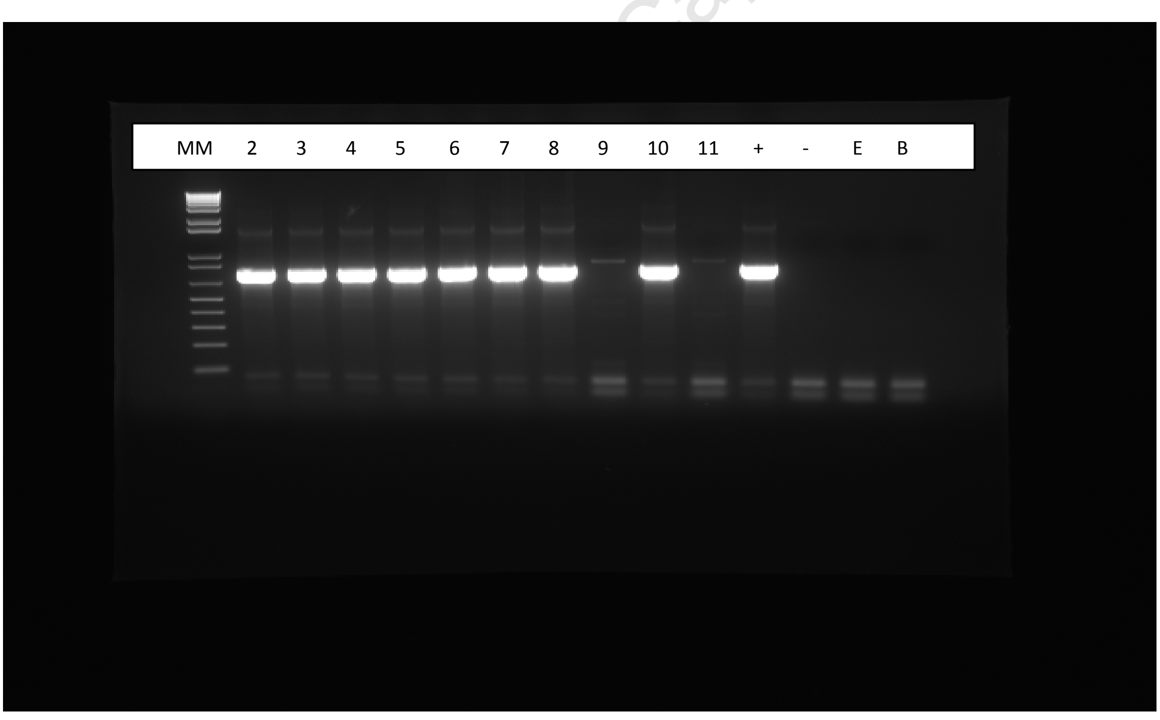
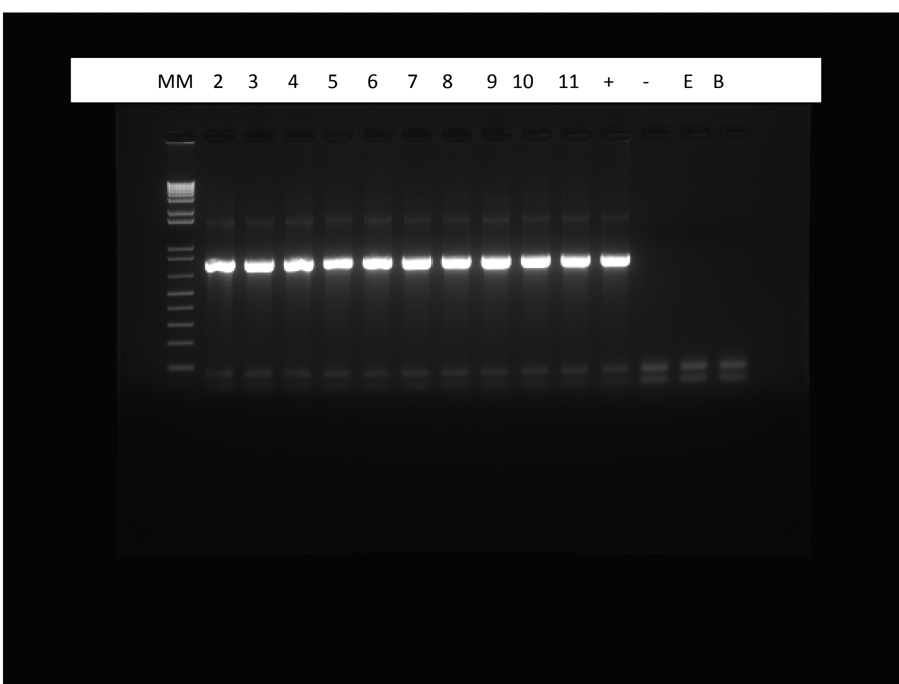
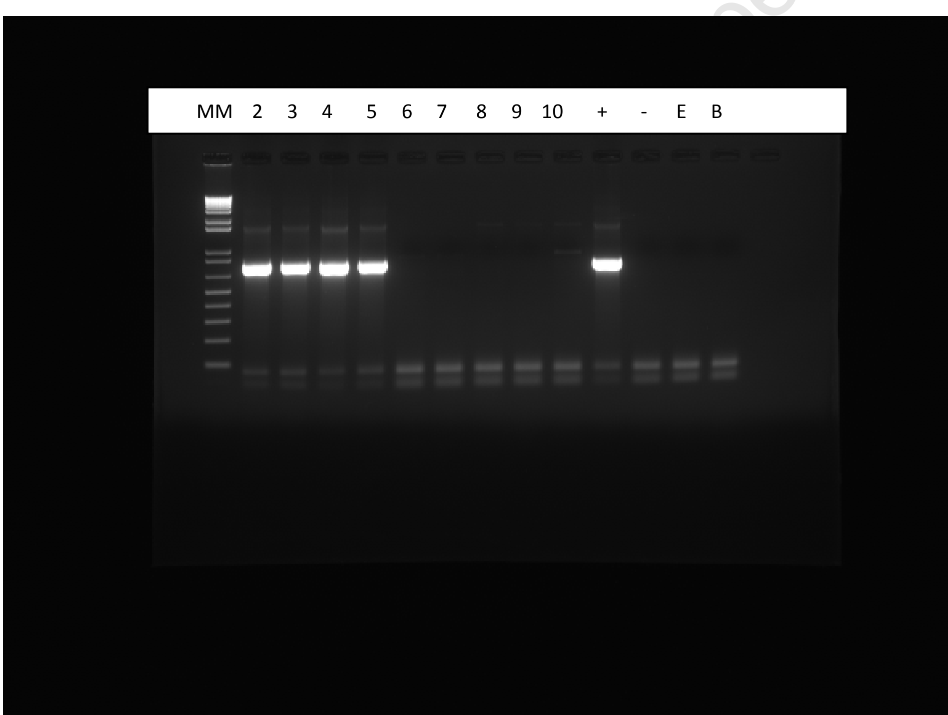


FIGURE 5: GEL 2
Lane 1: Molecular weight mark, Lanes 2-11: Specimens 11-20, Lane 12: Positive control, Lane 13: Negative control, Lane 14: Extraction control, Lane 15: Blank (water).



<u>FIGURE 6:</u> **GEL 3**

Lane 1: Molecular weight mark, Lanes 2-11: Specimens 21-30, Lane 12: Positive control, Lane 13: Negative control, Lane 14: Extraction control, Lane 15: Blank (water).



<u>Figure 7</u>**: GEL 4**

Lane 1: Molecular weight mark, Lanes 2-9: Specimens 31-39, Lane 11: Positive control, Lane 12: Negative control, Lane 13: Extraction control, Lane 14: Blank (water).

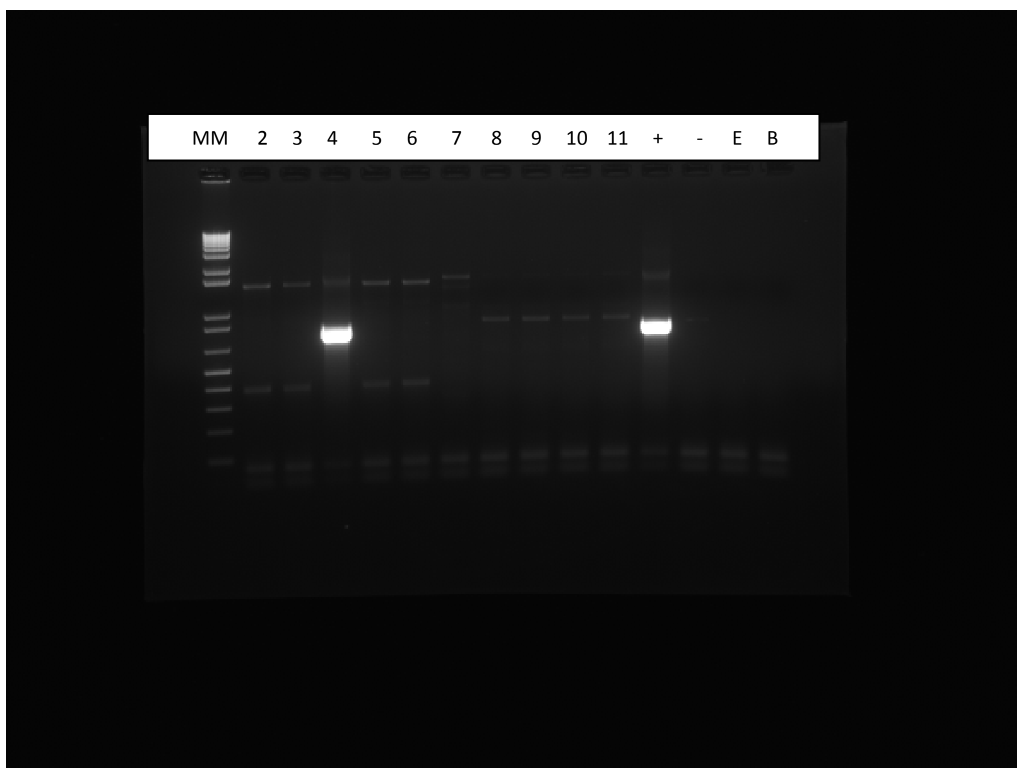


FIGURE 9: REPEAT GEL 1
Lane 1: Molecular weight mark, Lanes 2-11: Specimens 1-10, Lane 12: Positive control, Lane 13- Negative control, Lane 14- Extraction control, Lane 15- Blank (water).

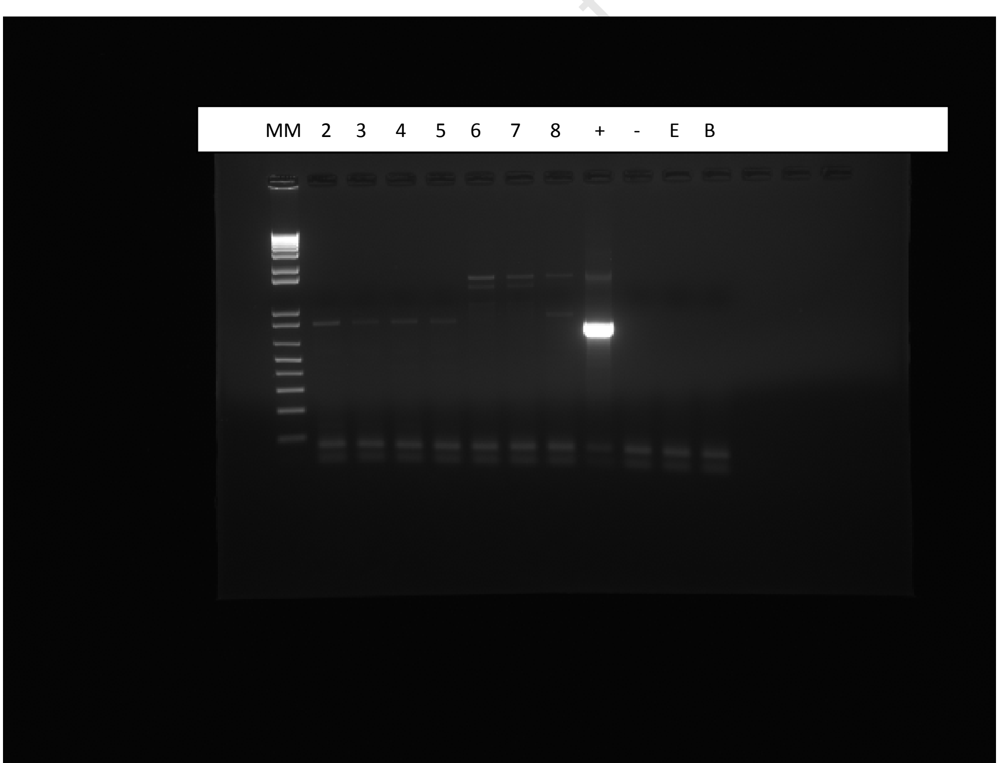


Figure 10: REPEAT GEL 2
Lane 1: Molecular weight mark, Lanes 2-8: Specimens 18, 20, 35-39, Lane 9: Positive control, Lane 10: Negative control, Lane 11: Extraction control, Lane 12: Blank.

SOP 5: 16S rRNA PCR (Adapted from NHLS SOP MIC0742) PCR Amplification and DNA Sequencing of 16srRNA and ITS2 r DNA for Fungal identification

PRINCIPLE

16S rRNA:

All bacteria have genes encoding the RNA for the 16S subunit of the ribosome. These genes are highly conserved (*i.e.* there are areas of the gene that do not differ between different genera of bacteria), allowing one set of primers to amplify DNA from a wide range of bacterial species. However, some differences between species do exist, so analysis of DNA sequence data from these genes can allow one to identify the organism from which the DNA was amplified.

16s rRNA Primers:

BAKF – 5' – AGA GTT TGA TCC TGG CTC AG 3'
BAKR – 3' – AAG GAG GTG ATC CAG CCG CA – 5'

(Ref: Edwards U *et al*. 1989. Isolation of a direct complete nucleotide determination of entire genes. Characterisation of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. 17: 7843 – 7853)

PROCEDURE:
Use 5µl of extracted DNA for your PCR reaction

PCR Reaction Mixture (Mastermix) 16s RNA:

PCR reaction mix for 10 samples

dNTPs 4x1µl each	4
Taq polymerase	3
MgCl	30
Buffer	50
Water	293
Total volume	380
Volume for each reaction	38 µl

Add 1µl of each forward (20pmol/µl) and reverse (20pmol/µl) primer from the working stock to each mastermix.

Use *E. coli* genomic DNA as a positive control, and always include both a blank control, and a water (negative) control.

PCR assay:

The conditions are programmed into the thermocycler – Programme **"16s rRNA PCR"**

The conditions are:
94°C for 2 minutes
94°C for 20 seconds; 47°C for 30 seconds, 72°C for 45 seconds – for 35 cycles
72°C for 7 minutes
Hold at 4°C

Detection of PCR product:

1. After completion of the PCR assay run 10µl of product on a 2% agarose gel.
2. Set the powerpack at 90 volts and run for approximately 30 to 40 minutes.
3. Once the products have run sufficiently visualise the products in the UV transilluminator.

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4. Place the gel on the glass surface of the transilluminator and take a photograph of the gel using the Uvitec Gel Documentation System (SOP CHE0627).
5. Capture the image and save in the S drive/laboratories/microbiology/common/read and write only/gel images/then the name and date the gel photo was taken.

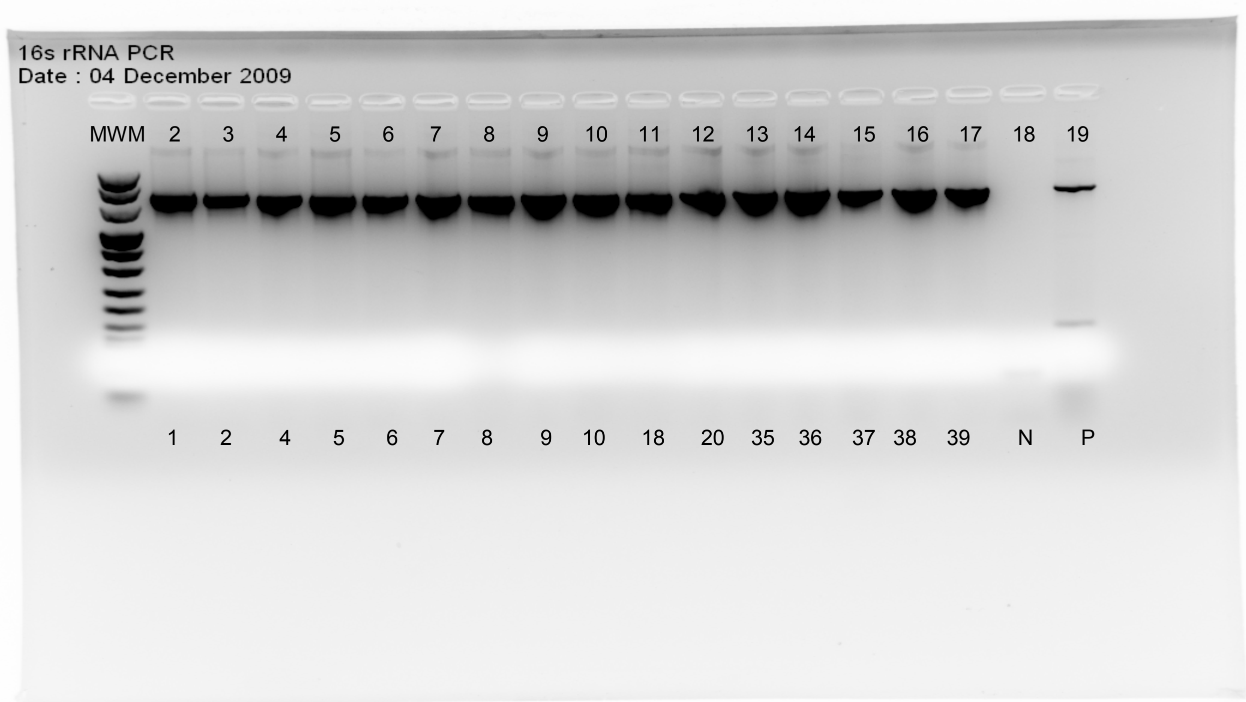

FIGURE 11:
Lane 1: MWM, Lanes 2-17: Isolates (1,2,4,5,6,7,8,9,10,18,20,35,36,37,38,39)with negative *aac(3)-IIa* PCR, N: Negative control, P: Positive Control

SEQUENCING SOP 6:

STANDARD OPERATING PROCEDURE

Title: SEQUENCING REACTION AND ANALYSIS

Document number: CHE0617

Version number: 2

(Changes from previous version highlighted)

Written by: S. Meldau

Checked by: N/A

Approved by: Dr E.P Owen

Active date: 19-02-2009

Date of next review	*Date reviewed*	*Reviewed by*	*Action*
19-02-2010			

Date withdrawn: ....

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SEQUENCING REACTION AND ANALYSIS

OBJECTIVE

To sequence PCR products and analysis the results

RESPONSIBILITY

Perform test: Technologist/medical scientist who is trained and deemed competent in molecular biology techniques.

REAGENTS AND CONSUMABLES

ABI PRISM BigDye™ Terminator v3.1 Ready Reaction Cycle sequencing kit **Catalogue No: 4390244**

Supplied with kit:
- Terminator Ready Reaction Mix
- pGEM-3Zf+ double stranded DNA control template, 0.2 µg/µl
- -21 M13 Control Primer (forward) 0.8 pmol/µl

PCR tubes (0.2ml)
PCR product to sequence
Correct primer 3.2 pmols/ul
H_2O

PROCEDURE

1. Quantitate the purified (either column or gel) PCR template using either an agarose gel method with visual estimate or by measuring the absorption at 260 nm (nanodrop spectrophotometer).
2. The copy number of the PCR product is important, so the longer the PCR product the greater the quantity of PCR product to add. – 10µl reaction volume

Template – PCR product	Quantity
100-200bp	0.5-1.5 ng
200-500 bp	1.5-5 ng
500-1000 bp	2.5-10 ng
1000-2000 bp	5-20 ng
>2000 bp	20-50 ng

3. Calculate the amount of PCR template to add to each reaction tube from the above table and write onto work sheet.
4. Check a thermocycler is available. Turn on to warm lid. Check program and set ready to run.
5. For each PCR template to be sequenced prepare a separate labeled PCR tube 0.2ml. Prepare the following on ice

Reagent	Quantity
Terminator ready reaction mix	1ul
10 x Buffer	0.6µl
Template	1-5µl
Primer 3.2pmols/ul	1 ul
H2O deionised	0-4µl
Total volume	10µl

6. Mix and spin Thermocycler SM 3.2.0
7. Immediately place in ~~PCR machine GeneAmp PCR system 9700~~
Program: BigDye

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See program called bigdye 50°C

- Rapid thermal ramp to 96°C
- 96°C for 15 sec
- Repeat the following for 25 to 30 cycles
    1. 96°C for 10 sec
    2. 50°C for 15 sec
    3. 60°C for 4 mins
- Followed by final step of 10 mins at 60°C
- Hold at 4°C until removed from the machine.

8. Place samples in deep freeze
9. Outsource samples for separation. See below

Stellenbosch University – DNA Sequencing Facility

Tel: (021) 808 5887

Fax: (021) 808 5833

e-mail: sequence@sun.ac.za

web page: www.sun.ac.za/saf

or

Genetics department

University of Cape Town.

Tel (021) 406 6456

Email Jeanne Rousseau, rousseau@cormack.uct.ac.za

10. Results will be emailed back.
11. Open the file in either Chromas, Chromas Lite, or Bioedit.
12. Print out the sequence (remember to reverse sequence if reverse primer used)
13. Check all bases for miss calling and possible heteroplasmy.
14. Sequences can be lined up with reference sequencing using Bioedit software.
15. Copy text sequence into a .txt file using notepad using the following format.
    >PDH E1a Exon 4 complete sequence:
    TTGCTTCTGGTTTGGGCCTTCCACTCTGTATTTTGGTGGAAAATAGCTACTTTCTCTGGTTATTAATGACAGGTTCTACTAGCCC
    ACATATTTCACTGTGGTCTAGGAAACGTTTTTATTTAGAAACA**T**GTATCATATTGCCTCATAGTTTCTCCTTCCTCT
    >exon 4:
    GAAGCTTGCTGTGTGGGCCTGGAGGCCGGCATCAACCCCACAGACCATCTCATCACAGCCTACCGGGCTCACGGCTTTACTT
    TCACCCGGGGCCTTTCCGTCCGAGAAATTCTCGCAGAGCTTACAG
    >F559 exon4:
    CCAANTNTTGGTGGAAATAGCTCTTTCTCTGGTTATTAATGACAGGTTCTACTAGCCCANATATTTCACTGTGGTCTAGG
    AAACGTTTTTATTTAGAAACATGTATCATATTGCCTCATAGTTTCTCCTTCCTCTAACACAGGAAGCTTGCTGTGTGGGC
16. Analyse the results and print.

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FORWARD PRIMER SEQUENCE(aacC2a) : 5'-3' : cgc gga agg caa taa c
REVERSE PRIMER SEQUENCE(aacC2a) : 5'-3' : gct tct caa gat agg tg

TABLE 20: ***Nanodrop results of isolates sent for sequencing***

SAMPLE	DATE	TIME	ng/µL	A260	A280	260/230	260/230
1	26/11/09	12h11	60.42	1.208	0.640	1.89	0.41
3	26/11/09	12h12	138.05	2.761	1.458	1.89	1.25
11	26/11/09	12h13	141.12	2.822	1.497	1.89	0.81
Forward Primer	09/11/09	08h54	81.19	1.624	0.741	2.19	2.29
Reverse Primer	09/11/09	08h56	70.59	1.412	0.818	1.73	2.39

Reaction mixture(per sample)

H_2O 23.5 µL
Buffer 10.0 µL
$MgCl_2$ 6 µL
dNTP 4 µL
Forward primer 2 µL
Reverse primer 2 µL
Taq polymerase 0.5 µL
Template 2 µL

Primer 1.0 µL (20pmoles/ µL)

Mix and place in thermocycler.

Program 96° 15", 30x : (96° 15", 50° 15", 60° 4'), 60° 10'.

TABLE 21: ***Reagents used for PCR***

REAGENT	LOT NUMBER	DATE	MANUFACTURER
5x Colourless GoTaq® Flexi Buffer	#25803910	17-06-2010	Promega Corporation
Magnesium chloride 25mM	#25803910	17-06-2010	Promega Corporation
GoTaq® Flexi DNA Polymerase	#25803910	17-06-2010	Promega Corporation
BigDye Terminator v3.1 Ready Reaction Cycle sequencing kit			AB Biosystems

EQUIPMENT	MANUFACTURER
Thermocycler 9	Hybaid Express

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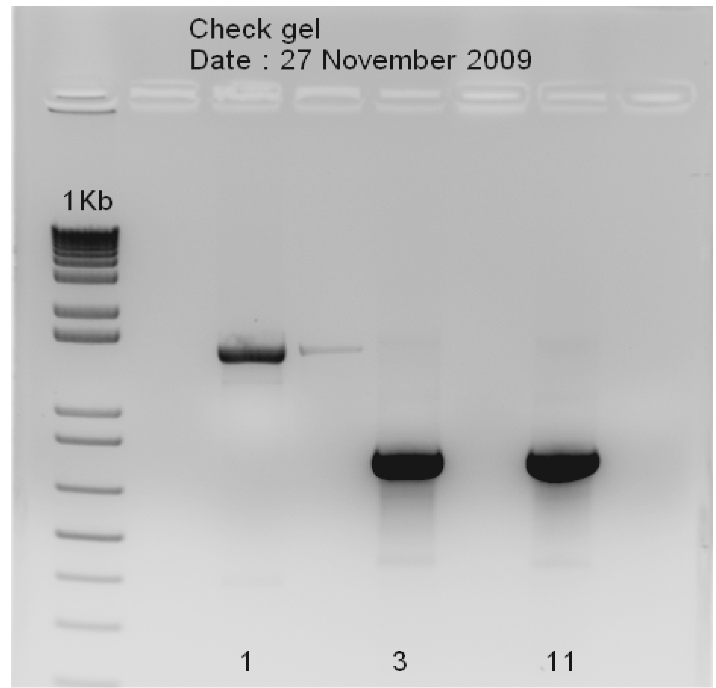

10µL run
on 2% gel

Product
size 786bp

<u>FIGURE 12:</u> Check gel of sequencing products
Lane 1: MWM, Lane 3-: Isolate 38, Lane 5: Isolate 28 , Lane 7- Isolate 17.

Three isolates were selected- 2 isolates with a positive PCR (isolate 17 and 28). Isolate 38 was also sequenced as an additional band at approximately 1.6 kb was visible on PCR for *aac(3)-IIa*.

The sequencing was performed at the DNA Sequencing Facility at the University of Stellenbosch using ABI genetic analyzers. The BLAST (Basic Local Alignment Search Tool) nucleotide collection database was searched using Megablast to find highly similar sequences. The results are shown below.

Chromas software was used to evaluate the quality of the sequencing using the chromatogram.

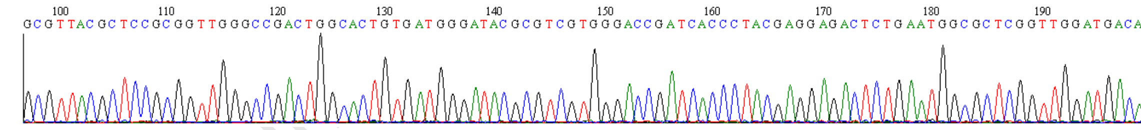

Figure 13: An example of the chromatogram obtained using Chromas Lite software.

Good chromatograms were obtained for isolates 17 and 38. However, the chromatogram for isolate 38 revealed evidence of a mixed sample.

SEQUENCE OF ISOLATE 38(FORWARD PRIMER) FASTA SEQUENCE:

TTGCAATGCGCTGCATATGYGAGMTGTACCGGTCATAGATCTGATCKCAGTGYTGMSATTGCAC
CTGTGGCGTTTTTTCACCGGATTTACCAMCTACTGCCTCTTCTATTTTGAAAAACKGSGCTTTCAC
CCATCTGATGCTTACAATTCTGGCTATGTCTTACATTGCCGCGRCGAARGCTTTTTGATCTGTTAT
TCTTACTTATGGTTCCTCTTGMCCTGCTGTGAATTTTTGKACCTTGTTTGTTGTGCRTGATTTTTGA
RGGGATTGRATTCSGAATACATACTACTTGYTCASARWGGKCCRCCTTAATCGRAMCKATGARC
CTCCTTGTTTCGTCTGCTTATTTCTGGWACGACTTCTCACCCTTGCCWGCCGCCTTCGATCATTA
CACCATTTATGATTATCTGACTGATTTCTGCCAGCCACGTGWATCACAACATTTTCTAAAGCTTTT
CACTGAATGAATGATTGGMCGTGKWCTGGAKGACACACSCGCMCGKCTTCYARWTGCWGTGW
RMMAKGTATCGTATTRCAARGACKAACCGCTATCKAAAAGATTGTGMTTGTGCTCGCGAKCSACT
TTGGWKGYWGKKYCKGCWKYAYCTTAYTGCAWGWTGAWYACGTGGAYGAAATTCATGTGGAAT
CAGATGAARATCWTSRTACKTTGCTTCGTCCGACTCTGTCTGMTCAAYASATTCRRACGATCAWA
CRWMTSMWGATCTGMATCTGWKACCCCWGAAYWKSWCCCAGAKWWCMATTTTCACGAAKRA

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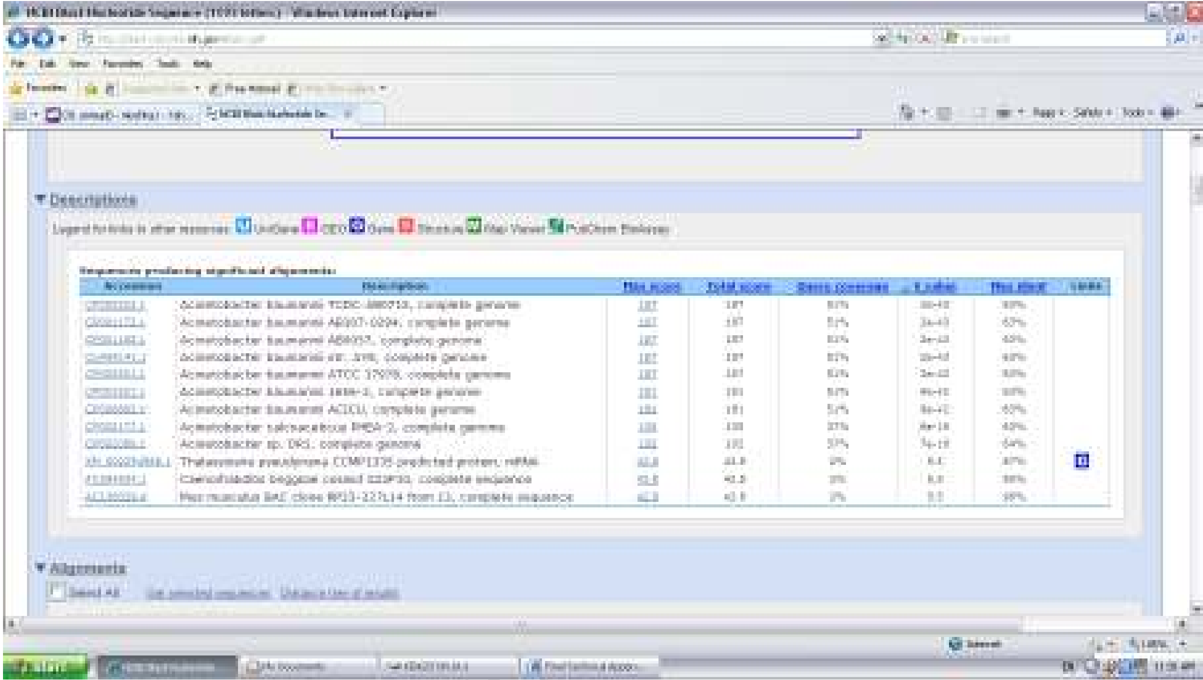
GCCAMWGCCGMAMCTGMAGCTGTCCCGMCCTAMRMTRRCWAMCTTTCCAKRGCYWTATTCTT
CGTATGMAWACYGSTRMTCGCRCTGTACSYSAYGCCGGGTWYSCSTCATAMCYGCWKCYMYYS
CCYACTSWKGGACATKWCTAAAYGACGTGMTCGTRCACCKGCTGCTGATCTTCAAMGAATGMY
CATMATKTGKSTKAKATSSARKSCCYGMCWGGGTGGAATRCSTKCCAMCACYMSCAGMAAGACT
WGAAGATCCYWCTGTCAATAGSTASCACTSTGACGATCRTGWTCCGACYYCTGCCCTCTGAAAT
CCGAGAACCGGRCGATACYACGCTWATGCTCTGGAAACTCGYGAATCWCGAACCTATGCCGAT
GAGCTATGAGCCATTCAAGGTTCGAAAKCGYGACTCGTGG

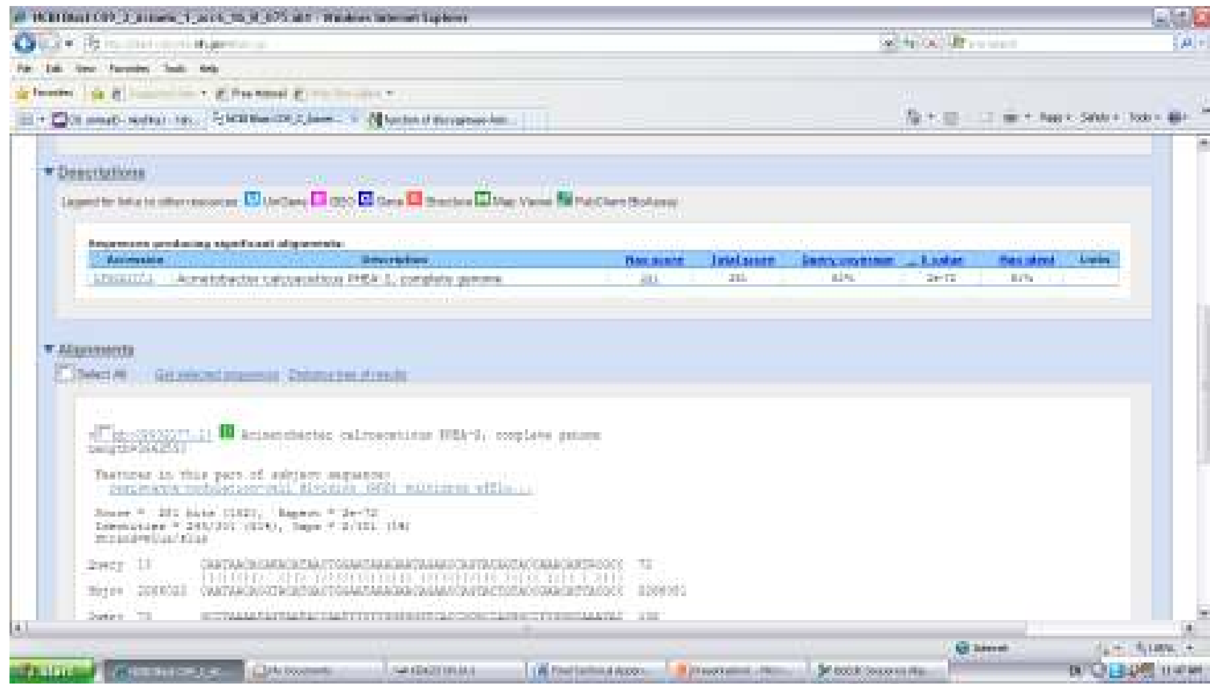
No result was obtain when Isolate 38 was blasted using MegaBlast or . However a result was obtained using Blastn (dissimilar). This sequence showed some similarity with the *A. baumannii* dioxygenase. This is an enzyme that is involved in iron binding. However, the association is weak.



SEQUENCE OF ISOLATE 38(REVERSE PRIMER) FASTA SEQUENCE:

TTTCGYGGAAGGCAATAACACAKACATAACTGGAATAAAGAATAGAACCAGTACAGTACCAAACA
KTRCGCCGCCTAAAATASTAATACCAATTTYTTGGSGGSTCACCGCRCCAGSGCCTTGGGCAAAT
ACAAGTGGAATCACACSKGCASCAAAGGCTAAAGAAGTCATTAAAATSGGTCTTAAACGCAAGCT
CGCACSTTATAAAGCGGCCKGAATAGCATTCTTTCCTTTSTCTTGGGSTAAKGCAGCAAACTCAA
CAATTAAAATTGSGTTKWTGCATGACAATMCAATCGTGGTTAGAAGTGCAATTCGCGGAAGGCAA
TAACACCCAGCTGMCACRCAGAMCAGMACAYAATWTT.

When this sequence was blasted using Blastn, a weak similarity was found with the RND multidrug efflux pump of *A. calcoaceticus* PKEA-2. The association was too weak to draw any further conclusions.



SEQUENCE OF ISOLATE 28(FORWARD PRIMER) FASTA SEQUENCE :

TAGYSASSTCGGGRGTCAACCGGTGACCTGTTGATGGTGCATGCCTCACTTAAAGCGATTGGTC
CGGTCGAAGGAGGAGCGGAGACGGTCGTTGCCGCGTTACGCTCCGCGGTTGGGCCGACTGGC
ACTGTGATGGGATACGCGTCGTGGGACCGATCACCCTACGAGGAGACTCTGAATGGCGCTCGG
TTGGATGACAAAGCCCGCCGTACCTGGCCGCCGTTCGATCCCGCAACGGCCGGGACTTACCGT
GGGTTCGGCCTGCTGAATCAATTTCTGGTTCAAGCCCCCGGCGCGCGGCGCAGCGCGCACCCC
GATGCATCGATGGTCGCGGTTGGTCCGCTAGCTGAAACGCTGACGGAGCCTCACGAACTCGGT
CACGCCTTGGGGAAAGGGTCGCCCGTCGAGCGGTTCGTCCGCCTTGGCGGGAAGGCCCTGCT
GTTGGGTGCGCCGCTAAACTCCGTTACCGCATTGCACTACGCCGAGGCGGTTGCGGATATCCC
CAACAAACGATGGGTGACGTATGAGATGCCGATGCTTGGAAGAAACGGTGAAGTCGCCTGGAAA
ACGGCATCAGAATACGATTCAAACGGCATTCTCGATTGCTTTGCTATCGAAGGAAAGCCGGATGC
GGTCGAAACTATAGCAAATGCTTACGTGAAGCTCGGTCGCCATCGAGAAGGTGTCGTGGGCTTT
GCTCAGTGCTACCTGTTCGACGCGCAGGACATCGTGACGTTCGGCGTCACCTATTTTKGAGAAG
C

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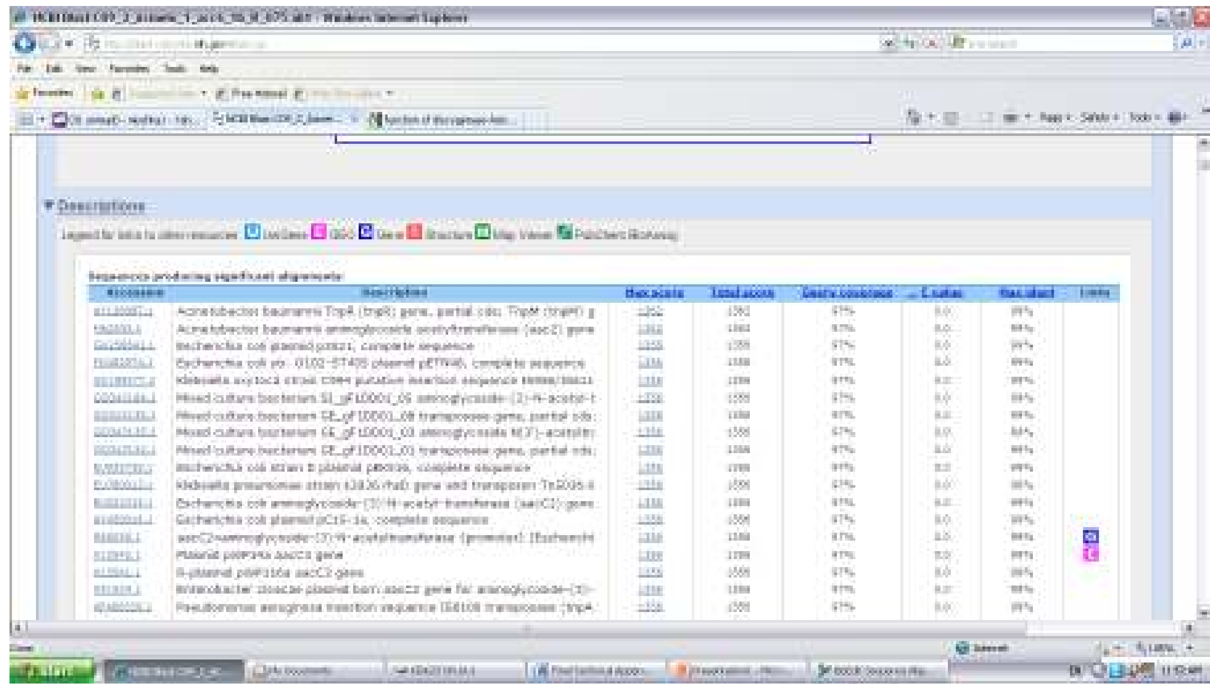

SEQUENCE OF ISOLATE 28(REVERSE PRIMER) FASTA SEQUENCE :

GCCSGSYKTWKAWCTGCGCGTCGACAGGTAGCACTGAGCAAAGCCCACGACACCTTCTCGATG
GCGACCGAGCTTCACGTAAGCATTTGCTATAGTTTCGACCGCATCCGGCTTTCCTTCGATAGCAA
AGCAATCGAGAATGCCGTTTGAATCGTATTCTGATGCCGTTTTCCAGGCGACTTCACCGTTTCTT
CCAAGCATCGGCATCTCATACGTCACCCATCGTTTGTTGGGGATATCCGCAACCGCCTCGGCGT
AGTGCAATGCGGTAACGGAGTTTAGCGGCGCACCCAACAGCAGGGCCTTCCCGCCAAGGCGGA
CGAACCGCTCGACGGGCGACCCTTTCCCCAAGGCGTGACCGAGTTCGTGAGGCTCCGTCAGCG
TTTCAGCTAGCGGACCAACCGCGACCATCGATGCATCGGGGTGCGCGCTGCGCCGCGCGCCG
GGGGCTTGAACCAGAAATTGATTCAGCAGGCCGAACCCACGGTAAGTCCCGGCCGTTGCGGGA
TCGAACGGCGGCCAGGTACGGCGGGCTTTGTCATCCAACCGAGCGCCATTCAGAGTCTCCTCG
TAGGGTGATCGGTCCCACGACGCGTATCCCATCACAGTGCCAGTCGGCCCAACCGCGGAGCGT
AACGCGGCAACGACCGTCTCCGCTCCTCCTTCGACCGGACCAATCGCTTTAAGTGAGGCATGCA
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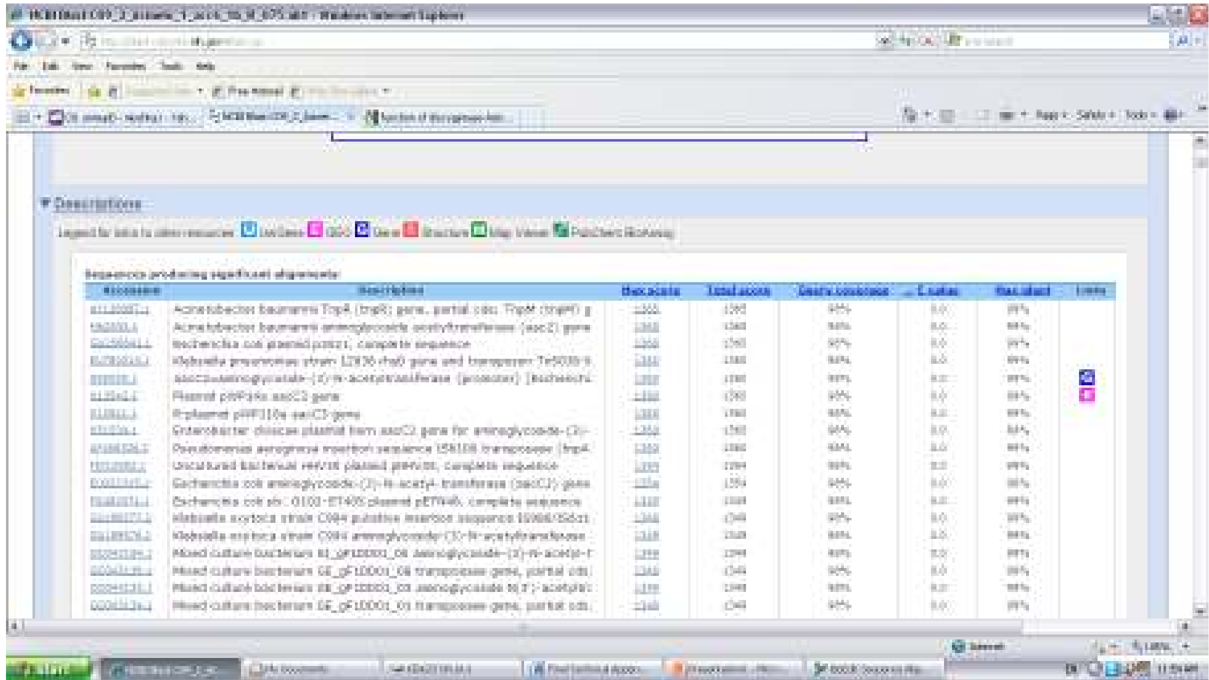
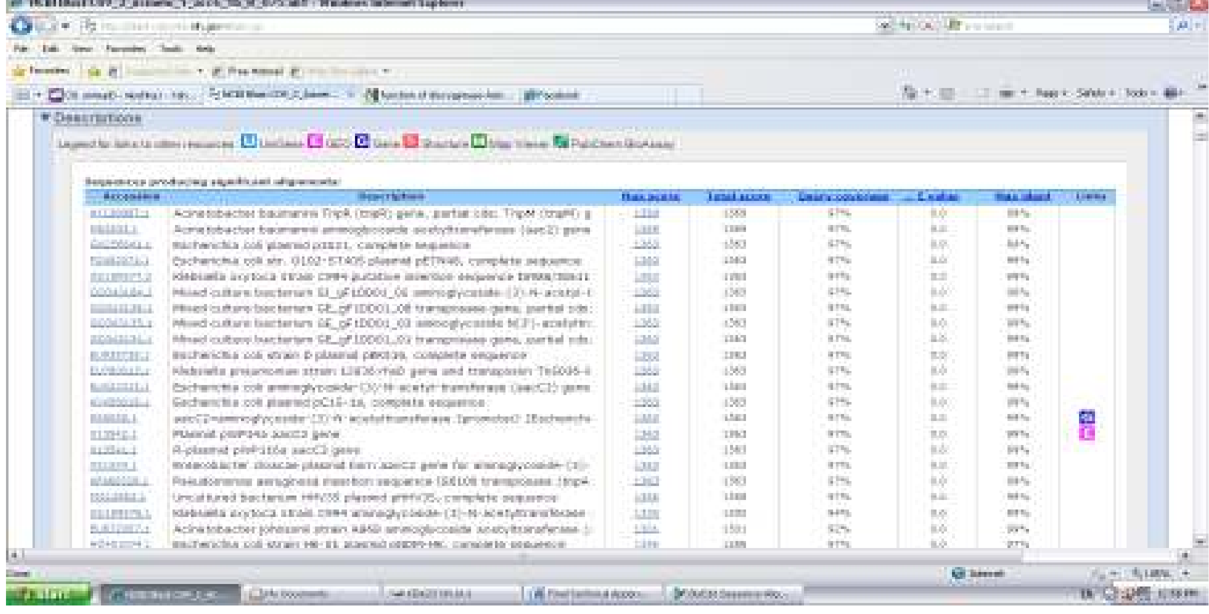


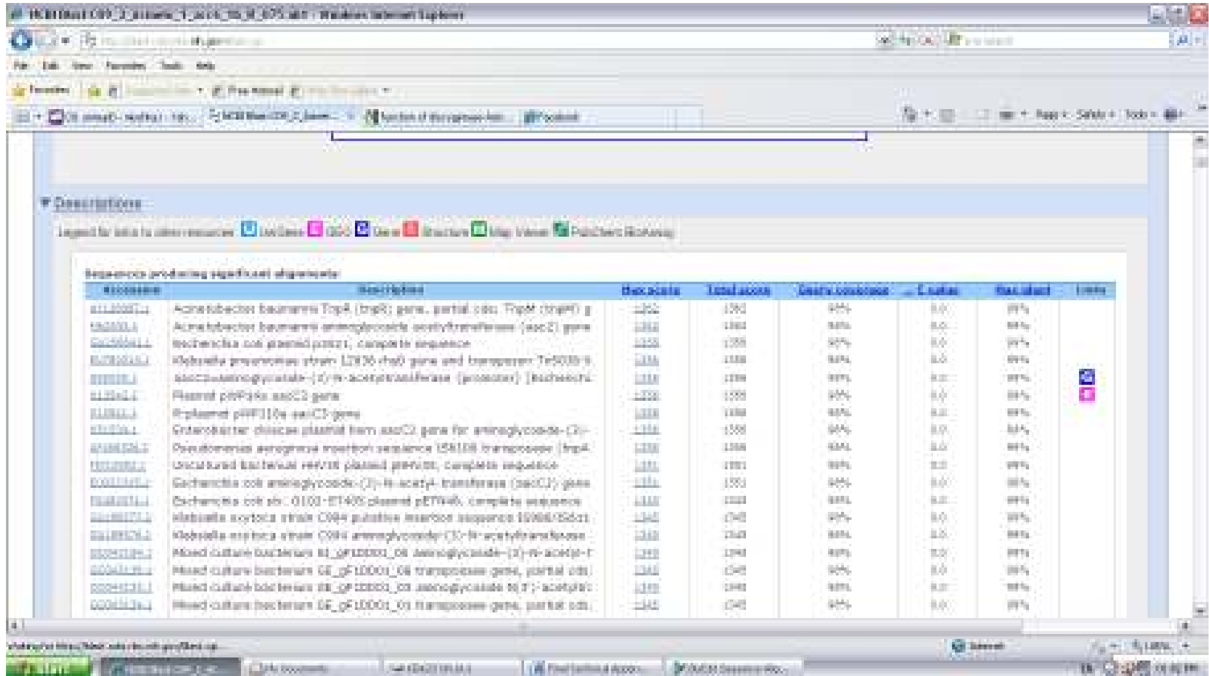
SEQUENCE OF ISOLATE 17(FORWARD PRIMER) FASTA SEQUENCE:

TWKYGRCYTCGGGAGTCAAACCGGTGACCTGTTGATGGTGCATGCCTCACTTAAAGCGATTGGT
CCGGTCGAAGGAGGAGCGGAGACGGTCGTTGCCGCGTTACGCTCCGCGGTTGGGCCGACTGG
CACTGTGATGGGATACGCGTCGTGGGACCGATCACCCTACGAGGAGACTCTGAATGGCGCTCG
GTTGGATGACAAAGCCCGCCGTACCTGGCCGCCGTTCGATCCCGCAACGGCCGGGACTTACCG
TGGGTTCGGCCTGCTGAATCAATTTCTGGTTCAAGCCCCCGGCGCGCGGCGCAGCGCGCACCC
CGATGCATCGATGGTCGCGGTTGGTCCGCTAGCTGAAACGCTGACGGAGCCTCACGAACTCGG
TCACGCCTTGGGGAAAGGGTCGCCCGTCGAGCGGTTCGTCCGCCTTGGCGGGAAGGCCCTGCT
GTTGGGTGCGCCGCTAAACTCCGTTACCGCATTGCACTACGCCGAGGCGGTTGCGGATATCCC
CAACAAACGATGGGTGACGTATGAGATGCCGATGCTTGGAAGAAACGGTGAAGTCGCCTGGAAA
ACGGCATCAGAATACGATTCAAACGGCATTCTCGATTGCTTTGCTATCGAAGGAAAGCCGGATGC
GGTCGAAACTATAGCAAATGCTTACGTGAAGCTCGGTCGCCATCGAGAAGGTGTCGTGGGCTTT
GCTCAGTGCTACCTGTTCGACGCGCAGGACATCGTGACGTTCGGCGTCACCTATYTTKGAGAAA
GMAA.



SEQUENCE OF ISOLATE 17(REVERSE PRIMER) FASTA SEQUENCE:

GGCSGATGTAWWAWCTGCGCGTCGACAGGTAGCACTGAGCAAAGCCCACGACACCTTCTCGAT
GGCGACCGAGCTTCACGTAAGCATTTGCTATAGTTTCGACCGCATCCGGCTTTCCTTCGATAGCA
AAGCAATCGAGAATGCCGTTTGAATCGTATTCTGATGCCGTTTTCCAGGCGACTTCACCGTTTCT
TCCAAGCATCGGCATCTCATACGTCACCCATCGTTTGTTGGGGATATCCGCAACCGCCTCGGCG
TAGTGCAATGCGGTAACGGAGTTTAGCGGCGCACCCAACAGCAGGGCCTTCCCGCCAAGGCGG
ACGAACCGCTCGACGGGCGACCCTTTCCCCAAGGCGTGACCGAGTTCGTGAGGCTCCGTCAGC
GTTTCAGCTAGCGGACCAACCGCGACCATCGATGCATCGGGGTGCGCGCTGCGCCGCGCGCC
GGGGGCTTGAACCAGAAATTGATTCAGCAGGCCGAACCCACGGTAAGTCCCGGCCGTTGCGGG
ATCGAACGGCGGCCAGGTACGGCGGGCTTTGTCATCCAACCGAGCGCCATTCAGAGTCTCCTC
GTAGGGTGATCGGTCCCACGACGCGTATCCCATCACAGTGCCAGTCGGCCCAACCGCGGAGCG
TAACGCGGCAACGACCGTCTCCGCTCCTCCTTCGACCGGACCAATCGCTTTAAGTGAGGCATGC
ACCATCAACAGGTCACCGGTTTGGACTCCGAGTTTTCGAATTGCCTCCGTTATGCCCYTTCCGCG
A



These sequences are identical to the *aac(3)-IIa* gene. The alignment with the original sequence is shown on the next page.

REFERENCES:

1. **Zhang, Z., S. Schwartz, L. Wagner, and W. Miller. 2000. *A greedy algorithm for aligning DNA sequences.* J Comput Bid. 7 (1-2): 203-214.**

MOLECULAR EPIDEMIOLOGY

University of Cape Town

PULSED FIELD GEL ELECTROPHORESIS OF *Acinetobacter baumannii* strains
(Adapted from Jacobson, R. **Association of IS1133 with an aminoglycoside resistance gene,*aacC2a*, in *Acinetobacter baumannii* isolates.** August 2007.**)**

STANDARD OPERATING PROCEDURE:

PLUG PREPARATION

1. A bacterial suspension (OD_{540} 1.8-2.0) should be prepared in cell suspension buffer (100mM Tris, 100mM EDTA, pH 8.0). The bacterial cells were obtained from a single colony plate that had been streaked out on 2% horse blood agar after 24 hour incubation at 37°C.
2. The suspension should be incubated at 55°C for 10min in a water bath.
3. Twenty-five microlitres of proteinase K (20mg/ml stock solution) and 5µL of lysozyme (100mg/ml) should be added to the cell suspension. The mix should be inverted 2-4X.
4. Five hundred microlitres of 1% Seakem (with 1% SDS) is added to each tube and the suspension was gently inverted 10-12X.
5. The plugs are then cast in plastic moulds and allowed to solidify at room temperature for 5min and at 4°C for another 15min.

LYSIS OF CELLS IN PLUGS AND WASHING

6. Five milliliters of cell lysis buffer (50mM Tris, 50 mM EDTA, pH 8.0+1 % sarcosine) together with 25µL proteinase K (20mg/ml stock solution) and 5µL lysozyme (100mg/ml).
7. The solution is incubated at 55°C in a shaking incubator for 2 hours.
8. The plugs are washed 2 X for 15 minutes in 10ml warmed distilled water at 55°C.
9. The plugs are then washed 3 X for 15 minutes in 10ml warmed TE buffer at 55°C.
10. The plugs are trimmed to the correct dimensions 2.0mmx 2.5mm
10. Store the plugs in 10ml fresh TE buffer at 4°C.

RESTRICTION DIGESTION

11. The plug slices are incubated in 179 µL distilled water, 20 µL Buffer A and 1 µL of bovine serum albumin (100 µg/ml) at 30°C for 15 minutes.
12. The buffer is replaced with fresh buffer and BSA, 30 IU Apa1 (restriction endonuclease) is added.
13. The mix is incubated at 30°C for 2 hours.

ELECTROPHORESIS

14. The plugs are washed with 200 µL 0.5X TBE, then incubated at 4°C for 10 minutes before being loaded onto a 1.5% agarose gel in 0.5X TBE buffer.
15. The PFGE machine is set at ramped pulses of 5-35s for 23 hours at 200V.

STAIN AND DESTAIN

16. The gel is then stained for 45 min in 0.5µg/ml of ethidium bromide (20 µL of 10mg/ml in 400 ml TBE)
17. The gel is destained in distilled water for an hour to achieve good contrast.
18. The gel is then photographed using a Fotodyne Inc. UV light box and a Kodak EDAS 290 camera, and an analysis using Gel Compare II software Version 4.6 (Applied Maths) was performed.

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REAGENTS:

	LOT NO	Expiry date	Manufacturer
SuRE/Cut Buffer A	14541900 CAT no 11417959001		Roche
Proteinase K (20mg/ml)	14950500	04-2010	Novagen
Pulsed Field Gel Electrophoresis Agarose	1620137		BioRad
Lysozyme(100mg/ml)			Roche
Seakem® Gold Agarose			Cambrex
Bovine Serum Albumin			Roche
ApaI	CAT no 10899208001		Roche
Blood agar plates	Nil	19/01/10	Greenpoint Laboratory
Lambda Ladder PFG Marker NO340S	1100804	04/10	New England Biolabs

EQUIPMENT:

Equipment		Manufacturer
PFGE	Gene Navigator®	Amersham Biosciences AB
Water bath		Memmert
Fridge		Kelvinator
Vortex	Vortex-Genie 2	Scientific Industries Incorporated
Spectrophotometer	General Purpose UV/Vis spectrophotometer DU720	Beckman-Coulter

PREPARATION OF BUFFERS:

Cell Suspension Buffer (100mM Tris, 100mM EDTA, pH 8.0)
10ml 1M Tris stock, pH 8.0
20ml 0.5M EDTA stock, pH 8.0
Dilute to 100ml with sterile ultra pure water

Cell Lysis Buffer (50mM Tris, 50mM EDTA, pH 8.0, 1% Sarcosine)
12.5 ml 1 M Tris stock
25 ml 0.5 M EDTA stock
12.5 ml 10% Sarcosyl (N-Lauryl-Sarcosine)
Dilute to 250 ml with sterile ultra pure water

TE Buffer
10mls 1M Tris 1.21g/L
2 mls 0.5M EDTA 0.37g/L
pH 8.0
Dilute up to 1000mls

TBE Buffer
0.89 M Tris

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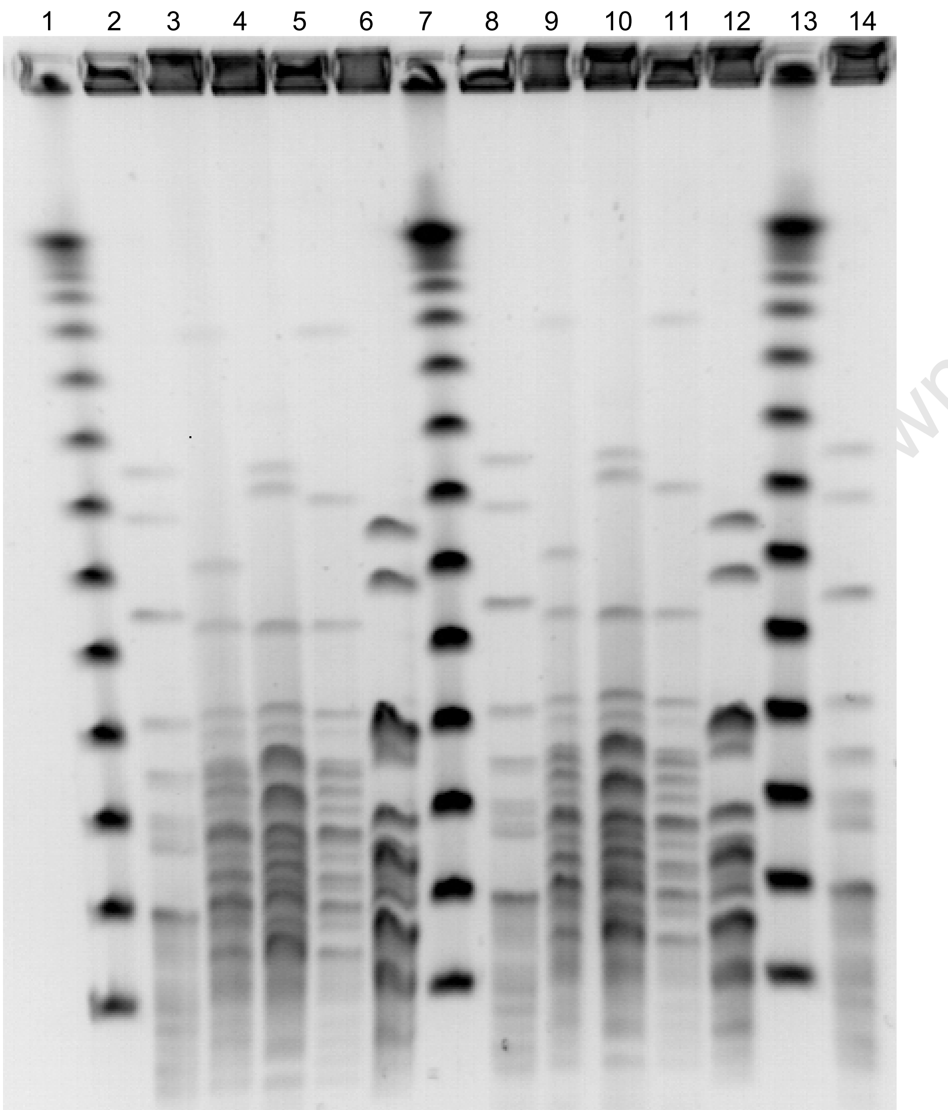
0.89 M boric acid
20 mM EDTA

RESULTS

Picture of original gel run (Fotodyne Inc. UV light box and a Kodak EDAS 290 camera)



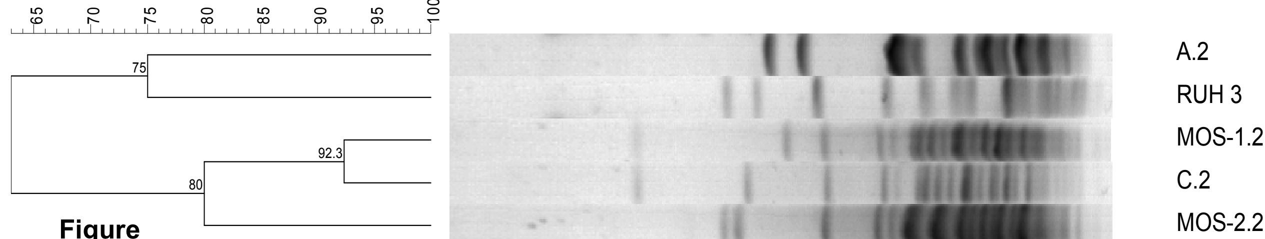
<u>Figure 14:</u> Pulse-field gel electrophoresis on selected isolates.

KEY:Lanes 1,7,13 Molecular weight marker; Lanes 2, 8, 14 Control strain RUH (European clone); Lanes 3, 9 MOS-1 strain; Lanes 4, 10 MOS-2 strain; Lanes 5, 11 Representative of Cluster C (Isolate 32); Lanes 6, 12 Representative of Cluster A (Isolate 16).

Results obtained using GEL Compare II Version 4.6 (Applied Maths) software are shown in Figure 15.

Dice (Opt:1.50%) (Tol 1.5%-1.5%) (H>0.0% S>0.0%) [0.0%-100.0%]
PFGE-ApaI

PFGE-ApaI



<u>Figure 15:</u> Pulsed field gel electrophoresis of selected isolates showing their relatedness. (KEY: A.2 Isolate 16, RUH 3- European RUH strain, MOS-1.2- MOS-1 strain, C.2- Isolate 32, MOS-2.2- MOS-2 strain).

STORAGE OF ISOLATES (See Package Insert.)

All isolates were stored on beads (Viabank VIM tubes, Abtek Biologicals Ltd).

Procedure for storage:
1. Label the vial.
2. Make a heavy Inoculation of the organism from a fresh, pure culture. Place into the vial.
3. Replace cap.
4. Distribute inoculated broth throughout the beads.
5. Decant the excess preservative fluid using a sterile pipette.
6. Replace cap.
7. Store at -70ºC.

Procedure for recovery of organisms:
1. Remove a bead with a sterile forceps.
2. Drop the bead on to the surface of a 2% blood agar plate, and streak across the surface.
3. Dispose of bead as per laboratory practice in a biohazard container.
4. Replace the vial in the -70ºC freezer.
5. Incubate the agar in conditions appropriate for the organism.

Lot GE 03, Expiry date May 2008.

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DiversiLab™ *Acinetobacter* Ki

48-Test Kit

Bacterial Barcodes, Inc
BBCI Catalog Number: DL-AB0
bioMérieux REF 27060

Not For Diagnostic Use.

Materials supplied in the kit

Rep-PCR Reagents Storage Range: -10 to -30° C

Cap Color	Name	Volume
Purple	Rep-PCR MM1	864 µL
Colorless	Primer Mix A	100 µL
Colorless	Positive Control C3	16 µL
Colorless	Negative Control	16 µL

Additional materials required, but not supplied

- Bacterial DNA isolation kit (UltraClean™ Microbial DNA Isolation Kit)
- *Taq* DNA polymerase* (AmpliTaq® DNA polymerase plus GeneAmp® 10X PCR Buffer [containing 15mM $MgCl_2$] by Applied Biosystems)
- Thermal cycler (GeneAmp® PCR System 9600 or 9700 by Applied Biosystems)
- Powder-free gloves, lab coat and eye protection
- Pipettes: 0.1-10 µL, 10-100 µL, 100-1000 µL, multi-channel or repeater pipette (optional)
- Aerosol resistant barrier pipette tips: 0.1-10 µL, 10-100 µL, 100-1000 µL
- Microcentrifuge tubes, 1.5 mL
- Microcentrifuge, variable speed
- Cooler Trays: 0.2 mL and 1.5 mL
- PCR tubes and caps: 0.2 mL
- PCR tray retainer set
- Rack to hold 0.2 mL and 1.5 mL tubes
- Vortex

Intended use

The DiversiLab™ DNA fingerprinting kits are designed to generate rep-PCR DNA fingerprints from pure-cultured microbial samples. These kits contain the reagents and buffers needed to set up PCR reactions using rep-PCR primers, starting from extracted genomic DNA from microbial isolates.

Introduction

The DiversiLab DNA fingerprinting kits use rep-PCR technology, which takes advantage of the non-coding repetitive sequences found interspersed throughout the genomes of all bacteria studied to date.[1,2,3] Key to the DiversiLab kit is primer sets that are complementary to these repetitive sequences. When PCR is performed using these primer sets, the DNA sequences between the repetitive elements are amplified. Thus, multiple fragments throughout the microbial genome are amplified simultaneously.

Principle of the DiversiLab System

The major steps involved in generating rep-PCR DNA fingerprints are as follows:

- Extract DNA from purified microbial cultures.
- Log sample information into the DiversiLab software (Internet-based).
- Perform rep-PCR amplification.
- Separate the DNA fragments using the Agilent® 2100 Bioanalyzer.
- Compare fingerprints using the statistical analysis feature of the DiversiLab software.

* NOTICE: This kit should only be used with AmpliTaq® DNA polymerase, a *Taq* DNA polymerase that may be covered by one or more patents, which has been obtained from an authorized, licensed source. Neither the offer to sell nor the sale of this kit nor these instructions constitutes an express or implied license to use AmpliTaq® or other *Taq* DNA polymerases. Additionally, the use of a thermal cycler may require a license under one or more patents held by third parties. This kit should only be used with an authorized, licensed thermal cycler. It is the sole responsibility of the user of this kit to ensure that use of the kit does not infringe the patent rights of third parties. Thus, the user of this kit has the responsibility to obtain any requisite license from parties other than the manufacturer and seller of this kit. Information on purchasing licenses to use AmpliTaq® may be obtained by contacting the Director of Licensing at Applied Biosystems, Inc. (www.appliedbiosystems.com).

This document provides the specific procedure associated with rep-PCR amplification. Refer also to the DiversiLab Software User's Guide and the DiversiLab Instructions for Use of LabChip® Devices insert.

Instructions for use

- This kit supplies sufficient reagents for the number of tests indicated. We recommend running a negative and positive control for each rep-PCR experiment performed.
- Do not use the components of this kit beyond the expiration date printed on the box.
- Use available preventative measures to avoid contamination and cross-contamination of PCR tubes and reagents, including but not limited to the following:
  - Separate the PCR workspace from other laboratory areas, especially those involving microbial samples or post-amplification products.
  - Clean the workspace with 10% bleach, followed by 70% ethanol.
  - Change gloves often and use dedicated lab coats and equipment for pre- and post-PCR areas.
  - Separate pre-PCR and post-PCR areas as much as possible. Use nuclease-free, disposable tubes and aerosol-resistant pipette tips.

Test limitations

- This kit may not produce useful fingerprints with some microbial samples. Although repetitive sequences have been found in all bacteria studied to date, it is possible that the primers supplied in this kit will not generate unique or discriminatory fingerprints with some microbial strains.
- The results obtained from using the DiversiLab kit may not be useful or reliable if the provided protocol is not followed exactly, or if the results are applied in a manner for which they were not intended.
- This product is not for diagnostic use and the results of this test should not be used to diagnose disease, or to cure, mitigate, treat, or prevent disease in a patient.

Collection and preparation of samples

- Proper purification of genomic DNA is critical for successful rep-PCR. Kit optimization has been performed with the UltraClean™ Microbial DNA Isolation Kit. The UltraClean™ kit is available through Bacterial Barcodes (catalog # BBMB-50 and BBMB-250).
- We recommend checking extraction consistency and DNA integrity by agarose gel electrophoresis.
- If a spectrophotometer is available, best results are obtained from purified genomic DNA with an A_{260}/A_{280} = 1.7-1.9.
- Use **2 µL** of genomic DNA at a concentration of 25-50 ng/µL in the rep-PCR protocol.

Procedure for the DiversiLab DNA fingerprinting kit

Step 1: Set up rep-PCR

■ **Prepare a master mix**

1. Allow the contents of the rep-PCR reagent box and the 10X PCR buffer to thaw completely.
2. Vortex briefly and centrifuge each reagent tube at 10,000 x g for 3 seconds.
3. In a nuclease-free 1.5 mL microcentrifuge tube, add the following amounts of reagents for each amplification to be performed:
   - Preparation must be set up on ice or in a PCR cooler tray.
   - We recommend adding reagents equivalent to **one extra** reaction for every 8-12 tests to be run (to compensate for volume loss during pipetting). The volumes of reagents provided are sufficient provided a minimum of 8 samples is run per setup.

Reagent	Volume/Reaction (µL)	Number of Reactions	Total Volume For Master Mix (µL)
Rep-PCR MM1	18.0	N	**18.0 x (N + extra)**
GeneAmp® 10X PCR Buffer	2.5	N	**2.5 x (N + extra)**
Primer Mix A	2.0	N	**2.0 x (N + extra)**
AmpliTaq® DNA Polymerase*	0.5	N	**0.5 x (N + extra)**
Total Volume	23.0	N	**23.0 x (N + extra)**

**Taq* should be the last reagent added to the master mix and should be kept cold at all times.

■ **Aliquot master mix**

1. Set up a nuclease-free PCR tube for each sample and control in a cold block or ice. Label the tubes according to the job worksheet.
2. Thoroughly mix the contents of the master mix tube. Centrifuge at 10,000 x g for 5 seconds.
3. Aliquot **23 µL** of the master mix into the bottom of each PCR tube.

■ **Add DNA to PCR tubes**

1. Add **2 µL** sample DNA (concentration of 25-50 ng/µL), Positive Control or Negative Control to the bottom of the corresponding PCR tube. Pipette up and down several times to mix.
2. Cap the PCR tubes securely. Gently tap tubes on benchtop to bring reagents to the bottom. Return the tubes to the cold block or ice.

Step 2: Perform rep-PCR amplification

1. Program the thermal cycler as shown below.

Rep-PCR Protocol – repPCR50		
Initial denaturation:	**94°C**	**2 min**
Repeat cycle:	**35 cycles**	
Denaturation:	**94°C**	**30 sec**
Annealing:	**50°C**	**30 sec**
Extension:	**70°C**	**90 sec**
Final extension:	**70°C**	**3 min**
Hold:	**4°C**	∞

2. For optimal amplification, pause the program after allowing the thermal cycler to warm to 94°C during initial denaturation.
3. Insert the PCR tubes into the preheated thermal cycler.

   Note: The temperature of the block will drop.
4. Resume the program **after** the thermal cycler temperature returns to 94°C.
5. Continue directly to DNA separation with the Caliper® LabChip device, or store rep-PCR product at 4°C or –20°C. See "Instructions for Use of LabChip® Devices," provided with the DNA chips and DNA Chip Reagents and Supplies.

Possible sources of error

- Pure microbial cultures must be used. Mixed or contaminated cultures may result in inconsistent fingerprints.
- Contamination of microbial cultures, incomplete lysis of cells, carryover of reagents used during DNA extraction, and fluctuations during rep-PCR amplification may cause the fingerprints to be weak or absent. Contact Bacterial Barcodes if problems are encountered.

References

1. Versalovic J, Schneider M, de Bruijn F, Lupski J. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Meth. Mol. Cell Bio.* 5:25-40.
2. Versalovic J, Koeuth T, Lupski J. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nuc. Acids Res.* 19(24): 6823-6831.
3. Versalovic J, de Bruijn F, Lupski J. 1998. Repetitive sequence-based PCR (rep-PCR) DNA fingerprinting of bacterial genomes. In: *Bacterial Genomes: Physical Structure and Analysis*. Chapman & Hall: New York.

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www.bacterialbarcodes.com

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Caliper and LabChip are registered trademarks of Caliper Technologies Corporation in the United States and/or other countries.
DiversiLab is a trademark of bioMérieux, Inc.
UltraClean is a trademark of MO BIO Laboratories, Inc.

43-03831 JULY 2007

RESULTS OF DIVERSILAB TYPING: This part of the experiment was performed by the R&D Microbiologie, bioMerieux, La Balme les Grottes.

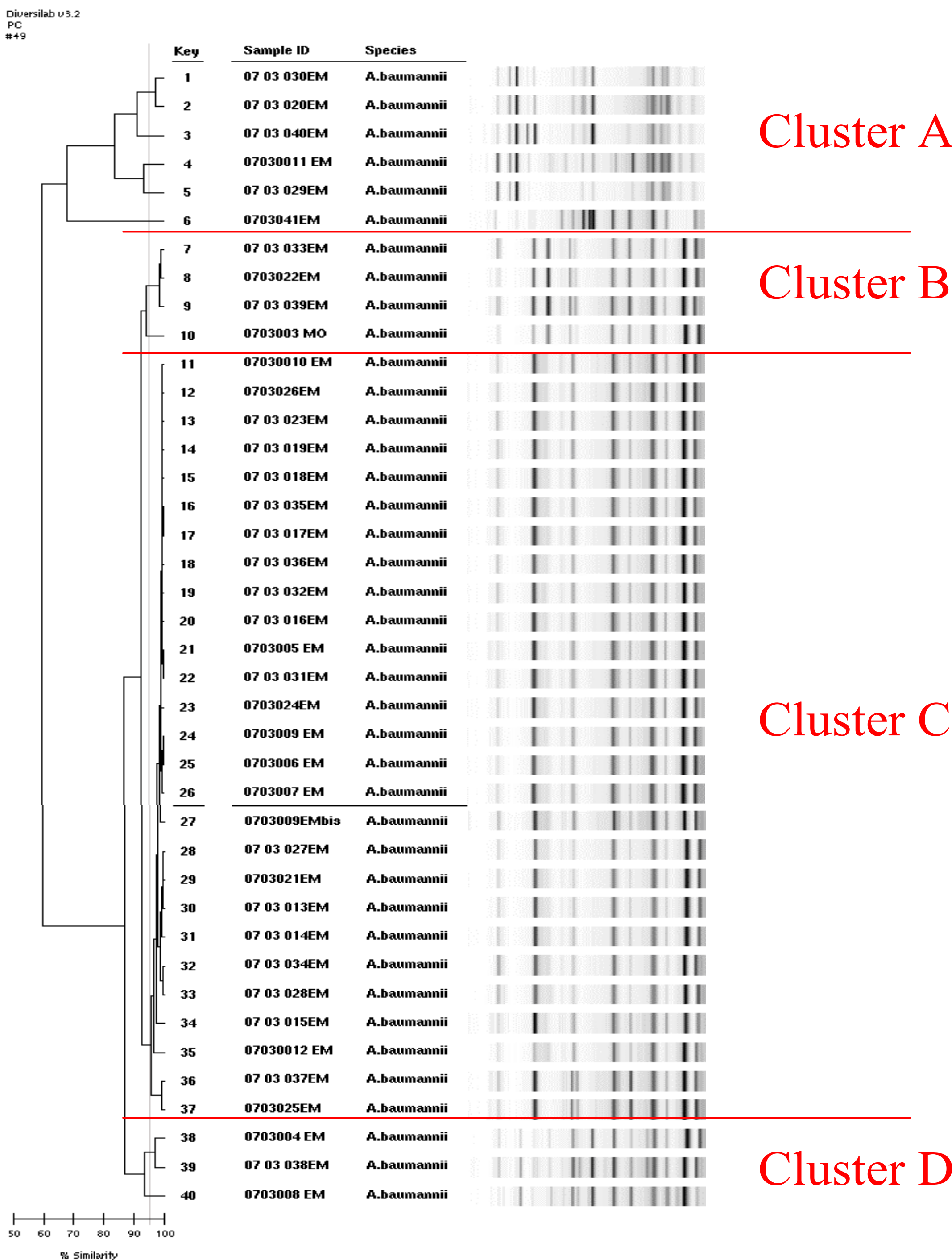

Figure 16: Phylogenetic relatedness of the isolates as determined by the Diversilab.

KEY:

KEY NO	ISOLATE
1	38
2	21
3	28
4	16
5	34
6	30
7	37
8	23
9	35
10	2
11	17
12	32
13	22
14	20
15	9
16	31
17	10
18	29
19	33
20	11
21	4
22	39
23	36
24	8
25	5
26	6
27	8
28	42
29	18
30	14
31	13
32	40
33	26
34	12
35	15
36	41
37	24
38	3
39	27
40	7

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JOURNAL OF CLINICAL MICROBIOLOGY

2011 INSTRUCTIONS TO AUTHORS*

SCOPE

The two principal attributes of papers published in the *Journal of Clinical Microbiology* (JCM) are relevance to the practice of clinical microbiology and quality science. JCM is devoted to the dissemination of new knowledge concerning the laboratory diagnosis of human and animal infections. In addition, JCM is an appropriate forum for the publication of information related to the role of the laboratory in both the management of infectious diseases and the elucidation of the epidemiology of infections. Manuscripts which present the results of original scientific investigations are encouraged. Case Reports will be considered if they are novel, add to existing knowledge, and are consistent with the primary objectives of the *Journal* as outlined above. (See "Case Reports" in the Organization and Format section for a description of the two different types of Case Reports published.)

JCM will consider manuscripts which describe truly novel molecular methods for use in the diagnosis or elucidation of infection. However, we discourage submission of manuscripts predicated on the application of previously well described methods (e.g., RT-PCR, RAPD analysis, PFGE, and real-time PCR, etc.) even though the application may be new. Papers employing established molecular methods will be considered only when the application is examined in comparison to some other existing diagnostic method(s). Such comparative studies should include information regarding assay sensitivity, specificity, and diagnostic accuracy.

To ensure the completeness of investigations in which the performance of various diagnostic assays or methods is presented, authors are encouraged to refer to the Standards for the Reporting of Diagnostic Accuracy (STARD) for guidance. The entire set of guidelines, including checklists, may be found at http://stard-statement.org/.

ASM publishes a number of different journals covering various aspects of the field of microbiology. Each journal has a prescribed scope which must be considered in determining the most appropriate journal for each manuscript.

(i) With respect to antimicrobial agents, JCM will consider clinically relevant manuscripts (a) that pertain to *in vitro* susceptibility test methods; (b) that are concerned with quality control procedures related to antimicrobial susceptibility tests; (c) that deal with investigations of test methods aimed at measuring levels of antimicrobial agents in clinical specimens; or (d) that describe the use of antimicrobial agents as tools in the isolation, identification, or epidemiologic assessment of microorganisms associated with disease. Manuscripts pertaining to other aspects of antimicrobial agents, such as their basic mechanisms of action, the elucidation of resistance determinants, pharmacokinetics and pharmacodynamics, and the development of new agents, will be considered for publication in *Antimicrobial Agents and Chemotherapy*.

(ii) Manuscripts that present the results of investigations with a primary focus on the basic mechanisms of pathogenesis of microorganisms or the pathophysiology of infections should be directed to *Infection and Immunity* (for bacteria, parasites, and fungi) or the *Journal of Virology* (for viruses).

(iii) Reports of clinical microbiology investigations or studies of the hospital population and the environment as they relate to nosocomial infections should be submitted to JCM. Manuscripts dealing with ecology or environmental studies or with the application of microorganisms to agricultural or industrial processes are more appropriate for *Applied and Environmental Microbiology*.

(iv) Papers involving immunologic assays for use in the diagnosis or elucidation of infection, vaccines, or the assessment and laboratory diagnosis of immunologic diseases (e.g., autoimmune diseases and primary immunodeficiencies) should be submitted to *Clinical and Vaccine Immunology* (formerly *Clinical and Diagnostic Laboratory Immunology*).

Questions about these guidelines may be directed to the editor in chief of the journal being considered.

If transfer to another ASM journal is recommended by an editor, the corresponding author will be contacted.

Note that a manuscript rejected by one ASM journal on scientific grounds or on the basis of its general suitability for publication is considered rejected by all other ASM journals.

EDITORIAL POLICY

Use of Microbiological Information

The Council Policy Committee (CPC) of the American Society for Microbiology affirms the long-standing position of the Society that microbiologists will work for the proper and beneficent application of science and will call to the attention of the public or the appropriate authorities misuses of microbiology or of information derived from microbiology. ASM members are obligated to discourage any use of microbiology contrary to the welfare of humankind, including the use of microbes as biological weapons. Bioterrorism violates the fundamental principles expressed in the Code of Ethics of the Society and is abhorrent to ASM and its members.

*Instructions to Authors are published annually in the January issue. A separate html version, which is updated throughout the year, is at http://jcm.asm.org/misc/ifora.dtl.

ASM recognizes that there are valid concerns regarding the publication of information in scientific journals that could be put to inappropriate use as described in the CPC resolution mentioned above. Members of the ASM Publications Board will evaluate the rare manuscript that might raise such issues during the review process. However, as indicated elsewhere in these Instructions, research articles must contain sufficient detail, and material/information must be made available, to permit the work to be repeated by others. Supply of materials should be in accordance with laws and regulations governing the shipment, transfer, possession, and use of biological materials and must be for legitimate, bona fide research needs. Links to, and information regarding, these laws and regulations can be found at http://www.asm.org/ under the Public Policy tab. We ask that authors pay particular attention to the NSAR Select Agents and Toxins list on the CDC website http://www.selectagents.gov/index.html and the NSABB criteria for identifying dual use research of concern in the report "Proposed Framework for the Oversight of Dual Use Life Sciences Research: Strategies for Minimizing the Potential Misuse of Research Information" on the Office of Biotechnology Activities website http://oba.od.nih.gov/biosecurity/pdf/Framework%20for%20transmittal%200807_Sept07.pdf (p. 17–22).

Ethical Guidelines

ASM requirements for submitted manuscripts are consistent with the Uniform Requirements for Manuscripts Submitted to Biomedical Journals, as last updated by the International Committee of Medical Journal Editors in April 2010 (http://www.icmje.org).

Authors are expected to adhere to the highest ethical standards. The following sections of these Instructions include detailed information about ASM's ethical standards. Failure to comply with the policies described in these Instructions may result in a letter of reprimand, a suspension of publishing privileges in ASM journals, and/or notification of the authors' institutions. Authors employed by companies whose policies do not permit them to comply with ASM policies may be sanctioned as individuals and/or ASM may refuse to consider manuscripts having authors from such companies. The ASM Publications Board wishes to clarify the following in particular.

Plagiarism. Misappropriating another person's intellectual property constitutes plagiarism. This includes copying sentences or paragraphs verbatim (or almost verbatim) from someone else's work, even if the original work is cited in the references. The NIH ORI publication "Avoiding Plagiarism, Self-Plagiarism, and Other Questionable Writing Practices: a Guide to Ethical Writing" (http://ori.dhhs.gov/education/products/plagiarism/) can help authors identify questionable writing practices.

Plagiarism is not limited to the text; it can involve any part of the manuscript, including figures and tables, in which material is copied from another publication without permission and attribution. An author may not reuse his or her own previously published work without attribution; this is considered self-plagiarism.

Primary publication. Manuscripts submitted to the journal must represent reports of original research, and the original data must be available for review by the editor if necessary.

By submission of a manuscript to the journal, the authors guarantee that they have the authority to publish the work and that the manuscript, or one with substantially the same content, was not published previously, is not being considered or published elsewhere, and was not rejected on scientific grounds by another ASM journal. It is incumbent upon the author to acknowledge any prior publication, including his/her own articles, of the data contained in a manuscript submitted to an ASM journal. A copy of the relevant work should be submitted with the paper as supplemental material. Whether the material constitutes the substance of a paper and therefore renders the manuscript unacceptable for publication is an editorial decision.

In brief, a paper is not acceptable for submission to an ASM journal if it, or its substance, has been published/posted in:

- A serial, periodical, or book
- A conference report or symposium proceedings
- A technical bulletin or company white paper
- A nonpersonal website
- Any other retrievable source

The following do not preclude submission to, or publication by, an ASM journal, as long as the posted data do not constitute the substance of a submission:

- Posting of a method/protocol on a nonpersonal website
- Posting of a limited amount of original data on a personal/university/corporate website or websites of small collaborative groups working on a problem
- Posting of unpublished sequence data on the Internet (the URL where the sequence is posted should be included in the text)
- Preliminary disclosures of research findings as meeting posters, webcast as meeting presentations, or published in abstract form as adjuncts to a meeting, e.g., part of a program
- Posting of theses and dissertations on a personal/university-hosted website

Availability of materials. By publishing in the journal, the authors agree that, subject to requirements or limitations imposed by laws or governmental regulations of the United States, any DNAs, viruses, microbial strains, mutant animal strains, cell lines, antibodies, and similar materials described in the article are available from a national collection or will be made available in a timely fashion, at reasonable cost, and in limited quantities to members of the scientific community for non-

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An author is one who made a substantial contribution to the overall design and execution of the experiments; therefore, **ASM considers all authors responsible for the entire paper.** Individuals who provided assistance, e.g., supplied strains or reagents or critiqued the paper, need not be listed as authors but may be recognized in the Acknowledgments section.

A study group, surveillance team, working group, consortium, or the like (e.g., the Active Bacterial Core Surveillance Team) may be listed as a coauthor in the byline if its contributing members satisfy the requirements for authorship and accountability as described in these Instructions. The names (and institutional affiliations if desired) of the contributing members only may be given in a footnote linked to the study group name in the byline or as a separate paragraph in the Acknowledgments section.

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Disputes about authorship may delay or prevent review and/or publication of the manuscript. Should the individuals involved be unable to reach an accord, review and/or publication of the manuscript can proceed only after the matter is investigated and resolved by the authors' institution(s) and an official report of such and signed statements of agreement are provided to ASM.

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Since ASM makes the final, typeset articles from its primary-research journals available free of charge on the ASM Journals and PMC websites 6 months after final publication, ASM recommends that when submitting the accepted manuscript to PMC or a similar public access site, the author specify that the **posting release date for the manuscript be no earlier than 6 months after publication of the typeset article by ASM.**

Use of Human Subjects or Animals in Research

The use of human subjects or other animals for research purposes is regulated by the federal government and individual institutions. Manuscripts containing information related to human or animal use should clearly state that the research has complied with all relevant federal guidelines and institutional policies. Copies of these guidelines and policy statements must be available for review by the editor if necessary.

Patient Identification

To protect the privacy of individuals mentioned in clinical studies, in case histories, and as sources of isolates, do not identify them by their initials, even as part of a strain designation. Change the initials to numerals or use randomly chosen letters. Do not give hospital unit

numbers; if a designation is needed, use only the last two digits of the unit. (Note: established designations of some viruses and cell lines, although they consist of initials, are acceptable [e.g., JC virus, BK virus, and HeLa cells].)

Provision of Requisite Information for Molecular Applications

Authors of manuscripts which contain quantitative real-time PCR applications are encouraged to consult the article concerning minimum information for publication of quantitative real-time PCR experiments (the MIQE guidelines) by Bustin et al. (Clin. Chem. 55:611–622, 2009) for guidance as to what information should be considered for inclusion in their submission.

Newly determined nucleotide and/or amino acid sequence data must be deposited and GenBank/EMBL/DDBJ accession numbers must be included in the manuscript no later than the modification stage of the review process. It is expected that the sequence data will be released to the public no later than the publication (online posting) date of the accepted manuscript. The accession numbers should be included in a separate paragraph at the end of the Materials and Methods section for full-length papers or at the end of the text for Notes. If conclusions in a manuscript are based on the analysis of sequences and a GenBank/EMBL/DDBJ accession number is not provided at the time of the review, authors should provide the sequence data as supplemental material.

It is expected that when previously published sequence accession numbers are cited in a manuscript, the original citations (e.g., journal articles) will be included in the References section when possible or reasonable.

Authors are also expected to do elementary searches and comparisons of nucleotide and amino acid sequences against the sequences in standard databases (e.g., GenBank) immediately before manuscripts are submitted and again at the proof stage.

Analyses should specify the database, and the date of each analysis should be indicated as, e.g., January 2011. If relevant, the version of the software used should be specified.

See "Presentation of Nucleic Acid Sequences" for nucleic acid sequence formatting instructions.

The URLs of the databases mentioned above are as follows: DNA Data Bank of Japan (DDBJ), http://www.ddbj.nig.ac.jp/; EMBL Nucleotide Sequence Database, http://www.ebi.ac.uk/embl/; and GenBank, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/.

Proper Use of Locus Tags as Systematic Identifiers for Genes

To comply with recommendations from the International Nucleotide Sequence Database (INSD) Collaborators and to avoid conflicts in gene identification, researchers should implement the following two fundamental guidelines as standards for utilization of locus tags in genome analysis, annotation, submission, reporting, and publication. (i) Locus tag prefixes are systematic gene identifiers for all of the replicons of a genome and as such should be associated with a single genome project submission. (ii) New genome projects must be registered with INSD, and new locus tag prefixes must be assigned in cooperation with INSD to ensure that they conform to the agreed-upon criteria. Locus tag prefixes that are currently in use may be searched at the NCBI locus tag database (http://www.ncbi.nlm.nih.gov/genomes/lltp.cgi).

Structural Determinations

Coordinates for new structures of macromolecules must be deposited in the Protein Data Bank and assigned identification codes must be included in the manuscript no later than the modification stage of the review process. It is expected that the coordinates will be released to the public no later than the publication (online posting) date of the accepted manuscript. Authors are encouraged to send coordinates with their original submission, however, so that reviewers can examine them along with the manuscript. The accession number(s) should be listed in a separate paragraph at the end of the Materials and Methods section for full-length papers or at the end of the text for Notes.

The URLs for coordinate deposition are http://rcsb-deposit.rutgers.edu/ and http://pdbdep.protein.osaka-u.ac.jp/en/.

Microarray Data

The entire set of supporting microarray data must be deposited in the appropriate public database (e.g., GEO, ArrayExpress, or CIBEX) and the assigned accession number(s) must be included in the manuscript no later than the modification stage of the review process. It is expected that the data will be released to the public no later than the publication (online posting) date of the accepted manuscript. Authors are encouraged to send the relevant data with their original submission, however, so that reviewers can examine them along with the manuscript. The accession number(s) should be listed in a separate paragraph at the end of the Materials and Methods section for full-length papers or at the end of the text for Notes.

The URLs of the databases mentioned above are as follows: Gene Expression Omnibus (GEO), http://www.ncbi.nlm.nih.gov/projects/geo/; ArrayExpress, http://www.ebi.ac.uk/microarray-as/ae/; and Center for Information Biology Gene Expression Database (CIBEX), http://cibex.nig.ac.jp/index.jsp.

Culture Deposition

JCM expects authors to deposit important strains in publicly accessible culture collections and to refer to the collections and strain numbers in the text. Since the authenticity of subcultures of culture collection specimens that are distributed by individuals cannot be en-

sured, authors should indicate laboratory strain designations and donor sources as well as original culture collection identification numbers.

MycoBank

New scientific names of fungi along with key nomenclatural and descriptive material must be deposited in MycoBank (http://www.mycobank.org) and the assigned accession number(s) must be included in the manuscript no later than the modification stage of the review process. It is expected that the data will be released to the public no later than the publication (online posting) date of the accepted manuscript. Authors are encouraged to send the relevant data with their original submission, however, so that reviewers can examine them along with the manuscript. The accession number(s) should be listed in a separate paragraph at the end of the Materials and Methods section for full-length papers or at the end of the text for Notes.

Supplemental Material

Supplemental material intended for posting by ASM should be restricted primarily to large or complex data sets or results that cannot readily be displayed in printed form because of space or technical limitations. Such material may include data from microarray, structural, biochemical, or video imaging analyses. In such cases, the manuscript submitted for review should include a distillation of the results so that the principal conclusions are fully supported without referral to the supplemental material.

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To ensure broad access, supplemental files should be submitted in the following standard formats:

(i) **Text.** Word, RTF, or PDF files.

(ii) **Figures.** TIFF, EPS, high-resolution PDF, JPEG, or GIF format. Supplemental figures should not be embedded in the manuscript text.

(iii) **Tables.** Word, RTF, or PDF files.

(iv) **Data sets.** Excel (.xls), RTF, TXT, or PDF files.

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SUBMISSION, REVIEW, AND PUBLICATION PROCESSES

Submission Process

All submissions to JCM must be made electronically. In 2011, the ASM journals are switching from Rapid Review to the eJournalPress manuscript submission and peer review system. Journals will be transitioned one by one over the course of several months, and the exact timing for JCM has not been determined. When the transition occurs, only new manuscript submissions will be made through the eJP system. If you are returning a modified manuscript and made the original submission in Rapid Review, please use Rapid Review. For up-to-date information about where to submit your manuscript, please refer to the separate html version of Instructions to Authors, http://jcm.asm.org/misc/ifora.dtl, which is updated throughout the year.

Review Process

All manuscripts are considered to be confidential and are reviewed by the editors, members of the editorial board, or qualified *ad hoc* reviewers.

To expedite the review process, authors must recommend at least three editorial board members who have expertise in the field, who are not members of their institution(s), who have not recently been associated with their laboratory(ies), and who could not otherwise be considered to pose a conflict of interest regarding the submitted manuscript.

Copies of in-press and submitted manuscripts that are important for judgment of the present manuscript should be included as supplemental material to facilitate the review.

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The corresponding author is notified, generally within 4 to 6 weeks after submission, of the editor's decision to accept, reject, or require modification. When modification is requested, the corresponding author must either submit the modified version within 2 months or withdraw the manuscript. A point-by-point response to the reviews must be provided with the revised manuscript, and a compare copy of the manuscript (without figures) should be included as supplemental material if the editor requested one.

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When an editor has decided that a manuscript is acceptable for publication on the basis of scientific merit, the author and the Journals Department are notified. A PDF version of the accepted manuscript is posted online as soon as possible (see "JCM Accepts").

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JCM Accepts

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1. **Alexander, T. W., et al.** 2008. Effect of subtherapeutic administration of antibiotics on the prevalence of antibiotic-resistant *Escherichia coli* bacteria in feedlot cattle. Appl. Environ. Microbiol. **74:**4405–4416.
2. **Cox, C. S., B. R. Brown, and J. C. Smith.** J. Gen. Genet., in press.* {*Article title is optional; journal title is mandatory.*}
3. **da Costa, M. S., M. F. Nobre, and F. A. Rainey.** 2001. Genus I. Thermus Brock and Freeze 1969, 295,[AL] emend. Nobre, Trüper and da Costa 1996b, 605, p. 404–414. *In* D. R. Boone, R. W. Castenholz, and G. M. Garrity (ed.), Bergey's manual of systematic bacteriology, 2nd ed., vol. 1. Springer, New York, NY.
4. **Elder, B. L., and S. E. Sharp.** 2003. Cumitech 39, Competency assessment in the clinical laboratory. Coordinating ed., S. E. Sharp. ASM Press, Washington, DC.
5. **Falagas, M. E., and S. K. Kasiakou.** 2006. Use of international units when dosing colistin will help decrease confusion related to various formulations of the drug around the world. Antimicrob. Agents Chemother. **50:**2274–2275. (Letter.) {*"Letter" or "Letter to the editor" is allowed but not required at the end of such an entry.*}
6. **Fitzgerald, G., and D. Shaw.** *In* A. E. Waters (ed.), Clinical microbiology, in press. EFH Publishing Co., Boston, MA.* {*Chapter title is optional.*}
7. **Forman, M. S., and A. Valsamakis.** 2003. Specimen collection, transport, and processing: virology, p. 1227–1241. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, J. H. Jorgensen, and R. H. Yolken (ed.), Manual of clinical microbiology, 8th ed. ASM Press, Washington, DC.
8. **Garcia, C. O., et al.** 1996. Detection of salmonella DNA in synovial membrane and synovial fluid from Latin American patients. Arthritis Rheum. **39**(Suppl.):S185. {*Meeting abstract published in journal supplement.*}
9. **Green, P. N., D. Hood, and C. S. Dow.** 1984. Taxonomic status of some methylotrophic bacteria, p. 251–254. *In* R. L. Crawford and R. S. Hanson (ed.), Microbial growth on C_1 compounds. Proceedings of the 4th International Symposium. American Society for Microbiology, Washington, DC.
10. **Odell, J. C.** April 1970. Process for batch culturing. U.S. patent 484,363,770. {*Include the name of the patented item/process if possible; the patent number is mandatory.*}
11. **O'Malley, D. R.** 1998. Ph.D. thesis. University of California, Los Angeles, CA. {*Title is optional.*}
12. **Rotimi, V. O., N. O. Salako, E. M. Mohaddas, and L. P. Philip.** 2005. Abstr. 45th Intersci. Conf. Antimicrob. Agents Chemother., abstr. D-1658. {*Abstract title is optional.*}
13. **Smith, D., C. Johnson, M. Maier, and J. J. Maurer.** 2005. Distribution of fimbrial, phage and plasmid associated virulence genes among poultry *Salmonella enterica* serovars, abstr. P-038, p. 445. Abstr. 105th Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, DC. {*Abstract title is optional.*}
14. **Stratagene.** 2006. Yeast DNA isolation system: instruction manual. Stratagene, La Jolla, CA. {*Use the company name as the author if none is provided for a company publication.*}

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1. **Charlier, D., and N. Glansdorff.** September 2004, posting date. Chapter 3.6.1.10, Biosynthesis of argi-

nine and polyamines. *In* R. Curtiss III et al. (ed.), EcoSal—*Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC. http://www.ecosal.org/. {*Note that each chapter has its own posting date.*}
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3. **Smith, F. X., H. J. Merianos, A. T. Brunger, and D. M. Engelman.** 2001. Polar residues drive association of polyleucine transmembrane helices. Proc. Natl. Acad. Sci. U. S. A. **98**:2250–2255. doi:10.1073/pnas.041593698.
4. **Winnick, S., D. O. Lucas, A. L. Hartman, and D. Toll.** 2005. How do you improve compliance? Pediatrics **115**:e718–e724.

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. . . similar results (R. B. Layton and C. C. Weathers, unpublished data).
. . . system was used (J. L. McInerney, A. F. Holden, and P. N. Brighton, submitted for publication).
. . . as described previously (M. G. Gordon and F. L. Rattner, presented at the Fourth Symposium on Food Microbiology, Overton, IL, 13 to 15 June 1989). {*For nonpublished abstracts and posters, etc.*}
. . . this new process (V. R. Smoll, 20 June 1999, Australian Patent Office). {*For non-U.S. patent applications, give the date of publication of the application.*}
. . . available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/index.html).
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and indicates that within that data set at least two groups differ from each other. The overall P value does not indicate which two groups are different. The main P value and the overall P value should be computed by using a *post hoc* test. For ANOVA, these *post hoc* tests are usually Dunnett's test (used to compare multiple experimental groups to a single control), the Fisher protected least significant difference (PLSD) test, the Tukey-Kramer test, and the Games-Howell test. Others may be used. Note that each *post hoc* test has certain underlying assumptions that may not be applicable to the data under analysis. For a Kruskal-Wallis nonparametric ANOVA, the Dunn procedure is appropriate to generate P values for two-group comparisons.

(vi) Data presented as endpoints (i.e., LD_{50} and ID_{50}, etc.) contain both the calculated value and a confidence interval with a statistical significance associated with it (95%, 99%, or similar confidence interval), calculated by logit or probit analysis. Simple LD_{50} values such as Reed-Muench calculations may not be used alone.

(vii) When samples are taken multiple times from one experimental entity (i.e., multiple serum samples from one animal, gross pathology scores measured for the same animal over time or growth curves, etc.), one cannot use analyses such as t tests, ANOVA, or the Mann-Whitney test, etc., because these tests assume that each measure is independent. An entity with a high score on day 1 is more likely to have a high score on day 2 than is an entity with a low score. It is likely that some expert statistical help will be needed for these situations, usually involving regression analysis or survival analysis, etc.

(viii) Statistical significance and biological significance are not the same. There is nothing magical about a P value of 0.05. When results from large sample sizes are compared, a P value of <0.05 will often be obtained, as P value is a function of both sample size and effect size. If sample sizes are large, then more-rigorous (i.e., smaller) P values may be desirable. If sample sizes are small, P values of >0.05 may still be important. There should be both statistical and biological significance to the results and conclusions in the manuscript.

For a review of some common errors associated with statistical analyses and reports, plus guidelines on how to avoid them, see the article by Olsen (Infect. Immun. **71**:6689–6692, 2003).

For a review of basic statistical considerations for virology experiments, see the article by Richardson and Overbaugh (J. Virol. **79**:669–676, 2005).

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For simple molecules, labeling is indicated in the chemical formula (e.g., $^{14}CO_2$, $^{3}H_2O$, and $H_2{}^{35}SO_4$). Brackets are not used when the isotopic symbol is attached to the name of a compound that in its natural state does not contain the element (e.g., ^{32}S-ATP) or to a word that is not a specific chemical name (e.g., ^{131}I-labeled protein, ^{14}C-amino acids, and ^{3}H-ligands).

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L-[*methyl*-^{14}C]methionine	*E. coli* [^{32}P]DNA
[2,3-^{3}H]serine	fructose 1,6-[1-^{32}P]bisphosphate
[α-^{14}C]lysine	[γ-^{32}P]ATP

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