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UNITE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCE DE LA VIE

****
DEPARTEMENT DE BIOCHIMIE

Laboratory for Public Health Research Biotechnology

Development of diagnostic algorithm for resistance monitoring of nosocomial infection causing bacteria: The case of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

THESIS

Defended for the obtention of Doctorate / PHD in Biochemistry

OPTION

Public Health Biotechnology

Written by

MADAHA LONGLA Estelle : 04S078

Msc in Health Sciences option Bacteriology

Supervisor

Wilfred FON MBACHAM

Professor

Director

Colins NJIE ATEBA

professor

Academic Year 2021-2022

UNIVERSITE DE YAOUNDE I
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FACULTE DES SCIENCES
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CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE, SCIENCE DE LA VIE, SANTE ET ENVIRONNEMENT (SVSE)
****
UNITE DE RECHEARCHE ET DE FORMATION DOCTORALE EN SCIENCES DE LA VIE
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DEPARTEMENT DE BIOCHIMIE

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CENTER FOR RESEARCH AND GRADUATE STUDIES IN LIFE, HEALTH AND ENVIRONMENTAL SCIENCES
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UNIT FOR RESEARCH AND GRADUATE STUDIES IN LIFE SCIENCES
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DEPARTMENT OF BIOCHEMISTRY

ATTESTATION DE CORRECTION DE LA THESE DE DOCTORAT/PHD

Nous soussignons : Prof **PENLAP NINTCHOM Véronique épouse BENG**, Présidente du jury et **Dr EYANGOH Sara Irène**, Membre du Jury, attestons que Mme **MADAHA LONGLA Estelle** a effectué les corrections conformément aux exigences du jury de soutenance de sa thèse de Doctorat / PhD en Biochimie option Biotechnologie de la Santé Public avec pour thème « Developement of diagnostic algorithm for resistance monitoring of nosocomial infections causing bacteria ; the case of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. » Cette thèse a été soutenue le 15 Décembre 2021.

En foi de quoi, la présente attestation lui est établi et délivré pour lui servir et valoir ce que de droit.

12 JAN 2022

Examinateur	Président du jury	Chef de Département
Dr Sara Eyangoh Directeur Scientifique Centre Pasteur du Cameroun	Veronique N. Penlap Beng Professor	Moundipa Fewou Pr ss Enzymology Toxi

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Department of Biochemistry
Le Chef de Département
Dépôt et Département
FACULTY OF SCIENCE
FACULTE DES SCIENCES

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DEPARTEMENT OF BIOCHEMISTRY

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UNIVERSITÉ DE YAOUNDÉ I **Faculté des Sciences** Division de la Programmation et du Suivi des Activités Académiques		THE UNIVERSITY OF YAOUNDE I **Faculty of Science** Division of Programming and Follow-up of Academic Affaires
LISTE DES ENSEIGNANTS PERMANENTS		**LIST OF PERMANENT TEACHING STAFF**

ANNÉE ACADEMIQUE 2021/2022

(Par Département et par Grade)

DATE D'ACTUALISATION 22 septembre 2021

ADMINISTRATION

DOYEN : TCHOUANKEU Jean- Claude, *Maître de Conférences*

VICE-DOYEN / DPSAA : ATCHADE Alex de Théodore, *Maître de Conférences*

VICE-DOYEN / DSSE : NYEGUE Maximilienne Ascension, *Professeur*

VICE-DOYEN / DRC : ABOSSOLO Monique, *Maître de Conférences*

Chef Division Administrative et Financière : NDOYE FOE Marie C. F., *Maître de Conférences*

Chef Division des Affaires Académiques, de la Scolarité et de la Recherche DAASR : AJEAGAH Gideon AGHAINDUM, *Professeur*

1- DÉPARTEMENT DE BIOCHIMIE (BC) (38)

N°	NOMS ET PRÉNOMS	GRADE	OBSERVATIONS
1	BIGOGA DAIGA Jude	Professeur	En poste
2	FEKAM BOYOM Fabrice	Professeur	En poste
3	FOKOU Elie	Professeur	En poste
4	KANSCI Germain	Professeur	En poste
5	MBACHAM FON Wilfried	Professeur	En poste
6	MOUNDIPA FEWOU Paul	Professeur	Chef de Département
7	NINTCHOM PENLAP V. épse BENG	Professeur	En poste
8	OBEN Julius ENYONG	Professeur	En poste
9	ACHU Merci BIH	Maître de Conférences	En poste
10	ATOGHO Barbara Mma	Maître de Conférences	En poste
11	AZANTSA KINGUE GABIN BORIS	Maître de Conférences	En poste

12	BELINGA née NDOYE FOE M. C. F.	Maître de Conférences	Chef DAF / FS
13	BOUDJEKO Thaddée	Maître de Conférences	En poste
14	DJUIDJE NGOUNOUE Marcelline	Maître de Conférences	En poste
15	EFFA NNOMO Pierre	Maître de Conférences	En poste
16	**EWANE Cécile Anne**	Maître de Conférences	En poste
17	MOFOR née TEUGWA Clotilde	Maître de Conférences	Inspecteur de Service MINESUP
18	NANA Louise épouse WAKAM	Maître de Conférences	En poste
19	NGONDI Judith Laure	Maître de Conférences	En poste
20	NGUEFACK Julienne	Maître de Conférences	En poste
21	NJAYOU Frédéric Nico	Maître de Conférences	En poste
22	TCHANA KOUATCHOUA Angèle	Maître de Conférences	En poste

23	AKINDEH MBUH NJI	Chargé de Cours	En poste
24	BEBEE Fadimatou	Chargée de Cours	En poste
25	BEBOY EDJENGUELE Sara Nathalie	Chargé de Cours	En poste
25	DAKOLE DABOY Charles	Chargé de Cours	En poste
26	DJUIKWO NKONGA Ruth Viviane	Chargée de Cours	En poste
27	DONGMO LEKAGNE Joseph Blaise	Chargé de Cours	En poste
28	FONKOUA Martin	Chargé de Cours	En poste
29	KOTUE KAPTUE Charles	Chargé de Cours	En poste
30	LUNGA Paul KEILAH	Chargé de Cours	En poste
31	MANANGA Marlyse Joséphine	Chargée de Cours	En poste
32	MBONG ANGIE M. Mary Anne	Chargée de Cours	En poste
33	Palmer MASUMBE NETONGO	Chargé de Cours	En poste
34	PECHANGOU NSANGOU Sylvain	Chargé de Cours	En poste

35	MBOUCHE FANMOE Marceline Joëlle	Assistante	En poste
36	OWONA AYISSI Vincent Brice	Assistant	En poste
37	WILFRIED ANGIE Abia	Assistante	En poste

2- DÉPARTEMENT DE BIOLOGIE ET PHYSIOLOGIE ANIMALES (BPA) (46)

1	AJEAGAH Gideon AGHAINDUM	Professeur	*DAARS/FS*
2	BILONG BILONG Charles-Félix	Professeur	Chef de Département
3	DIMO Théophile	Professeur	En Poste
4	DJIETO LORDON Champlain	Professeur	En Poste

5	**DZEUFIET DJOMENI Paul Désiré**	Professeur	En Poste
6	ESSOMBA née NTSAMA MBALA	Professeur	*Vice Doyen/FMSB/UYI*
7	FOMENA Abraham	Professeur	En Poste
8	KAMTCHOUING Pierre	Professeur	En poste
9	**KEKEUNOU Sévilor**	Professeur	En poste
10	NJAMEN Dieudonné	Professeur	En poste
11	NJIOKOU Flobert	Professeur	En Poste
12	NOLA Moïse	Professeur	En poste
13	TAN Paul VERNYUY	Professeur	En poste
14	TCHUEM TCHUENTE Louis Albert	Professeur	*Inspecteur de service Coord.Progr./MINSANTE*
15	ZEBAZE TOGOUET Serge Hubert	Professeur	*En poste*

16	BILANDA Danielle Claude	Maître de Conférences	En poste
17	DJIOGUE Séfirin	Maître de Conférences	En poste
18	JATSA BOUKENG Hermine épse MEGAPTCHE	Maître de Conférences	En Poste
19	**LEKEUFACK FOLEFACK Guy B.**	Maître de Conférences	En poste
20	MEGNEKOU Rosette	Maître de Conférences	En poste
21	MONY Ruth épse NTONE	Maître de Conférences	En Poste
22	NGUEGUIM TSOFACK Florence	Maître de Conférences	En poste
23	TOMBI Jeannette	Maître de Conférences	En poste

24	ALENE Désirée Chantal	Chargée de Cours	En poste
25	ATSAMO Albert Donatien	Chargé de Cours	En poste
26	BELLET EDIMO Oscar Roger	Chargé de Cours	En poste
27	DONFACK Mireille	Chargée de Cours	En poste
28	ETEME ENAMA Serge	Chargé de Cours	En poste
29	GOUNOUE KAMKUMO Raceline	Chargée de Cours	En poste
30	KANDEDA KAVAYE Antoine	Chargé de Cours	En poste
31	MAHOB Raymond Joseph	Chargé de Cours	En poste
32	MBENOUN MASSE Paul Serge	Chargé de Cours	En poste
33	MOUNGANG Luciane Marlyse	Chargée de Cours	En poste
34	MVEYO NDANKEU Yves Patrick	Chargé de Cours	En poste
35	NGOUATEU KENFACK Omer Bébé	Chargé de Cours	En poste
36	NGUEMBOK	Chargé de Cours	En poste
37	NJUA Clarisse Yafi	Chargée de Cours	Chef Div. UBA
38	NOAH EWOTI Olive Vivien	Chargée de Cours	En poste
39	TADU Zephyrin	Chargé de Cours	En poste
40	TAMSA ARFAO Antoine	Chargé de Cours	En poste
41	YEDE	Chargé de Cours	En poste

42	BASSOCK BAYIHA Etienne Didier	Assistant	En poste
43	ESSAMA MBIDA Désirée Sandrine	Assistante	En poste
44	KOGA MANG DOBARA	Assistant	En poste

45	LEME BANOCK Lucie	Assistante	En poste
46	YOUNOUSSA LAME	Assistant	En poste

3- DÉPARTEMENT DE BIOLOGIE ET PHYSIOLOGIE VÉGÉTALES (BPV) (33)

1	AMBANG Zachée	Professeur	Chef Division/UYII
2	BELL Joseph Martin	Professeur	En poste
3	DJOCGOUE Pierre François	Professeur	En poste
4	**MBOLO Marie**	Professeur	En poste
5	MOSSEBO Dominique Claude	Professeur	En poste
6	YOUMBI Emmanuel	Professeur	Chef de Département
7	ZAPFACK Louis	Professeur	En poste

8	ANGONI Hyacinthe	Maître de Conférences	En poste
9	BIYE Elvire Hortense	Maître de Conférences	En poste
10	KENGNE NOUMSI Ives Magloire	Maître de Conférences	En poste
11	MALA Armand William	Maître de Conférences	En poste
12	MBARGA BINDZI Marie Alain	Maître de Conférences	CT/ MINESUP
13	NDONGO BEKOLO	Maître de Conférences	*CE / MINRESI*
14	NGODO MELINGUI Jean Baptiste	Maître de Conférences	En poste
15	NGONKEU MAGAPTCHE Eddy L.	Maître de Conférences	En poste
16	TONFACK Libert Brice	Maître de Conférences	En poste
17	TSOATA Esaïe	Maître de Conférences	En poste

18	DJEUANI Astride Carole	Chargé de Cours	En poste
19	GOMANDJE Christelle	Chargée de Cours	En poste
20	MAFFO MAFFO Nicole Liliane	Chargé de Cours	En poste
21	MAHBOU SOMO TOUKAM. Gabriel	Chargé de Cours	En poste
22	NGALLE Hermine BILLE	Chargée de Cours	En poste
23	NGOUO Lucas Vincent	Chargé de Cours	En poste
24	NNANGA MEBENGA Ruth Laure	Chargé de Cours	En poste
25	NOUKEU KOUAKAM Armelle	Chargé de Cours	En poste
26	ONANA JEAN MICHEL	Chargé de Cours	En poste

27	GODSWILL NTSOMBAH NTSEFONG	Assistant	En poste
28	KABELONG BANAHO Louis-Paul-Roger	Assistant	En poste
29	KONO Léon Dieudonné	Assistant	En poste

30	LIBALAH Moses BAKONCK	Assistant	En poste
31	LIKENG-LI-NGUE Benoit C	Assistant	En poste
32	TAEDOUNG Evariste Hermann	Assistant	En poste
33	TEMEGNE NONO Carine	Assistant	En poste

4- DÉPARTEMENT DE CHIMIE INORGANIQUE (CI) (33)

1	AGWARA ONDOH Moïse	Professeur	*Chef de Département*
2	**DJOUFAC WOUMFO Emmanuel**	Professeur	En poste
3	Florence UFI CHINJE épouse MELO	Professeur	*Recteur Univ.Ngaoundere*
4	GHOGOMU Paul MINGO	Professeur	*Ministre Chargé deMiss.PR*
5	NANSEU Njiki Charles Péguy	Professeur	En poste
6	NDIFON Peter TEKE	Professeur	*CT MINRESI*
7	NDIKONTAR Maurice KOR	Professeur	*Vice-Doyen Univ. Bamenda*
8	NENWA Justin	Professeur	En poste
9	NGAMENI Emmanuel	Professeur	*DOYEN FS UDs*
10	NGOMO Horace MANGA	Professeur	*Vice Chancelor/UB*

11	**ACAYANKA Elie**	Maître de Conférences	En poste
12	BABALE née DJAM DOUDOU	Maître de Conférences	*Chargée Mission P.R.*
13	EMADACK Alphonse	Maître de Conférences	En poste
14	KAMGANG YOUBI Georges	Maître de Conférences	En poste
15	KEMMEGNE MBOUGUEM Jean C.	Maître de Conférences	En poste
16	KONG SAKEO	Maître de Conférences	En poste
17	NDI NSAMI Julius	Maître de Conférences	En poste
18	NJIOMOU C. épse DJANGANG	Maître de Conférences	En poste
19	NJOYA Dayirou	Maître de Conférences	En poste
20	**TCHAKOUTE KOUAMO Hervé**	Maître de Conférences	En poste

21	BELIBI BELIBI Placide Désiré	Chargé de Cours	CS/ ENS Bertoua
22	CHEUMANI YONA Arnaud M.	Chargé de Cours	En poste
23	KENNE DEDZO GUSTAVE	Chargé de Cours	En poste
24	KOUOTOU DAOUDA	Chargé de Cours	En poste
25	MAKON Thomas Beauregard	Chargé de Cours	En poste
26	MBEY Jean Aime	Chargé de Cours	En poste
27	NCHIMI NONO KATIA	Chargé de Cours	En poste

28	NEBA nee NDOSIRI Bridget NDOYE	Chargée de Cours	CT/ MINFEM
29	NYAMEN Linda Dyorisse	Chargée de Cours	En poste
30	PABOUDAM GBAMBIE A.	Chargée de Cours	En poste

31	NJANKWA NJABONG N. Eric	Assistant	En poste
32	PATOUOSSA ISSOFA	Assistant	En poste
33	SIEWE Jean Mermoz	Assistant	En Poste

5- DÉPARTEMENT DE CHIMIE ORGANIQUE (CO) (34)			
1	DONGO Etienne	Professeur	Vice-Doyen/FSE/UYI
2	GHOGOMU TIH Robert Ralph	Professeur	Dir. IBAF/UDA
3	NGOUELA Silvère Augustin	Professeur	Chef de Département UDS
4	NYASSE Barthélemy	Professeur	En poste
5	PEGNYEMB Dieudonné Emmanuel	Professeur	*Directeur/ MINESUP/* Chef de Département
6	WANDJI Jean	Professeur	En poste

7	Alex de Théodore ATCHADE	Maître de Conférences	Vice-Doyen / DPSAA
8	**AMBASSA Pantaléon**	Maître de Conférences	En poste
9	EYONG Kenneth OBEN	Maître de Conférences	En poste
10	FOLEFOC Gabriel NGOSONG	Maître de Conférences	En poste
11	FOTSO WABO Ghislain	Maître de Conférences	En poste
12	KEUMEDJIO Félix	Maître de Conférences	En poste
13	KEUMOGNE Marguerite	Maître de Conférences	En poste
14	KOUAM Jacques	Maître de Conférences	En poste
15	MBAZOA née DJAMA Céline	Maître de Conférences	En poste
16	MKOUNGA Pierre	Maître de Conférences	En poste
17	**MVOT AKAK CARINE**	Maître de Conférences	En poste
18	NGO MBING Joséphine	Maître de Conférences	Sous/Direct. MINERESI
19	NGONO BIKOBO Dominique Serge	Maître de Conférences	**C.E/** MINESUP

20	NOTE LOUGBOT Olivier Placide	Maître de Conférences	C.S/ MINESUP
21	NOUNGOUE TCHAMO Diderot	Maître de Conférences	En poste
22	TABOPDA KUATE Turibio	Maître de Conférences	En poste
23	**TAGATSING FOTSING Maurice**	Maître de Conférences	En poste
24	TCHOUANKEU Jean-Claude	Maître de Conférences	*Doyen /FS/ UYI*
25	TIH née NGO BILONG E. Anastasie	Maître de Conférences	En poste
26	YANKEP Emmanuel	Maître de Conférences	En poste
27	**ZONDEGOUMBA Ernestine**	Maître de Conférences	En poste

28	KAMTO Eutrophe Le Doux	Chargé de Cours	En poste
29	NGNINTEDO Dominique	Chargé de Cours	En poste
30	NGOMO Orléans	Chargée de Cours	En poste
31	OUAHOUO WACHE Blandine M.	Chargée de Cours	En poste
32	SIELINOU TEDJON Valérie	Chargé de Cours	En poste

33	MESSI Angélique Nicolas	Assistant	En poste
34	TSEMEUGNE Joseph	Assistant	En poste

6- DÉPARTEMENT D'INFORMATIQUE (IN) (25)

1	ATSA ETOUNDI Roger	Professeur	*Chef Div.MINESUP*
2	FOUDA NDJODO Marcel Laurent	Professeur	*Chef Dpt ENS/Chef IGA.MINESUP*

3	NDOUNDAM Réné	Maître de Conférences	En poste

4	ABESSOLO ALO'O Gislain	Chargé de Cours	En poste
5	AMINOU Halidou	Chargé de Cours	*Chef de Département*
6	DJAM Xaviera YOUH - KIMBI	Chargé de Cours	En Poste
7	**DOMGA KOMGUEM Rodrigue**	Chargé de Cours	En poste
8	EBELE Serge Alain	Chargé de Cours	En poste
9	KOUOKAM KOUOKAM E. A.	Chargé de Cours	En poste
10	MELATAGIA YONTA Paulin	Chargé de Cours	En poste
11	MONTHE DJIADEU Valery M.	Chargé de Cours	En poste
12	MOTO MPONG Serge Alain	Chargé de Cours	En poste
13	OLLE OLLE Daniel Claude Delort	Chargé de Cours	Directeur adjoint Enset. Ebolowa

14	TAPAMO Hyppolite	Chargé de Cours	En poste
15	TINDO Gilbert	Chargé de Cours	En poste
16	TSOPZE Norbert	Chargé de Cours	En poste
17	WAKU KOUAMOU Jules	Chargé de Cours	En poste

18	BAYEM Jacques Narcisse	Assistant	En poste
19	EKODECK Stéphane Gaël Raymond	Assistant	En poste
20	HAMZA Adamou	Assistant	En poste
21	JIOMEKONG AZANZI Fidel	Assistant	En poste
22	MAKEMBE. S . Oswald	Assistant	En poste
23	MESSI NGUELE Thomas	Assistant	En poste
24	MEYEMDOU Nadège Sylvianne	Assistante	En poste
25	NKONDOCK. MI. BAHANACK.N.	Assistant	En poste

7- DÉPARTEMENT DE MATHÉMATIQUES (MA) (30)

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2	EMVUDU WONO Yves S.	Professeur	*Inspecteur MINESUP*

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4	**MBANG Joseph**	Maître de Conférences	En poste
5	MBEHOU Mohamed	Maître de Conférences	En poste
6	**MBELE BIDIMA Martin Ledoux**	Maître de Conférences	En poste
7	NKUIMI JUGNIA Célestin	Maître de Conférences	En poste
8	NOUNDJEU Pierre	Maître de Conférences	*Chef service des programmes & Diplômes/FS/UYI*
9	TCHAPNDA NJABO Sophonie B.	Maître de Conférences	Directeur/AIMS Rwanda
10	**TCHOUNDJA Edgar Landry**	Maître de Conférences	En poste

11	AGHOUKENG JIOFACK Jean Gérard	Chargé de Cours	Chef Cellule MINPLAMAT
12	CHENDJOU Gilbert	Chargé de Cours	En poste
13	DJIADEU NGAHA Michel	Chargé de Cours	En poste
14	DOUANLA YONTA Herman	Chargé de Cours	En poste
15	FOMEKONG Christophe	Chargé de Cours	En poste
16	KIKI Maxime Armand	Chargé de Cours	En poste
17	MBAKOP Guy Merlin	Chargé de Cours	En poste
18	MENGUE MENGUE David Joe	Chargé de Cours	En poste

19	NGUEFACK Bernard	Chargé de Cours	En poste
20	NIMPA PEFOUKEU Romain	Chargée de Cours	En poste
21	POLA DOUNDOU Emmanuel	Chargé de Cours	En poste
22	TAKAM SOH Patrice	Chargé de Cours	En poste
23	TCHANGANG Roger Duclos	Chargé de Cours	En poste
24	TETSADJIO TCHILEPECK M. E.	Chargé de Cours	En poste
25	TIAYA TSAGUE N. Anne-Marie	Chargée de Cours	En poste

26	BITYE MVONDO Esther Claudine	Assistante	En poste
27	MBATAKOU Salomon Joseph	Assistant	En poste
28	MBIAKOP Hilaire George	Assistant	En poste
29	MEFENZA NOUNTU Thiery	Assistant	En poste
30	TCHEUTIA Daniel Duviol	Assistant	En poste

8- DÉPARTEMENT DE MICROBIOLOGIE (MIB) (18)

1	ESSIA NGANG Jean Justin	Professeur	*Chef de Département*
2	**NYEGUE Maximilienne Ascension**	Professeur	*VICE-DOYEN / DSSE*
3	**NWAGA Dieudonné M.**	Professeur	En poste

4	**ASSAM ASSAM Jean Paul**	Maître de Conférences	En poste
5	BOYOMO ONANA	Maître de Conférences	En poste
6	RIWOM Sara Honorine	Maître de Conférences	En poste
7	SADO KAMDEM Sylvain Leroy	Maître de Conférences	En poste

8	BODA Maurice	Chargé de Cours	En poste
9	BOUGNOM Blaise Pascal	Chargé de Cours	En poste
10	ESSONO OBOUGOU Germain G.	Chargé de Cours	En poste
11	NJIKI BIKOÏ Jacky	Chargée de Cours	En poste
12	TCHIKOUA Roger	Chargé de Cours	En poste
13	ESSONO Damien Marie	Assistant	En poste
14	LAMYE Glory MOH	Assistant	En poste
15	MEYIN A EBONG Solange	Assistante	En poste
16	NKOUDOU ZE Nardis	Assistant	En poste
17	SAKE NGANE Carole Stéphanie	Assistante	En poste
18	TOBOLBAÏ Richard	Assistant	En poste

9. DEPARTEMENT DE PYSIQUE(PHY) (40)

1	BEN- BOLIE Germain Hubert	Professeur	En poste
2	DJUIDJE KENMOE épouse ALOYEM	Professeur	En poste
3	EKOBENA FOUDA Henri Paul	Professeur	*Vice-Recteur. UN*
4	ESSIMBI ZOBO Bernard	Professeur	En poste

5	KOFANE Timoléon Crépin	Professeur	En poste
6	NANA ENGO Serge Guy	Professeur	En poste
7	**NANA NBENDJO Blaise**	Professeur	En poste
8	NDJAKA Jean Marie Bienvenu	Professeur	Chef de Département
9	NJANDJOCK NOUCK Philippe	Professeur	En poste
10	NOUAYOU Robert	Professeur	En poste
11	PEMHA Elkana	Professeur	En poste
12	TABOD Charles TABOD	Professeur	Doyen FS Univ/Bda
13	TCHAWOUA Clément	Professeur	En poste
14	WOAFO Paul	Professeur	En poste
15	ZEKENG Serge Sylvain	Professeur	En poste

16	BIYA MOTTO Frédéric	Maître de Conférences	DG/HYDRO Mekin
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NOMBRE D'ENSEIGNANTS					
DÉPARTEMENT	**Professeurs**	**Maîtres de Conférences**	**Chargés de Cours**	**Assistants**	**Total**
BCH	8 (01)	14 (10)	13 (05)	3 (02)	**38 (18)**
BPA	15 (01)	8 (06)	18 (05)	05 (02)	**46 (14)**
BPV	07 (01)	10 (01)	9 (06)	07 (01)	**33 (9)**
CI	10 (01)	10 (02)	10 (02)	03 (0)	**33 (5)**
CO	6 (0)	21 (05)	05 (02)	02 (0)	**34(7)**
IN	2 (0)	1 (0)	14 (01)	08 (01)	**25 (2)**
MAT	2 (0)	8 (0)	15 (01)	05 (02)	**30 (3)**
MIB	3 (0)	4 (02)	05 (01)	06 (02)	**18 (5)**
PHY	15 (0)	14 (02)	09 (03)	02 (0)	**40 (5)**
ST	7 (1)	15 (01)	18 (05)	02 (0)	**42(7)**
Total	**75 (5)**	**105 (29)**	**116 (31)**	**43 (10)**	**339 (75)**

Soit un total de **339 (**75**)** dont :

- Professeurs **75 (5)**
- Maîtres de Conférences **105 (**29**)**
- Chargés de Cours **116 (31)**
- Assistants **43** (10**)**

( ) = Nombre de Femmes **75**

Dedication

To my Mother

Mrs. KADJI KAMTA Octavie

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ABSTRACT

Nosocomial infections in human beings are frequently caused by non-fermentative bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Acinetobacter baumannii* (*A. baumannii).* This study, therefore, aimed at identifying virulence factors and antimicrobial resistance markers associated with both *P. aeruginosa* and *A. baumannii* isolates that have been directly implicated in human infection. A total of 77 and 27 presumptive strains of *P. aeruginosa* and *A. baumannii* respectively, isolated from clinical samples among which are pus, blood and broncho-alveolar lavage, were collected from Yaounde Teaching Hospital, Yaounde Central Hospital and Centre Pasteur du Cameroun. Identities of the isolates were further confirmed through PCR amplification of 16S rRNA the conserved region for *P. aerugimosa* and Internal Transcribed Spacer *(ITS)* for *A. baumannii*. Antimicrobial susceptibility testing (AST) was performed using the Kirby-Bauer disc diffusion method. Phenotypical expression of AmpC β-lactamases (AmpC), Extended Spectrum β-lactamases (ESBLs) and Metallo β-Lactamases (MBLs) was determined using combined disc method. Dye method was used to study biofilm formation ability of the isolates while genomes of the isolates were screened for the presence of β-lactamases bla_{TEM} and bla_{CTXM} genes using specific PCR primers. Pathogenicity of *P. aeruginosa* and *A. baumannii* was assessed through PCR amplification of a number of virulence genes such as *lasB, exoA, pslA, exoS, OmpA* and *csuE*. Whole genome sequencing (WGS) of two multi-drug resistant *P. aeruginosa* was done using illumina sequencing platform. Out of the 77 presumptive *P. aeruginosa* isolates, identities of 75 (97.4%) were positively confirmed. All the 27 strains of presumptive *A. baumannii* harbored the specific *ITS* gene fragment. Up to 25% of the *P. aeruginosa* strains produced ESBLs phenotypically, and more than 90% of these isolates were positive for the *lasB, exoA, pslA* and *exoS* genes. A large proportion (88-90%) of the *A. baumannii* isolates harbored *OmpA* and *csuE* genes. bla_{TEM} and bla_{CTXM} were detected in 17% and 4% of *P. aeruginosa* respectively while a much higher proportion (70% and 29%) of the *A. baumannii* isolates possessed these resistance determinants respectively. The genome sizes of the two multi-drug resistant strains of *P. aeruginosa*, UY1PSABAL and UY1PSABAL2 that we named after University of Yaounde I, were 7.02 and 6.26 Mb respectively. These genomes bore resistance markers for β-lactams, quinolones, aminoglycosides and even vancomycin at the same time together with transposases of several mobile genetic elements. Tremendous

numbers of virulence determinants were determined in these genomes. Our findings reveal the occurrence of both virulence and drug resistant determinants studied in the clinically relevant *P. aeruginosa* and *A. baumannii* during our study period. Among the different technologies applied in this studied, WGS revealed tremendous numbers of information concerning resistance and virulence. Nevertheless, phenotypical methods compare to genotypic method including PCR or WGS are of particular interest in terms of reduced costs, availability and simplicity.

Keywords: Resistance monitoring algorithm; *Pseudomonas aeruginosa*; *Acinetobacter baumannii*; resistance phenotype; resistance genotype

RESUME

Les infections nosocomiales sont fréquemment causées par les bactéries non fermentaires principalement *Pseudomonas aeruginosa* (*P. aeruginosa*) et *Acietobacter baumannii* Acinetobacter baumannii. Fort de cela, Cette étude visait, à identifier des facteurs de virulence ainsi que plusieurs marqueurs de résistance aux antibiotiques qui leurs sont relatifs. Un total de 77 et de 27 isolats suspects de *P. aeruginosa* et d'*A. baumannii* respectivement ont été collectés au Centre Hospitalier Universitaire de Yaoundé, à l'Hôpital Central de Yaoundé et au Centre Pasteur du Cameroun. Ceux-ci étaient isolés à partir de divers échantillons, comprenant le pus, le sang et le liquide de lavage broncho-alvéolaire. La charactérisation des isolats a été déterminée par amplification d'une portion de gène de l'ARN 16S spécifique de *P. aeruginosa* et de l'*Internal Transcribed Spacer* (*ITS*) spécifique d'*A. baumannii*. La méthode de diffusion ou méthode des disques sur milieux gélosés (méthode de Kirby-Bauer) a servi à réaliser les tests de sensibilité aux antibiotiques (AST). L'expression phénotypique des β-lactamases AmpC (AmpC), des β-lactamases à spectre élargi (BLSEs) et des Metallo β-lactamases (MBLs) a été déterminée par la méthode de diffusion des disques de β-lactamines combinés aux inhibiteurs de β-lactamases. Par la suite, la méthode de coloration par le cristal violet a été utilisée pour évaluer le potentiel de formation de biofilms de ces isolats. Nous avons utilisé des amorces de PCR spécifiques pour rechercher la présence de gènes de β-lactamases bla_{TEM} et bla_{CTXM} dans tous les échantillons de cette étude. La pathogénicité du *P. aeruginosa* et d'*A. baumannii* a été évaluée par amplification des gènes de virulence *lasB, exoA, pslA* et *exoS* ainsi que *OmpA* et *csuE* chez *P. aeruginosa* et *A. baumannii* respectivement. Le séquençage du génome complet (WGS) de deux souches de *P. aeruginosa* multirésistant a été effectué en utilisant la plateforme de séquençage illumina. Sur les 77 isolats suspects de *P. aeruginosa*, une grande proportion (75 ; 97,4%) a été identifiée et confirmé par PCR comme étant effectivement des *P. aeruginosa*. Tous (100%) les 27 présumés *A. baumannii* portaient le fragment du gène *ITS* spécifique d'*A. baumannii*. Vingt-cinq pour cent (25%) des isolats de *P. aeruginosa* présentaient le phénotype BLSEs tandis que plus de 90% de ces isolats étaient positifs pour les gènes *lasB, exoA, pslA* et *exoS*. Une grande proportion (88%) des isolats *A. baumannii* portaient les gènes *OmpA* et *csuE*. Les gènes bla_{TEM} et bla_{CTXM} ont été détectés respectivement dans 17% et 4% des isolats de *P. aeruginosa* tandis qu'une proportion plus élevée (70% et 29%) des isolats *A. baumannii* possédaient ces déterminants de résistance

respectivement. Le séquençage complet du génome de UY1PSABAL et UY1PSABAL2 a révélé une longueur de 7,02 et 6,26 Mb de chaque souche respective. Ces génomes portaient des marqueurs de résistance des β-lactames, des quinolones, des aminoglycosides et même ceux de la vancomycine. Les transposases de plusieurs éléments génétiques mobiles ont été détectées. Un nombre important de déterminants de la virulence ont été identifié dans ces différents génomes. Nos résultats révèlent la présence de déterminants de virulence et de résistance aux antibiotiques étudiés dans les isolats cliniques de *P. aeruginosa* et *A. baumannii* qui potentiellement circulaient au sein des établissements hospitaliers à Yaoundé, durant notre période d'étude. Au regard des différentes technologies utilisées dans cette étude, le séquençage du génome complet par une méthode de seconde génération : *Next Generation Sequencing* (NGS) a fourni le plus grand nombre d'informations en termes de déterminant de virulence et de résistance. Néanmoins, les méthodes phénotypiques par rapport aux méthodes génotypiques présentent un interêt certain en termes d'accessibilité, de coûts, de disponibilité et de simplicité.

Mots-clés Algorithme de monitoring de la résistance : *Pseudomonas aeruginosa* ; *Acinetobacter baumannii* ; phénotype de résistance ; génotype de résistance

LIST OF ABBREVIATIONS AND ACRONYMS

A. baumannii	:	*Acinetobacter baumannii*
6-APA	:	6-aminopenicillanic acid
6-FAM:	:	(6-carboxyfluorescein)
ABC	:	: ATP-binding cassette transporters
Ag+:	:	silver ion
AIDS	:	acquired immune deficiency syndrome
AIZD	:	Antibiotic inhibition zone diameter data
Al3+	:	Aluminum ion
Alg	:	Alginate
AlgL	:	Alginate lyase
AMB	:	l-2-amino-4-methoxy-trans-3-butenoic acid
AMKR	:	Amikacin Resistant
AmpC	:	Ampicillin-hydrolyzing cephalosporinase.
AntiSMASH	:	The antibiotics & Secondary Metabolite Analysis Shell
API 20 NE	:	analytical profile index with 20 tests for Non-Enterobacteriaceae
ArpA	:	Alkaline protease
AST	:	Antimicrobial susceptibility testing
BAL	:	Broncho-alveolar-lavage
blaTEM	:	**B**eta-**la**ctamase isolated from a patient named **TEM**oneira
blaCTX-M	:	**B**eta-**la**ctamase hydrolyzing cefotaxime
BHQ	:	Black Hole Quencher
BSAC	:	British Society for Antimicrobial Chemotherapy
CARD	:	Comprehensive Antibiotic Resistance Database
Cd2+	:	Cadmium ion
cDNA	:	complementary DNA
CDP	:	cyclodipeptide
CDPS	:	Cyclodipeptide synthases
CDT	:	Combined Disc Test

CFTR	:	Cystic fibrosis transmembrane conductance regulator
Cif	:	Cystic fibrosis
CLSI	:	Clinical and Laboratory Standards Institute
Co2+:	:	cobalt ion
CPA	:	cross-priming amplification
CPC	:	Centre Pasteur du Cameroun
Cr2+:	:	Chromium ion
CRISPR	:	Clustered Regularly Interspaced Short Palindromic Repeats
Ct	:	threshold cycle
Cu2+	:	Copper ion
DABCYL	:	4- (Dimethylaminoazo) benzene-4-carboxylic acid
DBO	:	Diazabicyclooctane
DDQ	:	2-3-Dichloro-5,6-dicyano-1,4-benzoquinone
DDST	:	double disc synergy test
DHFR	:	Dihydrofolate reductase
DNA	:	Deoxyribonucleotide acid
dNTPs	:	desoxy-nucleotide triphosohates
EDTA	:	Ethylene diamine tetra-acetic acid
ELISA	:	Enzyme-linked immunosorbent assay
EMB	:	eosin methionine blue
ERIC-PCR	:	Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction
ESBLs	:	Extended Spectrum β-lactamases
ESKAPE		*Enterococcus faecium, Staphylococcus aureus, Klebsiella Pneumoneae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter cloacaea*.
EUCAST	:	European Committee on Antimicrobial Susceptibility Testing
ExlA	:	Exolysine A
ExoS	:	Exotoxin S
ExoT	:	Exotoxin T
ExoU	:	Exotoxin U
ExoY	:	Exotoxin Y
FastQC	:	Fast quality control

Fe	:	iron ion
FODRUS Laboratory	:	Food and Drug Safety Laboratory
FRET	:	Fluorescence Resonance Energy Transfer
GENR	:	Resistant to gentamicin
GN card	:	Gram-negative card for fermenting and non-fermenting bacilli
HAD	:	Helicase-dependent amplification
HCAI	:	healthcare-associated infections
HEX	:	hexacholoro-6-carboxy-fluorescein
HVS	:	high vaginal swab and blood were
I	:	Intermediate
IFN	:	interferon
IMSA	:	isothermal multiple-self-matching-initiated amplification
IS	:	insertion sequence
ITS	:	Internal transcribed spacer
KPC-producing	:	*Klebsiella pneumoniae* carbapenemase-producing
LAMP	:	loop-mediated isothermal amplification
LasA	:	Elatase A
LasB	:	Elastase B
LB	:	Luria-Bertani
lpsB	:	Lipopolysaccharide B
MALDI-TOF-MS	:	Matrix-Assisted Laser Desorption/Ionization-time of flight-mass spectrometer
MBLs	:	Metallo β-Lactamases
MDR	:	multi drug resistance
MDR	:	multidrug resistant
MGE	:	Mobile genetic elements
MHA	:	Mueller Hinton agar
MHA	:	muller Hinton agar
MIC	:	Minimum Inhibitory Concentration
Mn2+	:	Manganese ion
MRSA	:	methicillin resitant Staphylococcus aureus
NAGGN	:	N-acetylglutaminylglutamine amide
NaOH	:	Sodium hydroxyl

NEAR	:	Nicking enzyme amplification reaction
NETR	:	Resistant to netilmicin
Ni2+	:	Nickel ion
NIs	:	Nosocomial infections
nm	:	nano meter
NRPSs	:	Non-ribosomal peptide synthetases
OD	:	optical density
Oligo-dT	:	deoxythymidylic acid residue
OmpA	:	Outer membrane protein
P. aeruginosa	:	*Pseudomonas aeruginosa*
PABA	:	Para-aminobenzoic acid
PATRIC	:	Pathosystems Resource Integration Center
Pb2+	:	Lead ion
pbpG	:	Penicillin-binding protein 7/8
PCR	:	polymerase chain reaction
PFGE	:	Pulsed-field Gel Electrophoresis
pH	:	Potential of hydrogen
PHASTER:	:	PHAge Search Tool Enhanced Release
PIP	:	piperacillin
PlcH:	:	Haemolytic phospholipase C
PlcN	:	Non-haemolytic phospholipase C
Pld	:	phospholipase D
Prp	:	Class IV protease lysine
***Pseudomonas Spp*.:**	:	*Pseudomonas species*
PSR	:	polymerase spiral reaction
ptk and epsA	:	Capsular polysaccharide
QS:	:	Quorum sensing
R	:	Resistant
RAST	:	Rapid Annotation using Subsytems Technology
RPA	:	recombinase polymerase amplification
rRNA	:	ribosomal RNA
RT-PCR	:	Reverse transcription PCR
S	:	Susceptible

SDA	:	strand displacement amplification
Sn2+	:	Tin ion
SP-A	:	surfactant protein A
T1SS	:	Type I secretion systems
T2SS	:	Type II secretion systems
T3SS	:	Type III secretion systems
T4SS	:	Type IV secretion systems
T5SS	:	Type V secretion systems
T6SS	:	Type VI secretion systems
T7SS:	:	Type VII secretion systems
TAMRA	:	6-carboxytetramethylrhodamine
Tb3+:	:	terbium ion
TCC	:	ticarcillin-clavulanic acid
TEM	:	β-lactamase named after Temoniera in Greece (the first patient who was infected by bacteria bearing TEM gene)
TET	:	tetrachloro-6-carboxy-fluocein
TIC	:	ticarcillin
Tl+	:	Thallium ion
Tm	:	melting temperature
Tn	:	transposable element
Tnp	:	transposase
TOBR	:	Resistant to tobramycin
ToxA and ExoA	:	Exotoxin A
TplE	:	Phospholipase A1
TSA	:	Triptic Soy Agar
TSB	:	Tryptic soy broth
Tth	:	Thermus thermophiles
TZP	:	piperacillin-tazobactam
WGS	:	Whole genome sequencing
WHO	:	world health organization
YCH	:	Yaoundé Central Hospital
YUTH	:	Yaoundé University Teaching Hospital
Zn2+	:	Zinc ion

INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) and *Acinetobacter baumannii* (*A. baumannii*) are important causative agents of nosocomial infections with more severe complications particularly in immunocompromised patients (Bassetti et al., 2018)**.** These pathogens are commonly isolated in wound infections, pneumonia and septicemia (Zeighami et al., 2019, Milivojevic et al., 2018). Infections due to *P. aeruginosa* and *A. baumannii* are difficult to eradicate because of their high intrinsic ability to acquire resistance to a variety of antimicrobial agents. Like other Gram-negative bacteria, *P. aeruginosa* and *A. baumannii* can express resistance through numerous mechanisms such as production of antibiotic modifying or degrading enzymes, active efflux pump or target modification (Breidenstein et al., 2011). Some strains can show resistance to all antibiotics families available. Based on evident report, β-lactam antibiotics are one of the most commonly used drugs worldwide (Chem et al., 2018a) and this also applies to the treatment of infections caused by *P. aeruginosa* and *A. baumannii* (Eze et al., 2018). The spread and persistence of β-lactam resistant and virulent bacteria strains in the environment most often results from the expanded use of this class of antibiotics (Bassetti et al., 2018). Enzymes such as penicillinases, oxacillinases, cephalosporinases, and carbapenmases contribute most frequently to β-lactam resistance. In addition, the potential to produce extracellular components such as exotoxin, phospholipase, alginase, elastase and biofilm forming determinants also enhance the pathogenicity of these bacteria (Newman et al., 2017). Multi-drug resistance (MDR): resistance to at least three classes of antimicrobials) and virulence determinants contribute significantly to the severity of infections. Studies conducted by De Angelis et al. (De Angelis et al., 2018) and Bassetti et al. (Bassetti et al., 2018) revealed the burden of infections caused by MDR strains. They showed how infections with such strains have led to increase hospitalization stay, increase cost of treatment and patient mortality rate. MDR strains are tremendous issue for public health stakeholders. MDR *P. aeruginosa* and *A. baumannii* are increasingly spreading in community and hospital environment leading to the severe infection cases resulting to the therapeutic impasses (Shlaes, 2010)**.** MDR persistence in the host environment is mainly due to various virulence factors expressed to reinforce their multiple resistance mechanism. The study of biochemistry of the virulence and resistance aims to elucidate the mechanisms of virulence and resistance. Thus, it can lead to develop molecules that inhibit this mechanism or to modify the existing antibiotics so that they escape this

virulence or resistance mechanism. A good understanding of leading mechanisms involved in bacteria host persistence can also help to find new target to thwart their spread. “I felt that if we understood how resistance spread, we sterm it. If we could understand the mechanisms by which the bacteria become resistant in detail we could find ways around them with new antibiotics” This statement of David M. Shlaes in his book entitled, *Antibiotics: the Perfect Storm* (Shlaes, 2010) is a perfect illustration of the need of better understanding of bacteria resistance mechanisms for their better management**.** To achieve the previous gold multidisciplinary studies have been conducted so far including Microbiology, Biochemistry, and Chemistry using Biotechnology and Bioinformatics resources. Various tools have been developed for this effect and most of them have brought relevant knowledge concerning bacteria persistence and resistance. Next Generation Sequencing (NGS) also called massive parallel sequencing is one of the latest approach of organisms’ genome study. It gives massive information on the genes present within considered organisms, since this technique is capable of processing multiple DNA sequences in parallel with high throughput, reduced cost and time (Schwarze et al., 2018). It is a powerful protocol, which has revolutionised the genome study landscape. Whole genome sequencing (WGS) protocol uses NGS methodology to study the complete DNA sequence of organisms (Schwarze et al., 2018). It has been used to investigate numerous genetic problems. To the best of our knowledge, there are few reports on the carriage, expression of resistance genes and virulence determinants among bacteria isolates especially *P. aeruginosa* and *A. baumannii* of clinical origin in Cameroon; there is no published data of *P. aeruginosa* whole genome sequencing from clinical sample in Cameroon. This study was aimed to develop an algorithm for resistance monitoring of nosocomial causing bacteria: the case of *P. aeruginosa* and *A. baumannii*.

CHAPTER 1: Problem Statement and Literature Review

1.1 Problem statement

Nosocomial infections (NIs) have become a great issue considered by public health stakeholders. According to WHO report, approximately 15% of hospitalized patients suffer from these infections (Khan et al., 2017). They account for 10 % of infection in developing countries (Khan et al., 2017). *P. aeruginosa* and *A. baumannii* are the most predominant bacteria incriminate in NIs among non-fermenting Gram-negative bacteria. *P. aeruginosa* contributes to 11% of all nosocomial infections (Lila et al., 2018). Independently of the type of infections, nosocomial or non-NIs, *P. aeruginosa* ventilator-associated pneumonia mortality is recognized to reach to 41.9%, (Micek et al., 2012) ; mortality rate of *A. baumannii* pneumonia can exceed 50% (Lannan et al., 2016). Those bacteria are highly resistant. As NIs occur during hospital stay, they cause prolonged stay, increase resistance of microorganisms to antimicrobials, disability, economic burden and excess death. To stem the spread of *P. aeruginosa* and *A. baumannii,* important etiological agents of NIs as described above, it is important to investigate and gather knowledge on determinants involved in their colonization and their persistence within the host environment. This knowledge is used for the development of more powerful antimicrobial agents or giving and insight to develop better protocol for the detection of certain strains displaying particular virulent and resistant features. A Cameroonian study carried out in a 200 beds hospital described a cumulative incidence of 19% of NI with 28 % of mortality rate (Nouetchognou et al., 2016b) This study also indicated that 75 % of surgical NIs were caused by *P. aeruginosa* and 12% of nosocomial urinary tract infections were due to *A. baumannii* (Nouetchognou et al., 2016a).When going through the literature, it is noted that, it lacks sufficient knowledge on determinant of virulence and resistance of strains collected in Cameroon. It is therefore important to study the characteristic of bacteria frequently isolated in nosocomial infection such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* in Yaounde environment to have a better understanding on which resistance and virulence determinant are present in this geographic area. Various tools have been developed for this effect and most of them have brought relevant knowledge concerning bacteria persistence and resistance within the host environ. The most common technics developed are biochemical tests (phenotypic tests) widely sprayed and simple to use. Molecular technics (PCR and Whole genome sequencing (WGS)) also were developed giving a deep insight to bacteria features.

Objectives

General objective

To develop an algorithm for the diagnosis of bacteria responsible for nosocomial infections: cases of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

Specific objectives

1. To identify the presumed *Pseudomonas aeruginosa* and *Acinetobacter baumannii* samples collected and the antimicrobial susceptibility testing.

2. To determine *P. aeruginosa* and *A. baumannii* biofilm formation ability and antimicrobial resistance enzymes phenotypic expression.

3. To characterize the virulence and resistance genes of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* samples

4. To design an algorithm for study for the surveillance of resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in routine Biology and Public Health laboratories

1.2 Literature review

Nosocomial infections and their pathogen

Nosocomial infections (NIs) are defined as hospital acquired infection developing at least 48-72 h after admission. Surgical wound and other soft tissue infections, urinary tract infections (UTI), Respiratory infections, Gastroenteritis and Meningitis are the common type of NIs. large number of microorganisms are responsible for hospital infections and any microbe may have the capacity/ability to cause an infection in the hospitalized patients. Ninety percent of the NIs is caused by bacteria, whereas mycobacterial, viral, fungal or protozoal agents are less commonly involved. The bacteria that commonly cause nosocomial infections include *Staphylococcus (S.) aureus, Streptococcus spp., Bacillus cereus, Acinetobacter spp.*, coagulase negative *staphylococci, enterococci, P.aeruginosa, Legionella* and members of the *Enterobacteriaceae* family such as *Escherichia coli, Proteus mirabilis, Salmonella spp., Serratia marcescens* and *Klebsiella pneumoniae.* But the most frequently reported nosocomial pathogens have been *E. coli, S. aureus, enterococci* and *P. aeruginosa*.

1.2.1 *Pseudomonas aeruginosa* description

- ***Pseudomonas aeruginosa* history and taxonomy**

Discovery of *P. aeruginosa* has followed several steps. In 1860, Fordos extracted the pigment responsible for the blue coloration in pus. Lucke was the first to associate this pigment with rod-shaped organisms in 1862. *P. aeruginosa* was successfully isolated from a pure culture during the study entitle "On the blue and green coloration of bandages" in 1882, conducted by Carle Gessard (Lister et al., 2009). Migula in 1894 described the genus *Pseudomonas* (Peix et al., 2009). This genus contains 254 species (http://www.bacterio.net/acinetobacter.html, last accessed January 2019), most of which are saprophytic. More than 25 species are associated with humans. Most *Pseudomonas species* known to cause disease in humans are associated with opportunistic infections. These include *P aeruginosa, P fluorescens, P putida, P cepacia, P stutzeri, P maltophilia, and P. putrefaciens.* Because of the frequency with which it is involved in human disease, *Pseudomonas aeruginosa* has received the most attention *Pseudomonas Spp.* is classified among *Pseudomonadaceae* family.

- ***Pseudomonas aeruginosa* biochemical and growth characteristics**

Pseudomonas is an aerobic, Gram-negative rod shaped bacteria with a monotrichous polar flagellum (Wisplinghoff, 2017). It is motile, non-fermentative, catalase and oxidase positive. *Pseudomonas spp's* colonies are easily identified on usual media such as nutrient agar where they produced characteristically blue-green and yellow-green water-soluble pigments. It

can also be cultivating on Gram-negative selective medium such as CLED giving green flat colonies with rough edges and turning the media; or Mac Conkey agar appearing as irregular, colorless and the agar surrounding the bacteria remains relatively transparent. *Pseudomonas aeruginosa* colonies lead to beta (β) hemolysis on blood agar. They are ubiquitous and frequently found in soil, food, wet environments, and hospital settings. It can survive with low levels of nutrients and grow in temperatures ranging from 4 °C to 42°C. These characteristics allow *Pseudomonas aeruginosa* to attach itself and survive on medical equipment and on other hospital surfaces, which favors the beginning of infections in immunocompromised patients.

- ***Pseudomonas aeruginosa* virulence factors and pathogenesis**

Virulence factors are molecules or component expressed by organisms such as bacteria that enable them to achieve colonization, immune-evasion of the host's immune response and obtain nutrition from the host. Some virulent factors are intrinsic to the bacteria like capsules and endotoxin, whereas, others are obtained from mobile genetic elements like plasmids or brought by bacteriophages such as some exotoxins. *P. aeruginosa* virulence factors include adherence factors, various exotoxin, and many enzymes catalyzing the lysis of host components. The combination of these virulence factors can lead this pathogen to cause pneumonia, urinary tract infections and bacteremia with consequently high morbidity and mortality. In the table below, are compiled some *P. aeruginosa* virulence factors with their activities and functions.

Table I: Virulence effectors of *Pseudomonas aeruginosa* (Fothergill et al., 2012; Klockgether and Tümmler, 2017)

Name	**Activity and function**
ArpA	Alkaline protease, zinc metalloprotease; degrades host immune complements C1q, C2, and C3 and cytokines interferon (IFN)- γ and tumour necrosis factor (TNF)-a
Cif	Cystic fibrosis transmembrane conductance regulator (CFTR) inhibitory factor, epoxide hydrolase; promotes sustained inflammation by hydrolysing the paracrine signal 14,15-epoxyeicosatrienoic acid that stimulates neutrophils to produce the proresolving lipid mediator 15-epi lipoxin A; Cif increases the ubiquitination and lysosomal degradation of some ATP-binding cassette transporters (ABC) including CFTR, P-glycoprotein, and TAP1

ExlA	Exolysin A, a pore-forming toxin that induces plasma membrane rupture in epithelial, endothelial, and immune cell
ExoS	Bifunctional toxin. It blocks the reactive oxygen species burst in neutrophils by ADP-ribosylation of Ras, thereby preventing the activation of phosphoinositide-3-kinase (PI3K), which is required to stimulate the phagocytic NADPH-oxidase
ExoT	Bifunctional toxin it impairs the production of reactive oxygen species burst in neutrophils and promotes the apoptosis of host cells by transforming host protein Crk by ADP-ribosylation into a cytotoxin and by activation of the intrinsic mitochondrial apoptotic pathway
ExoU	A phospholipase A2 (PLA2) inducing plasma membrane rupture and rapid cell necros
ExoY	Nucleotidyl cyclase with preference for cGMP and cUMP production; it becomes activated by binding to filamentous actin
Alg	Alginate is a polymer of mannuronic acid and glucuronic acid and overproduction leads to a mucoid phenotype, which is often associated with chronic infection in cystic fibrosis patients. Alginate has been implicated in multiple aspects of *P. aeruginosa* pathogenesis including adhesion, protection from phagocytosis and antibiotics and quenching of free radicals released from activated neutrophils
AlgL	Alginate lyase (AlgL) acts by cleavage of glycosidic bonds in the alginate polymer backbone.
LasA	Zinc metalloprotease of the M23A family; it enhances elastolytic activity of LasB
LasB	Zinc metalloprotease with strong elastolytic activity
PlcH	Haemolytic phospholipase C that releases phosphate esters from sphingomyelin and phosphatidylcholine
PlcN	Non-haemolytic phospholipase C that releases phosphate esters from phosphatidylserine and phosphatidylcholine
PldA	Trans-kingdom toxin, phospholipase D, facilitates intracellular invasion of host eukaryotic cells by activation of the PI3K/Akt pathway
PldB	Trans-kingdom toxin, phospholipase D, facilitates intracellular invasion of host eukaryotic cells by activation of the PI3K/Akt pathway

PrpL	Class IV protease, lysine endoproteinase, degrades proteins such as complement, immunoglobulins, elastin, lactoferrin, and transferrin
Pyocyanin	Redox-active zwitterion that is cytotoxic
Rhamnolipids	Chemically heterogeneous group of monorhamnolipids and dirhamnolipids that are also biosurfactants and cause haemolysis and lysis of immune effector cells
Tox A	Exotoxin A, a toxin with ADPRT activity; it mediates its entry into target host cells through its cell-binding domain, then ADP-ribosylates host elongation factor 2 (EF-2) to block protein synthesis through its enzymatic domain
TplE	Trans-kingdom toxin, phospholipase A1, disrupts the endoplasmic reticulum and thereby promotes autophagy by the activation of the unfolded protein response
Pyoverdine	Is a high-affinity siderophore that is released by *P. aeruginosa* in iron-limited conditions. This has the ability to bind iron and both convert it into a useable form and transport it into the bacteria.

1.2.2. *Acinetobacter baumannii description*

- ***Acinetobacter baumannii* history and taxonomy**

The genus *Acinetobacter* was discovery in 1911 by Beijerinck and was named *Micrococcus calcoaceticus* (Almasaudi, 2018) that was isolated from soil by enrichment in a calcium-acetate-containing minimal medium . *Acinetobacter* genus has gone through a long history of misclassification. Different names have been used to describe this organism before being named in 1954 by Brisou and Prevot as *Acinetobacter* (Almasaudi, 2018). It belongs to Moraxellaceae family. The most common names of which used were *Achromobacter anitratus*, *Achromobacter mucosus*, *Alcaligenes haemolysans*, Cytophaga, Diplococcus mucosus, *Bacterium anitratum*, *Herellea vaginicola*, *Lingelsheimia*, *Mimapolymorpha*, *M. calcoaceticus*, *Moraxella lwoffii* and *Neisseria winogradskyi* (Almasaudi, 2018). The *Acinetobacter* genus currently consists of 70 species **(http://www.bacterio.net/acinetobacter.html, last accessed 2020 February 18**) with several provisionally species termed genomic species delineated by DNA-DNA hybridization. Although *A. baumannii* is the most frequently identified nosocomial pathogen in the genus, several other species cause occasionally infections in humans including

A. nosocomialis, *A. pittii*, and less frequently *A. ursingii*, *A. haemolyticus*, *A. lwoffii*, *A. parvus*, and *A. junii* (Touchon et al., 2014)

- ***Acinetobacter* biochemical and growth characteristics**

Acinetobacter baumannii like others species of its genus are non-fastidious non-fermenteting bacteria. They produce a pale yellow to white greyish pigment on the nutrient agar even in triptic soy agar. On eosin methylene blue agar, *Acinetobacter* species appear bluish to bluish gray color. *A. baumannii* strains grow well on usual culture media and produced whitish and opaque colonies 1-2 mm in diameter after 18-24 h incubation at 37 ˚C (Percival et al., 2014). They appear blue-green on CLED media as lactose negative colonies. The cell wall of *Acinetobacter* is a typical Gram-negative bacteria wall structure; however, acetone-alcohol de-staining is difficult because it keeps the crystal violet and this can lead to erroneous detection as Gram-positive cocci (Almasaudi, 2018). Colonies suspected as *A. baumannii* are catalase positive, oxidase negative, Simmon-citrate positive, strictly aerobic coccobacilli like and non-motile. *Acinetobacter species* are usually present in pairs or long chains of variable length (Almasaudi, 2018)

- ***Acinetobacter* virulence factors and pathogenesis**

Infections caused by *Acinetobacter spp.* emerged during the 1960s and 1970s in parallel with increasing use of complex intensive care. *Acinetobacter spp.* was initially considered a commensal opportunist, a low-virulence pathogen. In subsequent decades, the intensification of mechanical ventilation, central venous and urinary catheterization, and antibacterial therapy has caused a rapid increase in the frequency and severity of *Acinetobacter spp.* infections. Several virulence factors have been identified by genomic and phenotypic analyses, capsular polysaccharides, proteases, phospholipases, lipopolysaccharides (LPS), including outer membrane porins, protein secretion systems, and iron-chelating systems (McConnell et al., 2013). Some of them are presented in the table below.

Table II: Identified *Acinetobacter baumannii* virulence factors (McConnell et al., 2013).

Virulence factor (gene)	**role in pathogenesis**
Porin (OmpA, Omp33-36, Omp22, CarO, OprD-like)	Induction of apoptosis in host cells, adherence and invasion of epithelial, cells, biofilm formation, surface motility, serum resistance
Lipopolysaccharide (lpsB)	Evasion of the host immune response, triggering the host inflammatory

	response
Capsular polysaccharide (ptk and epsA)	Evasion of the host immune response, growth in serum
Phospholipase (A1S_2989)	Serum resistance, bacterial dissemination, in vivo bacterial survival
Penicillin-binding protein 7/ 8(pbpG)	Peptidoglycan biosynthesis, cellular stability, growth in serum
Outer membrane vesicles	Delivery of virulence factors to the cytoplasm of host cells, transfer of genetic material between bacterial cells
Acinetobactin-mediated iron acquisition system	Provides iron needed to persist in the host, causes cell apoptosis
Pili	Adherence, biofilm formation
Type II protein secretion system	In vivo survival
Type VI protein secretion system	Killing of competing bacteria, host colonization
Type V protein secretion system	Adherence, biofilm formation

1.3 Antibiotics

Antibiotics are molecules that act in low concentration on bacteria by interacting between specific targets on the surface or inside the bacteria cell. It can either inhibit the growth or kill the bacteria cell. Antibiotics able to inhibit the bacteria growth are bacteriostatic; those causing bacterial cell death are bactericidal. Some antibiotics can display bacteriostatic activity in some circumstances and bactericidal activity in others (Walsh, 2003). Antimicrobial compound can be either natural, synthetic or semi-synthetic. The discovery and production of antibiotics in the 1990s has been one of greatest achievements in medicine. The first antibiotic discovered was penicillin (Penicillin G) by Alexander Fleming in 1928 and first available commercially in 1942 (Marek and Timmons, 2019). Nowadays numerous different classes of antimicrobial agents are known and can be classified based on their mechanisms of action or according to their structure. Antimicrobial commonly used for *Pseudomonas aeruginosa* and *Acinetobacter baumannii* therapy are β-Lactams, aminoglycosides, quinolones, additional

tetracycline and trimethoprim-sulfamethoxazole antibiotics are recommended on *Acinetobacter baumannii* treatment (Bassetti et al., 2019).

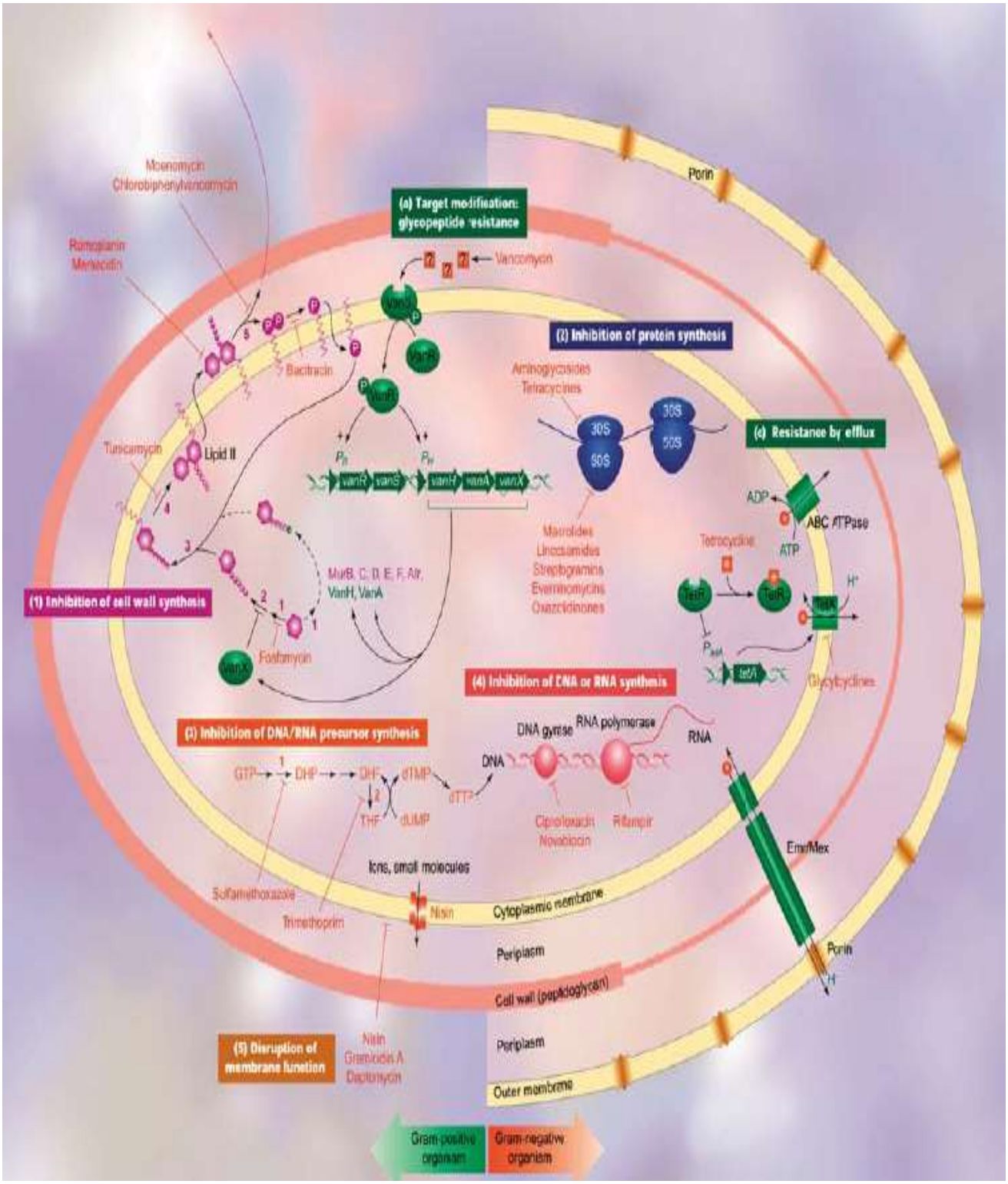


Figure 1: Schematic representation of antimicrobial cell target (Walsh, 2003)

1.3.1. β-Lactams

β-Lactam antibiotics are antibacterial agents interrupting cell wall biosynthesis through covalent binding to Penicillin-Binding Proteins (PBPs) enzymes. Consequently, during the last step of cell wall building, the peptidoglycan cross-linking will not occur, leading to the bacterial cell death. All β-lactam antibiotics carry the same β-lactam ring which can be substituted by some biochemical groups or carry on an additional ring; the considered modification will determine whether the agent is classified as a penam, penem, cephem, carbapenem, or monobactam. These molecules have a low toxicity, which explain their popularity in the infectious therapy . It is considered below, each β-lactam subclasses in terms of structure and spectrum of activity.

- **Penams**

Most penams are semisynthetic derivatives of the penicillin nucleus, 6-aminopenicillanic acid (6-APA) which consists of a thiazolidine ring bound to a β-lactam ring (Ball et al., 1978), prepared synthetically by the addition of acyl side chains at the 6-amino group (Finch et al., 2010). The semisynthetesis of penicillin from 6-APA aimed to improve the spectrum, activity and stability of the parent compound (Maddison et al., 2008). Penicillins include natural penicillins, penicillinase-resistant agents, aminopenicillins, carboxypenicillins, and ureidopenicillins.

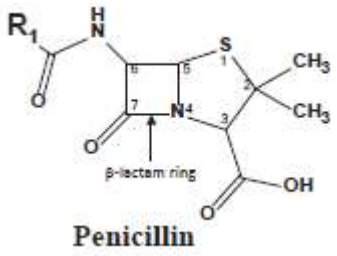
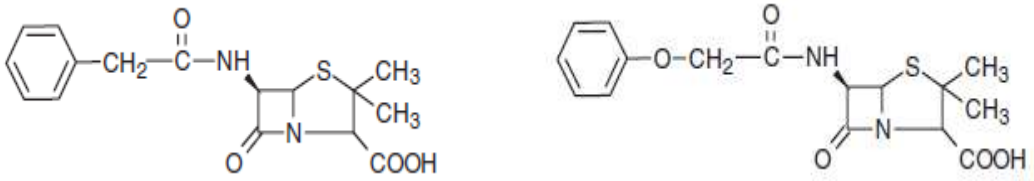


Figure 2: The penam molecule figure (Zagursky *et al.*, 2018)

- **Natural penicillins**

Penicillins belonging to this group are essentially two, benzylpenicillin (Penicillin G), Phenoxymethylpenicillin (penicillin V) or amoxicillin. They are both natural biosynthetic products from *Penicillium chrysogenum* by fermentation (Ball et al., 1978). There are actives against Gram-positive cocci such *streptococcus pneumonia*, *streptococcus agalactiae*, *streptococcus pyogenes*, non β-lactamase *staphylococci*, Gram-negative bacteria such as *Neisseria gonorrheae, Neisseria meningitidis*. Gram-Positive bacilli such as *Bacillus anthracis*

or *Corynebacterium diphtheria,* Anaerobic species such as *Peptostreptococcus spp*., *Clostridium spp.* are also among bacteria targeted by natural penicillins (Doi and Chambers, 2015).



Benzylpenicillin (Penicillin G) Phenoxymethylpenicillin (Penicillin v)

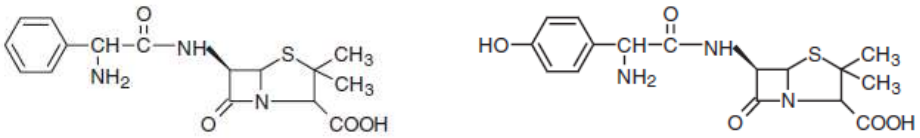
Figure 3: Benzylpenicillin (Penicillin G) and Phenoxymethylpenicillin (penicillin V) molecules, figures from (Vardanyan and Hruby, 2006).

- **Penicillinase-resistant penicillins**

All penicillinase-resistant penicillins display the same antibacterial activity. They are active against methicillin-susceptible strains of staphylococci, non-β-lactamase resistant streptococci. None is active against aerobic gram-negative cocci or bacilli. Among Penicillinase-resistant penicillins are: Methicillin (2,6-dimethoxyphenylpenicillin) the first to be developed, Nafcillin (2-ethoxy-1-naphthylpenicillin). Others are classified under the terminology of isoxazolyl penicillins. There are oxacillin, cloxacillin, dicloxacillin, and flucloxacillin.

- **Aminopenicillins**

Aminopenicillins have a free amino group at the alpha position on β-lactam ring and consequently increasing their ability to enter the Gram-negative membrane (Finch et al., 2010). They all have similar antimicrobial spectra similar to penicillin G, though aminopenicillin are slightly more active against *Enterococci* (Doi and Chambers, 2015). They are inactive against β-lactamase producers either Gram-positive or Gram-negative (Doi and Chambers, 2015). Ampicillin, amoxicillin and hetacillin are examples of aminopenicillins. Aminopenicillins are commonly used together with β-lactamase inhibitors.



Ampicillin Amoxicillin

Figure 4: The ampicillin and amoxicillin structure figure from (Vardanyan and Hruby, 2006)

- **Carboxypenicillins and ureidopenicillins**

Some derivate of penicillin G and ampicillin exhibit good activity against *Pseudomonas aeruginosa* (Finch et al., 2010). They are usually termed as antipseudomonal penicillin (Bassetti et al., 2018). They include carboxypenicillins and ureidopenicillin. Among carboxypenicillins are Carbenicillin and ticarcillin (Doi and Chambers, 2015). The last antibiotic are no longer used in clinic because of their great toxic potential due to the high doses required for treatment (Doi and Chambers, 2015). Ureidopenicillins are other antipseudomonal group of antibiotics. They include piperacillin, azlocillin, and mezlocillin. The two last or later have been withdraw from the market (Doi and Chambers, 2015). It is argued that the great activity of the ureidopenicillins against *Pseudomonas aeruginosa* may be due to a combination of better penetration characteristics and their greater affinity for PBPs (Finch et al., 2010). Carboxypenicillins and ureidopenicillins commercialised nowadays are usually combined with β-lactamase inhibitors, thus instead of ticarcillin alone it is now used the combined ticarcillin with clavulanic acid and piperacillin combined with tazobactam instead of piperacillin alone.

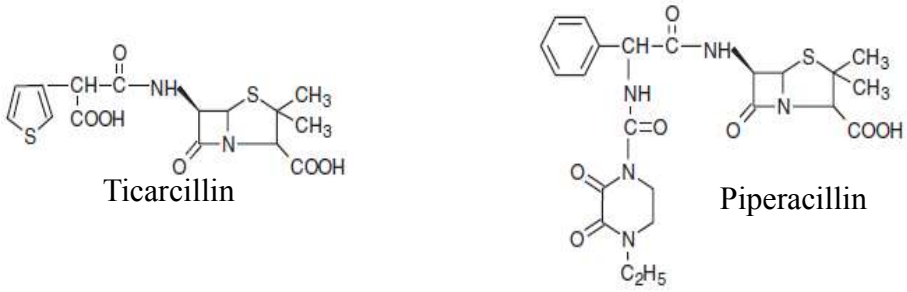


Figure 5: The ticarcillin and Piperacillin molecule figure from (Vardanyan and Hruby, 2006)

- **Cephems**

Cephem can be classified either chemically or according to microbial spectrum. Chemically, the cephems may be divided into three or five groups depending on authors according to the atom in position 1 of the hexa-atomic ring it is found: cephalosporins (sulfur), carbacephems (methylene), and oxa-1-cephems (oxygen), cephamycins and miscellaneous (Fernandes et al., 2013).

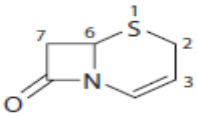
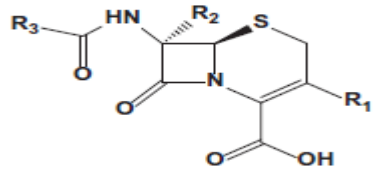


Figure 6: The cephalosporin skeleton from Finch et al 2010 (Finch et al., 2010)

- **Cephalosporins**

During the 1950s, the discovery of the naturally occurring penicillinase-stable cephalosporin C opened a new era onto the development of hundreds of new cephalosporins (Bush and Bradford, 2016). All cephalosporins are based on cephalosporin C. In all cephalosporins the β-lactam ring is binded to a six-membered dihydrothiazine ring replacing the five-membered thiazolidine ring of penicillins (Finch et al., 2010).



Cephalosporin

Figure 7: The cephalosporin skeleton from Bush and Bradford 2016 (Bush and Bradford, 2016)

- **First generation cephalosporins**

First-generation cephalosporins are very active against Gram-positive cocci, except enterococci and methicillin-resistant *staphylococci*, *listeria* and moderately active against some Gram-negative rods primarily *Escherichia coli*, *Proteus*, and *Klebsiella*. None of the first-generation drugs penetrate the central nervous system, and they are not drugs of first choice for any infection (Fernandes et al., 2013).

- **Second-generation cephalosporins**

The second-generation cephalosporins are generally active against organisms covered by first-generation drugs with an extended activity against Gram-negative bacilli, including Klebsiella but not against *Enterococci*, *Listeria, Pseudomonas aeruginosa*. Often, cephamycins are considered as second-generation cephalosporins for its clinical use (Fernandes et al., 2013). They got greater stability against β-lactamases producers of Gram-negative bacteria.

- **Third generation cephalosporins**

Third generation cephalosporins have an enhanced activity against Gram-negative. Ceftazidime and cefoperazone have good activity against *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Latter specie's cell wall is also well damaged by ceftriaxone and cefotaxime. This class of antimicrobials is very useful in the treatment of nosocomial Gram-negative bacteremia (Fernandes et al., 2013).

- **Fourth generation cephalosporins**

Cefepime and cefpirome are the only fourth generation cephalosporins in the market (Fernandes et al., 2013). Fourth generation cephalosporins in vitro activity is enhanced against *Enterobacter* and *Citrobacter* species that are resistant to third-generation cephalosporins.

Cefepime has a similar activity as ceftazidime against *Pseudomonas aeruginosa* (Fernandes et al., 2013).

- **Fifth generation cephalosporins**

Fifth generation cephalosporins were developed specifically target against resistant strains of bacteria such as *Haemophilus influenzae, Moraxella catarrhalis Enterococcus faecalis* or methicillin-resistant *Staphylococcus aureus* (Fernandes et al., 2013). Ceftobiprole displays potent activity against *Pseudomonas aeruginosa*. Ceftaroline in the other hand exhibits limited activity against most non-fermentative Gram-negative bacilli such as *Pseudomonas aeruginosa* or *Acinetobacter spp.* Cephalosporins are very useful antibiotics family with increasing Gram-negative activity in each higher generation.

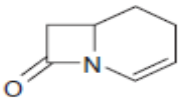
Table III: group of cephalosporins according to their antimicrobial activity (Fernandes et al., 2013).

First-generation	Second-generation	Third-generation	Fourth-generation	Fifth-generation
Cephalothin	Cefamandole	Cefotaxime	Cefepime	Ceftobiprole
Cephapirin	Cefuroxime	Ceftizoxime	Cefpirome	ceftaroline
Cefazolin	Cefonicid	Ceftriaxone		
Cephalexin[a]	Ceforanid	Ceftazidime		
Cephradine[a]	Cefoxitin[b]	Cefoperazone		
Cefadroxil[a]	Cefmetazole[b]	Cefixime[a]		
	Cefminox[b]	Ceftibuten[a]		
	Cefotetan[b]	Cefdinir[a]		

[a]Oral cephalosporins; all the others are parental cephalosporins. [b]Besides being cephamycins (chemical classification), they are usually included in the microbiological classification as second-generation cephems.

▪ Carbacephems

The Carbacephems are a class of synthetic antibiotics, based on the structure of cephalosporin with a carbon replacing sulfur on the β-lactam ring (Finch et al., 2010). Loracarbef the only carbacephems ave for the clinical used and is often classified within second generation of cephalosporin (Fernandes et al., 2013).

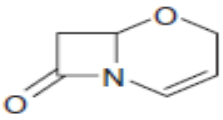


Carbacephem

Figure 8: Picture of carbacephem from Finch et al 2010 (Finch et al., 2010)

- **Oxa-1-cephems**

The oxacephems is caractrised by oxygene molecule in position 1 . Moxalactam and latamoxef are include among oxacephem class. They have similar antimicrobial activity to the cephalosporin of third and fourth generations (Bush and Bradford, 2016). Oxacephems have a good stability to hydrolysis by β-lactamases. They are more active against *Pseudomonas aeruginosa* than cephotaxime.

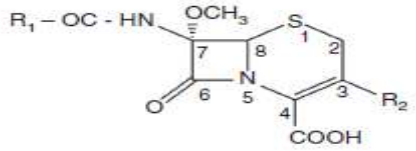


Oxacephem

Figure 9: Oxacephem skeleton from Finch et al 2010 (Finch et al., 2010)

- **Cephamycins**

Cephamycins are cephem frequently classified among second-generation because of their close related spectrum of activity (Fernandes et al., 2013). They are characterized at 7 α position by a methoxy group (Bryskier, 2005). Cefoxitin and cefotetan are part of cephamycin. They are active against *Bacteroides fragilis* but present lesser activity against *Pseudomonas aeruginosa* and *Acinetobacter species* (Ward and Richards, 1985). Cephamycins spray stability to the TEM-type β-lactamases, including ESBLs (Bush and Bradford, 2016).



Cephamycine

Figure 10: The cephamycin from (Finch et al., 2010)

- **Carbapenem**

Carbapenems is similar to penicillin with a carbon replacing sulfur at 1 position and a sulfur atom is linked to C2 (Bush and Bradford, 2016). More than ten of carbapenems have been isolated from fermentation products of various streptomycetes (Finch et al., 2010). Because of its chemical instability thienamycin the first potent carbapenem identified had never

entered the market of therapeutic agent but instead, the stabilized variant made by adding the N-formimidoyl group to the 2-position got a better success (Bush and Bradford, 2016). Later modification on thienamycine led to the synthesis of imipenem, which displays good activity against Gram-positive, Gram-negative either aerobic or anaerobic. Carbapenems show a notable stability to most β-lactamases, exception on the emerging carbapenemases found primarily in Gram-negative bacteria (Bush and Bradford, 2016). Doripenem, ertapenem, imipenem and meropenem are countable amount carbapenem groups of antibiotics. Each carbapenem is different to other in one or two aspects. That is the case of doripenem which displays an enhance stability compare to imipenem and meropenem (Bush and Bradford, 2016). Imipenem in contrary to others carbapenem requires dehydropeptidase inhibitor in the dosing regimen (Bush and Bradford, 2016, Finch et al., 2010). Ertapenem differs to doripenem, imipenem and meropenem on its less efficacity against *Pseudommonas aeruginosa* (Finch et al., 2010). Carbapenems are considered as last resort antibotics.

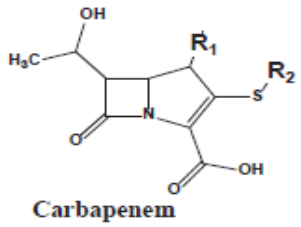


Figure 11: The carbapenem skeleton from (Zagursky et al., 2018)

- **Penems**

Penems are penicillins differing from penams by the presence of a double bond between C-2 and C-3 (Finch et al., 2010). Only one penem, faropenem, has entered limited clinical use (Finch et al., 2010). Faropenem demonstrates broad spectrum in vitro antimicrobial activity against many Gram-positive and negative aerobes and anaerobes, and is resistant to hydrolysis by nearly all β-lactamases, including extended-spectrum beta lactamases and AmpC beta-lactamases (Schurek et al., 2007).

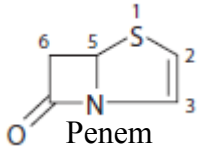


Figure 12: The penem skeleton from Finch et al 2010 (Finch et al., 2010)

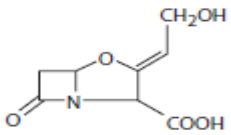
▪ β-Lactamase inhibitors

- **β-lactams inhibitors structurally made with β-lactam ring**

Therapeutic failure following the β-lactams treatment incriminated to β-lactamase production led in the mid-1970s (Bush and Bradford, 2016) to development of β-Lactamase inhibitors which are combined to available β-lactams to act synergistically with them (Finch et al., 2010). Most of the β-Lactamase inhibitors are derivatives of β-lactams molecules. That is the case of clavulanic acid (natural product) or the synthetic sulfones, sulbactam and tazobactam.

- **Clavulanic acid**

Clavulanic acid is an oxapenam (clavam) produced by *Streptomyces clavuligerus* (Finch et al., 2010). It is usually combined with amoxicillin and ticarcillin (Finch et al., 2010). The only notable member of the clavam family at present is clavulanic acid, a compound that possesses its therapeutic rolbility to inhibit many class A (functional group 2) bacterial β-lactamases, extended spectrum β-lactamases (ESBLs) and to a lesser extend serine carbapenemase (Finch et al., 2010, Bush and Bradford, 2016).

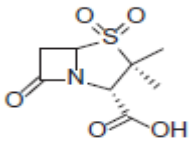


Clavulanic acid

Figure 13: The clavulanic acid structure from (Finch et al., 2010)

- **Sulbactam**

Sulbactam as tazobactam is a sulfone β-lactamase inhibitor with a broader spectrum of activity than clavulanic acid (Doi and Chambers, 2015). Sulbactam display notable activity against *N. gonorrhoeae*, *N. meningitidis* and *Acinetobacter baumannii* (Finch et al., 2010). It is a moderate inhibitor of the TEM enzymes of groups 2b and 2be but show little effect against group 1, group 2br or group 3 β-lactamases . Sulbactam is commonly combined with ampicillin (Bush and Bradford, 2016). Some report presented ampicillin-sulbactam to be more efficient than polymixins for treatment of infections caused by carbapenem-resistant *Acinetobacter baumannii* strains (Doi and Chambers, 2015).

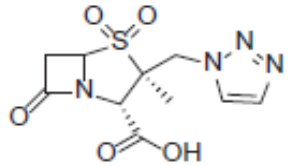


Sulbactam

Figure 14: The sulbactam structure from (Bush and Bradford, 2016)

- **Tazobactam**

Tazobactam is a sulfone β-lactamase inhibitor as sulbactam. Its spectrum is similar to clavulanic acid. It is generally combined with piperacillin and, more recently, with cefoperazone and ceftolozane for nosocomial-infections, including those caused by *Pseudomonas aeruginosa* (Bush and Bradford, 2016).



Tazobactam

Figure 15: The tazobactam structure from (Bush and Bradford, 2016)

- **Non-β-lactam ring β-lactamase inhibitors**
- **Diazabicyclooctane (DBO) type**

A novel β-lactamase inhibitor has emerged from diazabicyclooctane (DBO) derivate, avibactam with a broader spectrum of activity than clavulanic acid. In addition to clavulanic acid activity spectrum, avibactam is able to inhibit bacterial class C cephalosporinases and some class D oxacillinases (Bush and Bradford, 2016). Avibactam has been approved for therapeutic use in combination with ceftazidime, and is under development for ceftaroline-avibactam or aztreonam-avibactam (Bush and Bradford, 2016). Relebactam is also a derivate of DBO, structurally related to avibactam; it is another β-lactamase inhibitor. Relebactam inhibits the activity of class A, and C beta-lactamase, but does not have activity against metallo-β-lactamases (MBL) and class D carbapenemases (Bassetti et al., 2018).

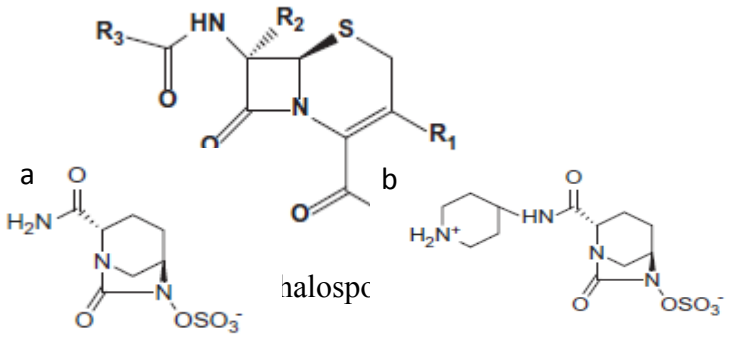


Figure 16: The avibactam (a) and relebactam (b) structures from (Bush and Bradford, 2016)

- **The boronic acid inhibitor**

The increasing dissemination of carbapenemases in Gram-negative led searchers to launch the development of new serine β-lactamase inhibitors containing a boronic acid. Hecker and his team developed, RPX7009 a promising novel class of synthetic none-β-lactam β-lactamase inhibitors , with no mammalian serine proteases inhibition potential (Hecker et al., 2015). RPX7009 is particularly active against *Klebsiella pneumoniae* carbapenemase (KPC). RPX7009 it is being developed in combination with meropenem to target pathogens producing serine carbapenemases.

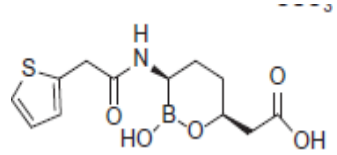


Figure 17: The RPX7009 a boronic acid base β-lactamase inhibitor structure from (Bush and Bradford, 2016)

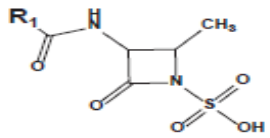
- **New combinations of β-lactams and β-lactamases inhibitor**

With the increasing rate of multidrug resistant strains, many others combination have been proposed. In case *Pseudomonas aeruginosa* infection is of suspected, Bassetti and collaborators suggested an early regimen of ceftolozane-tazobactam administrate with aminoglycosides or fluoroquinolone (Bassetti et al., 2018). Ceftolozane-tazobactam is being developed to overcome *Pseudomonas aeruginosa* antimicrobial mechanisms of resistance, ceftazidime-avibactam and meropenem-vaborbactam have also been developed for complicated *Pseudomonas aeruginosa* urinary tract infections (Bassetti et al., 2018).

Combination of aztreonam and avibactam showed a potent in-vitro activity against extended spectrum β-lactamase (ESBL), class C β-lactamase, metallo-β-lactamase (MBL), and KPC-producing strains with an activity 10 times that of aztreonam alone (Bassetti et al., 2019). Imipenem (imipenem-cilastatin) combined with relebactam a diazabicyclooctan (DBO) has shown a significant activity against a wide spectrum of MDR Gram-negative pathogens including *Pseudomonas aeruginosa*, *KPC-producing Klebsiella pneumoniae* and *Enterobacter spp.* (Bassetti et al., 2018).

- **Monobactam**

In contrast to penicillins and cephalosporins commonly produced by fungi and actinomycetes, monobactams are produced by bacteria *Chromobacterium violaceum* (Finch et al., 2010). Monobactams are structurally particular in a way that they only bear β-lactam ring not fused to any other ring. Monocyclic β-lactam antibiotics, such as monobactams, are active in vitro against Gram-negative bacteria, including *Pseudomonas aeruginosa* (Finch et al., 2010). The simplicity of their structure allows total synthesis of monobactam. Monobactams are hydrolyzed poorly by many serine β-lactamases and all metallo-β-lactamases, but can be hydrolysed by ESBLs and serine carbapenemases (Finch et al., 2010).

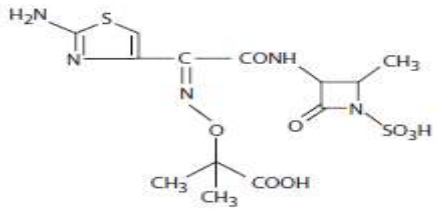


Monobactams

Figure 18: The monobactam skeleton from (Zagursky et al., 2018).

- **Aztreonam**

Aztreonam has been approved in 1986 for treatment of Gram-negative infection (Macor, 2011). It is the only available monobactam antibiotic (Finch et al., 2010). It is not hydrolysed by metallo β-lactamase (MBLs), but instead by Ambler class A β-lactamases including ESBLs (Bassetti et al., 2019). Aztreonam showed activity against common Gram-negative organisms such as *Escherichia coli*, *Pseudomonas aeruginosa* and even *Acinetobacter baumannii* (Finch et al., 2010).



Aztreonam

Figure 19: The aztreonam skeleton from (Finch et al., 2010)

1.3.2. Quinolones

The quinolone antibiotics arose in the early 1960s, with the isolation of 7 -chloro-l-ethyl-l, 4-dihydro-4-oxoquinoline-3-carboxylic acid, a chloroquine preparation by-product (Sheehan and Chew, 2003). This compound was found to have anti-bacterial activity and was subsequently modified to produce nalidixic acid, a 1, 8-naphthyridine the first quinolone synthetized (Finch et al., 2010). Unsuitable for systemic treatment, nalidixic acid (figure 1.21. a) was mostly used for urinary tract infection (Sheehan and Chew, 2003). By the early 1980's, advancements in quinolone chemistry resulted in the development of second generation compounds (figure 21) with considerably improved activity and pharmacokinetics. The most critical change to the quinolone nucleus was the introduction of a fluorine atom at the C-6 position to create fluoroquinolone. This substitution dramatically enhanced potency against DNA gyrase and promoted drug uptake by bacterial cells and giving them more potent antibiotic action and a broader spectrum of activity. In contrast to most other anti-infective drugs, quinolones do not kill bacteria by inhibiting a critical cellular process. Rather, they exert their actions by inhibiting bacterial nucleic acid synthesis through disruption of the enzymes topoisomerase IV and DNA gyrase, inducing the cells kill by generating high levels of double-stranded DNA breakage (Pham et al., 2019). Since their discovery quinolones and fluroquinolones have gained increasing importance as key therapies to treat both community-acquired and severe hospital-acquired infections (Pham et al., 2019). Fluoroquinolones remain the backbone of therapy for *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections, although reports on resistance have emerged (Bassetti et al., 2019). Norfloxacin, a second-generation quinolone was the first clinical relevant fluoroquinolone. It displayed a much important activity against Gram-negative bacteria than nalidixic acid with a modest activity against some Gram-positive species. However, due to poor tissue distribution and low serum levels, norfloxacin was limited to the treatment of urinary tract infections and several sexually transmitted diseases (Anderson and Osheroff, 2001). Ciprofloxacin and ofloxacin, others second generation quinolone were the first family members to display significant activity on

infections outside the urinary tract. Thereafter, a third generation of quinolone antibacterial (figure 22) was then produced with almost the same structure as the second generation, with an advantage of increased activity against Gram-positive bacteria (Anderson and Osheroff, 2001).

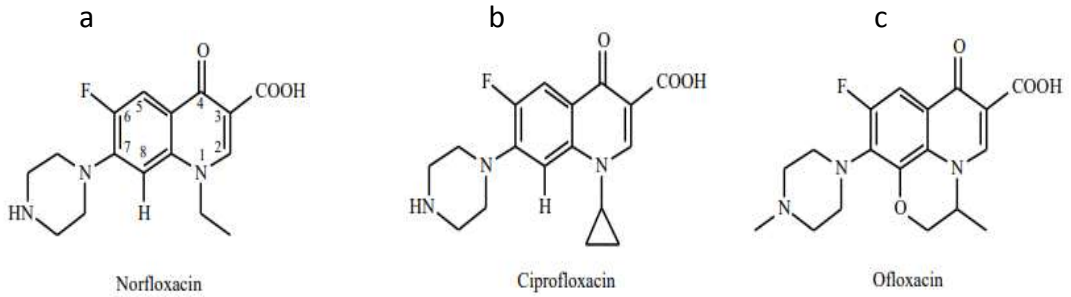


Figure 20: first generation quinolone from Anderson and Osherof 2001(Anderson and Osheroff, 2001)

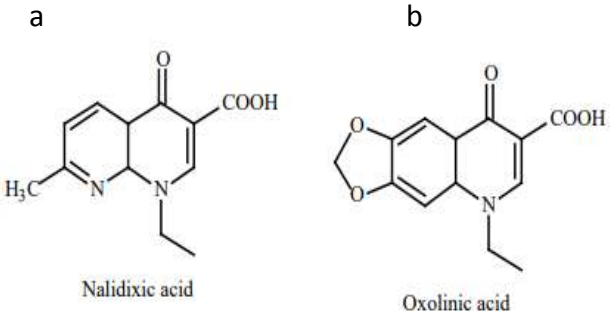


Figure 21: Second-generation quinolone (Fluoroquinolone), from (Anderson and Osheroff, 2001)

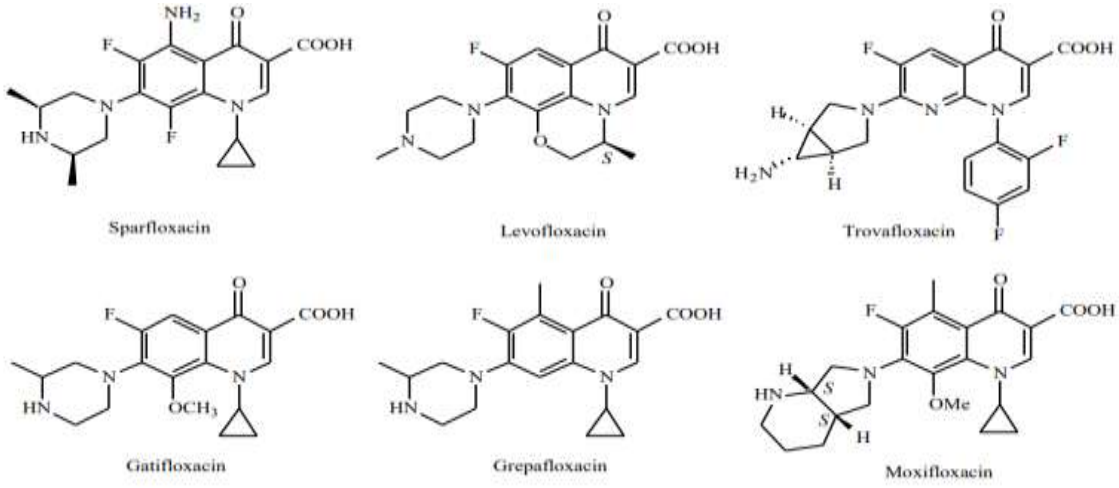


Figure 22: Third generation quinolone (Fluoroquinolone), fromAnderson and Osheroff, 2001)

1.3.3. Aminoglycosides

Selman Waksman's team discovered streptomycin, the first aminoglycoside antibiotic in 1944 from a natural product of a soil bacterium, *Streptomyces griseus* displaying good activity against *Mychobacterium tuberculosis* (Finch et al., 2010). Structurally, they consist of a core aminocyclitol that can be streptamine, streptidine, and 2-deoxystreptamine (2-DOS) connected to at least one amino-sugar via a glycosidic linkage. Aminoglycosides are antimicrobial agents that inhibit bacterial protein synthesis through binding to specific sites on the 30S ribosomal subunit with consequently disrupt the protein biosynthesis through of interruption of translocation event (Finch et al., 2010). The discovery of streptomycin was followed by the discovery in 1949 of neomycin isolated from *Streptomycete fradiae* by the same research group (Krause et al., 2016). Thereafter, kanamycin was isolated from a new strain of actinomycete named *Streptomyces kanamyceticus* by Umezawa and colleagues in 1957 (Umezawa, 1958). Gentamicin, other natural aminoglycosides was reported for the first time in 1963 isolated from *Micromonospora purpurea*, tobramycin was the isolated in 1967 from *Streptomyces tenebrarius* (Krause et al., 2016). Sisomicin was discovered a decade after in 1976 by Weinstein and colleague from *Micromonospora inyoensis* (Weinstein et al., 1976). Beside the natural aminoglycosides, researches on new aminoglycosides focused on the chemical modification of existing molecules (Finch et al., 2010). Aminoglycosides such as netilmicin in 1967 was derived from sisomicin and amikacin in 1972 was synthetized from kanamycin and later one, were developed arbekacin and plazomicin aminoglycosides with a potential to overcome common aminoglycoside resistance mechanisms (Ban et al., 2020). Aminoglycosides have a broad spectrum of antibacterial activity. They are used primarily to treat aerobic, some gram-negative bacilli infections or in synergistic combinations with cell wall-active, antimicrobial agents like penicillin, ampicillin, vancomycin; against some resistant, gram-positive bacteria, such as enterococci (Aminov, 2017). Aminoglycoside uptake required active electron transport, therefore, the class inherently lacks activity against anaerobic bacteria (Krause et al., 2016).This class also has good activity against *Staphylococcus aureus* methicillin-resistant and vancomycin-intermediate and -resistant isolates, *Pseudomonas aeruginosa* and to a lesser extent *Acinetobacter baumannii* (Krause et al., 2016).

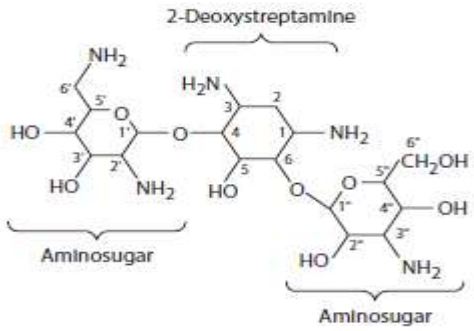


Figure 23: Aminoglycoside structure from kanamycin and gentamicin groups, in which the aminocyclitol here the 2-deoxystreptamine is 4,6-disubstituted figure from (Finch et al., 2010)

1.3.4. Tetracyclines

Tetracycline is a family of antibiotics naturally produced from *Streptomyces spp*. and including semisynthetic derivatives. Tetracycline structure is made with linear fused tetracyclic molecule, 6-deoxy-6-demethyltetracycle. (Finch et al., 2010).

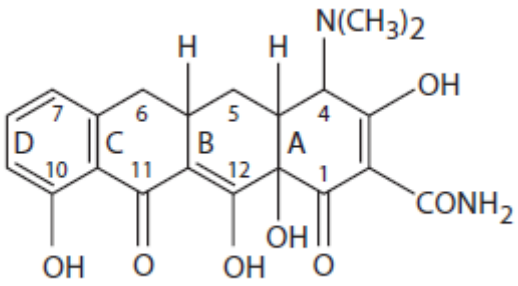


Figure 24: Tetracycline structure from (Finch et al., 2010)

Tetracyclines preferentially bind to bacterial ribosomes and interact with a highly conserved 16S ribosomal RNA (rRNA) target in the 30S ribosomal sub-unit (Grossman, 2016). The first compound discovered was chlortetracycline, in 1948 by Duggar and his colleague from *Streptomyces aureofaciens* (Grossman, 2016). Tetracyclines rich the cell in form of calcium-magnesium complex which bind to the ribosome and prevent the protein biosynthesis by triggering the bacteriostatic effect (Borghi and Palma, 2014). However the bactericidal activity of tigecycline has been demonstrated (Grossman, 2016). After penicillin, tetracyclines are the second family of antibiotics produced and the more consumed worldwide especially because of their growth promoting ability used in food-producing animals (Borghi and Palma, 2014). Natural tetracyclines include chlortetracycline, oxytetracycline, tetracycline and demeclocycline (demethychlortetracycline) (Finch et al., 2010). Semisynthetic derivatives include doxycycline, minocycline, methacycline, lymecycline, rolitetracycline and tigecycline,

a glycylcycline that has been specifically developed to overcome problems of bacterial resistance to earlier tetracyclines (Finch et al., 2010). In general, tetracyclines are active against many Gram-positive and Gram-negative bacteria, *Chlamydiae, Mycoplasma, Rickettsiae, Coxiellae, Spirochete* and some *Mycobacteria*. None is active against *Pseudomonas aeruginosa, Proteus spp.* or *Providencia spp.*, but *Burkholderia pseudomallei* and *Stenotrophomonas maltophilia* are usually susceptible (Finch et al., 2010). *Acinetobacter baumannii* retains a good susceptibility to tigecycline where other tetracyclines present very weak activity (Bassetti et al., 2019, Meshkat et al., 2017b).

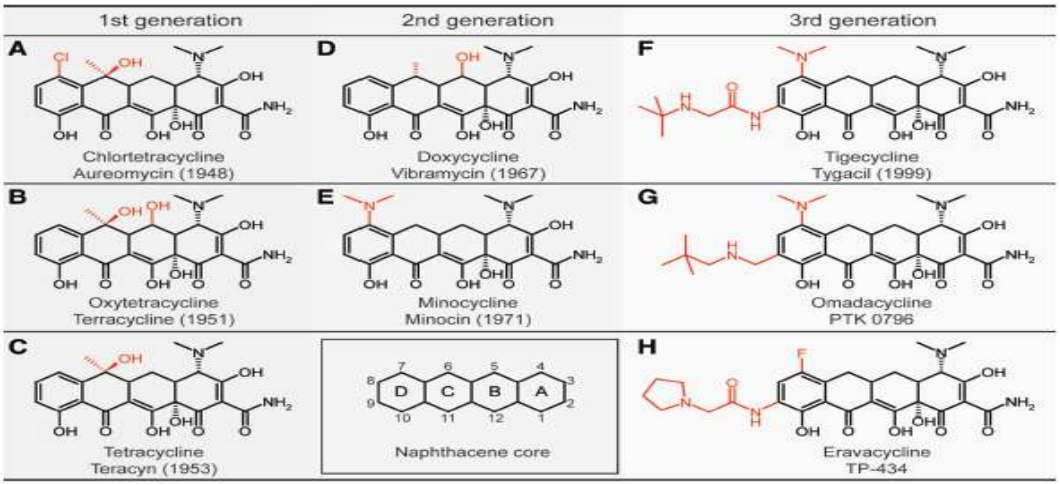


Figure 25: Biochemical structure of few tetracycline from (Nguyen et al., 2014).

1.3.5. Trimethoprime-sulfamethosazole

Trimethoprim was first used for the treatment of infections in humans in 1962, and it was registered for clinical use, in combination with sulfonamides, in 1968 (Eliopoulos and Huovinen, 2001). In 1935, they were the first class of true antimicrobial agents with life-saving potency (Eliopoulos and Huovinen, 2001). In contrary to human being, bacteria are folate synthesizer. Combination of trimethoprim-sulfamethoxazole inhibit the synthesis of tetrahydrofolate. Tetrahydrofolate is a necessary component for the synthesis of purines required for DNA and protein production. Each of the drug molecules blocks a step in folic acid metabolism. Sulfamethoxazole is an analogue of para-aminobenzoic acid (PABA), thus acts in competition through blockage of the enzyme dihydrofolate synthase in folate biosynthetic pathway. While, trimethoprim inhibits dihydrofolate reductase (DHFR), and consequently the production of tetrahydrofolate acid (Maddison et al., 2008). They are bacteriostatic when used separately, however, in combination they work synergistically in bactericidal manner (Maddison et al., 2008). Trimethoprim-sulfamethoxazole has been used for the treatment of

patients with urinary tract infections and for AIDS patients with *Pneumocystis carinii* infections (Walsh, 2003). This drug combination is effective strategy in curing bacterial infections, Gram-negative and Gram-positive aerobic bacteria, some protozoa infections, Toxoplasma and Neospora, for example (Maddison et al., 2008). Sulfonamide-trimethoprim is not effective against *Pseudomonas aeruginosa* , however some *Acinetobacter* strains are sensitve (Maddison et al., 2008).

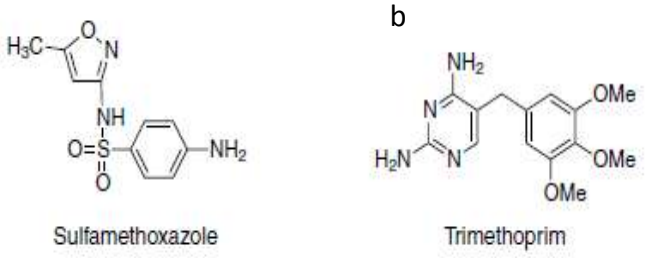


Figure 26: Trimethoprim (a)-sulfamethoxazole (b) combination structure

1.4. Antimicrobial resistance mechanisms

Bacteria resistance refers to the ability of some bacteria to survive to antibacterial agent or to the natural host response. Some bacteria are naturally resistance to some antimicrobial agent such as almost all non-fermentative bacteria like *P. aeruginosa* and *Acinetobacter baumannii* to first and second generation cephalosporin and ertapenem (CASFM / EUCAST, Société Française de Microbiologie: recommandations 2016). Others thanks to the genome plasticity acquired new genome material that carry antimicrobial resistance genes. Resistance mechanisms to antibiotics include decreased uptake, expression of efflux pumps, enzymatic or non- enzymatic modification or drug modifying enzymes, acquisition of alternative metabolic pathways to those inhibited by the drug and structurally target alteration. *P. aeruginosa* and *Acinetobacter baumannii* exhibit most of these resistance mechanisms through both intrinsic chromosomally encoded or genetically imported resistance determinants affecting the major classes of antibiotics including β-lactams, aminoglycosides, quinolones, tetracyclins and trimethoprim-sulfamethoxazole. Bacteria can combine the above-mentioned mechanisms to exhibit resistance towards several antibiotics.

1.4.2.Antimicrobial resistance mechanisms against β-lactams

Enzymes inactivation is one of the most common mechanisms incriminated in the drug resistance. There are hundreds of enzymes or others proteins, which are chromosomal or plasmid-encoded, which can confer resistance to a wide range of antibiotics. Hundreds of β-lactamases, enzymes hydrolyzing β-lactams have been characterized till date based on their structure, classified as molecular Ambler's classification (Ambler, 1980). Few decades later,

other authors classified β-lactamases according to their functional activities and that classification is referred as updated Bush Jacoby and Meideros classification (Bush et al., 1995b). Ambler classification divided β-lactamases into four structural groups. Class A, C, D, which are characterized by presence of serine residue within their active site, and Class B which contains zinc within the active site, a bivalent metal, which confers the name metallo β-lactamases to enzymes belonging to this class (Ambler, 1980). Major groupings generally correlate the functional classification with Ambler's classification. Functional classification includes group 1 (Ambler class C) gathering enzymes hydrolysing cephalosporins (cephalosporinases); group 2 (Ambler classes A and D) represent the largest group of β-lactamases. It includes penicilinase, extended-spectrum β-lactamases (ESBL) and serine carbapenemases. Finally, group 3 (Ambler class B), grouping the metallo-β-lactamases enzymes characterized by their ability to hydrolyze carbapenems.

P. aeruginosa wild-type strain encodes an inducible group 1 (molecular class C) AmpC cephalosporinase usually not inhibited by β-lactam inhibitors such as clavulanic acid, tazobactam and sulbactam (Bush and Jacoby, 2010, Bassetti et al., 2018). The AmpC cephalosporinase usually exhibits a low level expression which, together with low membrane permeability and multiple efflux systems, confers resistance to aminopenicillins alone or in combination with β-lactam inhibitors, first and second generation cephalosporins (C1G, C2G), cephamycins, the two third generation cephalosporins (C3G), cefotaxime and ceftriaxone, as well as the carbapenem, ertapenem (Bassetti et al., 2018). *A. baumannii* characteristically produces an AmpC-type cephalosporinase recognized as *Acinetobacter*-derived cephalosporinases (ADCs).

Group 2 of functional classification gathers penicillinases (Ambler's class A), oxacillinases (Ambler's class D) and extended spectrum β-lactamases (ESBLs) (Bush and Jacoby, 2010). ESBLs are part of two subgroups of group 2, the subgroups 2be (Ambler's class A enzymes) and 2de (Ambler's class D ESBLs). ESBLs retain the activity against penicillins and cephalosporins and in addition, they hydrolyze cefotaxime, ceftazidime, and aztreonam and are inhibited by clavulanic acid or tazobactam; whereas carbapenems remain always active towards strains carrying these β-lactamases types (Bush and Jacoby, 2010). TEM family have been identified within various bacteria species including *P. aeruginosa* and *A. baumannii* (Alyamani et al., 2015b); they can either be part of penicilinase or ESBLs. CTX-M enzymes types are essentially ESBLs, and are among the most frequent ESBLs found in *P. aeruginosa* and *A. baumannii* (Celenza et al., 2006b). Class D enzymes (oxacillinases) or OXA-type

enzymes are distinguished by their ability to hydrolyze cloxacillin or oxacillin (Bush and Jacoby, 2010). OXA-type enzymes are imported in bacteria following horizontal transfer of mobile genetic elements exception with OXA-50, which is naturally occurring oxacillinase of *P. aeruginosa* (Bassetti et al., 2018) and OXA-51 intrinsic to *A. baumannii* strains (Feizabadi et al., 2008b).

Group 3 (Ambler's class B) contains essentialy metallo-β-lactamases (MBLs); they differ structurally from the other β-lactamases by their requirement for a zinc ion at the active site. In contrast to the serine β -lactamases, the MBLs have poor affinity or hydrolytic capability for monobactams and are not inhibited by clavulanic acid or tazobactam (Bush and Jacoby, 2010). Instead, they are inhibited by metal ion chelators such as EDTA and this characteristic is used in the phenotypical identication protocol.

Other factors besides enzymes production contribute to bacteria resistance to β-lactams among them are low membrane permeability and active efflux pumps. Membrane impermeability or reduced permeability is a mechanism known to lead to resistance towards several antibiotic classes such as aminoglycosides, β-lactams and quinolones. Concerning β-lactams, the porine OprD is known to promote the uptake of imipenem and, to some extent, meropenem but not of other β-lactams (Bassetti et al., 2018). Thus, the modification of OprD structure and/or the reduction of its expression confer a reduced susceptibility to imipenem (Bassetti et al., 2018).The natural resistance of *P. aeruginosa* to several antibiotics classes is partly due to the combination of low membrane permeability and active efflux pumps (Bassetti et al., 2018). Because of their broad substrate specificities, active efflux pumps display resistance against different classes of antibiotics, which are chemically unrelated.

1.4.2. Antimicrobial resistance mechanisms against quinolone

Bacteria can intrinsecly express resitance to quinolone through various effux pump mechanisms. The well-studied representatives of multidrug efflux pumps responsible for intrinsic resistance to quinolones include MexAB-OprM of *P. aeruginosa* (Li, 2005). For decades, acquired resistance to quinolones were believed to be only mediated by chromosomal mutations (Van Hoek et al., 2011). These mutations either decreased outer-membrane permeability related to porin loss or over expression of the multidrug efflux pumps for which quinolones are substrates or alter the targets (30S subunit of bacteria ribosomes) of the quinolone drugs. The ribosome alteration occurs through mutations at specific "quinolone resistance determining regions" (QRDR) in the genes subunits *gyrA, gyrB* of DNA gyrase, and

parC, *parE* gene subunits encoding topoisomerase IV. (Li, 2005). However, later on, three plasmid-mediated quinolone resistance (PMQR), mechanisms were reported (Van Hoek et al., 2011). PMQR have been conspicuously rare in non-fermenting bacteria but have occasionally been reported in *P. aeruginosa*, other *Pseudomonas spp.*, *A. baumannii* (Jacoby et al., 2015) . The first identified PMQR, a *qnr* determinant,which encodes for a qnr protein that protects DNA gyrase and type IV topoisomerase from quinolone inhibition, was revealed in 1998 after a conjugason experiement of a broad host range plasmid pMG252 into *K pneumoniae* (Martínez-Martínez et al., 1998). Up to the date seven families of qnr genes have been reported; qnrA, qnrB, qnrC, qnrD, qnrS, qnrVC and qnrE1 (Albornoz et al., 2017). qnrA1 variant has been reported to be borne by *P. aeruginosa* and *A. baumannii* (Wang et al., 2012); qnrD variant was identified in Nigeria within one *Pseudomonas* isolate (Ogbolu et al., 2011).

The second type of plasmid located quinolone resistant gene is a cr variant of aac(6')-Ib, aac(6')-Ib-cr, responsible for low-level ciprofloxacin resistance (Van Hoek et al., 2011). It encodes anaminoglycoside acetyltrans ferase, called AAC (6')-Ib-cr that has two aminoacid changes, Trp102Arg and Asp179Tyr.These substitutions are responsible for the enzyme ability to acetylate ciprofloxacin (Van Hoek et al., 2011). Up to the date, they still have no evidence of occurrence of this type of AAC(6')-Ib-cr resistance among *Pseudomonas aeruginosa* and *Acinetobacter bamanniii* (https://card.mcmaster.ca/ontology/38947; last consulted 10/04/2020).

The third mechanism is enhanced efflux produced by plasmid genes for pumps QepAB and OqxAB (Jacoby et al., 2015), which can extrude hydrophilic fluoroquinolones such as ciprofloxacin and enrofloxacin (Van Hoek et al., 2011).

Antimicrobial resistance mechanisms against aminoglycosides

Several aminoglycoside resistance mechanisms have been recognized including active efflux pump, decreased permeability, ribosome modification, rarely, nucleotide substitution of the target molecule and drugs inactivation by aminoglycoside-modifying enzymes (AMEs) (Van Hoek et al., 2011). The latter is the most common mechanism for clinical resistance to aminoglycosides (Kotra et al., 2000, Van Hoek et al., 2011). AMEs are classified into three major classes according to the type of modification: aminoglycoside phosphotransferases (APHs), aminoglycoside nucleotidyltransferases (ANTs), and aminoglycoside acetyltransferases (AACs) (Kotra et al., 2000). The existance of bifunctional enzymes such as AAC(6')-APH(2") have been demonstrated (Van Hoek et al., 2011). aph(3')-IIb and aph(3')-VIa were the most prevalent AME genes in *P. aeruginosa* and *A. baumannii* respectively in an

Iranian study (Aghazadeh et al., 2013). In many cases, resistance to aminoglycosides is caused by ribosomal protection through methylation of specific nucleotides within the A-site of 16S rRNA, which hampers binding of aminoglycosides to the 30S ribosomal subunits and serves as a means of self-protection (Doi and Arakawa, 2007). Clinical strains of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* that produced 16S rRNA methylases were reported in 2003; these enzymes were found to confer extraordinarily high levels of resistance to clinically useful aminoglycosides, such as amikacin, tobramycin, and gentamicin (Doi and Arakawa, 2007) except streptomycin (Nie et al., 2014). 16S rRNA methylases *rmtA* and *armA* genes are well documented within *P. aeruginosa* and *A. baumannii* isolates respectively (Doi and Arakawa, 2007, Aghazadeh et al., 2013).

1.4.3. Antimicrobial resistance mechanisms against tetracycline

The resistance mechanisms for the tetracycline class of antibiotics are shared into four categories: mutations in rRNA subunits, ribosomal protection proteins (RPPs), energy-dependent efflux pumps and enzymatic inactivation (Van Hoek et al., 2011, Grossman, 2016). The *tet*, *otr* and *tcr* genes contribute to these resistance mechanisms (Van Hoek et al., 2011). Target-based mutations in rRNA conferring tetracycline resistance are usually found in bacteria with low rRNA gene copy numbers (Grossman, 2016). Mutations in the rpsJ, encoding changes or deletions in residues 53-60 in the 30S ribosomal subunit protein S10, have been linked to *A. baumannii* tetracycline or tigecycline resistance during an *in vitro* studies. Ribosomal protection is considered as one of the important and wider mechanisms related to tetracyclines resistance and mainly spraid through horizontal transfer (Sheykhsaran et al., 2019). Ribosomal protection proteins (RPPs) promote ribosome conformational changes which induced on the one hand rapid binding of the amino acid-tRNA translation complex, enabling translation to continue in the presence of tetracycline and on the other hand prevents rebinding of tetracyclin (Grossman, 2016, Nguyen et al., 2014). These proteins, as well as efflux pump mechanism have little or no effect on the potency of third generation tetracyclines such as tigecycline and the fourth generation such as eravacycline and omadacycline (Nguyen et al., 2014). The most common tetracycline-specific efflux pumps are members of the major facilitator superfamily (MFS) of transporters; however, there have been rare reports of non-MFS pumps (Grossman, 2016). Enzymatic inactivation has emerged as a new concern for the third and fourth generation tetracyclines (Markley and Wencewicz, 2018). The enzymatic inactivation mechanism can be attributed to three *tet* genes (Van Hoek et al., 2011). The *tetX* tetracycline resistance determinants encode FAD-requiring monooxygenases that confer resistance to tetracyclines

through modification of the drug (Nguyen et al., 2014). The *tet37 tet34, tet47, tet48, tet49, tet50, tet51, tet52, tet53, tet54, tet55, and tet56* are suggested to encode other enzyme-inactivating tetracycline (faculty.washington.edu/marilynr last update 06/04/2020). Several of the tetX harboring pathogens are on the CDC's list of ESKAPE pathogens including *P. aeruginosa* and *A. baumannii* (Markley and Wencewicz, 2018). *Acinetobacter* tetracycline resistance genes for ribosomal protection and/or efflux and/or enzymatic alteration tet(A)(B)(C)(D)(G)(H)(L)(M)(O)(W) (X)(Y)(39) (faculty.washington.edu/marilynr: last update 06/04/2020). *Pseudomonas* tetracycline resistance genes for ribosomal protection and/or efflux and/or enzymatic alteration tet(A)(B)(C)(D)(E)(G)(K)(L)(M)(O)(T)(W)(X)(Y)(34)(39)(42) (faculty.washington.edu/marilynr last update 06/04/2020).

1.4.4. Antimicrobial resistance mechanisms against trimethoprim-sulfamethoxazol

Trimethoprim-sulfamethoxazol inhibit the synthesis of tetrahydrofolate, essential component for the purines synthesis. Resistance to trimethoprim, mostly low level, can for example occur via non-allelic and drug-resistant variants of the chromosomal *folA* gene encoding the bacterial Dihydrofolate Reductase (DHFR) (Van Hoek et al., 2011). Although, high-level resistance to trimethoprim is generally achieved by a bypass mechanism through the action of an acquired gene which is a non-allelic and drug-insusceptible variant of a chromosomal DHFR (Van Hoek et al., 2011). *dfrAl*, *dfrA12, dfrA14,* genes coding for non-susceptible variant of DHFR were identified within non aeruginosa *Pseudomonas* species collected from salmonid fishes (Domínguez et al., 2019). A *dfrA28* variant of non-susceptible variant of DHFR was isolated within *Acinetobacter johnsonii* Strain from River Mahananda, India (Kumar et al., 2010). Genes coding for non-susceptible variant of DHFR seems to be scarce within clinical samples of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Chromosomally mediated resistance to sulfamethoxazol develops slowly and gradually and results from impairment of drug penetration, production of an insensitive dihydropteroate synthase (DHPS) (target of sulfonamide drugs) or hyperproduction of Para-aminobenzoic acid (PABA) (Maddison et al., 2008). Plasmid mediated resistance is far more common and can involve impairment of drug penetration or production of additional, sulfonamide-resistant dihydropteroate synthetase enzymes (Maddison et al., 2008). Currently, four different types of DHPS genes (*sul1, sul2, sul3* and *sul4*) have been described update but *sul4* has not yet been detected in clinical isolates (Sánchez-Osuna et al., 2019). The *sul1* gene was identified among

P. aeruginosa isolates and the *sul1* and *sul2* genes among *Acinetobacter spp* isolates according to a study by shin and colleagues (Shin et al., 2015).

1.5. Diagnostic methods

Bacteria identification, antimicrobial resistance and virulence mechanisms can be done either, through phenotypical technics involving biochemical analysis, antibiotyping or chemical based assays such as matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) or by molecular technics.

1.5.1. Phenotypical methods

- **Biochemical test**

Biochemical identification of bacteria relies on their capacity to use a particular pathway or to hydrolyze a particular substrate. *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are typically known like non-fermentative bacteria since they can not use sugar through a fermentation pathway. The most common phenotypical assay designed for routine biochemical testing in medical analysis laboratories for *Pseudomonas aeruginosa* and *Acinetobacter baumannii* identification is API 20 NE (bioMérieux, marcy l'étoile, France). API 20 NE is a miniaturized system of twenty microtubes containing lyophilized reagents for biochemical tests. VITEK 2 is another well-known phenotypical technology using particular cards with 64 wells corresponding each to substrates involved in measuring various metabolic activities such as acidification, alkalinisation, enzyme hydrolysis, and growth in the presence of inhibitory substances. At the end of the reaction, each sample shows a bio-pattern which is then compared to the database of reactions for each taxon, and a numerical probability calculation is performed to give the isolate identity.

- **Spectrophotometry based technics**

Matrix-Assisted Laser Desorption Ionization-Time of flight mass spectrometry (MALDI-TOF-MS) MS is a method used to analyse biomolecules such as DNA, carbohydrates, proteins, and peptides by their ability to become ionized and enter gas phase and then measuring their time of flight. A molecular signature is given by the ratio of mass out to charge of each biomecule analysed. This signature is then compared to the database to identify the microorganism MALDI TOF is also a useful tool used for detection of antimicrobial resistance determinants since there is a characteristic differences in the spectra of susceptible and resistant strains of a species.

- **Serotyping**

A serotyping is a diagnostic assay, which characterizes organism using its antigens set. Lipopolysaccharide (LPS) is a complex glycolipid located on the outer bacterial membrane. It is required for cell viability and pathogenicity. The somatic (O) antigen is one of the constituent LPS. The variability of O-antigens is the primary basis for the serotyping schemes of many gram-negative bacteria. According to various studies, some *Pseudomonas aeruginosa* serotypes have been proved more virulent than others thus enhance the need of use of serotyping as diagnostic assay. Considering variability of O-antigen, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* the International Antigenic Typing Scheme (IATS) defined 20 *Pseudomonas aeruginosa* serotypes (Lu et al., 2014). A study made by Gonçalves and colleagues have highlighted 34 *Acinetobacter baumannii* serotypes (Goncalves et al., 2000). Serotyping protocol involved an agglutination test between bacteria suspension with a specific prepared antiserum. The positive test is observed with the agglutination formation. O:6 serotype was predominantly identified among typed samples of *Pseudomonas aeruginosa* by (Faure et al., 2003).

- **Antimicrobial susceptibility testing: Antibiotyping**

Antibiotyping consist of bacteria typing considering their sensitivity or most commonly, their resistance against antibiotics set through antimicrobial susceptibility testing. Antibiotype is generally termed as sensitivity or resistance phenotype. In addition, some resistance pattern can be used to predict a resistance mechanism involved. There is a wide literature presenting the potential linkage between resistance patterns with resistance mechanism. Bush and co-authors (Bush et al., 1995b) in her minirevew presented the preferable β-lactam substrates for some β-lactamases. Thus, antimicrobial resistance typing can be used like preliminary assay to select resistance genes to be targeted in a molecular diagnostic test. The initial step for antibiotyping is antimicrobial susceptibility testing. Various technics are used for this purpose. The most common is Kirby-Bauer method also called agar disk-diffusion method. Kirby-Bauer method consist on agar plates inoculation with a standardized inoculum of the test microorganism. Then, filter paper discs of about 6 mm in diameter containing the test compound at a desired concentration are placed on the agar surface. The Petri dishes are incubated under suitable conditions. Generally, antimicrobial agent diffuses into the agar and inhibits germination and growth of the test microorganism and then the diameters of inhibition growth zones are measured.

There is also broth dilution methods or minimal inhibitory concentration (MIC) methods. The procedure involves preparing two-fold dilutions within the tests tube (macro-dilution method) or within the micro titration plates (micro-dilution method); the inoculum with

the same turbidity is then added in each tube or well. The preparation is the checked and incubated at the appropriate temperature. After incubation, follows determination of the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in tubes or microdilution wells as detected by the unaided eye also called minimal inhibitory concentration.

Antimicrobial gradient method (Etest) is a variety of MIC method. In the procedure, a strip impregnated with an increasing concentration gradient of the antimicrobial agent from one end to the other is deposited on the agar surface, previously inoculated with the microorganism tested. This method is used for the MIC determination of antibiotics.

Many states cooperation have defined break points to characterise sensitivity or resistance of each bacteria specie against each antimicrobial agent. Among them are: the clinical and laboratory standards institute (CLSI) from United States, British society for antimicrobial chemotherapy (BSAC) and European committee on antimicrobial susceptibility testing (EUCAST) joined by French committee of antiprogram. After antimicrobial susceptibility testing, studied bacteria resistance pattern represent the antibiotyping.

1.5.2. Molecular Diagnostic Techniques

Molecular diagnostic has taken a prominent place and has shown advantages in the clinical diagnostic laboratory for routine detection, fingerprinting, and epidemiologic analysis of infectious microorganisms (Chen et al., 2018). There are many different technologies available, which include whole-genome sequencing and metagenomics, PCR-based techniques, hybridization technics such as DNA microarray, Southern and Northern blotting. It is also included various molecular typing technics such as ERIC-PCR, PFGE finger print analyses (Anjum et al., 2018).

- **PCR-based methods**

Conventional PCR

PCR is a technique that was developed in 1983s by Kary Mullis and has revolutionized molecular biology, enabling rapid and exponential amplification of target DNA sequences (Anjum et al., 2018). PCR is an in vitro reproduction of DNA replication. All PCR type requires a thermostable DNA polymerase as far as these reactions require high temperatures up to 95˚C for DNA denaturation. PCR also, requires DNA template, primers, dNTPs, and Mg2+ ion as fundamental reagents. PCR principle lies on enzymatique action of Taq-polimerase. In fact, DNA polymerase directs the synthesis of DNA from deoxyribonucleotide substrates on single-stranded DNA template. It catalyses an addition of deoxyribonucleotides to the 3’ end of primer

when it is annealed on the template DNA. The reaction takes place in the thermocycler. This automate, allows a temperature variations necessary to move from one reactional stage to another. Identically with DNA replication, PCR process start by a separation of the double strand DNA, followed by annealing of primers, which allows the elongation of a new DNA strand to take place. In short, conventional PCR described by Mullis is proceed as follows. The DNA template of target DNA is heated to 94˚C or 95˚C to break the hydrogen bonds that maintain each double strand DNA together, so that DNA strands can separate; this process is termed denaturation. The mixture is cooled in temperature between 50-70˚C to allow the primers to bind to their complementary DNA. The temperature is then heated to 72˚C so that *Taq* DNA polymerase can optimally proceed the addition of dNTPs to form a new DNA strand. Each new double stranded DNA will serve as a template for a new reaction cycle from denaturation to elongation. Depending of DNA targeted, the PCR can go until 30 or 45 cycles. Conventional PCR offer an opportunity for multiplexing and therefore several points should be considered.

Others Type of PCR

After Mullis's discover, many PCR models have been developed with different approaches and different specificities. Here are some others PCR types.

Real time PCR

Real time PCR is a polymerase chain reaction process in which the target DNA is amplified and quantified simultaneously in real time. Higuchi et al. (Higuchi et al., 1993) one of the first to develop a quantitative assay using a video camera to analyse the kinetic of PCR (Higuchi et al., 1993). This quantification was done by measuring fluorescence of ethidium bromide intercalating the double strands DNA during annealing and extention phase of PCR reaction (Higuchi et al., 1993). Real time PCR protocol like described by Higushi *et al.,* includes a denaturation phase, primers annealing and extension phase (Higuchi et al., 1993). Specialized thermocyclers equipped with fluorescence detection modules are used. Optimized real-time PCR assays are highly sensitive, detecting as few as one to ten copies of a target gene (Loftis and Reeves, 2012). Reverse transcriptase PCR and mutation detection can also be carry out with real time PCR devices. The ability of real time PCR to simultaneous amplificaty and quantify DNA makes useless the electrophoresis gel running. Though real time PCR is a very sensitive and rapid, there are some drawbacks; the most striking is that it allows the amplification of only small DNA fragments, optimally150 bp amplicons (Anjum et al., 2018). However, conventional PCR has the ability to amplify larger gene fragments (Anjum et al., 2018). The high cost of devices for real time PCR is one of the limitation of its expansive use.

In the early hours of real time PCR, detection based upon binding dyes were used as presented above. Nowadays, it has been developed various others detections dyes; including binding dyes others than ethidium bromide, fluorescent probes such as hydrolysis probes and hybridization probes (Loftis and Reeves, 2012).

Nested PCR

Nested PCR is a PCR method that involves two primer sets. The most common is "two step/two tubes" protocol where the first set of primer is used for the first amplification round. Subsequently, a second set is used to amplify a smaller fragment from the former amplicon. Nested PCR can also use the "two steps/one tube" method involving two PCR rounds using one tube (Cribb et al., 2002). This type of nested PCR operate like follow. Mixture for normal conventional PCR is made with the first set of primer intendent to amplify a larger fragment of DNA. After that, the second mixture containing the second set of primer is added straight to the product of the first amplification for the second PCR round. Nested PCR is used to reduce unspecific amplification due to polymerization of unexpected DNA sequence. Because of the used of two sets of primers, sensitivity and specificity of nested PCR is improved compare to conventional PCR method (Adzitey et al., 2013). The main drawback of nested PCR is a high probability of contamination compare to conventional or real time PCR (Adzitey et al., 2013). To overcome the contamination issue, a third nested PCR protocol has been developed and engaged "one closed tube" protocol. There are several protocols available for a nested "one closed tube" PCR. da Sylva and colleagues (da Silva et al., 2013) developed a nested PCR protocol which involved two primers sets with inner (second) primers immobilized on interior of the tube lid by means of adsorption microtubes. After the first PCR round, PCR tube is removed from the thermocycler and inverted several times to dissolve the inner primer before returning to the thermocycler for the second PCR round. Nevertheless, da Silva and colleagues (da Silva et al., 2013) obtained a reduce sensitivity with their modifyed nested PCR protocol compared to the conventional one. Sun et al and colleagues (Sun et al., 2017) also developed an "one closed tube" nested PCR with all primers included in the starting mixture. Unlike da Silva and colleagues (da Silva et al., 2013), Sun and colleagues (Sun et al., 2017) obtained a very high sensitivity with his protocol despite the complexity of their PCR mixture. we notice that the "one closed tube" nested PCR developed by these authors use a very short number PCR cycles for the first round 15 for da Sylva and colleagues (da Silva et al., 2013) 10 for Sun and colleagues (Sun et al., 2017). The second round for both of them is longer, 35 for da Sylva and colleagues (da Silva et al., 2013) and 40 for Sun and colleagues (Sun et al., 2017). Each round

was obviously set with different PCR conditions, as far the primers used were different. In blood and urine samples, Das and colleagues (Das et al., 2015) identify with nested PCR protocol *Pseudomonas aeruginossa* and *Acinetobacter baumannii*. There are much more others amplification protocols. In the two last decades, has emerged a new type of nucleic acid polymerisation occurring at isothermal conditions.

- **Isothermal amplification assays**

Isothermal amplification is a nucleic acid amplification assay that allows DNA amplification at a constant temperature. Several variants have been developed. The most common is LAMP standing for loop-mediated isothermal amplification. Are also included polymerase spiral reaction (PSR), recombinase polymerase amplification (RPA), cross-priming amplification (CPA), isothermal multiple-self-matching-initiated amplification (IMSA) (Yang et al., 2018) strand displacement amplification (SDA), Helicase-dependent amplification (HDA), Nicking enzyme amplification reaction (NEAR)**.** Isothermal amplification technology is an easy assay, straightforward to perform and do not require specialized instruments such as thermocycler (Yang et al., 2018).

Loop mediated isothermal amplification (LAMP)

LAMP one of most common isothermal amplification method, was developed by Notomi and colleagues in japan in 2000 (Notomi et al., 2000). LAMP assay uses DNA polymerase, with four different primers including two inner and two outer designed to recognize six particular sequences on the target DNA. At the beginning of the reaction, all four primers are used, later during the reaction only inner primers are used. The mixture should firstly be heated for 5 min at 95˚C and then cooled down on ice, LAMP reaction is then initiated by adding a Bst DNA polymerase, a DNA polymerase with strand displacement activity. This mixture is incubated at 65˚C for one hour. Up to 10^9 copies can be accumulated in one-hour experiment. Reaction is finally terminated by heating at 80˚C for 10 min. LAMP can also be used for gene expression study by using reverse transcriptase, coupled with DNA polymerase. Detection can be done by naked eyes or by analizing the turbidity, or color changing. LAMP is ten-fold more sensitive than conventional PCR. LAMP successfully saved as base protocol in identification of OprL and OprD2 genes in imipenem-resistant *Pseudomonas aeruginosa* (Wang et al., 2016). Carbapenemase *bla*-$_{\text{NDM-1}}$ and *bla*-$_{\text{KPC}}$ were identified among carbapenem-resistant *Acinetobacter baumannii* with higher sensitivity than with conventional PCR using LAMP protocol (Solanki et al., 2013).

Recombinase polymerase amplification (RPA)

Piepenburg and his collaborators developed recombinase polymerase amplification (RPA) methodology (Piepenburg et al., 2006). In RPA protocol, primer with recombinase activity targets DNA template at 3' end allowing strand exchange at cognate site, subsequently strand-displacement DNA polymerase catalyzes extension of new DNA strand. Recombinase polymerase amplification do not need to achieve 95˚C denaturation step, which is a preliminary to LAMP. RPA requires less than 30 min to be done. A lateral flow detection is used to detect the reactional products. Real time detection can also be performed using a simple handheld fluorometer to monitor the fluorescence. Piepenburg *et al.* (2006) used RPA method to identify three methicillin-resistant-*Staphylococcus aureus* MRSA types. ESKAPE bacterial pathogens, which include *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were identifying using this protocol (Renner et al., 2017).

Polymerase spiral reaction (PSR)

Polymerase spiral reaction (PSR), as RPA uses DNA polymerase with strand displacement activity and does not need 95˚C denaturation. This technic was developed by (Liu et al., 2015). Unlike most of isothermal PCR reaction, PSR need only one pair of primers and one enzyme. The PSR products can be detected using two methods: turbidity or direct visual detection within one hour. PSR detection method was used on *Pseudomonas aeruginosa* DNA extract for identification of *toxA* virulence gene (Dong et al., 2015). Isothermal nucleic acid polymerisation methods are very suitable for point-of-care-testing for low resources settings, because they required inexpensive equipment. Molecular diagnostic methods include also a wide range of hybridization technics, among them are microarray, blotting technics like southern blotting and northern blotting.

- **Hybridization technics**

DNA microarray assay

DNA microarray or gene chips is a gene expression profiling technic, which has been used for resistance genes identification. This molecular method can measure tens to thousands genes at once. It is a highly computerised technology. Three type of gene chips have been designed: spotted arrays on glass, in-situ synthesized arrays and self-assembled arrays (Bumgarner, 2013). To construct a customized gene chips, a single strand of chromosomal DNA (cDNA) or genomic DNA probes is printed within each chip with and internal positive control included (Call et al., 2003) while commercialised gene chips use sophisticated technology like photolithography to generate probe particle *in situ* on the chip's glass (Bumgarner, 2013). A target cDNA or DNA extract is treated according to each manufacturer protocol to generate a single stranded labelled DNA. Gene chips hybridized present generally

a multi-coloured image, which is captured by a specific scanner followed by a quantification made by an appropriate software. This protocol is highly used in resistance gene characterisation. Batchelor and colleagues (Batchelor et al., 2008) used this assay to identify various resistance genes including, extended-spectrum β-lactamases, aminoglycoside modifying enzymes, tetracycline resistance gene among Gram negative bacteria (Call et al., 2003), identified 90 tetracycline resistance genes among Gram positive using gene chips assay. Commercialised microarray such as Identibac developed by Alere (Alere Technologies GmbH, Jena, Germany) was designed to assess antimicrobial resistance in both Gram negative and Gram positive (Anjum et al., 2018).

Southern blotting

Southern blotting is a hybridization technique described by Edwin Southern in 1975. It is based on identification of particular target DNA fragment from gel electrophoresis through its hybridization with a complementary DNA probes generally radioactively labelled, coated on the nylon membrane. Prior to transfer on the nylon membrane electrophorese gel goes through an alkaline treatment. The experiment result is revealed by autoradiography.

- **DNA next generation sequencing (NGS)**

Bacteria strain characterisation, required to monitor epidemiological hospital outbreaks to prevent or to document transmission, requires additional laboratory techniques with a higher discriminatory performance than conventional methods used for the identification at the species level (Bassetti et al., 2018). Several molecular techniques have been used during the past decades including PCR with its multiple variants or isothermal amplifications or micro-array protocols. However, these techniques may be gradually put aside in the profit of high-throughput sequencing technologies allowing rapid genome sequencing with a significant higher discriminatory power than conventional molecular techniques (Bassetti et al., 2018). This new technology can be used in hospital epidemiology settings to prevent transmission, and also to analyse strains resistomes (genes and operons involved in antibiotic resistance) and/or to follow the evolution of the population of bacteria strain in chronic disease (Bassetti et al., 2018). Next generation sequencing (NGS), massively parallel or deep sequencing are related terms that describe a DNA sequencing technology which has revolutionised genomic research. It has been introduced to the market in 2005 (Chen et al., 2018). NGS can allow the sequencing in the single day where the Sanger method can take up to a year to decipher the same genome. NGS can be used to sequence entire genomes (the whole genome sequencing) or coding genes (a whole exome) or small numbers of individual genes. Next Generation Sequencing (NGS)

technology is based on shearing/cutting DNA into small fragments, and their massive parallel sequencing. The multiple overlapping segments termed "reads" are assembled into a contiguous sequence. To reduce sequencing errors, every genome region should be sequenced several dozen times. In general the workflow for Next-Generation Sequencing includes the DNA extraction from the pure bacteria colony in case we are dealing with bacteria sample, followed by DNA shearing, library preparation by PCR amplification, sequencing, and DNA sequence analysis (Anjum et al., 2018). The schematicatical representation of NGS pipeline is shown in the figure 1.28.

- **DNA Shearing or fragmentation**

Fragment length depends to be generated should take into account the next generation platform to be used. Shearing methods are divided into two categories: the enzymatic protocol and the physical protocols including nebulization, sonication, covaris or acoustic shearing and Fragmentation Through Polymerization (FTP)

Enzymatic Shearing

Enzymatic shearing yields fragments through simultaneous digestion of both DNA strands, or by generation of nicks on each strand of double strand DNA to produce dsDNA breaks. Fragmentase (New England Biolabs, Ipswich MA) is a mix of two enzymes, one a non-specific nuclease randomly generating nicks in the double strands DNA and the other one cutting the strand opposite to the nicks. Nextera, an illumina protocol uses a transposase tagmentation reaction to simultaneously fragment and insert adapters into double strand DNA (Ignatov et al., 2019). Enzymatic digestion is simple and very efficient, but it may introduce an enzymatic bias, such as insertions and deletions (Ignatov et al., 2019), physical fragmentations therefore, are often the preferred methods for shearing.

Nebulization

Nebulization is a physical technique commonly used during the early years of sequencing. Compressed nitrogen or air forces the DNA sample to cross the thin hole of nebulizer unit, resulting in the formation of random mechanically sheared fragments leading to a heterogeneous mix of double-stranded DNA molecules containing 3' or 5' overhangs as well as blunt ends (Knierim et al., 2011). Nebulization is easy, quick, and requires only small amounts of DNA (0.5-5 μg) with the range of sizes between are 700 and 1330 (Sambrook and Russell, 2006) bp.

Sonication: During sonication, samples are subjected to ultrasonic waves, whose vibrations produce gaseous cavitations in the liquid that shear or break high molecular weight DNA

molecules through resonance vibration (Knierim et al., 2011). Most sonicators will not shear DNA to a size of less than 300-500 bp but, 700 bp in length can be obtained in certain conditions.

Covaris or acoustic shearing

Acoustic energy is focused into a small glass vial containing the sample, which induces cavitation of the aqueous sample (Ignatov et al., 2019). Covaris shearing uses acoustic wave energy transmitted into a closed tube containing an aqueous DNA solution. This results in formation and collapse of air bubbles, which generate microscale water jets that cause physical shearing of the nucleic acid (Voelkerding et al., 2010). This method shows little or no genome bias compared to above-mentionned methods, this makes Covaris shearing the preferred and gold standard method for most NGS users (Ignatov et al., 2019).

Fragmentation Through Polymerization (FTP)

Fragmentation Through Polymerization is a new type of fragmentation proposed since 2019 by Ignatov and colleagues (Ignatov et al., 2019). FTP consists on adding to a required amount of DNA a SD polymerase (a Taq DNA polymerase mutant that has strong 5'-3 strand displacement and 5'-3' polymerase activities (Ignatov et al., 2014)) and other PCR ingredient as described by the authors. The fragmentation of DNA is then carried out by two-step incubation: 20 minutes at 30˚C and then 20 minutes at 70˚C (Ignatov et al., 2019). This method generates double-stranded DNA fragments that are suitable for direct use in NGS library construction and allows the elimination of the additional step of reparation of DNA ends (Ignatov et al., 2019).

After shearing, the fragmented DNA is end repaired and ligated at both blunt ends of each fragment with specific adaptors, tags, barcodes, which serve as primer-binding sites for amplification (Voelkerding et al., 2010). Alternatively, Illumina's fragmentation technology, called Nextera Tagmentation, can be implemented using a transposase enzyme to simultaneously fragment and insert adapter sequences into the ds DNA and thereby reduce sample handling and preparation time. The adapters contain specific sequences designed to interact with the NGS platform (Head et al., 2014). The adaptor-ligated DNA fragments formed in the previous step are size-selected through agarose gel electrophoresis or with paramagnetic beads; at this step the ligation duplicates are removed. Subsequently DNA fragments are melted, and the single-stranded DNAs are immobilized either on planar solid surfaces of a flow cell (Illumina sequencers), or on the surface of micron-scale beads (454-Roche and SOLiD sequencers), or on ionized spheres (Ion Torrent sequencers) ready for library preparation

(Voelkerding et al., 2010). Comparative table of most common NGS platform is shown in table I.4.

- **Library preparation**

There are several important considerations when preparing libraries from DNA samples, including the amount of starting material and whether the application is for resequencing (in which a reference sequence is available to align reads to) or de novo sequencing (in which the reads will need to be assembled to create a new reference sequence) (Head et al., 2014). Library amplification is performed on a solid surface or on beads while isolated within miniature emulsion droplets or arrays. Clonal amplification of the library, can be made by either cluster generation for Illumina or microemulsion PCR for Ion Torrent (Head et al., 2014). Nucleotide incorporation is monitored directly by luminescence detection or by changes in electrical charge during the sequencing procedure. NGS generates many millions of nucleotide short reads in parallel in a much shorter time than by the Sanger sequencing method. The read types generated by NGS are digital and therefore enable direct quantitative comparisons. Either single or pair end reads can be obtained at fragment ends.

- **Sequencing**

Finally, sequencing is achieved by detecting the emission of light or hydrogen ions from every dot on the solid surface or spheres, during enzymatic attachment of complimentary nucleotides to the clusters of identical single stranded DNA fragments (Poptsova et al., 2014).

- **DNA sequence analysis**

For unambiguous determination of the whole genome sequence, the overall length of the sequenced reads has to exceed the genome size by a dozen of times (Poptsova et al., 2014). The multiple overlapping segments termed reads in a fastq or fasta format are uploaded into specialised software to generate contiguous sequence (Poptsova et al., 2014) during a protocol called assembling, that contiguous sequence constitute scafold for annotation step. After the stage of scafolds building the gap fling is performed to improve the accuracy of the scafolds. Then, the annotation stage is done. There is structural annotation and functional annotation. In structural annotation, repeated sequences in the assembled scafolds are masked. Then, a gene prediction tool is used to locate genes within the scafolds, and the structures of introns, exons, and untranslated regions (UTRs) constituting these genes are determined. Finally, in functional annotation, homology search and ontology mapping are performed using structure-annotated sequences in order to determine the functions of the genes.

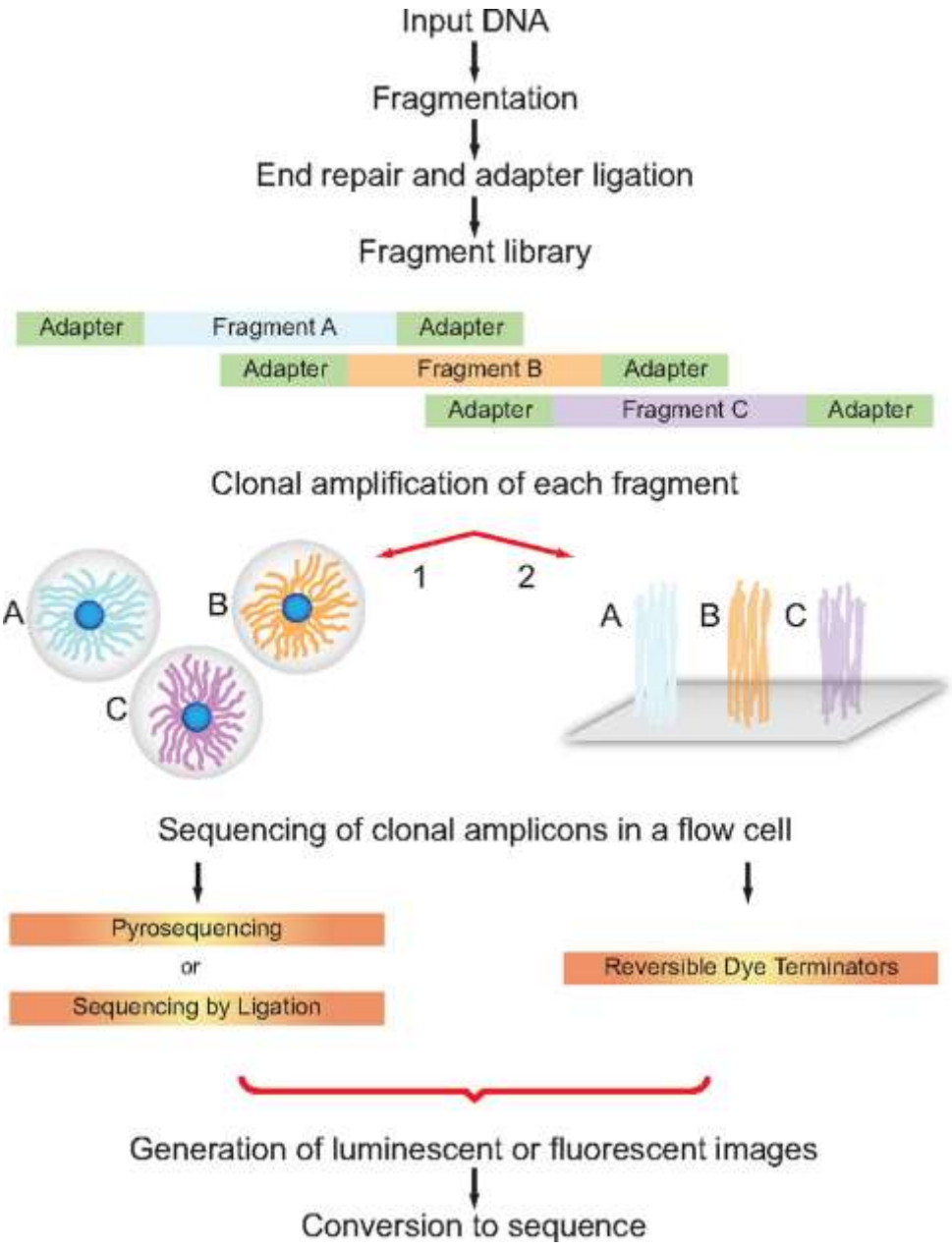


Figure 27: Next generation sequencing process steps for platforms requiring clonally amplified templates (Roche 454, Illumina and Life Technologies).

Input DNA is converted to a sequencing library by fragmentation, end repair, and ligation to platform specific oligonucleotide adapters. Individual library fragments are clonally amplified by either (1) water in oil bead-based emulsion PCR (Roche 454 and Life Technologies) or (2) solid surface bridge amplification (Illumina). Flow cell sequencing of clonal templates

generates luminescent or fluorescent images that are algorithmically processed into sequence reads. Figure from Voelkerding and colleagues (Voelkerding et al., 2010)

Table IV: The main features and performances of Sanger sequencing and four commonly used second-generation sequencing. Information drawn from the following authors: [(Kulski, 2016; Loman et al., 2012)

NGS platforms/company/max output per run	Read length per run (bp)	Positional separation of fragments	Detection of nucleotide incorporation	Advantage of technology	drawback of technology
First generation					
Sanger/Life Technologies /84 kb	800		**Dideoxy terminator**	Low investisment for devices	Relatively low data output,
Second generation					
HiSeq/ Illumina/1500 Gb	2x150 (pair-end)	Oligonucleotides on flow cell immobilize fragments	'Sequencing by synthesis' - fluorescently-labelled **reversible chain terminating** dideoxynucleotides	Cost-effective when high throughput	Short reads Long run time
MiSeq/Illumina/15Gb	2x300 (pair-end)	Oligonucleotides on flow cell immobilize fragments	'Sequencing by synthesis' - fluorescently-labelled **reversible chain terminating** dideoxynucleotides	Cost-effective when high throughput	lower quality assemblies produced
Ion Torrent/ (Thermo Fisher) / 1Gb	400	Fragments clonally amplified on beads in individual 'wells'	Individual nucleotides flooded sequentially, **pH change detected** if incorporated. Wells act as individual	Cost-effective when low throughput Short run times	Relatively low data output

			'pH meters' for each fragment.		
SOLiD (Applied Biosystems)/ 120Gb	50	Fragments clonally amplified on beads in emulsion. Beads covalently attached to flow cell slide.	**Ligation** based sequencing with Fluorescently-labeled dibase probes.	Low error rate	Short reads Long run time

CHAPTER 2: Material and Methods

2.1. Sample collection

1.2.2 Description of the samples collection sites

- **Description of the Yaounde University Teaching Hospital (YUTH)**

YUTH is located in the Yaounde III sub-Division in the MELEN neighbourhood. This institution shares the same fence with the University of Yaounde I and the Faculty of Medecin and Biomedical Sciences. It is one of the referral hospitals in the city of Yaounde. Created by the Decree No. 78/741 of June 24, 1978. The YUTH is a public health setting with financial autonomy. The Clinical Biology pole is located in the first and second cellars and gathers 05 large separate services. The Bacteriology service, is located in the second cellar, a large room equipped with benches where the analyzes are mainly carried out.

- **Description of the Yaounde Central Hospital**

The Yaounde Central Hospital is a 650-bed hospital located in the Central region in the Mfoundi Division and Yaounde II sub-Division. It is located on the edge of large structures such as the Centre Pasteur du Cameroon and Mother and Child of the Chantal Biya Foundation. The Yaounde Central Hospital was created in 1933, initially as a day care hospital, it has undergone several structural changes and is today a second category care health setting, which provides patients with a specialized medical and paramedical team. It contains several services including medical analysis laboratories.

Description of *Centre Pasteur du Cameroun*

Le *Centre Pasteur du Cameroun* (CPC) is located in the Centre region in the Mfoundi Division in the neighborhood of the Mother and Child Center of the Chantal BIYA Foundation. *Centre Pasteur du Cameroun* is a technical hand of the Cameroon Ministry of Public Health. It is a public administrative health institution with financial autonomy. It was created in 1959 in Yaounde. The CPC is a member of the International Network of Pasteur Institut (INIP). CPC shares with INIP the main mission of fighting against infectious diseases. To this end, it provides four missions: medical analyzes and vaccination, public health, research and formation.

Justification for the choice of YUTH, YCH and CPC as the collection sites.

The YUTH, YCH and CPC were chosen for their high attendance level which was a major asset in obtaining the desired number of sample. In addition, these sites have an adequate technical platform allowing reliable identification of bacterial strains.

A cross sectional study was carried out with convenace sampling. 104 bacteria isolates were collected from different laboratory settings independently to age and gender of patients; comprising of 77 and 27 presumptive *P. aeruginosa* and *A. baumannii* respectably isolated. Out of the 77 presumptive *P. aeruginosa* 9 were collected from YUTH, 6 were from YCH and 62 from CPC. Out of 27 presumed *Acinetobacter baumannii* 4 were from YUTH and 23 from CPC. clinical specimens, included pus, urine, sputum, bronco-alveolar lavage (BAL), sperm, high vaginal swab (HVS) and blood were collected from Yaounde University Teaching Hospital (YUTH), Yaounde Central Hospital (YCH) and *Centre Pasteur du Cameroun* (CPC) between January 2015 to March 2016. Presumptive identification of the isolates was performed in each collection site using either API 20 NE or VITEK 2. Bacteria isolates were revived on tryptic soy agar (TSA) and transported to the Food and Drug Safety (FODRUS) Laboratory, Yaounde, Cameroon.

2.2. Phenotypic identification

The isolates were further identified using the catalase, oxidase, mannitol and citrate Simmons agar test. The Hajna-Kliger media was used to assess lactose and glucose fermentation as well as H_2S production potentials of the isolates. All presumptive *P. aeruginosa* and *A. baumannii* isolates were then stored in brain heart infusion broth at -20°C. Isolates were later transported to the Antimicrobial Resistance and Phage Biocontrol Research Laboratory at the North West University, South Africa for further analysis for further analysis.

2.3. Molecular identification

2.3.1. DNA extraction and quantification

Each isolate was cultured on nutrient agar and incubated aerobically at 37°C for 24 hours. Pure bacteria colonies were inoculated in 15 mL of Luria-Bertani (LB) broth (Merck, South Africa) and incubated at 37°C for 24 hours. Chromosomal DNA was extracted from exponential phase broth cultures using a DNA extraction kit (Zymo Research, CA) according to the manufacturer's instructions. Prior to PCR, DNA was quantified using a NanoDrop TM 1000 spectrophotometer (Thermo Fischer Scientific, USA).

2.3.2. *P. aeruginosa* and *A. baumannii* PCR identification tests

Pseudomonas species-specific 16S ribosomal RNA and *P. aeruginosa* specific 16S ribosomal RNA gene sequences were amplified in all the isolates using oligonucleotide primer sequences as listed in Table V Amplification Intergenic Space sequences (*ITS*) specific to

Acinetobacter baumannii was used to identify the isolates. PCRs were prepared as standard 25 µL volumes comprising 12.5 μl of 2X Master mix [0.05 U/µL *Taq* DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dATP, dCTP, dGTP and dTTT], 0.25μl of each primer (1μM), 1μl template DNA (20-30 ng/μl) and 11 μl nuclease free water. Each amplification was carried out following PCR conditions provided by authors in Table V with slight modification on annealing temperature.

The oligonucleotide primers for the different target sequences and amplification conditions are shown in Table V. PCR was performed using a Thermal cycler (model C1000 Touch) supplied by BIO-RAD, California, USA. Reference strains *Escherichia coli* (ATCC 25922) and *Pseudomonas eruginosa* (ATCC 27853) were respectively used as negative and positive controls.

Table V: List of Primers and PCR

Target gene	Primer sequences (5' to 3')	Annealing temperature	Amplicon size bp	References*
***Pseudomonas aeruginosa* Identification genes**				
16S gene for *Pseudomonas spp.*	F:GACGGGTGAGTAATGCCTA R:CACTGGTGTTCCTTCCTATA	53.6 ˚C	618	(Spilker et al., 2004)
16S gene for *Pseudomonas aeruginosa*	F:GGGGGATCTTCGGACCTCA R:TCCTTAGAGTGCCCACCCG	53.6˚C	956	(Spilker et al., 2004)
***Pseudomonas aeruginosa* virulence genes**				
lasB	F:GGAATGAACGAAGCGTTCTCCGAC R:TGGCGTCGACG*AACA*CCTG	55 ˚C	284	(Fazeli and Momtaz, 2014)
exoA	F:TGCTGCACTACTCCATGGTC R:ATCGGTACCAGCCAGTTCAG	60 ˚C	190	(Ghadaksaz et al., 2015)
pslA	F: TCCCTACCTCAGCAGCAAGC R: TGTTGTAGCCGTAGCGTTTCTG	60 ˚C	656	(Ghadaksaz et al., 2015)

exoS	F:CGTCGTGTTCAAGCAGATGGTGCTG R: CCGAACCGCTTCACCAGGC	55 ˚ C	444	(Ghadaksaz et al., 2015)
	***Acinetobacter baumannii* identification gene**			
ITS	F:CATTATCACGGTAATTAGTG R:AGAGCACTGTGCACTTAAG	55˚ C	208	(Feizabadi et al., 2008a)
	***Acinetobacter baumannii* virulence genes**			
OmpA	F:GTTAAAGGCGACGTAGACG R:CCAGTGTTATCTGTGTGACC	56˚C	578	(Azizi et al., 2016)
csuE	F:CATCTTCTATTTCGGTCCC R:CGGTCTGAGCATTGGTAA	59˚C	168	(Azizi et al., 2016)
	Resistance genes			
bla$_{TEM}$	F:ATGAGTATTCAACATTTCCG R:TTACCAATGCTTAATCAGTGAG	50˚C	861	(Celenza et al., 2006a)
bla$_{CTX\ M}$	F:CGCTTTGCGATGTGCAG R:ACCGCGATATCGTTGGT	56.9˚ C	550	(AL-Kadmy et al., 2018)

*references are articles describing amplification conditions of each gene studied

2.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) of the isolates was determined by Kirby-Bauer disc diffusion method according to the CASFM/EUCAST guidelines. Bacterial suspensions of 24 hours cultures were prepared in 0.8% (w/v) normal saline solution to achieve the turbidity equivalent to 0.5 Mc McFarland standard, then.inoculated spread-plated on Mueller Hinton agar (MHA) plate by swabbing-plate technique. The following antibiotics piperacillin (30 µg[a] and 100 µg[b]), piperacillin-tazobactam (30-6 µg[a] and 100-10 µg[b]), ticarcillin (75 µg[a,b]), ticarcillin-clavulanic acid (75-10 µg[a,b]), cefepime (30 µg[a,b]), cefotaxime (30 µg[b]), ceftazidime (10 µg[a] and 30 µg[b]), ceftriaxone (30 µg[b]),imipenem (10 µg[a,b]), meropenem (10 µg[a,b]), ciprofloxacin (5 µg[a,b]), levofloxacin (5 µg[a,b]), amikacin (30µg[a,b]), gentamicin (10 µg[a,b]), netilmicin (10 µg[a,b]), tobramycin (10 µg[a,b]), doxycycline (30 µg[b]),minocycline (30 µg[b]),

tetracycline (10 μg[b]) and trimethoprim-sulfamethoxazole (1-25 μg[a] and 23-75μg[b]) (Liofilchem s.r.l., Via Scozia, Italy) were placed on the inoculated plates. Antibiotics concentrations with superscripts "[a]" and "[b]" were used in the screening of PSA and ACB respectively.

Plates were incubated aerobically at 37 °C for 24 h. Antibiotic inhibition zone diameter data (AIZD) of the different antibiotics were measured in mm and results were used to uil a dendogram. TIBCO Statistica version 13.3 (StatSoft, TIBCO Software Inc., USA) software was used to cluster organisms based on their AIZD data. Bacteria reference strains *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) were used antibiotics quality control. Multidrug Resistant (MDR) patterns where defined as resistance to at least three or more antimicrobial agents belonging to different classes (Magiorakos et al., 2012).

2.5. Phenotypic determination of resistance enzymes

The potential of *P. aeruginosa* and *A. baumannii* to produce enzymes such as hyperproduced cephalosporinase (AmpC), extended spectrum β-lactamases (ESBL), and metallo β-lactamases (carbapenemases) was determined by comparing the inhibition zone diameter data produced by the different antibiotics with and without a β-lactamase inhibitor.

2.5.1. Detection of hyper-produced cephalosporinase AmpC phenotype

All isolates were routinely subjected to the phenotypic test for cephalosporinase AmpC using the cefoxitin-cloxacillin combined disc as described by Tan et al (Tan et al., 2009). The test is based on the inhibitory effect of cloxacillin on the activity of the AmpC cephalosporinase. Cefoxitin (30 μg) discs supplemented with 200 μg of cloxacillin and only cefoxitin (30 μg) disc were used in the analysis. Isolates were considered AmpC producer if the antibiotic inhibition zone diameter (AIZD) produced by the cefoxitin+cloxacillin discs differ by 4 mm higher when compared to those of the cefoxitin discs alone.

2.5.2. Detection of extended-spectrum β-lactamase phenotype (ESBLs)

The combined disc method and the double disc synergy test (DDST) as recommended by CASFM/EUCAST (CASFM / EUCAST : Société Française de Microbiologie, 2016), were used to screen all isolates for the production of extended-spectrum β-lactamases.

- **The Double Disc Synergy Test (DDST)**

Double disc synergy test (DDST) for detection of ESBL is based increase in zone of inhibition around cefepime, cefotaxime, ceftazidime, ceftriaxone and aztreonam towards ticarcillin-clavulanic acid disc due to the ESBL inhibition potential of

clavulanic acid. Antibiotic discs ceftazidime, ceftriaxone, cefotaxime, cefepime and aztreonam each were placed about 30 mm around ticarcillin + clavulanic acid disc in pre-inoculated MHA plates. The plates were incubated aerobically at 37°C for 24 hours. The production of heart-shaped clear zones that distorts the zone of inhibition indicates positive results for ESBLs production

- **Combination Disc Method**

Ceftazidime (30 µg) and clavulanic acid (10 µg) combined disc and Ceftazidime (30 µg) were placed searately on pre-inoculated MHA plates, and which were later incubated aerobically at 37°C for 24 hours. Isolates were positive for the test if the AIZD produced by ceftazidime with clavulanic acid compared to that of ceftazidime alone is ≥ 5 mm.

2.5.3. Detection of the Metallo β-Lactamase (MBLs) phenotypes

All isolates that were categorized as intermediate resistant (I) or resistant (R) to imipenem and/or meropenem were subjected to the MBLs phenotypic test using the imipenem- Ethylene Diamine Tetra-Acetyl (EDTA) inhibition method as described by Yong et al. (Yong et al., 2002). The principle of the test is based on the potential of EDTA to inhibit metallo β-lactamase activity. In order to perform the test, EDTA was prepared by adding 24.193 x 10^3 µg of EDTA-$2H_2O$ disodium in 130x 10^3 µl of sterile distilled water so that 4 µl of the solution corresponds to 750 µg of 0.5 M EDTA per imipenem disc (Yong et al., 2002). The pH of the EDTA solution was adjusted to 8 with NaOH solution. Bacterial suspensions were prepared in 0.8% (w/v) saline solution to obtain a turbidity equivalent to 0.5 McFarland's standard and aliquots of 100 µL were inoculated on Muller Hinton agar (MHA) plates by spread-plate technique. Two imipenem (10 µg) discs were placed at 30 mm apart on the inoculated MHA plates. An aliquot of 4 µl EDTA solution corresponding to 750 µg of EDTA was added on one of the discs (Yong et al., 2002). The inoculated plates were incubated aerobically at 37°C for 24 hours. The AIZD produced by imipenem discs (10 µg) alone and imipenem + EDTA (10 + 750) µg discs were measured and used to determine the potential of isolates to produce MBLs. For MBLs producers, the AIZD from imipenem + EDTA must be ≥7 mm that produced by imipenem alone.

2.6. Biofilm formation assay

A total of 102 well identified isolates were selected based on their virulence gene and antibiotic resistance profiles, and screened for their potential to form biofilms using a standard method (Papa et al., 2018). Bacterial strains were grown aerobically at 37 ºC for 24 hours in Tryptic soy broth (TSB) and later diluted 1:100 in TSB. Aliquots of 200 µl of the diluted broth cultures were transferred into the wells of 96-well polystyrene microtitre plates in triplicates. The plates were incubated at 25°C and 37 º C for 24 hours. TSB broth without bacteria cultures was used as a negative control. After incubation, plates were washed twice with Phosphate-buffered saline (PBS) buffer to removed unattached cells and cells were stained with 200 µl of 1% (w/v) crystal violet for 1 hour. Plates were washed five times with sterile distilled water to remove excess crystal violet stain, drained and air-dried. An aliquot of 200 µl of 95% (v/v) alcohol (>99%, Sigma-Aldrich) added to each well in order to dissolve crystal violet bound to biofilms. The optical density ($OD_{630\ nm}$) was measured using an ELISA reader (HEALES MB-580). The mean OD of each sample was compared to the optical density of the negative control. Bacteria strains were classified as non-biofilm formers (ODs<ODc), weak biofilm formers (ODc<ODs<2ODc), moderate biofilm formers (2ODc<ODs<4ODc) and strong biofilm formers (ODs>4ODc) (Papa et al., 2018).

2.7. PCR-based detection of virulence and resistance determinants

The presence of *P. aeruginosa* and *A. baumannii* virulence determinants (*lasB*, *exoA*, *pslA* and *exoS*) and (*OmpA* and *csuE)* respectively were assessed by PCR assay with specific primers as mentioned in **Table 1**. The cycling conditions were based on previous protocols with slight modifications after protocols optimization. β-lactam resistance genes bla_{TEM} and bla_{CTXM} were also amplified using previously described protocols. PCR reactions were prepared as standard 25 µl volumes comprising 12.5 µL of 2X DreamTaq Green Master Mix, 0.25 µM of each primer, 1 µL of template DNA and RNase-nuclease free PCR water. Amplifications were performed using a Bio-Rad C1000 Touch^{TM} thermal cycler. All the PCR products were kept at 4°C before they were separated by electrophoresis on 1% (w/v) agarose gel. PCR amplicons were visualised using a Chemi-Doc Imaging System (BIO-RAD ChemiDoc^{MP} Imaging System, Hercules, California, USA).

2.8. Data analysis

Excel 2016 software was used to organise and draw diagrams. TIBCO Statistica v13.3 (StatSoft, TIBCO software Inc., USA) was used to build dendograms using AIZD. Khi-square test was made on SAS v. 9.4 to study the link between biofilm–formation ability of isolates and growth temperature 25˚C and 37˚C. Briefly, two variables were created using optical density obtained after biofilm formation assay carried out either at 37˚C or at 25˚C. A variable "group" refers to growth temperature and variable “biofilm” indicates the biofilm-formation ability. “Group A” was samples optical density (OD) obtained after incubation at 37˚C, and group B was samples optical density obtained after incubation at 25˚C. When there was biofilm formation value “1” was attributed and if there was, no biofilm formation value “0” was given. Each sample’s OD higher than blank OD was considered as a biofilm former. The hypothesis were as follows:

H0: There is independence between the two qualitative variables "group" and “biofilm”

H1: There is a link between the two qualitative variables "group" and “biofilm”

2.9. Whole genome sequencing

Two bacteria selected base on their MDR profil previously collected from Centre Pasteur du Cameroun were choosen for better understanding of the disparity between phenotypic observation and PCR based genotypic charactrsation of resistance mecanisms. They were named after University of Yaounde 1 (UY1), sample species (PSA) and after the sample origine (Broncho Aveolar Lavage): UY1PSABAL and UY1PSABAL2. They were revived on nutrient agar media prior to sequencing. UY1PSABAL and UY1PSABAL2 strains described in this report were multidrug resistant (MDR) and had respectively the following resistance pattern, PIP^R TIC^R TCC^R FEP^R CIP^R LEV^R AMK^R GEN^R NET^R TOB^R and PIP^R TZP^R TCC^R FEP^R CAZ^R MEM^R CIP^R as it was observed after antimicrobioal susceptibility testing.

2.9.1. Library preparation and sequencing

The draft genome of *Pseudomonas aeruginosa* UY1PSABAL and UY1PSABAL2 were obtained with whole-genome shotgun sequencing using an Illumina paired-end library with an average insert size of 300 bp. We used 50 ng of the DNA sample to prepare the library with the Nextera DNA sample preparation kit (Illumina). The sample was fragmented with an ultrasonication approach (Covaris). The resulting DNA fragments were size selected (300 to

800 bp) with AMPure XP beads. The fragments were then end repaired, and Illumina-specific adapter sequences were ligated to each fragment. The sample was indexed, and a second size selection step was performed. The sample was then quantified with a fluorometric method, diluted to a standard concentration (4 nM), and then sequenced on Illumina's MiSeq X Five platform according to the kit protocol.

2.9.2. Quality control, trimming, assembling and annotation

After sequencing, quality control was made using FastQC v.0.11.5 (Andrews, 2010). Sequenced genome was trimmed using Trimmomatic v.0.36 (Bolger et al., 2014), *de novo* genome assembling was performed with SPAdes genome assembler v.3.13 (Bankevich et al., 2012). The assembled contigs were annotated using Prokka v.2.1.1 annotation pipeline (Seemann, 2014) compared to RAST (Rapid Annotation using Subsytems Technology) server v.2.0 (Overbeek et al., 2013) and PATRIC v.3.6.2 (Wattam et al., 2016) annotation. Identification of the virulome, resistome, mobile genetic elements (MGEs) and secondary metabolites

Virulence and antimicrobial resistance determinant genes were retrieved by browsing features in Prokka (Seemann, 2014), RAST (Overbeek et al., 2013) and PATRIC (Wattam et al., 2016) annotations; ResFinder (Zankari et al., 2012) and Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2016) were also used for the same purposes. PHAge Search Tool Enhanced Release (PHASTER) web server (Arndt et al., 2016) was used for the rapid identification and annotation of prophage sequences within bacterial genomes. Predictive secondary metabolites were identified with AntiSMASH v.5.0 (Blin et al., 2019). Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and *cas* genes regions were investigated with the CRISPRone (Zhang and Ye, 2017) and to corroborate the results CRISPRFinder server (Grissa et al., 2007) were also used.

2.9.3. Phylogeny

Phylogenetic tree of UY1PSABAL and UY1PSABAL2 and twenty-four others randomly selected *P. aeruginosa* isolated from Homo sapiens between 2014 and 2016 in Africa, America, Asia and Europe was built to study evolutionary relation between those strains. PATRIC Fastree service (Wattam et al., 2016) following the codon tree protocols was used for this purpose. The newick result file was then opened in Figtree v1.4.3 software for tree view and annotation. Tips of generated tree were labelled according to the codified name, which consisted on strain's country of collection_Genbank accession number.

2.10. Ethical Approval

This study was approved by the Central Region Ethical comity. The approval number is 0493/CRESHC/2019.

CHAPTER 3: Results

3.1. Identification and distribution of isolates

Out of 79 presumed *Pseudomonas aerugonosa* collected, 77 presented both Gram morphology and biochemical characteristics of *Pseudomonas aerugonosa* and 27 presumed *Acinetobacter baumannii* showing *Acinetobacter baumannii* biochemical features. Out of the previous 77 presumed *Pseudomonas aerugonosa* 76 were confirmed by PCR as *Pseudomonas spp*. and out the 76 confirmed *Pseudomonas spp*. 75 were finally confirmed as *Pseudomonas aerugonosa*. Out of the 75 confirmed *P. aeruginosa* 9 were collected from YUTH, 6 were from YCH and 60 from CPC. All the 27 presumed *Acinetobacter baumannii* were confirmed with PCR as *Acinetobacter baumannii*, 4 were from YUTH and 23 from CPC.

Proportion of isolation of presumptive *P. aeruginosa* and *A. baumannii* isolates according to gender was identical either within female or within male (figure 28). The identities of a large proportion (75/77; 97.4%) of the *P. aeruginosa* and all (27/27; 100%) of the *A. baumannii* were positively confirmed by specific PCR as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* respectively. *P. aeruginosa* was frequently isolated in pus (33; 44%), urine (23; 30%), and broncho-alveolar-lavage (BAL) (13; 17%). On the contrary, *A. baumannii* was more frequently detected in blood (16; 59%), urine (5; 18%) and pus (5; 18%) table VI.

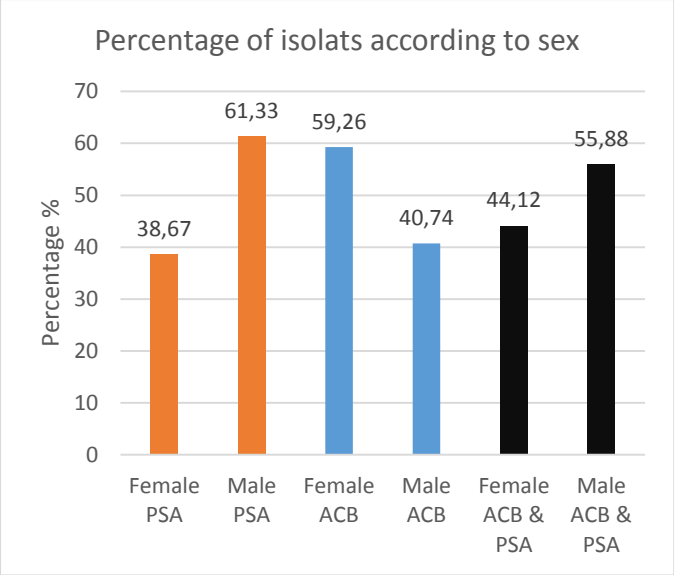


Figure 28: Percentage of isolation according to patient gender

Table VI: Frequency of isolates according to specimen origin

	specimen source	BAL	*HVS	Pus	Blood	Sperm	Urine	Total
Pseudomonas aeruginosa	frequency	13	0	33	5	1	23	75
	Percentage (%)	**17.33**	0	**44**	6.66	1.33	**30.66**	100
Acinetobacter baumannii	frequency	0	1	5	16	0	5	27
	Percentage (%)	0	3.7	**18.51**	**59.25**	0	**18.51**	100
Total	Effectives	13	1	38	21	1	28	102
	Percentage (%)	12.75	0.98	**37.25**	**20.59**	0.98	**27.45**	100

*HVS: High Vaginal Swab, BAL: Broncho-alveolar lavage

The frequency of isolation of *P. aeruginosa* and *A. baumannii* from patients was also determined using age groups as variables. There was generally no significant difference in the proportion of isolates detected in patients from the different sexes. On the contrary, *P. aeruginosa* and *A. baumannii* were more frequently isolated in young (≤ 15 years old) and elderly (≥ 60 years old) individuals (Figures 29.a and 29.b**)**.

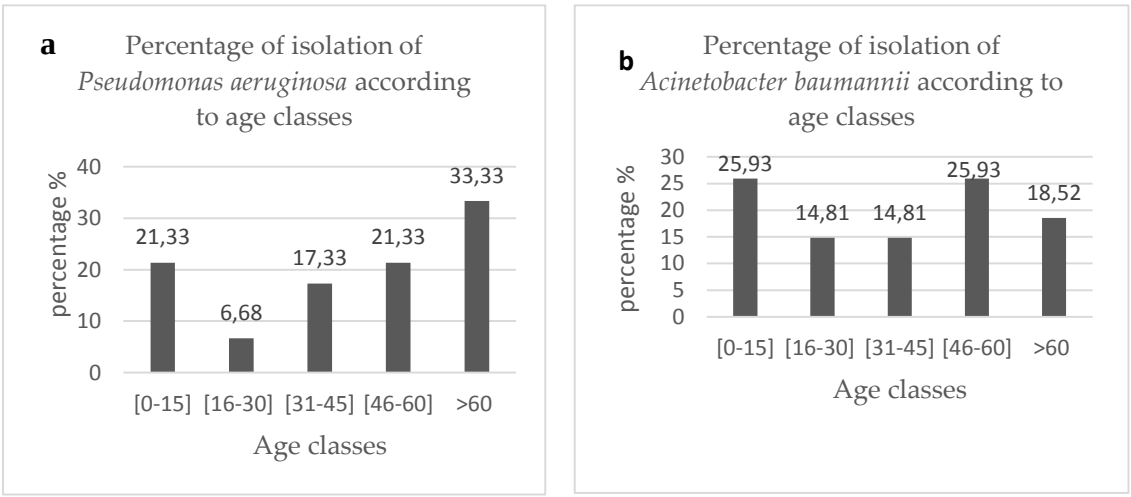


Figure 29: Percentage of bacteria isolation according to age classes. **a.** *Pseudomonas aeruginosa*; **b.** *Acinetobacter baumannii*

3.2. Biofilm formation assay

Large proportions (82%) of *P. aeruginosa* and (70 %) of *A. baumannii* were able to form biofilms at 37˚C and this ability was affected by a reduction of incubation temperature to 25˚C as the proportion of biofilm formers reduced especially for *A. baumannii* (Figures 30 and 31). Twenty-two percent of *P. aeruginosa* lost their potentials to form biofilms when the incubation temperature was decreased to 25˚C from 37˚C while 13% of *P. aeruginosa* also lost their biofilm forming capacity when the temperature was increased from 25˚C to 37˚C. Similar, fluctuations in biofilm forming potentials was observed for *A. baumannii* at the different incubation temperatures (37˚C to 25˚C) and (25˚ C to 37˚ C). Irrespective of the incubation temperature, most of the *P. aeruginosa* and *A. baumannii* isolates were moderate and weak biofilm formers respectively.

The p-value of the khi-square test between the variable "group" and the variable "biofilm" is 0.0056 in *A. baumannii*. Since this p-value is below the 5% threshold, the null hypothesis of independence between temperature (37˚C or 25˚C) and biofilm formation is rejected. There is therefore a link between biofilm formation and temperature for *A. baumannii*. Probability for *A. baumannii* to form biofilm at 37˚C is 65% and this probability is 27% at 25˚C. Biofilm ability of *A. baumannii* is then higher at 37˚C. On the other hand, concerning *P. aeruginosa*, the p-value is 0.0761, which is greater than the threshold of 5%; the null hypothesis of independence between the variables "group" and "biofilm" in this case is not rejected. Thus, In *P. aeruginosa*, there is no link between the “group” and “biofilm” formation. In others words, the biofilm ability of *P. aeruginosa* is not link to growth temperature.

This experiment showed that either at room temperature or body temperature *P. aeruginosa* and *A. baumannii* are able to build biofilm aggregats.

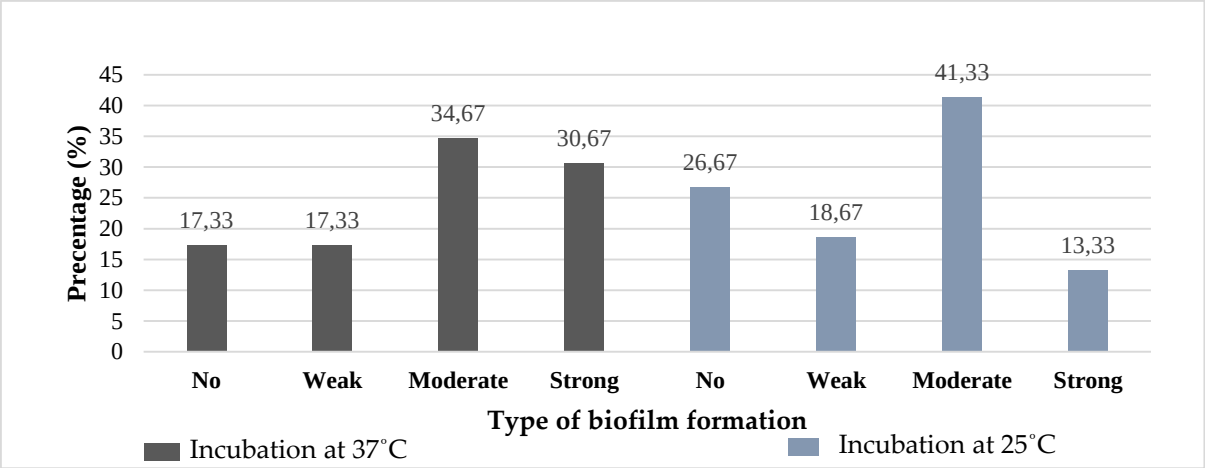


Figure 30: Percentage of *Pseudomonas aeruginosa* according to biofilm formation ability

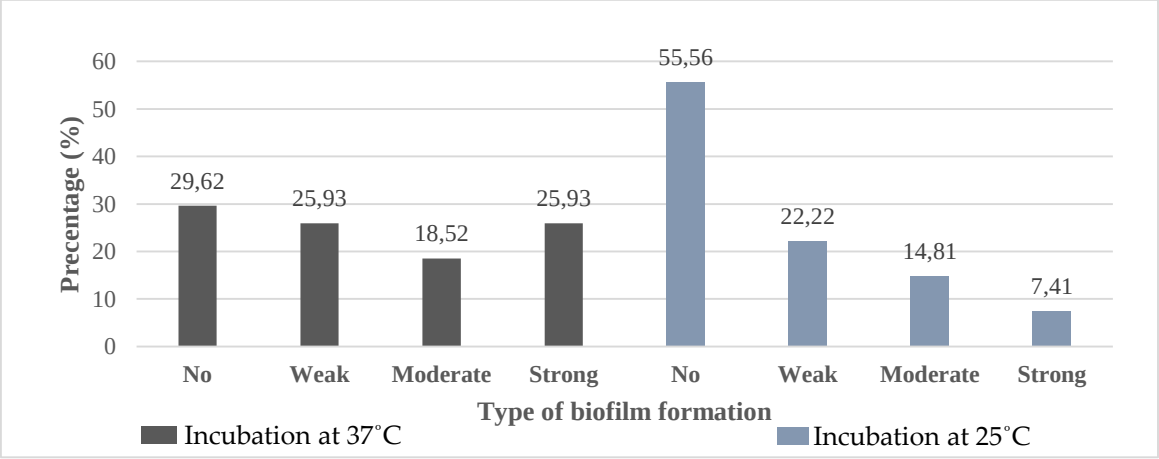


Figure 31: Percentage of *Acinetobacter baumannii* according to biofilm formation ability

3.3. Phenotypic cluster analysis of isolates based on AIZD

In order to determine the phenotypic relationships based on AIZD of *P. aeruginosa* and *A. baumannii*, isolates were subjected to a cluster analysis. The dendrograms in Figures 32 and 33 reveal 2 major clusters (1 and 2) for each species and each cluster is sub-divided in 2 sub-clusters (A and B) (Figure 32 and 33). The clusters were analysed for patterns of associations of isolates from the sources and data is presented in Table VII. A large proportion 60 (80%) of *P. aeruginosa* belong to cluster 1. Distribution of *P. aeruginosa* on its dendogram was matched with isolates source (Table VII), it was observed that 25(75%) of pus isolates, 11(84%) of BAL are found in cluster 1. This observation reveals also that 4(80%) of blood isolates belong to sub cluster 1A and 15(65%) of urine are found in cluster 1B. When *P. aeruginosa* dendogram distribution was then matched with results of biofilm formation assay (incubation done at 37˚C)

(Table VII) it reveals that 53 % of non- biofilm formers were included in one sub-cluster; sub-cluster 1B.

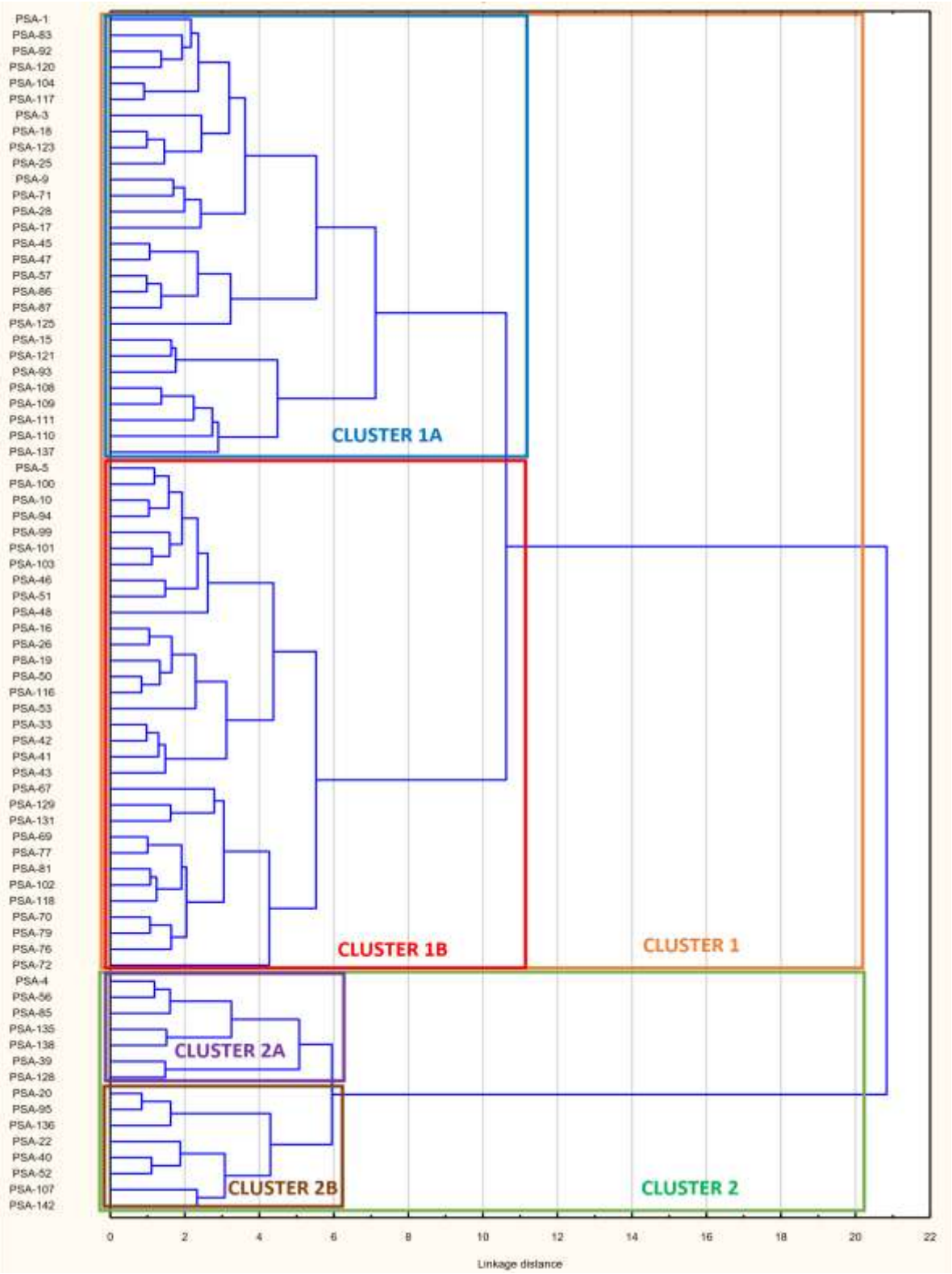


Figure 32: Relationship between *P. aeruginosa* Isolates based on their antibiotic inhibition zone diameter (AIZD)

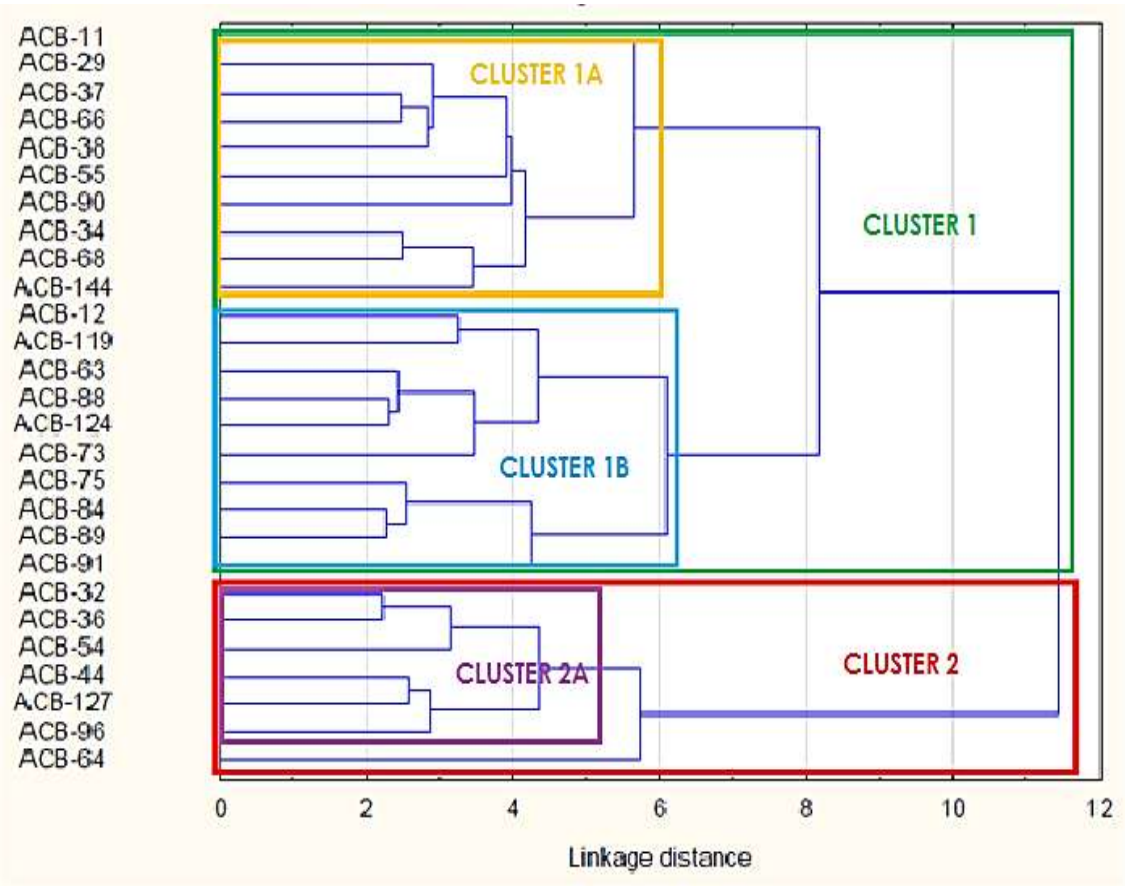


Figure 33: Relationship between *A. baumannii* Isolates based on their antibiotic inhibition zone diameter (AIZD)

The clusters represent isolates with similar antimicrobial resistance or susceptivity pattern.

The generated dendrogram was analysed and the results are shown in Table VII

Table VII: *P. aeruginosa* designations based on sampling origin and biofilm formation ability

Cluster 1A N: 28 (37%)	Isolates N* (%)**	**Pus: 14(42)** **Blood: 4(80)** **BAL: 7(53)** **Urine: 3(13)**	**Cluster 2A** N: 7(9%)	Isolates N (%)	Pus: 2(3) Blood: 0(0) Urine: 4(17) BAL: 1(7)
	Biofilm formation type at 37˚C	S: 9(39) M: 12 (46) W: 5 (38) No: 2(15)		Biofilm formation type at 37˚C	S: 4(17) M: 1 (3) W: 1(7) No: 1(7)
Cluster 1B N: 32 (42%)	Isolates N **(%)**	**Pus: 11(33)** Blood: 1(20) Urine: 15(65) **BAL: 4(30)** Sperm: 1(100)	**Cluster 2B** N: 8(10%)	Isolates N:(%)	Pus: 6(18) Blood: 0(0) Urine: 1(4) BAL: 1(7)
	Biofilm formation type at 37˚C	S: 7(30.23) M: 11(42) W: 7(53) **No: 7(53%)**		Biofilm formation type at 37˚C	S: 3(11) M: 2(7) W: 0(0) No: 3(23)

N*: number; (%)**: percentage of the considered observation out of the total number of this observation ; S: strong; M: moderate; W: weak; No: no biofilm formation ability.

3.4. Antibiotic Resistance Profiles

In this study, approximately 50 % of *P. aeruginosa* isolates showed resistance to antibiotics belonging to the penam and cefem subfamilies. Resistance to carbapenems, fluoroquinolones and aminoglycosides was observed among 26%, 34% and 27% of the isolates, respectively. *P. aeruginosa* showed lowest level of resistance to meropenem 22%. The majority (13 of 20) of antibiotics used for AST of *A. baumannii* recorded above 70% resistance frequency while

minocycline showed the lowest resistance rate with a frequency of 46 %. It was observed that 40% of the isolates were resistant to carbapenem, more than 70 % were resistant to fluoroquinolone and aminoglycoside and about 60 % of resistance to tetracycline family. Figure 34 and 35 present percentage of *P. aeruginosa* and *A. baumannii* isolates antibiotics resistance respectively

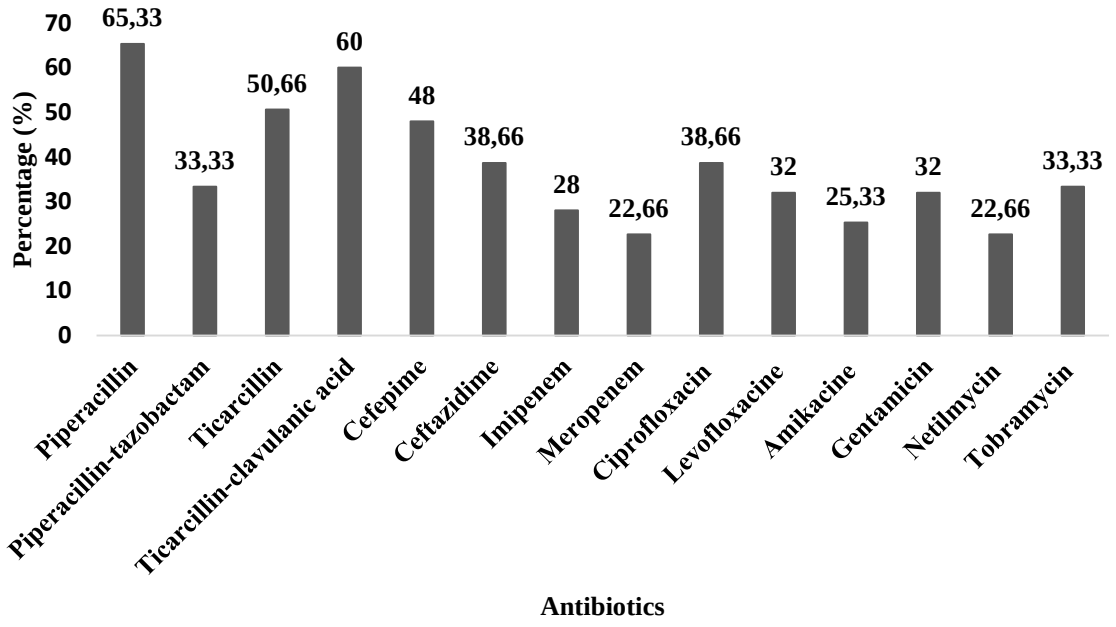


Figure 34: Antibiotic Resistance of *Pseudomonas aeruginosa*

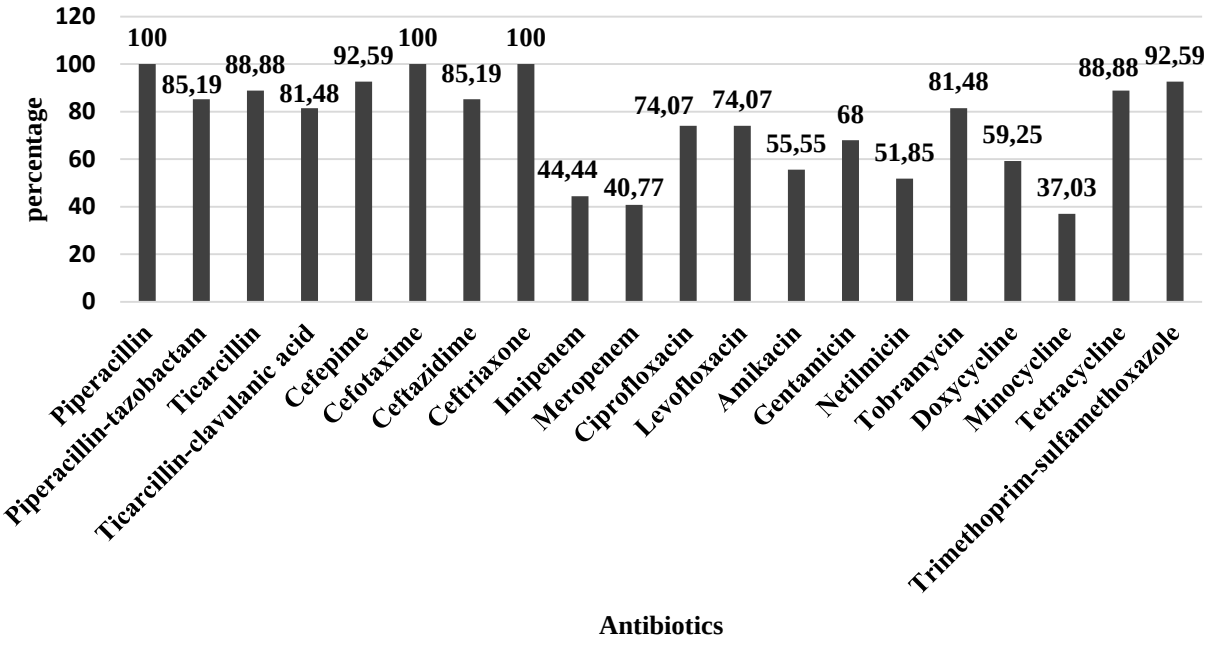


Figure 35: Antibiotic Resistance of *Acinetobacter baumannii*

3.5. Drug modifying-enzymes

While 11 % of *P. aeruginosa* produced the AmpC enzyme none of the *A. baumannii* was positive for AmpC (figure 36) production, higher proportions (25 %) and (18%) of the *P. aeruginosa* and *A. baumannii* were positive for phenotypic production of ESBLs. ESBLs production was detected only with combined disc method (figure 37), no positive result was found with DDST. Despite that, 6% of the *P. aeruginosa* were positive for MBLs (figure 38), none of the *A. baumannii* produced these enzymes. A large proportion (66%) of *P. aeruginosa* and all (100%) of the *A. baumannii* displayed multidrug-resistance phenotypes.

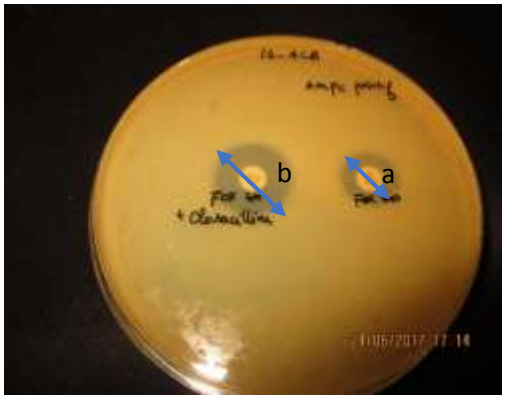


Figure 36: AmpC positive strain: a = 9 mm; b = 13 mm

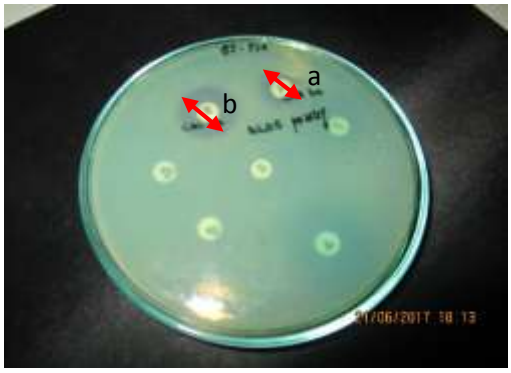


Figure 37: ESBL Positive strain with combined disc method a = 7 mm; b = 12 mm

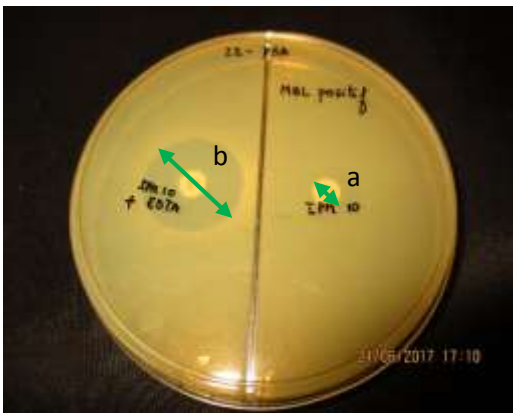
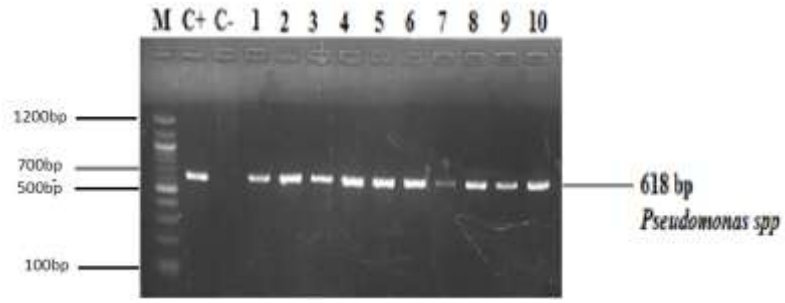


Figure 38: MBL positive strain a = 6 mm; b = 14 mm

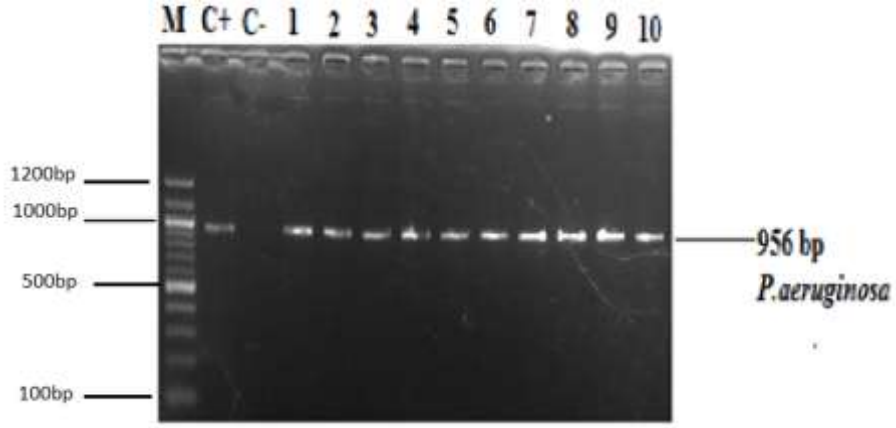
3.6. Virulence genes identification

lasB, *exoA*, *pslA* and *exoS* virulence genes were amplified in more than 90% of *P. aeruginosa*. *OmpA* and *csuE* virulence genes were identify on 88% of *A. baumannii*. Figures 39 to 49 show the gel electrophoresis images of identification genes and virulence genes amplified in *P. aeruginosa* and/or *A. baumannii* on 1% agarose gel.



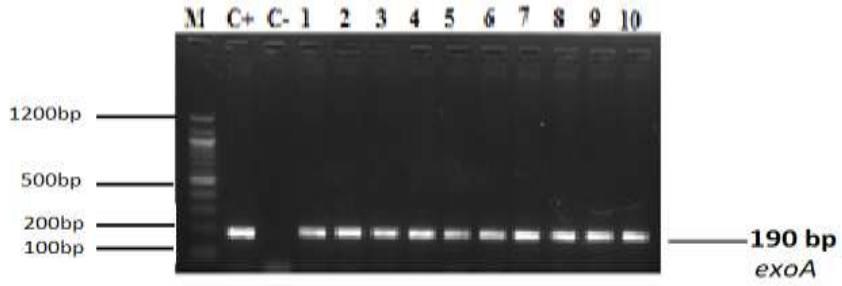
M: 100 bp ladder, C+: Positive control; C-: negative control; from 1 to 10 are 10 representative samples

Figure 39: Gel electrophoregram of the *Pseudomonas Spp* amplicon



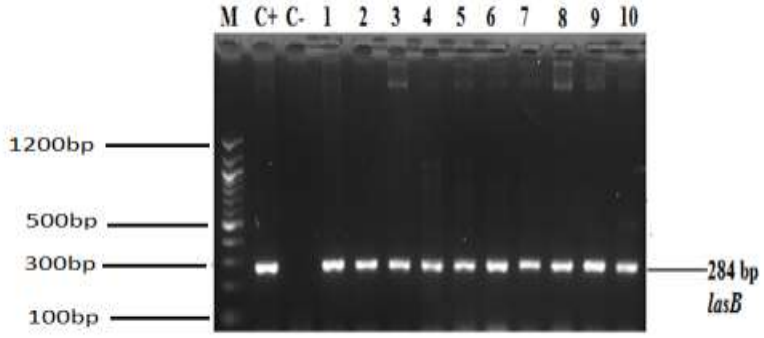
M: 100 bp ladder, C+: Positive control; C-: negative control; from 1 to 10 are 10 representative samples

Figure 40: Gel electrophoregram of the *Pseudomonas aeruginosa* amplicon



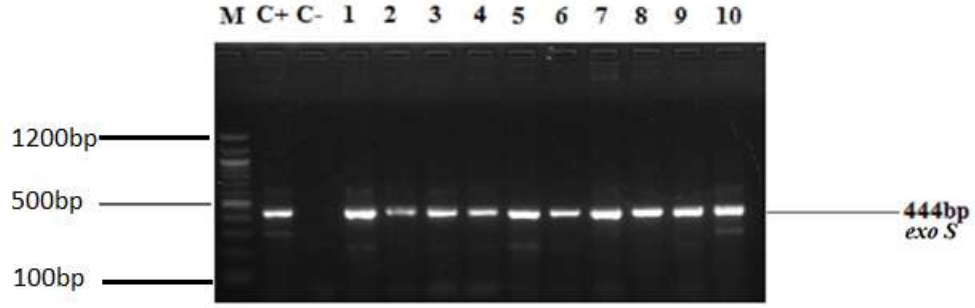
M: 100 bp ladder, C+: Positive control; C-: negative control; from 1 to 10 are 10 representative samples

Figure 41: Gel electrophoregram of the *exoA* amplicon



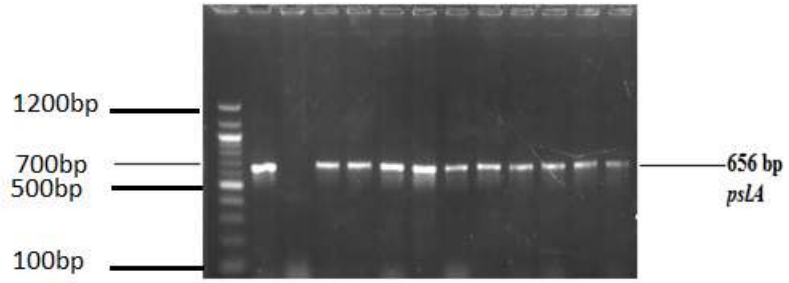
M: 100 bp ladder, C+: Positive control; C-: negative control; from 1 to 10 are 10 representative samples

Figure 42: Gel electrophoregram of the *lasB* amplicon



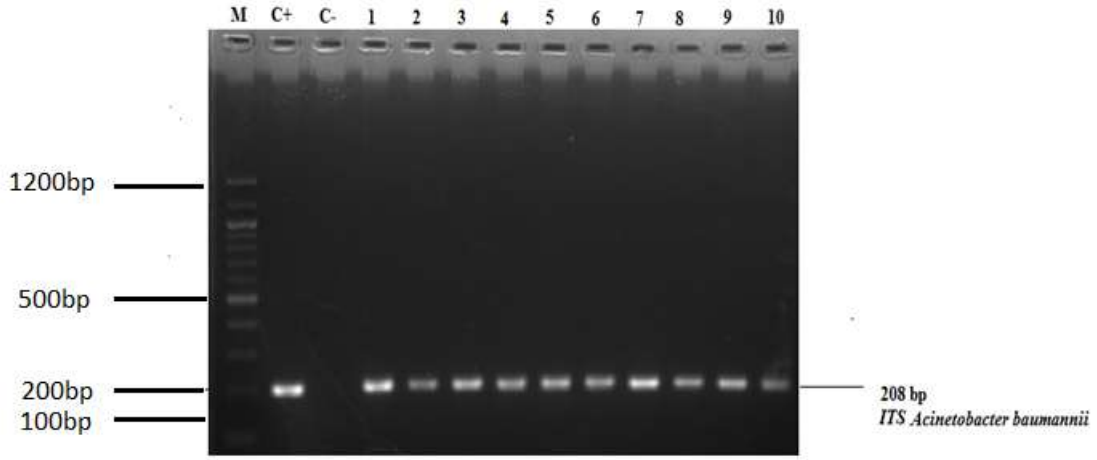
M: 100 bp ladder, C+: Positive control; C-: negative control; from 1 to 10 are 10 representative samples

Figure 43: Gel electrophoregram of the *exoS* amplicon



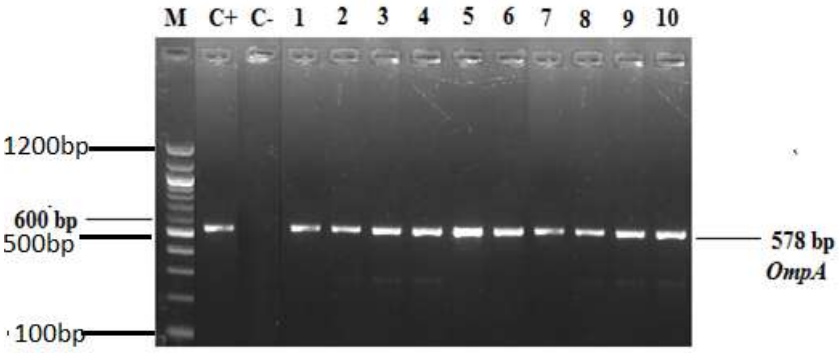
M: 100 bp ladder, C+: Positive control; C-: negative control; from 1 to 10 are 10 representative samples

Figure 44:Gel electrophoregram of the *pslA* amplicon



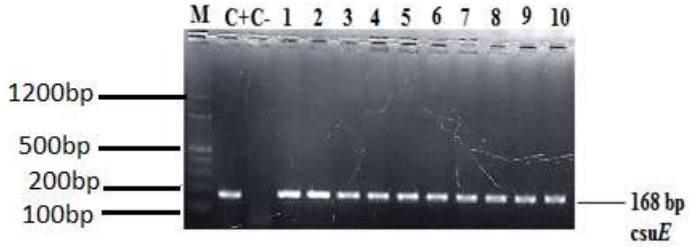
M: 100 bp ladder, C+: Positive control; C-: negative control; from 1 to 10 are 10 representative samples

Figure 46: Gel electrophoregram of the *ITS* specific for *Acinetobacter baumannii* amplicon



M: 100 bp ladder, C+: Positive control; C-: negative control; from 1 to 10 are 10 representative samples

Figure 47: Gel electrophoregram *OmpA* amplicon

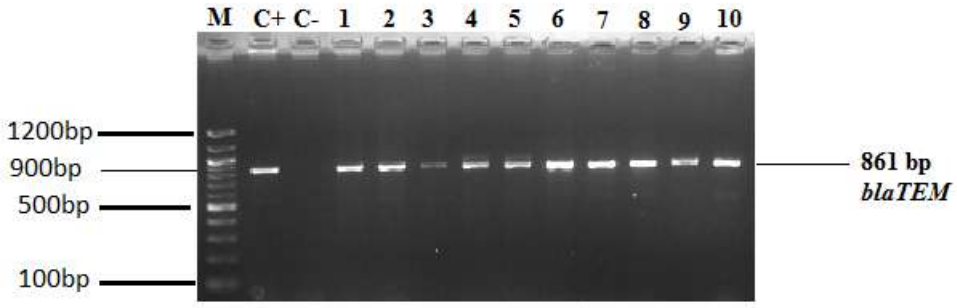


M: 100 bp ladder, C+: Positive control; C-: negative control; from 1 to 10 are 10 representative samples

Figure 48: Gel electrophoregram *csuE* amplicon

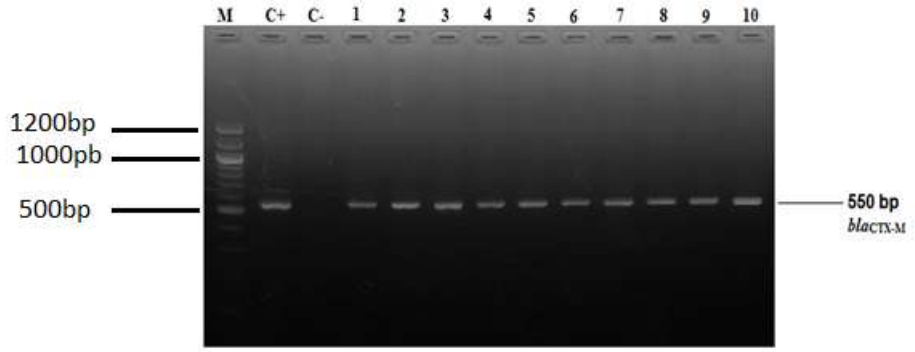
3.7. Resistance genes identification

Resistance genes bla_{TEM} and bla_{CTX-M} were respectively found among (13)17% and (3)4% *P. aeruginosa*. Those genes were present among *A. baumannii* with a proportion of (19)70% of bla_{TEM} and (8)29% of bla_{CTXM}.



M: 100 bp ladder, C+: Positive control; C-: negative control; from 1 to 10 are 10 representative samples

Figure 49: Gel electrophoregram of the *blaTEM* amplicon



M: 100 bp ladder, C+: Positive control; C-: negative control; from 1 to 10 are 10 representative samples

Figure 50: Gel electrophoregram of the *bla*$_{CTX-M}$ amplicon

3.8. Whole genome sequencing

3.8.1. Genome annotation features

The draft assembly of entire genome for UY1PSABAL and UY1PSABAL2 were developed using Spades and annotated using RAST, Prokka and Patric.

Summary of draft genome assembly features of UY1PSABAL and UY1PSABAL2 are shown in the table VIII below and the circular genome representing the entire assembly is shown in figure 50.

Table VIII: Summary of the draft genome assembly of UY1PSABAL and UY1PSABAL2

	Size	**GC content**	**Number of contigs**	***CDSs**	**number RNAs**
UY1PSABAL	7 029 327	66.1%	136	6907	63
UY1PSABAL2	6,354,435	66.48%	96	6046	63

*CDSs: coding sequences

UY1PSABAL

UY1PSABAL2

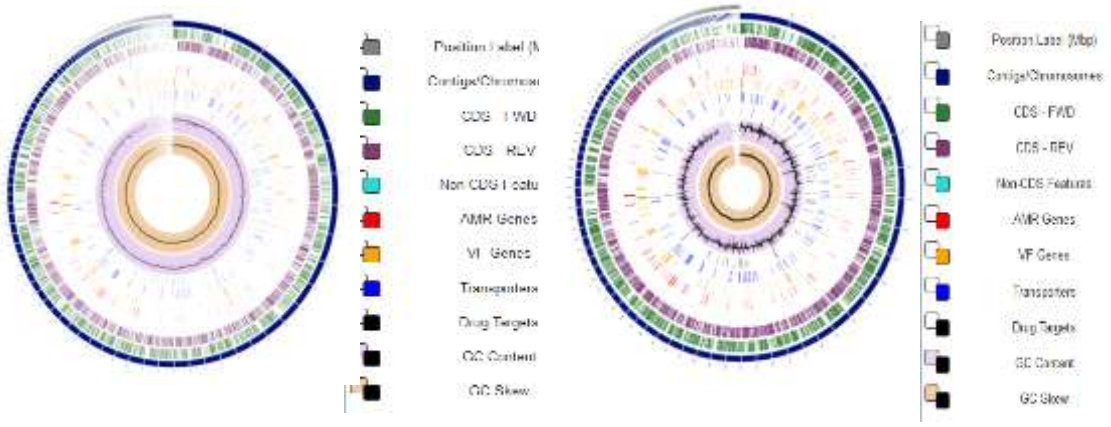


Figure 51: Circular genome map of the UY1PSABAL and UY1PSABAL2. The circular genome was generated with PATRIC sever 3.6.2

3.8.2. Identification of the virulome

The protein encoding sequences putative for flagella protein biosynthesis, adherence motility, endotoxin, Type IV pili Adherence Twitching motility, ion uptake, Antiphagocytosis (Serum resistance), Type I secretion system, Type II secretion system, Type III secretion system and Type VI secretion system among the others were found common to both isolates (table IX).

Table IX: Major findings of virulence genes within UY1PSABAL and UY1PSABAL2 genomes

Virulence factors genes	Function	Strains
motA, , motB, motD, flgB, flgI, fliF fliR, fliE, fliQ, flgC, flgF, fliM, fliO, fleN, fliL, fliJ, flgG, fliG, flgK, fleR, flhF, flgE.	Flagelle motor protein biosynthesis; Flagellar M-ring protein FliF; Adherence, motility	UY1PSABAL UY1PSABAL2
flgP, fleH, fpvA	Adherence, motility	UY1PSABAL
fliD, fliH, flgJ,flgH, flgL, fleQ, fliN flhA, flhB, flgD,	Adherence, motility	UY1PSABAL2
waaP, waaA, waaG, waaC, waaF,	Adherence, endotoxin	UY1PSABAL UY1PSABAL2
pilH, pilS, pilC, pilG, pilF, pilK, pilM, xcpA/pilD, pilP, pilJ, chpD, pilT,, , chpA, pilR, pilA, chpB, chpC pilU, pilN, pilO, pilX, pilE pilI, pilB, pilW, fimU	Type IV pili Adherence Twitching motility	UY1PSABAL UY1PSABAL2
chpE ,fimV, pill	Type IV pili Adherence Twitching motility	UY1PSABAL2
aprF	protease exporter	UY1PSABAL UY1PSABAL2
PhzA	Phenazine biosynthesis protein	UY1PSABAL UY1PSABAL2
AlgR	Alginate biosynthesis two-component system response regulator AlgR	UY1PSABAL UY1PSABAL2
AlgK	Alginate export system AlgK/AlgE, periplasmic component AlgK	UY1PSABAL UY1PSABAL2
AlgQ	Alginate regulatory protein AlgQ, positive transcriptional regulator of AlgD	UY1PSABAL UY1PSABAL2

PhzC 1	2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase II PhzC	UY1PSABAL UY1PSABAL2
estA	Phospholipase/lecithinase/hemolysin	UY1PSABAL UY1PSABAL2
modA	Molybdenum ABC transporter, substrate-binding protein ModA	UY1PSABAL UY1PSABAL2
LasR	homoserine lactone-binding transcriptional activator involved in quorum sensing	UY1PSABAL UY1PSABAL2
narG	(Respiratory nitrate reductase alpha chain) for Biofilm formation	UY1PSABAL
pchR , pchA, pchC, , pchF, fptA, pchH, pchD, pvdA, pvdS,	ion uptake	UY1PSABAL UY1PSABAL2
pvdD, pvdE, fpvA,	ion uptake	UY1PSABAL
pchI, pchG, pchE, pchB,	ion uptake	UY1PSABAL2
algL, , alg8, algD, algJ, alg44, algG, mucB, algA, algE, algK, algQ, , algU, algF, mucC, algl, algB, algR, algZ, algX,	Antiphagocytosis (Serum resistance)	UY1PSABAL UY1PSABAL2
mucA	Antiphagocytosis (Serum resistance)	UY1PSABAL
algP/algR3, algE	Antiphagocytosis (Serum resistance)	UY1PSABAL2
exoT, toxA,	Toxin, Type III translocated protein,Intracellular toxin,ADP-ribosyltransferase and GTPase activating	UY1PSABAL UY1PSABAL2
exoU, exoY		UY1PSABAL
exoS		UY1PSABAL2
aprF, LapC, ATP-binding component PrtD/AprD, LapB, LapE,	Type I secretion system	UY1PSABAL UY1PSABAL2
aprF	Type I secretion system	UY1PSABAL

outer membrane component PrtF/AprF,	Type I secretion system	UY1PSABAL2
xcpX, xcpV, xcpW, xcpY, xcpS, xcpZ,	Type II secretion system	UY1PSABAL UY1PSABAL2
xcpT,	Type II secretion system	UY1PSABAL
xcpR, xcpP, xcpQ, xcpU	Type II secretion system	UY1PSABAL2
pscJ , pscD, popN, pscQ, pscK, pscP, pcr2, , exsD, pscF, pscB, exsA, pscN, exsB, pcrG, pcr3, pcr4, pcrV, popD, pscC, pscG, pscE, exsC, pscU, pcrR, pscS, pscR, pcrH	Type III secretion system	UY1PSABAL UY1PSABAL2
pcr1, lip1, pscI, xcpX, pcrD,	Type III secretion system	UY1PSABAL
pscL, pscH, popB, pscL, pscT, psc1, exsE, pscO,	Type III secretion system	UY1PSABAL2
tagR, vgrG1a, pppA, fha1, ppkA, hsiF1, HsiC1/vipB, hsiG1, hsiE1, hsiH1, icmF1/tssM1, lip1, hsiA1, hcp1, tagF/pppB, hsiJ1, clpV1, dotU1, tagS, tagT, hsiB1/vipA, PA0082	Type VI secretion system	UY1PSABAL UY1PSABAL2
lasI, rhlI	Regulation, Quorum sensing system	UY1PSABAL UY1PSABAL2
PqsE	*quinolone signal response protein,* Quorum sensing system	
lasA, lasB	extracellular zinc protease	UY1PSABAL UY1PSABAL2
aprA	Metallo-proteinase	UY1PSABAL

		UY1PSABAL2
rhlA, rhlB	rhlA rhlB TDP-rhamnosyltransferase 1 (Biosurfactant)	UY1PSABAL
		UY1PSABAL2
PhzH, PhzM	Phenazine-specific methyltransferase PhzH, PhzM	UY1PSABAL
		UY1PSABAL2
metE	homocysteine methyltransferase	UY1PSABAL
		UY1PSABAL2
phzG1	Pyridoxamine 5'-phosphate oxidase PhzG	UY1PSABAL
		UY1PSABAL2
PhzD	2-amino-2-deoxy-isochorismate hydrolase PhzD	UY1PSABAL
		UY1PSABAL2
PlcH	Haemolytic PlcH	UY1PSABAL
		UY1PSABAL2

3.8.3. Identification of the resistome

A number of resistance genes harbored within the genomes of the two strains were identified through functional annotation generated from different platforms and as well as ResFinder. Nine predicted regions for levofloxacin resistance were detected and other genes that confer resistance to quinolones such as *crpP*, *gyrA (*variant/mutant), *parE* (variant/mutant) were detected within UY1PSABAL genome; while on the other hand *basS* was found in the genome of UY1PSABAL2 (table X). Both organisms were found bearing resistance gene to β-lactams aminoglycosides, tetracyclines, fosfomycins and glycopeptides resistance genes.

Table X: Different resistance genes found within UY1PSABAL and UY1PSABAL2 genomes

Antibiotics	**Putative resistance gens**	
	***P. aeruginosa* UY1PSABAL**	***P. aeruginosa* UY1PSABAL2**
β-lactams	*blaPAO*, *blaOXA*-395	*blaPAO*, *blaOXA*-486, *blaOXA*-50
Quinolone	*crpP*, *gyrA* (variant/mutant), *parE* (variant/mutant) and nine regions for levofloxacin resistance	None
Aminoglycoside	*aph(3')*-IIb, *aph(3'')*-Ib,*aph(6')*-Id, *rmtB*	*aph(3')*-IIb

Tetracycline	*tet(G)*, Class B [*tet(A)_2*, *tetR*], Class C [*tet(A)_1*, *tet(A)_3*]	Class B [*tet(A)_2*], Class C [*tet(A)_1*]
Fosfomycin	*fosA*	*fosA*
Glycopeptide	Vacomycin type-B: *vanW*	Vacomycin type-B: *vanW*
Phenicol	*catB7*	*catB7*
Sulphonamide	*sulI*	*sulI*
Peptide antibiotic	None	*basS*
Efflux pump	*emrE*, *cpxR*, *muxB*, *muxC*, *opmB*, *soxR*, *oprM*, *mexA*, *triB*, *mexG*	*emrE*, *cpxR*, *muxC*, *soxR*, *oprM*, *mexG*, *pmpM*, *opmH*, *mexC*, *mexJ*, *mexL*, *armR*, *mexA*, *oprN*, *mexF*, *triC*, *triA*, *bcr-1*, *mexH*, *mexI*, *opmD*

3.8.4. Identification of the Mobile genetic elements (MGE)

- **Transposable elements**

Transposase InsN, InsO for IS911 and IS407 were found in various position on UY1PSABAL and UY1PSABAL2. Tn4652 transposase was only found in UY1PSABAL Presence of these transposase means that on UY1PSABAL and UY1PSABAL2 harboured insertion sequence and transposon for UY1PSABAL2 particularly. The list of transposase found within the two isolates are shown in table XI.

Table XI: Major findings of transposases genes within UY1PSABAL and UY1PSABAL2 genomes

Transposase	**Node**	**Length (bp)**	**start**	**stop**	**Strand**
UY1PSABAL					
Transposase InsN for IS911	102	60	2578	2637	-ve
Transposase	108	368	1213	846	-ve
Transposase	114	65	3	63	-ve
Transposase	117	819	55	765	+ve
Transposase InsO for IS911	119	782	666	117	-ve
Transposase	134	3	518	516	-ve
Transposase	15	55615	55454	162	-ve

Transposase	15	56437	55628	810	-ve
Transposase	16	298	2	297	+ve
Transposase	1	365468	366343	876	+ve
Transposase	22	22495	22259	237	-ve
Transposase	27	2195	5161	2967	-ve
Transposase	36	64700	67585	2886	+ve
Transposase InsN for IS911	38	63	1	63	+ve
Transposase	38	15372	16307	936	+ve
Transposase	3	1405	2	1404	-ve
Transposase and inactivated derivatives	3	172849	172316	534	-ve
Transposase and inactivated derivatives	44	11073	11774	702	+ve
Phage transposase	46	20617	18833	1785	-ve
Transposase InsO for IS911	47	53445	53329	117	-ve
Tn4652, Transposase	64	23385	20371	3015	-ve
Transposase InsO for IS911	69	3	1847	1845	+ve
Transposase InsO for IS911	70	16012	15896	117	-ve
Transposase	71	65	3	63	-ve
Transposase InsN for IS911	73	63	1	63	-ve
Transposase InsN for IS911	78	7963	8022	60	+ve
Transposase	83	5966	5175	792	-ve
Transposase Insertion element IS407	8	198848	199111	264	+ve
TniA putative Transposase	99	2805	2404	402	-ve

Transposase InsO for IS911	9	33	860	828	-ve
Transposase and inactivated derivatives UY1PSABAL2	9	170968	170366	603	-ve
Transposase InsN for IS911	11	182212	182415	204	+ve
Transposase InsN for IS911	17	122464	122523	60	+ve
Transposase and inactivated derivatives	1	228928	229362	435	+ve
Transposase InsN for IS911	1	570256	570140	117	-ve
Transposase InsN for IS911	22	98495	98554	60	+ve
Transposase and inactivated derivatives	29	28904	28302	603	-ve
transposase-like protein	35	2730	3008	279	+ve
Transposase InsN for IS911	45	60	1	60	-ve
Transposase InsO for IS911	46	35	229	195	+ve
Transposase InsO for IS911	46	25622	25428	195	-ve
Transposase	54	6148	6351	204	+ve
Transposase InsO for IS911	72	1118	1002	117	-ve
Transposase InsO for IS911	78	369	1	369	-ve
Transposase InsO for IS911	78	629	402	228	+ve

transposase-like protein	7	232153	232254	102	+ve
Transposase InsO for IS911	88	3	119	117	+ve
	9	189289	188981	309	+ve
Insertion element IS407 (Burkholderia multivorans) transposase	9	190540	190277	264	-ve

- **Prophage and CRISPR arrays**

Prophage search by PHASTER showed the presence of coding sequences (CDS) which are predictive for five intact, three questionable and two incomplete prophages in UY1PSABAL (figure 3.23). On the other hand, only one putative CDS was found each for intact, questionable and incomplete phages within the genome of UY1PSABAL2 (figure 3.24). We searched for prophage related genes in RAST annotation report and we found two hundreds and sixty-three phage related genes in UYPSABAL and eighty-three only identified on UYPSABAL2.

The analysis of our strains with CRISPRone showed the presence of one questionable CRISPR array on UY1PSABAL and two confirm CRISPR arrays UY1PSABAL2 with *cas* associate genes. The result of CRISPRone is consigned in the table XII.

Figure 52: Genome mapping of the UY1PSABAL showing the region and position occupied by the different types of prophages identified as as intact (), incomplete () and questionable ()

Figure 53: Genome mapping of the UY1PSABAL2 showing the region and position occupied by the different types of prophages identified as intact (), incomplete () and questionnable ().

The CRISPR-One software described besides CRSIPR system six cas protein genes cas1, cas3, cas8f, cas5f, cas7f and cas6f within UY1PSABAL2 genome.

Table XII: Putative CRISPR-Cas sequences found within UY1PSABAL and UY1PSABAL2 genomes

Contig no	CRISPR location	No of repeats	Spacer counts	Status	Strain identity
20	12208 - 12312	2	1	Questionable	
44	14030-14177	2	1	Questionable	UY1PSABAL
31	6895 - 7248	7	6	Questionable	
2	276731 - 276833	2	1	Questionable	
21	12226 - 12339	2	1	Questionable	
5	62269 - 62897	11	10	Confirmed	UY1PSABAL2
6	182441 - 183789	23	22	Confirmed	
6	192664 - 194308	27	31	Confirmed	

3.8.5. Secondary metabolites

Determination of secondary metabolites within the two isolates were carried out using AntiSMASH v.5.0 (Blin et al., 2019). Nine different secondary metabolites were found distributed on seventeen gene clusters and fifteen gene clusters within the draft genome UY1PSABAL and UY1PSABAL2 respectively. Many of the identified secondary metabolites are shared by both isolates. The secondary metabolite found common to the isolates were NRPS–like betalactone cluster, Homoserine lactone, bacteriocin, Non-ribosomal peptide synthetase cluster (NRPS), NRPS–like, N-acetylglutaminylglutamine amide (NAGGN), thiopeptide and phenazine. The two of NRPS found in both isolates were identified with 100% similarity, to be 2-amino-4-methoxy-*trans*-3-butenoic acid and pyochelin. Cyclodipeptide synthetase (CDPS) is the secondary metabolite, which is found only in UY1PSABAL2.

3.8.6. Phylogeny

Evolutionary similarity between UY1PSABAL and UY1PSABAL2 with other selected strains of *Pseudomonas aeruginosa* are shown in figure 3.25.

Analysis of generated phylogenetic tree showed that UY1PSABAL (Cameroon_VLHS01000000) and Malaysia_MPCQ00000000 belong to the same clade and both have about 0.9% divergence compared to common ancestor. UY1PSABAL2 (Cameroon_VLHR01000000) strain and China_VWQQ01000000 belong to the same clade. They are closely derived from the common ancestor with less divergence percentage (about 0.3%). Comparing to strains sampled for this phylogenetic tree building, the present study strains are closely related to Asian strains.

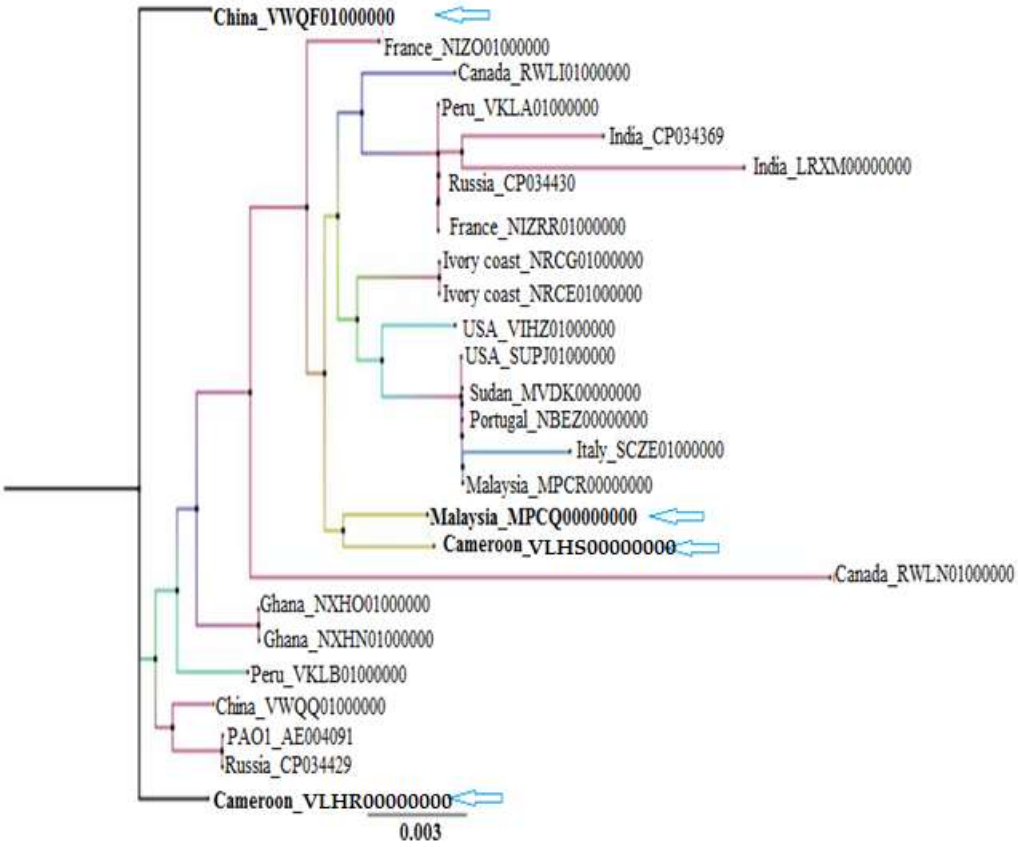


Figure 54: The phylogenetic tree showing the evolutionary similarity between UY1PSABAL and UY1PSABAL2 and other selected strains of *Pseudomonas aeruginosa.* The tree was generated using PATRIC

3.9. Data availability

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession VLHS00000000 and VLHR00000000 for UY1PSABAL and UY1PSABAL2 respectively. The version described in this thesis is the first version. Raw sequencing data sets have been registered in the NCBI Sequence Read Archive.

Table XIII: Sequence strains NCBI credentials

Items	UY1PSABAL	UY1PSABAL2
Biosample	SAMN12261616	SAMN12273497
Bioproject	PRJNA554269	PRJNA554541
SRA	SRP214447	SRP214569
Accession Number	VLHS00000000	VLHR00000000

CHAPTER 4: Discussion

Pseudomonas aeruginosa and *Acinetobacter baumannii* are nowadays become important pathogen especially in health care associated infections. There have been incriminated in surgical wounds infections (Streeter and Katouli, 2016) and in pulmonary infection especially within nosocomial cases (Lee et al., 2019). Highest isolation rate of *P. aeruginosa* observed in pus and urine (44% and 30%) in this study corroborated with the results got in studies made in Ethiopia and Cameroon (Gangoue-Pieboji et al., 2006, Alemayehu et al., 2019). However, *A. baumannii* isolation in this report isolation rate was higher in blood samples and pus / urine (59% and 18%). This finding differs from Alemayehu et al., (Alemayehu et al., 2019) results who isolated *A. baumannii* frequently in urine and contrast with the findings of Alkasaby et al. (Alkasaby and El Sayed Zaki, 2017a) in Egypt who rather recorded higher isolation rate in endotracheal secretion. *P. aeruginosa* and *A. baumannii* are known to be opportunistic pathogens; it was therefore coherent to isolate them in patients within the age groups 0-15 years, 46-60 years, 60 years and above. Patients belonging to these age groups specifically for those between [0-1] and >60 are more susceptible to the pathogens due to their low immune system. Previous studies have reported a spread of resistant strains of *P. aeruginosa* and *A. baumannii*, this report presents a high resistance level of these species in Cameroon with a particular emphasis to penam and cephem sub-families. In this study, we assessed the AST of our samples to penam. More than 50% *P. aeruginosa* were resistant to penam (TIC, TCC and PIP) except to piperacillin-tazobactam whereof 33% were resistant. Lower resistance rate to piperacillin-tazobactam in South Africa (19%) (Surveillance for Antimicrobial Resistance and Consumption of Antibiotics in South Africa, 2018) and France (21%) were reported (Llanes et al., 2013). Resistance to penam (TIC, TCC, PIP and TZP) was found among 88% of *A. baumannii* isolates in this study and similar compared to Alkasaby and colleagues (Alkasaby and El Sayed Zaki, 2017b) finding in Egypt, where 89% of penam resistance were recorded. *P. aeruginosa* presented 43% resistance to cephem, this result is lower than Tanzania study where they got 63% resistance to cephem (Mushi et al., 2014) and higher than Israëlian report (Dickstein et al., 2019) where they got 13% resistance to cephem in 2017 report. 95% *A. baumannii* isolates showing resistance to cephem, this result is in accordance with Alkasaby's report in Egypt (Alkasaby and El Sayed Zaki, 2017a) and Mushi's study (Mushi et al., 2014) in Tanzania. Carbapenems are considered last resort antibiotics. Resistance to carbapenems seems to be increasing in Cameroon since 2006. In Gangoue et al's study (Gangoue-Pieboji et al., 2006) only 6% of *P. aeruginosa* were resistance to carbapenems, nine years later Gonsu et al., (Gonsu et al., 2015) recorded 8%. In this study, 24% of *P. aeruginosa* isolates were resistant to carbapenems. Similar result was found in Spain, Italy, Latvia and Lithuania (Moghnieh et

al., 2019). Resistance level of *A. baumannii* was higher than that of *P. aeruginosa* and followed the same increasing trend. In 2006, no case among *A. baumannii* isolate was resistant to carbapenems in Cameroon (Gangoue-Pieboji et al., 2006), however in this study, 42% of *A. baumannii* showed resistant to cabapenems. Spain and Hungary got similar result (Moghnieh et al., 2019). Whereas studies from Italy, South Korea and South Africa presented 80-86% of *A. baumannii* resistant to carbapenems (Lowings et al., 2015). A survey conducted in several countries including Cameroon had shown an increase in Carbapenem and colistin consumption (Van Boeckel et al., 2014) which are considered to be the last resort antibiotics. This increase has exposed bacteria to a strong selection pressure during these years and may have led to the dissemination of resistant strains at the expense of sensitive ones.

Antimicrobial susceptibility testing of *P. aeruginosa* isolates using quinolone showed that they have low resistance level to it. Israelian and Tanzanian studies report 17% of *P.aeruginosa* quinolone resistance whereas in this study, 35% of *P. aeruginosa* isolate were resistant to quinolone. Concerning *A. baumannii* isolates, up to 74% of isolates showed resistance to this family. Our finding is higher than Tanzania report where 40% of *A. baumannii* were resistance to quinolone (Mushi et al., 2014) but lower than Egyptian study where 92% of *A. baumannii* were resistant to quinolones. However, our finding is similar to the South African's report. (De Angelis et al., 2018)

The mean of aminoglycosides resistance frequency was 28% among PSA, however, in 2015 there were less than 10% *P. aeruginosa* resistant to aminoglycosides in Cameroon (Gonsu et al., 2015). The resistance rate to aminoglycosides in this study is lower than that of many others studies with both *A. baumannii* and PSA. Seventy percent (70%) of *A. baumannii* was resistant to aminoglycosides. This finding though high, remains lower than Egyptian report. There, they got 91% *A. baumannii* resistant to aminoglycosides (Alkasaby and El Sayed Zaki, 2017a). Among four aminoglycosides tested in this study, *A. baumannii* and *P. aeruginosa* presented the highest resistance frequency against tobramycin and the lowest was with netilmicin. This result disagrees with the findings of some other authors who reported a lowest resistance rate with tobramycin (Llanes et al., 2013)**.**

A. baumannii recorded 89% rate of resistance to tetracycline. This finding was similar to report of Meshkat et al (Meshkat et al., 2017a) who recorded 90% of resistant isolates to this antibiotic. *A. baumannii* showed lower resistance rate to doxycycline and minocycline than to tetracycline as well as Meshkat et al (Meshkat et al., 2017a) report. Tigecycline among tetracyclines antibiotics seems to be the most effective. AST done in highly resistant strains in Egypt showed a very low resistance rate. Less than 2% of *A. baumannii* strains were resistant to tigecycline

(Alkasaby and El Sayed Zaki, 2017a). Thus, this antibiotic may be used like a rescue antibiotics against MDR *Acinetobacter baumannii* (Bassetti et al., 2019).

Resistance to trimethoprim-sulfamethoxazole

Thirteen years ago in Cameroon, 30% of *A. baumannii* isolates were resistant to trimethoprim-sulfamethoxazole (Gangoue-Pieboji et al., 2006), in this study there was a breakneck increase of resistance to trimethoprim-sulfamethoxazole. In fact, 92% of *A. baumannii* were resistant to this antibiotic. The results of this study is higher than that of Alkasaby et al (Alkasaby and El Sayed Zaki, 2017a). The resistance frequency of *A. baumannii* against trimethoprim-sulfamethoxazole observed in this study may be due to the dissemination of the selected resistant clone which may have emerge because of expanded consumption of this antibiotic in Cameroon (Chem et al., 2018b).

Looking across this report, globally, there is an increase of resistance frequency of both *Pseudomonas aeruginosa* and *Acinetobacter baumannii* against all antimicrobial family since a decade. This increase may be subsequent to either poor infection control practices or misuse of antibiotics. In actual fact, a *Cameroonian report on practices and attitudes on antibiotics use* made by Ekambi et al (Ekambi et al., 2019) showed that 47% of antibiotics purchase are without prescription. Unfortunately, in Cameroon antibiotics consumption are not under a tight control; thus, enhancing emergence of resistant strains.

Biofilm formation is one of the most frequent virulence mechanism, in fact, Sixty-five (65) % of all healthcare-associated infections (HCAI) originate from biofilms (Percival et al., 2014). In this study, 82% of *P. aeruginosa* were biofilm formers at 37˚C. This result differ from Iran and Serbia studies which found more than 90% of biofilm formers among *P. aeruginosa* isolates (Milivojevic et al., 2018). The previous reports and ours, showed that a great percentage of *Pseudomonas aeruginosa* strains have biofilm ability on abiotic environment at 37˚C. Previous study on *P. aeruginosa* biofilm ability in different temperature condition showed that the production of biofilm differed without any association with the growth temperature (Hoštacká et al., 2010). In this study, we found no significant relation of biofilm formation ability of *P. aeruginosa* according to growth temperature after varying temperature from 37˚C to 25˚C and from 25˚C to 37˚C. This result is in accordance with Hoštacká et al (Hoštacká et al., 2010) conclusion. Molecular study of gene marker associated with biofilm formation confirmed the phenotypic observation. In fact, 100% of *P. aeruginosa* isolate bearing biofilm related gene *pslA* were biofilm former and 80% of *pslA* negative strains were "No biofilm former" at 37˚C. Seventy (70 %) of *A. baumannii* were biofilm formers at 37˚C, this finding is lower than results reported by Zeighanmi et al. in Iran and Yang et al in Taiwan. They found respectively 100%

and 93% biofilm formers among *A. baumannii* isolates (Zeighami et al., 2019, Yang et al., 2019). Biofilm related genes *OmpA* and *csuE* were found in all *A. baumannii* biofilm former isolates, the finding corroborates Longo et al., report in Italy who showed that *A. baumannii* isolates biofilm adherence on abiotic depends to *cusE* expression (Longo et al., 2014). In this study, the khi square test showed biofilm formation ability of *A. baumannii* isolates varied significantly with culture temperature. M'hamedi et al (M'hamedi et al., 2014) in Algeria also depicted in their report the variability of *A. baumannii* biofilm ability according to growth temperature. They showed that *A. baumannii* biofilm formation were substantial at 30˚C than at 37˚C which is different to our report where 37˚C were more suitable for biofilm formation than the lower temperature. Irrespectively to the temperature, most of *P. aeruginosa* isolates were moderate biofilm formers, which is in accordance with carelash et al (Corehtash et al., 2015) report. *A. baumannii* isolates were most frequently weak biofilm formers irrespective of the temperature; the observation differs to Zeighami et al., report who got more strong biofilm formers (Zeighami et al., 2019). Knowing that biofilm formation increases the survival rate of some bacteria species on dry surfaces (Espinal et al., 2012), it is therefore important to increase hygiene in health setting to stem bacteria propagation and persistence in the hospital environment in view to control and reduce nosocomial infections and outbreaks. Many protocols are developing nowadays for health device hygiene and there is more to be done in this area to ensure the safety in health practice.

Clustering based on inhibition zone diameters of *P. aeruginosa* and *A. baumannii* showed that there is a wide diversity of *P. aeruginosa* isolates and *A. baumannii* isolates considering antimicrobial resistance patterns. *P. aeruginosa* dendogram when matched with isolates source shows that more than 75% of pus and BAL isolates are found in cluster 1 and 65% of urine isolates are found in cluster 1B. This observation suggests that *P. aeruginosa* from same origin (either pus or BAL or blood) have almost the same antimicrobial sensitivity/resistance behavior. When *Pseudomonas aeuginosa* dendogram was then matched with 37˚C biofilm ability, this matching reveals that 53 % of no biofilm formers were found to be included in the same sub-cluster; sub-cluster 1B. This result suggests that *P. aeruginosa* isolates unable to build biofilm at 37˚C ("No biofilm former") have close antimicrobial sensitivity pattern.

Previous studies have reported a spread of resistant strains of *P. aeruginosa* and *A. baumannii* with a particular emphasis on occurrence of enzymatic resistance markers (Gupta et al., 2016, Abrar et al., 2019). The present study highlights expression of AmpC, ESBLs and MBLs resistance enzymes.

From this study, no *P. aeruginosa* isolates showed hyper-produced AmpC phenotype; on the contrary, Gupta et al., reported 50% of *P. aeruginosa* strains exhibiting this phenotype (Gupta et al., 2016). The difference between these two studies is due to the interpretation criteria. Gupta et al. (2016) in India considered as AmpC producers, any isolate resistant to cefoxitin. However, 11% of *A. baumannii* presented hyperproduced AmpC phenotype though Rynga et al (Rynga et al., 2015) recorded 99%. The difference between the former report and this could be attributed to the disparity of the protocols used in each study.

ESBLs are serious threat for antibiotherapy since they lead to bacteria resistance to all antibiotics among penicillin, cephalosporin and monobactam subfamilies (Bush et al., 1995a). In this study, *P. aeruginosa* showed 24 %. A report from Kauer et al. (Kaur and Singh, 2018) in India showed 17% of ESBLs production phenotype among *P. aeruginosa* isolates which is lower than our report. In this report, 9% of *A. baumannii* showed ESBLs producer phenotype. Two studies carried out in India showed higher ESBLs production rate compare to this report. They got 38% (Rynga et al., 2015) and 12% (Litake et al., 2015). Two different methods were used to identify production of ESBLs, the Double Disc Synergy Test (DDST) and the Combined Disc Test (CDT). This report found no positive result with DDST indicating that CDT is the appropriate phenotypic protocol of ESBLs identification compared to DDST. This observation corroborates Litake et al. (Litake et al., 2015) and Uddin et al. (Uddin et al., 2018) conclusions. In this study, no *P. aeruginosa* isolates, showed MBLs production phenotype unlike, Gupta et al (Gupta et al., 2016) who found 10%; although we recorded 6% MBLs production among *A. baumannii* isolates. Our finding remains lower than India and Pakistan's studies where they got 29% and 12% MBLs production phenotype respectively among *P. aeruginosa* and *A. baumannii* isolates (Gupta et al., 2016, Uddin et al., 2018). In this work, it was noticed that 80% to 100% isolates showing AmpC, ESBLs or MBLs expression are also MDR. This can be due to the carriage by these isolates of genetic mobile elements that bear most of the time multiple genes encoding resistance to diverse antibiotics families (Naas et al., 1999).

Among *P. aeruginosa* isolates, 66% showed MDR. This report is higher than the Italy report, which recorded 35% MDR *P. aeruginosa* (Giulia et al., 2018) but is similar to India result (Gupta et al., 2016). *A. baumannii* resistance pattern according to many studies all over the world showed high resistance level to almost all antibiotics families. In our study, 100% of *A. baumannii* isolates showed MDR pattern, this result is in accordance to Iran, Italy and India studies, which reported 97%, 94% and 91% MDR pattern respectively among *A. baumannii* (Salehi et al., 2018, Giulia et al., 2018, Rynga et al., 2015).

Among more than 90% of *P. aeruginosa lasB, exoA, pslA* and *exoS* virulence genes were amplified. The frequency of *lasB* (98.6%) in the present study is higher compare to Asadpour et al (Asadpour, 2018a) report in Iran; they recorded 91 %. The exotoxin A is considered as the major virulence factor playing a key role in cell death. In this study, we got 90% of *P. aeruginosa* harboring the gene coding for this toxin, the result is lower compared to Ahmed et al. and Ruiz-Roldán results who recorded 100% and 98% respectively *P. aeruginosa* bearing *exoA* gene (Ahmed et al., 2019, Ruiz-Roldán et al., 2018). Cilia is expressed on the surface of *P. aeruginosa* by *pslA* gene, which is responsible for surface adherence. In this study, 92% *P. aeruginosa* bearing *pslA* were recorded; the result is higher than Iran report who got 87.5% (Asadpour, 2018b). An exoS, type-III cytotoxin is involved in bacteria evasion, which is one of the important stage of bacteria pathogenesis. This toxin gene was amplified among 98% *P. aeruginosa* and that was higher compared to Ruiz-Roldan et al. report in Spain and Ahmed et al report in Tunisia, they got 65% and 77% respectively (Ruiz-Roldán et al., 2018, Ahmed et al., 2019). In this study, a high rate of virulence genes was recorded among *Pseudomonas aeruginosa* species suggesting a high pathogenicity level of isolates tested in this work. *OmpA* and *csuE Acinetobacter baumannii* biofilm formation related genes were identified among 88% *A. baumannii* isolates. Zeighami et al. got 81% of *A. baumannii* bearing *OmpA* gene which is closer to this study report, but in contrary, among their *A. baumannii* isolates, all (100%) were positive to *csuE* amplification (Zeighami et al., 2019).

β-lactams are the most prescribed and consumed antibiotics in Cameroon (Chem et al., 2018a) and the world at large. Its usage most of the time are not limited by a medical prescription. Several studies have highlighted the role of bla_{TEM} and bla_{CTXM} in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* β-lactams resistance. In the current study, 17% of bla_{TEM} were found among *P. aeruginosa* isolates. This record was higher than Abrar et al report who got 0% bla_{TEM} among *P. aeruginosa* (Abrar et al., 2019). Four (4%) of *P. aeruginosa* isolates in this study were positive to bla_{CTXM} gene amplification which is lower to Abrar et al who reported 50% of *P. aeruginosa* with bla_{CTXM} gene in Pakistan. bla_{CTXM} is one of the most spread ESBLs among Gram negative rod bacteria (Cantón et al., 2015). Its low incidence compares to phenotypical observation (24% *P. aeruginosa* with ESBLs production phenotype) among *P. aeruginosa* isolates in this study suggest that others enzymes and/or determinants are involved for ESBLs activity in our isolates. Those genes were present among *A. baumannii* in higher proportion. We recorded 70% of bla_{TEM}. This finding corroborate Alyamani et al. report in Saudi Arabia who found 71% of *A. baumannii* isolates bearing bla_{TEM} gene (Alyamani et al., 2015a). ESBLs bla_{CTXM} were amplified among 29% of *A. baumannii*. This report is higher than the observations

recorded in Egypt where only 1.8% of *Acinetobacter baumannii* isolates were bearing bla_{CTXM} gene (Alkasaby and El Sayed Zaki, 2017b).

This study identified antibiotic resistance genes, virulence markers, mobile genetic elements (MGEs) and phylogenetic relationship between UY1PSABAL and UY1PSABAL2 with others samples isolated from Homo sapiens origin across continent. PCR amplification of the 16S region was employed to identify the isolates under investigation. Genotype-based identification methods help in solving the problem of variable phenotype and provide more accurate species identification (Spilker et al., 2004) . Among these genotype-based identification methods, 16S rRNA has suitable features allowing it to be the most commonly used identification marker, however, the limitations of using molecular identification by 16S rRNA have been described by several authors (Srinivasan et al., 2015). This study further sequenced the entire genome of the two isolates with a view to access the genetic make-up of the pathogens under study. Next-generation sequencing (NGS) approach entry within the biological technology field opened the opportunity of retrieving huge number of information including better identification of pathogens and offered fast and cost effective way to get accurate details on genes or genes variant carried by a specific specie. The NGS approach allows the complete genome sequencing, which is currently, regarded as the best way to characterize pathogenicity of a microbial strain (Schwarze et al., 2018).

The whole genome sequencing of UY1PSABAL and UY1PSABAL2 showed numerous virulence factor genes. Those genes encoded various pathogenicity markers. Among them were determinant involved in motility, bacteria adherence, type I, type II, III and VI secretion systems, type III toxins, alginate production/regulation, antiphagocytosis (Serum resistance), quorum sensing, ion uptake and protease production.

Flagella are the most common apparatus used by bacteria for motility. Numerous flagella biosynthetic related genes were observed within the current isolates, which improve their motility and consequently their pathogenicity. Moreover, bacteria equipped by flagella are found to be more resistant to surfactant protein A (SP-A) (Zhang et al., 2007). SP-A is an important lung innate immune protein that kills microbial pathogens by opsonization and membrane permeabilization (Tan et al., 2014), thus, being equipped by flagella has increased UY1PSABAL and UY1PSABAL2 resistance to opsonization by the cells of lung where those strains were collected. Besides the flagella some particular pili are recognize to play a role in motility, a twitching motility in particular. This motility ability of pili type is observed with type IV pili. Type IV pili has also been shown to participate to *P. aeruginosa* resistance to antimicrobial activity of SP-A action (Tan et al., 2014). They can achieve diverse other array

of functions, including its primary function which is the attachment to surfaces, electrical conductance, acquisition of DNA and secretion of a broad range of structurally distinct protein substrates. Moreover, type IV pili has been proved to be involve in DNA transfer through conjugation. Pili of *P. aeruginosa* appeared to cause severe cases of pneumonia, bacteraemia, and increased mortality than the nonpiliated strains (Tang et al., 1995). UY1PSABAL and UY1PSABAL2 showed wide range of pili especially type IV pili determinant. This observation reinforces the evidence shown above where authors (Tan et al., 2014) described through in vivo experiments the importance of pili in *P. aeruginosa* infection and severity of such piliated *P. aeruginosa* strains in lung infection.

Gram negative bacteria unlike Gram positive have two membranes protecting the cytoplasm, the inner and the outer membrane. Seven secretion systems have been described up to the date achieving transport of substrates across the outer membrane (Jain, 2011). Type I secretion systems (T1SSs) which mediate the secretion of various protein such as Pseudolysin (lasB, a metalloprotease) from the cytoplasm into the extracellular environment. Such secretion system found within our isolates has contributed to reinforce their virulence through its ability to facilitate the secretion of the pathogenic factor lasB a metalloprotease amplified on both isolates during this study. Type II secretion systems that secrete folded proteins such as pseudolysin (lasB), phospholipase C (PlcH) or lipase (LipA) from the periplasm into the extracellular milieu has also been identified among UY1PSABAL and UY1PSABAL2 contributing to such strains to achieve the above-mentioned function. Type III secretion systems (T3SSs), the most robust virulence factor in animal models and human are specialized protein delivery machineries embedded within the double-membrane. T3SS allows Gram-negative bacteria such as *P. aeruginosa* to translocate a specific subset of bacterial effector proteins into the host cell cytosol or the plasma membrane. The presence of a functional T3SS was associated with bacterial persistence in the lungs, higher relapse rates, and increased mortality in patients with acute respiratory infections caused by *Pseudomonas aeruginosa*. T3SS apparatus are promising target for new therapeutic studies. All these features displayed by T3SS borne by the current isolates, gave them a strong equipment for their pathogenicity. Type IV secretion system (T4SSs) in addition to proteins transfer have the unique ability among secretion systems to mediate the translocation of DNA into bacterial or eukaryotic target cells. According to various studies, *P. aeruginosa* does not bear T4SS (Filloux, 2011). Our finding is coherent to literature on T4SS; in fact, no T4SS was found within UY1PSABAL and UY1PSABAL2. Type V secretion systems (T5SSs) also called the auto transporter system due to the fusion between the substrate and its secretion pore to form a single polypeptide; T5SS can though drive its own secretion

through the outer membrane (Meuskens et al., 2019). In contrary to literature report on *Pseudomonas aeruginosa* (Meuskens et al., 2019) UY1PSABAL and UY1PSABAL2 lack T5SS. Type VI secretion systems (T6SS) is a cell envelope-spanning machine that translocate toxic effector proteins into eukaryotic and prokaryotic cells and though play a crucial role in pathogenesis and bacterial competition. The T6SS is broadly distributed among *Proteobacteria* including *Pseudomonas aeruginosa* (Costa et al., 2015). The seventh (T7SS) type of secretion system is only found in mycobacteria, such as Mycobacterium tuberculosis (Costa et al., 2015). Our isolates displayed TISS, T2SS, T3SS and T6SS as reported in *P. aeruginosa* secretion system literature. Those secretion systems are channels by which the bacteria uptake or extrude molecules or toxins. They give to UY1PSABAL and UY1PSABAL2 wide range of means to infect and persist in the host cell; thus, they can serve as important antimicrobial target.

Type III toxins are exo-enzymes (Exo) excreted through T3SS and are believe to be directly injected into the cytosol of target cells. Among those enzymes are ExoU or ExoS ExoT and ExoY. UY1PASBAL and UY1PSABAL2 borne either *exoU* or *exoS* gene exclusively. This observation is in accordance with previous studies (Subedi et al., 2018) which described mutual exclusion between *exoU* and *exoS* genes. Other authors (Lyczak et al., 2000) described the major role of type III virulence factors such as ExoS, ExoT and ExoU on lung cells, this feature explained how pathogenic were UY1PASBAL and UY1PSABAL2.

Alginate serves as an extracellular matrix component involved in biofilms formation, it confers resistance to antibiotics and prevent phagocytosis by the immune system of the host (Rehman and Rehm, 2013). The synthesis of alginate is a complex mechanism involving twelve genes which belong to the algA-algD operon (Pulcrano et al., 2012). Alginate production result in *P. aeruginosa* conversion from the non-mucoid to mucoid phenotype. Mucoid *P. aeruginosa* is then associated with a significant clinical deterioration. Beside alginate produced by the current strains, others antiphagocytosis genes equipment is found within UY1PSABAL and UY1PSABAL2 genomes. Those armatures represent for these isolates an important asset for their pathogenesis feature. There are novel therapeutic treatment targeting alginate synthesis.

Like human been, bacteria can display different behaviour depending on their number in the considered environment. The intercellular signalling process depending on bacteria population density is termed as quorum sensing (QS). *rhlI lasI* homoserin lactones synthase and *PqsE Pseudomonas* quinolone system gene belonging to *rhl*, *las* and *Pqs* system the QS system genes and lasR a transcriptional regulator were found within UY1PSABAL and UY1PSABAL2 genomes. QS plays a major role in virulence factor production within *P. aeruginosa* (Fothergill et al., 2012). It allows *P. aeruginosa* for example, in the presence of high bacterial numbers to

produce two *P. aeruginosa* toxins found within these isolates: an elastase (protease) and a pyocyanin (syderophore), which will subsequently harm their host. QS Is considered as one of the best targets for future therapeutics (Fothergill et al., 2012).

Protease are enzymes that have activity on protein or peptide hydrolysis. A zinc metalloprotease such as *lasA* and *lasB* have an elastolytic activity on human tissue and especially lung tissue (Fazeli and Momtaz, 2014). Others hydrolysis enzymes, phospholipases C encoded by *plcH* and *plcN* genes can also be active on lung cells biomolecules such as Phospholipids contained in surfactants (Fazeli and Momtaz, 2014). Among the phospholipases produced by PSA, the thermostable hemolysin PlcH has the particularity of attenuating the radical oxygen species (ROSs) produced by neutrophils. UY1PSABAL and UY1PSABAL2, which exhibited the above-mentioned phospholipases C genes as well as zinc metalloprotease virulence factor genes, were thus well equipped to hydrolyse host cell proteins and avoid ROSs produced by host immune cells given to the current strains a powerful asset to invade and persist in the host environment.

Biological metal ions, including Co, Cu, Fe, Mg, Mn, Mo, Ni and Zn ions, are necessary for the survival and the growth of all microorganisms. They can serve as a cofactor in various biological mechanism and are essential for microbial pathogenicity. *Pseudomonas aeruginosa* expresses multiple ions uptake systems. Ions uptake of microorganism can be made through various pathway. They can either be uptaken via specific transporter mediated by ion chelators or directly go through porins (Braud et al., 2009). Iron Fe^{3+} is an essential element for living organisms. It is involved in numerous cellular processes various organisms including prokaryotes as well as eukaryotes. Thus, the maintenance of a proper intercellular level is critical. *Pseudomonas aeruginosa* secretes two siderophore: pyochelin (Pch) and pyoverdine (Pvd) observed within UY1PSABAL and UY1PSABAL2 that help in iron assimilation. pyochelin (Pch) and pyoverdine (Pvd) chelate iron in the extracellular medium and transports it into the cell via a specific outer membrane transporter, FptA and FpvA respectively that were also found in the current strains (Schalk and Cunrath, 2016).

The antibiotic resistome is the collection of all the antibiotic resistance genes, including those usually associated with pathogenic bacteria isolated in the clinics, non-pathogenic antibiotic producing bacteria and all other resistance genes. The current isolates after a phenotypical study were characterized as multidrug resistant (MDR). That phenotypical observation was confirmed by the concomitant presence of numerous resistance genes; namely, β-lactams, aminoglycosides, tetracyclins, fosfomycins, glycopeptides, phenicols resistance genes found

within both strains genome. UY1PSABAL harboured additionally quinolone and sulphonamide resistance genes.

Several published studies on *Pseudomonas aeruginosa* genome characterization showed prevalence of *blaPAO* and *blaOXA50* which are classified as β-lactamases resistance genes (Hussain et al., 2017); and this does not make a difference in *Pseudomonas aeruginosa* strain examine during the course of this study. The OXA-type carbapenemases OXA-50, seems to be naturally occurring enzymes in the *P.aeruginosa* species (Bassetti et al., 2018). Clonage assay made by inserting *blaOXA-50* onto *P. aeruginosa* host carried out by Girlich and collaborators in 2004 showed that, this gene conferred decreased susceptibility to ampicillin, ticarcillin, moxalactam and meropenem (Girlich et al., 2004). Thus, presence of *blaOXA50* within UY1PSABAL2 genome could explain it resistance to meropenem. Genes *blaOXA-395* and *blaOXA-486* present respectively within UY1PASBAL and UY1PSABAL2 are commonly acquired resistance genes found within *P. aeruginosa* genome sequence (Hussain et al., 2017, Taiaroa et al., 2018).

Moreover, quinolones resistance genes were also found common to both isolates. UY1PSABAL genome sequence showed nine predicted regions to levofloxacin and plasmid encoded ciprofloxacin resistance gene *crpP* which has been demonstrated to induce resistance to ciprofloxacin was also identified. *gyrA* variant/mutant and *parE* variant/mutant were observed within the same strain. UY1PSABAL as seen, borne wide range of quinolone resistance markers and that explain its resistance to whole antipseudomonadal quinolone tested in this study namely ciprofloxacin and levofloxacin compared to UY1PSABAL2, which did not carry any of these resistant genes or resistant variants, which showed no resistance to quinolone antibiotics.

In addition, rmtB is a 16Sr RNA methylases encoded by *rmtB* gene conferring resistance to almost all aminoglycosides except streptomycin (Nie et al., 2014). This ability was verified with UY1PSABAL which borne *rmtB* gene and displayed resistance to all aminoglycosides (amikacin, gentamicin, gentamicin and tobramycin) tested represented by the following pattern AMK^R GEN^R NET^R TOB^R. Apart from *rmtB* gene, aminoglycosides modifying enzymes (AMEs) were found within UY1PASBAL and UY1PSABAL2 genome, and, it was exclusively aminoglycoses phosphotransferases (APHs). APHs confer most of the time resistance to amikacin and isepamicin (Poole and Chemotherapy, 2005). *aph(3')-IIb* variant found in both UY1PASBAL and UY1PSABAL2 genome has been observed among MDR *Pseudomonas aeruginosa* from various studies around the world (Grandjean et al., 2018).

Identification of Class B and C tetracyclin; fosfomycin *fosA;* vancomycin B-type (*VanW*); and Phenicols *catB7* resistance genes within UY1PASBAL and UY1PSABAL2 genome suggest that these strains can expressed resistance to considered antibiotic families. The worrisome presence of vancomycin resistance gene within these genomes is particularly frightening because of the possibility of interspecies transfer (Finch et al., 2010) of this gene to *Staphylococcus aureus* which vancomycin is the front line treatment antibiotic (Amina and Amin, 2010).

Efflux pump systems are displayed within five different classes. In accordance to literature (Breidenstein et al., 2011), the Resistance-Nodulation-Division family (RND) was the most represented within our isolate. In coherence with literature (Bassetti et al., 2018), numerous efflux pump gene determinants were found within our isolated; this occurrence can explain the multi-drug resistance of the two isolates. It is important to note that efflux pump resistance determinant were found predominant within UY1PSABAL2 strain.

Bacteria genomic diversity is in part, due to the acquisition of a new genetic material into the chromosome (Qiu et al., 2009). The resulting clusters of genes called genomic islands (GIs) contain genes specifying virulence or resistance traits as well as genes that can foster it adaptability within a specific environment (Qiu et al., 2009). Among mobile genetic elements are transposable elements and phages.

Transposable elements include Insertion sequence (IS) and transposons (Tn). Depending on it carriage of IS elements or not Tn is composite or none composite transposon respectively. Some Tn are also integrated by a genetic element called integron. ISs play major role in the prokaryote genomes plasticity. ISs are generally small mobile elements that typically carry one and sometimes two transposase (tnp) genes. Transposase InsO, for IS911 and InsN transposase of IS407 (*Burkholderia multivorans*) were found within UY1PSABAL and UY1PSABAL2. IS elements such as IS911 are often located upstream of several β-lactamases (Vandecraen et al., 2017) suggesting that UY1PSABAL and UY1PSABAL2 borne β-lactamase resistance genes and such suggestion is in accordance with findings on these bacteria resistome. IS407 has been demonstrated to promote the expression of silent lac operon in Transposons in *Burkholderia cepacia*. Tns as others MGEs are DNA segments that can move from one genetic location to another. Tn4652 (Transposon 4652) identified within UY1PSABAL genome is a derivative of the toluene degradation transposon Tn4651 that belongs to the Tn3 which is a non-composite transposon bearing β-lactamase resistance gene family of transposons (Hõrak and Kivisaar, 1998). The transposable elements found within the current isolates bring to them resistance genes that are the assets to resist to antimicrobial agents especially β-lactams.

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated proteins cas is an adaptive immunity against mobile genetic elements, especially phages (Alkhnbashi et al., 2019). The combined results of PHASTER, CRISPRone and RAST annotation showed high number of intact prophages and related prophages genes on UY1PSABAL proving that various bacteriophage residues have infected this strain. Infection of UY1PSABAL have been probabily easier due to its lack of the "immune system" against bacteriophages depicted by the absence of confirm CRISPR-cas system. Counter to UY1PSABAL, UY1PSABAL2 showed less prophage related genes and a confirmed CRISPR-cas system, which has certainly protected UY1PSABAL2 from bacteriophages infection that is confirmed by the fewer number of prophage found within this strain. A recent study showed that about 36% of 122 *Pseudomonas aeruginosa* clinical isolates harboured a CRISPR-cas (Cady et al., 2011) systems meaning that presence of CRISPR-cas systems is not very common within *P. aeruginosa* isolates.

Secondary metabolites are naturally produced compounds that serve as defensive apparatus and homeostasis drive for the organisms producing them. Various gene clusters of Non ribosomal peptide synthetases (NRPSs), a non-proteinogenic amino acid which have toxicity for prokaryotes and eukaryotes (Rojas Murcia et al., 2015a) were revealed within the two isolates. Two NRPS clusters appeared to match with 100% similarity to clusters encoding 2-amino-4-methoxy-trans-3-butenoic acid and pyochelin within both UY1PSABAL and UY1PSABAL2 genomes. Some NRSPs were similar to pyoverdine cluster with less than 100% similarity. Pyoverdine is one of the common siderophore found within *P. aeruginosa* species, it could represent a novel drug or vaccine target (Fothergill et al., 2012). The *Pseudomonas aeruginosa* toxin l,2-amino-4-methoxy-trans-3-butenoic acid (AMB) is a non-proteinogenic amino acid, which is toxic for prokaryotes and eukaryotes (Rojas Murcia et al., 2015b). As described previously, pyochelin acts as a siderophore.

N-acetylglutaminylglutamine amide gene (NAGGN) clusters was also observed. NAGGN plays a key role in cell osmoprotection. It is the most abundant solute at high salt concentration within *Pseudomonas aeruginosa* genome (Sagot et al., 2010). β-lactones are another class of natural product found within these isolates. They have a unique reactivity among natural products and very suitable for the inhibition of hydrolases, transferases, ligases, and oxidoreductases unlike β-lactams which primarily target penicillin-binding proteins (Kreitler et al., 2019). β-lactones can belong to various natural product classes, including terpenoids or nonribosomal peptides (Kreitler et al., 2019) such as those found within the current strains. Phenazine is a secondary metabolite produced by *Pseudomonas aeruginosa* acting by altering

the expression of immunomodulatory proteins by human airway epithelial cells it is thus coherent having observed it gene cluster within these isolates, which have been sampled, from broncho-alveolar-lavage.

We saw previously how bacteria communicate to sense population density through the phenomenon called quorum sensing (QS). Homoserine lactone is the most studied QS auto-inducer molecule involved in the *las* and *rhl* systems. Homoserine lactone gene clusters with highlighted QS system were predicted to be borne by UY1PSABAL and UY1PSABAL2 genome.

Additionally, bacteriocins heat-stable ribosomally synthesized antibacterial peptides that are active by either killing or inhibiting the growth of other bacteria, which are commonly the close related bacteria was predicted to be borne within these genomes. It can also intervene as a probiotic and can serve as signalling peptide in the QS (Perez et al., 2014).

Thiopeptide cluster is a biosynthetic gene clusters for a class of antibiotics widely distributed in genomes and metagenomes of the human microbial ecosystem (Donia et al., 2014). Thiopeptides are known to be inactive against Gram negative bacteria, nevertheless, thiostrepton; a thiopepetide antibiotic, was found to stimulate *P. aeruginosa* biofilm formation (Ranieri et al., 2019). Among secondary metabolite gene clusters, biosynthetic enzymes tRNA-dependent cyclodipeptide synthases gene clusters (CDPS) were identified within UY1PSABAL2. The CDPS code for cyclodipeptide (CDP) are known antimicrobial agents, hence, the bacteria that are producing CDP have competitive edge against neighbouring pathogens.

After analysing the phylogenetic relation between the current study isolates with others collected around the world, at the same period, it appeared that our strains were closely related to Asian (Malaysian and Chinese) strains. The explanation can be drawn from the result of the population dynamic. In fact, this last two decades was characterize by the increase of economic exchange between Asia and Africa, leading to the increase of population rate of Asian in Africa and vice versa (Sullivan et al., 2018). When population move, they carry with them pathogens and cross exchange of genetic material can occur. The phylogeny proximity of our isolates to Malaysian and Chinese strains can be explained by improved economic migration and social interaction between different regions.

RESEARCH PRODUCT

1. Algorithm of the study of *P. aeruginosa* and *A. baumannii* for resistance surveillance in the routine and public Health Laboratory

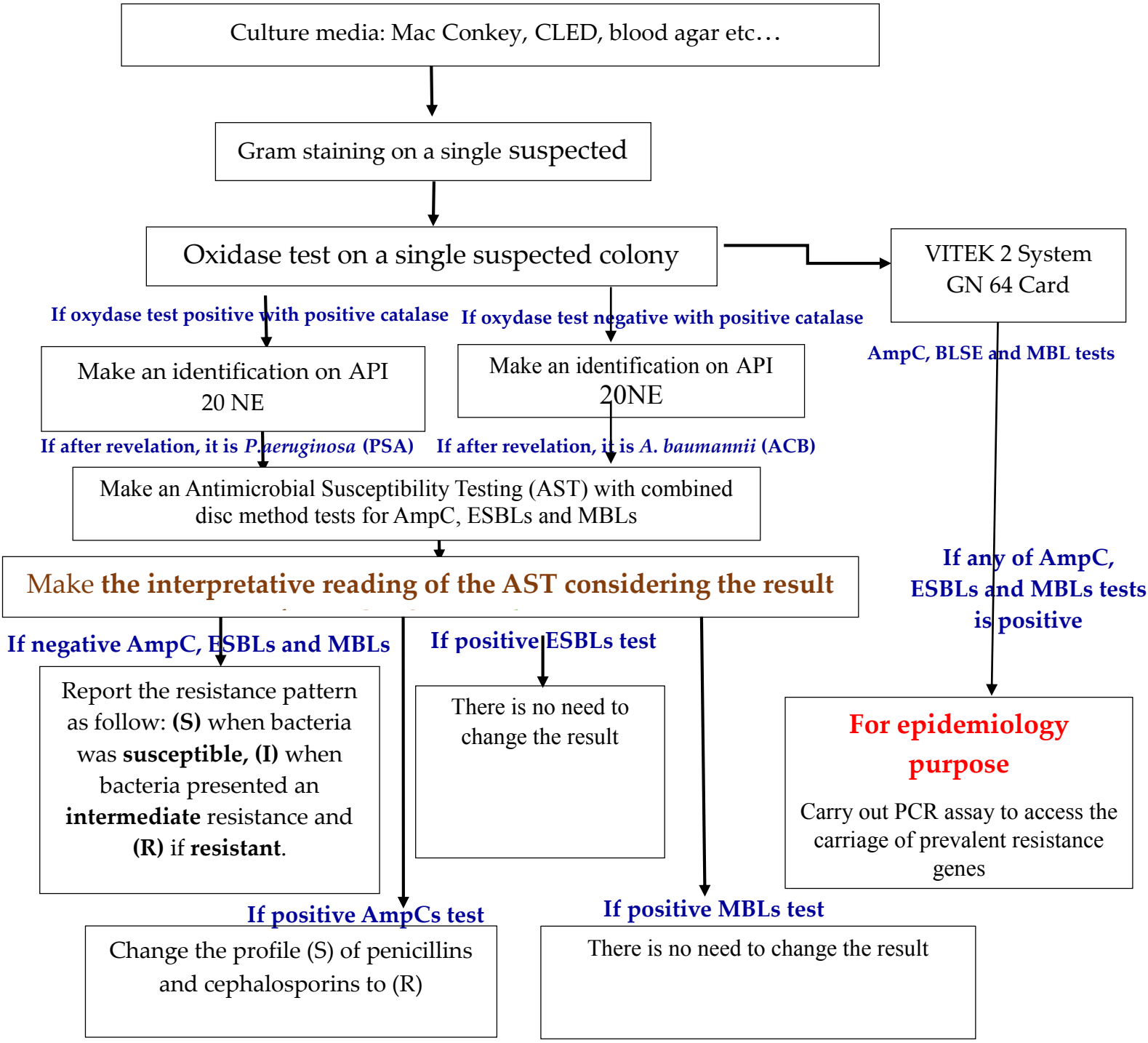


Figure 1: Procedural protocol for the detection of virulent and resistant *P. aeruginosa* and *A. baumannii* for clinical diagnostic institution and institutions engaged in antimicrobial stewardship

2. Standard Operating Procedures (SOPs)

2.1. *Pseudomonas aeruginosa* and *Acinetobacter baumannii* collection and conservation SOP

STANDARD OPERATING PROCEDURE		
Doc. No.: **SOP- 01** Version N°: 01	***Pseudomonas aeruginosa* and *Acinetobacter baumannii* collection and conservation**	**LAPHER-BIOTECH-FODRUS Laboratory**
Written by: **MADAHA LONGLA Estelle**	Verified by: **GONSU KAMGA Hortense**	Approved by: **Prof Wilfred FON MBACHAM**
Visa: *Date* :	***Visa:*** *Date :*	*Visa:* *Date:*

1. **Purpose**

This SOP outlines the procedure how to collect ready identified isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* from isolation sites.

Sample collection is one of the first step of research project. It requires appropriate conditions of conservation and transport, which take into account growth and survival environment of the considered bacteria.

2. **Applicability**

This SOP applies to all Laboratory Scientists, Technologists, Technicians and other assigned researcher willing to carry out study on *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

3. **Procedure**

- **Transfer of the transport media from Food and Drugs safety (FODRUS). Laboratory of Biotechnology Centre/University of Yaoundé 1 to collection site laboratories**

1ml Tryptic soy agar with 200-µl paraffin oil contained in 1.8 ml cryovials are put into box, the box is then placed insight the cooler box with cold accumulator and then transported to the collection site laboratories and conserved at room temperature aside of light.

- **Bacteria conservation in the collection sites**

Pure colonies of identified *Pseudomonas aeruginosa* or *Acinetobacter baumannii* are then conserved in the transport media each time there are isolated.

- **Transfer of collected bacteria from collection site laboratory to Food and Drugs Safety Laboratory (FODRUS) of Biotechnology Centre/University of Yaoundé 1.**

Pure colonies conserved in the tryptic soy agar with paraffin oil should be put back into box, the box will then be placed insight the cooler box with cold accumulator and then transported to FODRUS Laboratory.

- **Bacteria conservation at the FODRUS Laboratory**

Pseudomonas aeruginosa or *Acinetobacter baumannii* conserved into the transport media are then conserved in the same transport media for maximum of eight months. On the other hand, bacteria are sub-cultured in the nutrient agar and conserved within the brain-heart-infusion broth with 30% glycerol for maximum of one year at -20◦C.

1. MATERIALS AND EQUIPMENTS

- Loop
- Gloves
- Tryptic soy media
- Parrafin
- Petri dish
- Nutrient agar
- Brain hearth infusion agar
- Glycerol
- Bunsen burner
- Incubator of 37◦C
- refrigerator of -20◦C
- gaz and matches

2. REFERENCES

LaBauve, A.E., Wargo, M.J., Growth and laboratory maintenance of Pseudomonas aeruginosa, Current protocols in microbiology 25, 6E. 1.1-6E. 1.8, (2012).

2.2. *Pseudomonas aeruginosa* and *Acinetobacter baumannii* antimicrobial susceptibility testing SOP

STANDARD OPERATING PROCEDURE		
Doc. No.: **SOP- 02** Version N°: 01	***Pseudomonas aeruginosa* and *Acinetobacter baumannii* antimicrobial susceptibility testing**	**LAPHER-BIOTECH-FODRUS Laboratory**
Written by: **MADAHA LONGLA Estelle**	Verified by: **GONSU KAMGA Hortense**	Approved by: **Prof Wilfred FON MBACHAM**
Visa: *Date* :	***Visa:*** *Date :*	*Visa:* *Date:*

1. **Purpose**

Susceptibility testing is used to determine which antimicrobials will inhibit the growth of the bacteria or fungi causing a specific infection. The results from this test will help a healthcare practitioner determine which drugs are likely to be most effective in treating a person's infection.

2. **Applicability**

This SOP applies to all Laboratory Scientists, Technologists, Technicians and other assigned researcher willing to carry out study on *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

3. **Procedure**

- **Preparation of 0.5 Mc Farland suspension**

Bacterial suspensions of 24 hours cultures is prepared in 0.8% (w/v) normal saline solution to achieve the turbidity equivalent to 0.5 Mc McFarland standard. Practically one colony or a half is put inside screw tube containing 10ml of normal saline solution; the mixture is then shake using a vortex. The turbidly is then compare to 0.5 Mc Farland standard. The turbidity when necessary is adjust by adding bacteria isolate to increase turbidity or by adding normal saline to decrease turbidity.

- **Bacteria suspension seeding**

0.5 Mc Farland bacteria suspension is seed using swabbing method on Muller Hinton media. Practically, inoculate the dried surface of a Muller Hinton agar plate by streaking the swab three times over the entire agar surface; rotate the plate approximately 60 degrees each time to ensure

an even distribution of the inoculum and finally swab the inner edge of the Petri dish, to allow the bacteria suspension to be seeded around the edges. Allow the culture to dry for 15 minutes.

- **Antibiotics discs distribution**

Each antibiotics is moved from the antibiotic cartridge; using forceps and laid under the surface of seeded Muller Hinton 30 mm edge to edge. The forceps is sterilised on the flame of Bunsen burner in between each usage.

- **Incubation**

After distribution of antibiotics on the surface of Mueller-Hinton agar plates, the Petri dish is then put insight the Incubator and temperature set at 37◦C, for 24 hours in aerobic condition.

1. **<u>Materials and equipment</u>**

- Sterile saline in 145-ml tubes
- 0.5 McFarland standard
- Mueller-Hinton agar plates, 90 mm
- Caliper or ruler
- Forceps
- Bunsen burner, matches
- Antibiotic discs according to CASFM-EUCAST 2016 (antibiotics piperacillin (30 μg^{a} and 100 μg^{b}), piperacillin-tazobactam (30-6 μg^{a} and 100-10 μg^{b}), ticarcillin (75 $\mu g^{a,b}$), ticarcillin-clavulanic acid (75-10 $\mu g^{a,b}$), cefepime (30 $\mu g^{a,b}$), cefotaxime (30 μg^{b}), ceftazidime (10 μg^{a} and 30 μg^{b}), ceftriaxone (30 μg^{b}),imipenem (10 $\mu g^{a,b}$), meropenem (10 $\mu g^{a,b}$), ciprofloxacin (5 $\mu g^{a,b}$), levofloxacin (5 $\mu g^{a,b}$), amikacin (30$\mu g^{a,b}$), gentamicin (10 $\mu g^{a,b}$), netilmicin (10 $\mu g^{a,b}$), tobramycin (10 $\mu g^{a,b}$), doxycycline (30 μg^{b}),minocycline (30 μg^{b}), tetracycline (10 μg^{b}) and trimethoprim-sulfamethoxazole (1-25 μg^{a} and 23-75μg^{b}) with superscripts "a" and "b" for screening of PSA and ACB respectively
- Incubator of 37◦C.

2. **<u>References</u>**

Hudzicki, J., *Kirby-Bauer disk diffusion susceptibility test protocol*, (2009)

2.3. Combined disc test (CDT) for AmpC, Extended spectrum β-lactamases, Metallo- β-lactamases identification SOP

STANDARD OPERATING PROCEDURE		
Doc. No.: **SOP- 03** Version N°: 01	**Combined disc test (CDT)for AmpC, Extended spectrum β-lactamases, Metallo- β-lactamases identification**	**LAPHER-BIOTECH-FODRUS Laboratory**
Written by: **MADAHA LONGLA Estelle**	Verified by: **Gonsu Kamga Hortense**	Approved by: **Wilfred FON Mbacham**
Visa: *Date :*	***Visa:*** *Date :*	*Visa:* *Date:*

1. **Purpose**

β-lactams are the most used antibiotics agent in the bacteria infection treatment. Many bacteria strains have developed resistance to this family of antibiotic by producing inhibitory enzymes against β-lactams, called β-lactamases. It is thus, helpful to know as soon as possible if the bacteria strains isolated in a considered patient borne any of AmpC, Extended spectrum β-lactamases, Metallo- β-lactamases, in order to adjust antimicrobial prescription for an efficient treatment.

2. **Applicability**

This SOP applies to all Laboratory Scientists, Technologists, Technicians and other assigned researcher willing to carry out study on *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

3. **Procedure**

- **Preparation of 0.5 Mc Farland suspension**

Bacterial suspensions of selected isolates of 24 hours cultures is prepared in 0.8% (w/v) normal saline solution to achieve the turbidity equivalent to 0.5 Mc McFarland standard. Practically one colony or a half is put inside screw tube containing 10ml of normal saline solution; the mixture is then shake using a vortex. The turbidly is then compare to 0.5 Mc Farland standard. The turbidity when necessary is adjust by adding bacteria isolate to increase turbidity or by adding normal saline to decrease turbidity.

- **Bacteria suspension seeding**

0.5 Mc Farland bacteria suspension is seed using swabbing method on Muller Hinton media. Practically, inoculate the dried surface of a Muller Hinton agar plate by streaking the swab three times over the entire agar surface; rotate the plate approximately 60 degrees each time to ensure

an even distribution of the inoculum and finally swab the inner edge of the Petri dish, to allow the bacteria suspension to be seeded around the edges. Allow the culture to dry for 15 minutes.

- **Antibiotic discs distribution for AmpC detection**

All bacteria resistant to Cefoxitin (30 µg) are subject to the test. The test is based on the inhibitory effect of cloxacillin on the activity of the AmpC cephalosporinase described by Tan et al (Tan et al., 2009). Cefoxitin (30 µg) discs supplemented with 200 µg of cloxacillin and only cefoxitin (30 µg) disc are placed on the surface of seeded Muller Hinton plate.

- **Antibiotics discs distribution for ESBLs detection**

All studied bacteria are subject to the test. The test is based on incisory activity of clavulanic acid against ESBLs according to Bush *et al.,* 1995. The Ceftazidime (30 µg) and clavulanic acid (10 µg) combined disc and Ceftazidime (30 µg) are placed separately on pre-inoculated Muller Hinton agar plates. (CASFM-EUCAST 2016).

- **Antibiotics discs distribution for MBLs detection**

All isolates categorized as intermediate resistant (I) or resistant (R) to imipenem and/or meropenem are subjected to the MBLs phenotypic test using the imipenem- Ethylene Diamine Tetra-Acetyl (EDTA) inhibition method as described by Yong et al. (Yong et al., 2002). Two imipenem (10 µg) discs are placed at 30 mm apart on the inoculated Muller Hinton agar plates. An aliquot of 4 µl EDTA solution corresponding to 750 µg of EDTA is added on one of the discs (Yong et al., 2002).

- **Incubation**

After distribution of antibiotics on the surface of Mueller-Hinton agar plates, the Petri dish is then put insight the Incubator and temperature set at 37◦C, for 24 hours in aerobic condition.

- **Interpretation criteria**

Isolates are considered AmpC producer if the antibiotic inhibition zone diameter (AIZD) produced by the cefoxitin+cloxacillin discs differ by 4 mm higher when compared to those of the cefoxitin discs alone.

Isolates are positive for the test if the AIZD produced by ceftazidime with clavulanic acid compared to that of ceftazidime alone is ≥ 5 mm.

The AIZD produced by imipenem discs (10 µg) alone and imipenem + EDTA (10 + 750) µg discs is measured and used to determine the potential of isolates to produce MBLs. For MBLs

producers, The AIZD from imipenem + EDTA must be ≥7 mm that produced by imipenem alone.

1. **Materials and equipment**

- Sterile saline in 145-ml tubes
- 0.5 McFarland standard
- Mueller-Hinton agar plates, 90 mm
- Caliper or ruler
- Forceps
- Bunsen burner, matches
- Antibiotic discs (cefoxitin (30 µg), imipenem (10 µg), cetazidime (30 µg) and ceftazidime (30 µg) + (10 µg) clavulanic acid.
- Cloxacillin solution
- EDTA solution P^H8

2. **References**

- Hudzicki, J., Kirby-Bauer disk diffusion susceptibility test protocol, (2009).
- CASFM / EUCAST, (Société Française de Microbiologie: recommandations 2016).
- Tan, T.Y., Ng, L.S.Y., He, J., Koh, T.H., Hsu, L.Y., *Evaluation of screening methods to detectbplasmid-mediated AmpC in Escherichia coli, Klebsiella pneumoniae, and Proteus mirabilis*, Antimicrobial agents chemotherapy 53, 146-149, (2009).
- Yong, D., Lee, K., Yum, J.H., Shin, H.B., Rossolini, G.M., Chong, Y., Imipenem-EDTA disk method for differentiation of metallo-β-lactamase-producing clinical isolates of Pseudomonas spp. and *Acinetobacter spp*, Journal of clinical microbiology 40, 3798-3801, (2002).

2.4. Biofilm ability assay SOP

STANDARD OPERATING PROCEDURE		
Doc. No.: **SOP- 04** Version N°: 01	**Biofilm ability assay**	**LAPHER-BIOTECH-FODRUS Laboratory**
Written by: MADAHA LONGLA Estelle	**Verified by: GONSU KAMGA Hortense**	**Approved by: Prof Wilfred FON MBACHAM**
Visa: *Date* :	*Visa:* *Date :*	*Visa:* *Date:*

1. Purpose

A major reason for bacterial persistence during chronic infections is the survival of bacteria within biofilm structures, which protect cells from environmental stresses, host immune responses and antimicrobial therapy. It has been shown that with the formation of bacterial biofilms, it becomes extremely difficult to eradicate the infection. Since the treatment tend to take into consideration the bacteria biofilm ability, it is therefore important to assess the biofilm ability of each bacteria, prior to any treatment.

1. Applicability

This SOP applies to all Laboratory Scientists, Technologists, Technicians and other assigned researcher willing to carry out study on *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

2. Procedure

- Conserved bacteria are refreshed by seeding a nutrient agar plate by agar or broth of the conservation media
- The plate is then incubated at 37 ºC for 24 hours
- A 0.5 Mc Farland bacteria suspension with fresh colony is prepared in the Tryptic soy broth (TSB)
- The broth is then incubated aerobically at 37 ºC for 24 hours
- After incubation, broth is diluted with 1:100 dilution in TSB

- Aliquots of 200 µl of the diluted broth cultures is transferred into the wells of 96-well polystyrene microtitre plates in triplicates.
- TSB broth without bacteria cultures is used as a negative control.
- The plates is incubated at 37 º C for 24 hours.
- After incubation, plates is washed twice with Phosphate-buffered saline (PBS) buffer to removed unattached cells
- Potential biofilm formed is stained with 200 µl of 1% (w/v) crystal violet
- Incubation for 1 hour at room temperature.
- After incubation, plate is washed five times with PBS to remove excess crystal violet stain, drained and air-dried.
- An aliquot of 200 µl of 95% (v/v) alcohol (>99%, Sigma-Aldrich) is added to each well in order to dissolve crystal violet bound to biofilms.

➢ **Interpretation criteria**

The optical density (OD630 nm) is measured using an ELISA reader (HEALES MB-580).

The mean OD of each sample should be compared to the optical density of the negative control. Bacteria strains is classified as non-biofilm formers (ODs<ODc); weak biofilm formers (ODc<ODs<2ODc);, moderate biofilm formers (2ODc<ODs<4ODc) and strong biofilm formers (ODs>4ODc)

With OD: sample optical density

ODc: blank optical density

3. Materials and equipment

- Loop
- 1.5 ml Eppendorf tubes
- Racks
- 0.5 McFarland standard
- Nutrient broth
- ELISA reader
- Microtitration plate
- Cristal violet 1%
- Alchool 95%
- PBS buffer P^H 8
- Oven for 37◦C
- Micropipete of 200 µl and 1000 µl

4. References

- Macia, M., Rojo-Molinero, E., Oliver, A**.**, *Antimicrobial susceptibility testing in biofilm-growing bacteria*, Clinical Microbiology and Infection 20, 981-990, (2014).
- O'Toole, G.A., Pratt, L.A., Watnick, P.I., Newman, D.K., Weaver, V.B., Kolter, R., [6] Genetic approaches to study of biofilms, Methods in enzymology. Elsevier, pp. 91-109, (1999).

2.5. Biosample submission of Whole Genome Sequence SOP

STANDARD OPERATING PROCEDURE		
Doc. No.: **SOP- 05** Revision N°: 01	**Biosample submission of Whole Genome Sequence**	**LAPHER-BIOTECH-FODRUS Laboratory**
Written by: MADAHA LONGLA Estelle	**Verified by:** Kaseem Adekunle Alayande	**Approved by:** Wilfred FON Mbacham
Visa: *Date :*	*Visa:* *Date :*	*Visa:* *Date:*

1. **Purpose**

The BioSample database stores submitter-supplied descriptive information, or metadata, about the biological materials from which data stored in NCBI's primary data archives are derived. The BioSample database promotes the use of structured and consistent attribute names and values that describe what the samples are as well as information about their provenance, where appropriate. This information is important for providing context to the derived data so that it may be more fully understood; it adds value, promotes re-use, and enables aggregation and integration of disparate data sets, ultimately facilitating novel insights and discoveries across a wide range of biological fields.

2. **Applicability**

This SOP applies to all Laboratory Scientists, Technologists, Technicians and other assigned researcher willing to carry out study on *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

Procedure

- **Create an NCBI account**

- Go to https://www.ncbi.nlm.nih.gov/
- Click on button Sign in to NCBI on the top right of web page
- Press on Register for an NCBI account button appearing at the bottom of the dialog box situated at left of the web site
- Fill the dialog box appearing and press on the button create account
- Save the NCBI username and the password for further usage in the save note book.

- **Submit**

- Go to https://www.ncbi.nlm.nih.gov/
- Press on “Submit”, situated in the middle of the web page, a submission portal will be opened
- Press on “My submissions”
- Go to the box written “Start a new submission” and Press on BioProject
- Press on “New Submission” a new page with seven steps to cross
- **The first step is to fill the dialog boxes appearing with your credentials**
- Press on “continue”
- **The second step is to fill the “General info” information,**
- Press on the option “Release immediately following processing” to answer to the question “When this submission should be released to the public?”
- Press on “Single BioSample” to Specify if you are submitting a single sample or a file containing multiple samples
- Press on “continue” button
- **The third step is about the “sample type”**
- Tick on the button on “Microbe” to select the package that best describes your samples
- Press on “continue” button
- **The fourth step is about the “attributes”**
- Fill the sample name by the name that you choose for the sample.
- Fill the space provided for “organism” which correspond to the species name
- Fill the “strain’s” name, which correspond to the same name as “sample name”
- Fill the space provide for the “isolate” which correspond to the identification or description of the specific individual from which this sample was obtained
- Fill the host name such “Homo sapiens” if the host is human

- Fill the isolation source, which describes the physical, environmental and/or local geographical source of the biological sample from which the sample was derived. Example, blood, urine, stool, …
- Fill the collection date
- Fill the geographic location where the sample were collected
- Fill the sample type, here it is the assembled genome
- Press on “continue” button
- **The fifth step in thon of “BioProject”**
- If the is an existing BioProject, fill the space provide
- Press on “continue” button
- If there is yet no existing BioProject, click on “delate”
- Press on “continue” button
- **The sixth step is the “Description” step**, an auto generated description will have been generated for the description, just press on “continue’ button to move to the next step
- **The seventh step is the “Review & Submit” step**, to review each information filled previously and the submit by pressing on the button “Submit”
- You may for several hours or days to receive a message from NCBI giving the BioSample number for sequence.

➢ **Interpretation criteria**

NA (Not applicable)

3. **Materials and equipment**

- Computer
- Internet

4. **References**

- https://www.ncbi.nlm.nih.gov/
- Barrett, T., Clark, K., Gevorgyan, R., Gorelenkov, V., Gribov, E., Karsch-Mizrachi, I., Kimelman, M., Pruitt, K.D., Resenchuk, S., Tatusova, T., BioProject and BioSample

databases at NCBI: facilitating capture and organization of metadata, Nucleic acids research 40, D57-D63, (2012).

2.6. BioProject submission of Whole Genome Sequence SOP

STANDARD OPERATING PROCEDURE		
Doc. No.: **SOP- 06** Version N°: 01	**BioProject submission of Whole Genome Sequence**	**LAPHER-BIOTECH-FODRUS Laboratory**
Written by: **MADAHA LONGLA Estelle**	Verified by: **Kaseem Adekunle Alayande**	Approved by: **Prof Wilfred FON Mbacham**
Visa: *Date :*	***Visa:*** *Date :*	*Visa:* *Date:*

1. **Purpose**

A BioProject is a collection of biological data related to a single initiative, originating from a single organization or from a consortium. A BioProject record provides users a single place to find links to the diverse data types generated for that project.

2. **Applicability**

This SOP applies to all Laboratory Scientists, Technologists, Technicians and other assigned researcher willing to carry out study on *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

3. Procedure

- **Create an NCBI account**

- Go to https://www.ncbi.nlm.nih.gov/
- Click on button Sign in to NCBI on the top right of web page
- Press on Register for an NCBI account button appearing at the bottom of the dialog box situated at left of the web site
- Fill the dialog box appearing and press on the button create account
- Save the NCBI username and the password for further usage in the save note book.

- **Submit**

- Go to https://www.ncbi.nlm.nih.gov/
- Press on “Submit”, situated in the middle of the web page, a submission portal will be opened
- Press on “My submissions”
- Go to the box written “Start a new submission” and Press on BioProject
- Press on “New Submission” a new page with with seven steps to cross
- **The first step is to fill the dialog boxes appearing with your credentials**
- Press on “continue”
- **The second step is to fill the “project type” information,**
- On project data type item, press on “Genome sequencing and assembly”
- Press on continue
- **The third item is “Target”**
- Fill on “Organism name” with the name chosen for the strain
- Press on “continue” button
- **Fill the fourth step general info step**
- Press on “continue” button
- Press on the option “Release immediately following processing” to answer to the question “When this submission should be released to the public?”
- Fill the “Public description” box where we should provide a description (a paragraph) of the study goals and relevance (we can just write the title of the project)
- Press on “continue” button
- **On the fifth step which is step of add the BioSample credential , if there is any add if not press on “delate”**
- Press on “continue” button

- **The sixth step is the step on information about "Publication"**
- Fill the item about the "PubMed ID" or "DOI" if the available
- Press on "continue" button
- **The seventh step is the "Review & Submit" step,** to review each information filled previously and the submit by pressing on the button "Submit"

➢ **Interpretation criteria**

NA (Not applicable)

1. **Materials and equipment**

- Computer
- Internet

2. **References**

- https://www.ncbi.nlm.nih.gov/

2.7. SRA submission of Whole Genome Sequence SOP

STANDARD OPERATING PROCEDURE		
Doc. No.: **SOP- 07** Version N°: 01	**SRA submission of Whole Genome Sequence**	**LAPHER-BIOTECH-FODRUS Laboratory**
Written by: **MADAHA LONGLA Estelle**	Verified by: **Kasseem Adekunle Alayande**	Approved by: **Wilfred Fon Mbacham**
Visa: *Date :*	*Visa:* *Date :*	*Visa:* *Date:*

1. **Purpose**

Archives raw sequencing data and alignment information from high-throughput sequencing platforms, including Roche 454 GS System®, Illumina Genome Analyzer®, Applied Biosystems SOLiD System®, Helicos Heliscope®, Complete Genomics®, and Pacific Biosciences SMRT®.

Makes sequence data available to the research community to enhance reproducibility and allow for new discoveries by comparing data sets.
SRA accepts data from all kinds of sequencing projects including clinically important studies that involve human subjects or their metagenomes, which may contain human sequences. These data often have a controlled access via dbGaP (the database of Genotypes and Phenotypes).

2. **Applicability**

This SOP applies to all Laboratory Scientists, Technologists, Technicians and other assigned researcher willing to carry out study on *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

3. **Procedure**

- Create an NCBI account

- Go to https://www.ncbi.nlm.nih.gov/
- Click on button Sign in to NCBI on the top right of web page
- Press on Register for an NCBI account button appearing at the bottom of the dialog box situated at left of the web site
- Fill the dialog box appearing and press on the button create account

- Save the NCBI username and the password for further usage in the save note book.

➢ Submit

Prior to this submission the BioSample and BioProject should already be available

- Go to https://www.ncbi.nlm.nih.gov/
- Press on “Submit”, situated in the middle of the web page, a submission portal will be opened
- Scroll down until the middle of web page where it is written SRA, and press on “Submit”
- Then press on “new submission”
- There is five steps to cross
- The first step is to fill the dialog boxes appearing with your credentials
- Press on “continue”
- The second step is to fill the “General information”,
- Include BioSample number in the space provided
- Tick “release immediately” at the space provided at the last dialog box
- And leave as default other items
- Press on “continue” button
- The third is the “SRA METADA”
- Click on use the” Use built-in table editor” and fill the spaces provided (fill the first and the second line, since there are two different files for the same sequence)
- Fill BioSample Assession Number
- Fill the Library ID with the name
- Fill the “Title” space with the title of the reseach work
- Fill the “strategy” by selecting WGS
- Fill the Library source by selecting the “Genomic”
- Fill the library selection by “PCR”
- Fill the “library layout” by pair end
- Fill the “platform” use for sequencing. Example: “ILLUMINA”
- Choose the “Instrument model” example: “Illumina Miseq”
- Fill the “Design descript” by explaining the sequence protocol

- Fill the “file type”
- Fill the “file name.fq”
- Press on “continue” button
- The fourth step is the file step
- Select “I have all files preloaded for this submission” (best method for all submissions)
- Select preload folder to upload the fastq format
- Press on “continue” button
- The fifth step is the “Review & Submit” step, to review each information filled previously and the submit by pressing on the button “Submit”

➢ **Interpretation criteria**

NA (Not applicable)

1. **Materials and equipment**

- Computer
- Internet

2. **References**

- https://www.ncbi.nlm.nih.gov/

2.8. Genebank submission of Whole Genome Sequence SOP

STANDARD OPERATING PROCEDURE		
Doc. No.: **SOP- 08** Version N°: 01	**Genebank submission of Whole Genome Sequence**	**LAPHER-BIOTECH-FODRUS Laboratory**
Written by: **MADAHA LONGLA Estelle**	Verified by: **Kasseem Adekunle Alayande**	Approved by: **Wilfred Fon Mbacham**
Visa: *Date :*	***Visa:*** *Date :*	*Visa:* *Date:*

1. Purpose

Most journals require DNA and amino acid sequences that are cited in articles be submitted to a public sequence repository (DDBJ/ENA/Genbank - INSDC) as part of the publication process. Data exchange between DDBJ, ENA and GenBank occurs daily so it is only necessary to submit the sequence to one database, whichever one is most convenient, without regard for where the sequence may be published. Sequence data submitted in advance of publication can be kept confidential if requested. GenBank will provide accession numbers for submitted sequences, usually within two working days. This accession number serves as an identifier for your submitted your data, and allows the community to retrieve the sequence upon reading the journal article. The accession number should be included in your manuscript, preferably in a footnote on the first page of the article, or as required by individual journal procedures.

2. Applicability

This SOP applies to all Laboratory Scientists, Technologists, Technicians and other assigned researcher willing to carry out study on *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

3. Procedure

- **Create an NCBI account**

- Go to https://www.ncbi.nlm.nih.gov/
- Click on button Sign in to NCBI on the top right of web page
- Press on Register for an NCBI account button appearing at the bottom of the dialog box situated at left of the web site
- Fill the dialog box appearing and press on the button create account
- Save the NCBI username and the password for further usage in the save note book.

- **Submit**

Prior to submission, make sure to have register and obtained the BioProject, BioSample, number, your sequenced genome assembled and available in Fasta or (or sqn) files of the

genome sequences. Get your sequencing protocol written and the name you have chosen for your strain available.

- Press on “Submit”, situated in the middle of the web page, a submission portal will be opened
- Go to the dialog box where it is written on the top “Register for an NCBI account”
- Select “Genome” among the “suggest tool”
- Press again on “genome” (Submit assembled complete or incomplete/draft prokaryotic and eukaryotic genomes - do not submit viral genomes, organelle genomes, or plasmids by themselves.
- Scroll down to the middle of the web page until it is seeing the box on the right site of the web page where it is written: “Submit assembled eukaryotic and prokaryotic genomes (WGS or Complete)”
- Press on “Submit” button.
- Press on “submission” button
- Tick on the “single genome” task option
- Press on “continue”
- There are six steps to cross in the submission process
- The first step is to fill the dialog boxes appearing with your credentials
- Press on “continue”
- The second is to fill the “general info” information including Bioproject and Biosample number
- Then fill the third until the sixth

- **Interpretation criteria**

NA (Not applicable)

4. Materials and equipment

- Computer
- Internet

5. References

- https://www.ncbi.nlm.nih.gov/

CONCLUSION

The current study was carried out in view to characterize both phenotypicaly and genotypicaly the *Pseudomonas aeruginosa* and *Acinetobacter baumannii species* collected in some Yaounde health settings.

Sample identification was performed using both biochemical tests and PCR assays. Ninety-seven (97) % and 100 % biochemicaly identified were confirmed by PCR to be *Pseudomonas aeruginosa* and *Acinetobacter baumannii* respectively. Thus, identification trough biochemical tests remains a good option for *Pseudomonas aeruginosa* and *Acinetobacter baumannii* identification in a clinical laboratory for routine diagnostic. However, for an epidemiological purpose PCR protocol stays the gold standard. Concerning resistance to antimicrobial agents, antimicrobial susceptibility testing of *Acinetobacter baumannii* strains results show resistance in a range of 80% to 100% against β-lactams except cabapenems which resistance was observed among 40% to 44% of the isolates, showing the threat of each *Acinetobacter baumannii* infection.

More than 70% of either *Pseudomonas aeruginosa* or *Acinetobacter baumannii* had the biofilm formation ability and displayed the trend of better biofilm expression at 37◦C compared to incubation at 25◦C. A clustering of *Pseudomonas aeruginosa* isolates according to their antimicrobial susceptibility pattern showed that at 37◦C most of the biofilm non-former belong to the same cluster, confirming the impact of biofilm formation ability on antimicrobial susceptibility. These results advocate the necessity of introduction in the public health research laboratories of the study of antimicrobial susceptibility of MDR bacteria in biofilm condition to optimize the antimicrobial susceptibility testing and ultimately antimicrobial therapy. Phenotycal identification of ESBLs carried out in this study has shown 24% positive test among *Pseudomonas aeruginosa* samples. Production of β-lactamases observed phenotypicaly was confirmed by the identification of bla_{TEM} and $bla_{CTX\text{-}M}$ resistance genes amplified among (17% and 4%) and (70% and 29%) *P. aeruginosa* and *A. baumannii* respectively. Given the fact that those resistant genes are frequently borne by genetic mobile element, the risk of their spreading among community or hospital bacteria is increasing. It is thus, more than ever urgent to initiate a sustainable surveillance of the resistance pattern and genetic markers involved in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* resistance for a good management of infections due to these pathogens.

This work focused also on the characterisation of two multidrug resistant (MDR) *P. aeruginosa* strains isolated from broncho-alveolar-lavage of lung-infected patients. The whole genome sequencing (WGS) approach was used to obtain a deeper insight into the UY1PSABAL and

UY1PSABAL2 strains. Numerous resistance and virulent genes observed in the genome of these isolates indicate their potential severity of infections they could cause in humans and the difficulties in the management of these complications. The diverse mobile genetic elements (MGEs) identified in both strains coupled with different CRISPR DNA fragments, demonstrate how dynamic each strain was during its evolutionary history. UY1PSABAL and UY1PSABAL2 strains are well equipped with secondary metabolites, which serve as important asset for these isolates to invade and persist in the host environment.

Further studies are required to have a wider understanding of genetic features of *P. aeruginosa* and much more *Acinetobacter baumannii* isolates circulating within communities in Cameroon. This could ultimately bring about informed government policy and adequate community awareness toward curbing the transmission and continuous emergence of such dangerous pathogens.

PERSPECTIVES

For further researches, we recommende to investigate the following erea:

- Carry out a survey to access in Cameroonian hospitals the types of bacteria manly incriminated among nosocomial infections;
- Carry out further assays on mechanisms influencing biofilm formation both in vitro and in vivo;
- Made a comparative genomic to screen for potential new variants found within the strains collected in Cameroonian environment;
- Develop both phenotypical and genotypic diagnostic tests, which will take into account Cameroonian's bacteria variants.

References

ABRAR, S., AIN, N. U., LIAQAT, H., HUSSAIN, S., RASHEED, F. & AND RIAZ, S. 2019. Distribution of blaCTX − M, blaTEM, blaSHV and blaOXA genes in Extended-spectrum-β-lactamase-producing Clinical isolates: Athree-year multi-center study from Lahore, Pakistan. *Antimicrobial Resistance and Infection Control,* 8**,** 1-10.

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Webography

http://www.bacterio.net/acinetobacter.html

https://card.mcmaster.ca/ontology/38947

Appendixes

Appendix 1 : Api20 NE protocol

REF 20 050

376156 - fr - 2009/11

api® 20 NE

IVD

Système d'identification des bacilles à Gram négatif non entérobactéries et non fastidieux

INTRODUCTION ET OBJET DU TEST

API 20 NE est un système standardisé pour l'identification des bacilles à Gram négatif non entérobactéries et non fastidieux (ex. *Pseudomonas, Acinetobacter, Flavobacterium, Moraxella, Vibrio, Aeromonas*, etc.) combinant 8 tests conventionnels, 12 tests d'assimilation, et une base de données. La liste complète des bactéries qu'il est possible d'identifier avec ce système est présente dans le Tableau d'identification en fin de notice.

PRINCIPE

La galerie API 20 NE comporte 20 microtubes contenant des substrats déshydratés.
Les tests conventionnels sont inoculés avec une suspension bactérienne saline qui reconstitue les milieux. Les réactions produites pendant la période d'incubation se traduisent par des virages colorés spontanés ou révélés par l'addition de réactifs.
Les tests d'assimilation sont inoculés avec un milieu minimum et les bactéries cultivent seulement si elles sont capables d'utiliser le substrat correspondant.
La lecture de ces réactions se fait à l'aide du Tableau de Lecture et l'identification est obtenue à l'aide du Catalogue Analytique ou d'un logiciel d'identification.

PRESENTATION (coffret de 25 tests)

- 25 galeries API 20 NE
- 25 boîtes d'incubation
- 25 ampoules d'API AUX Medium
- 25 fiches de résultats
- 1 notice

COMPOSITION

Galerie

La composition de la galerie API 20 NE est reportée dans le tableau de lecture de cette notice.

Milieu

API AUX Medium 7 ml	Sulfate d'ammonium	2 g
	Agar	1,5 g
	Solution de vitamines	10,5 ml
	Solution d'oligo-éléments	10 ml
	Phosphate monosodique	6,24 g
	Chlorure de potassium	1,5 g
	Eau déminéralisée	qsp 1000 ml
	pH final : 7,0-7,2	

REACTIFS ET MATERIEL NECESSAIRES MAIS NON FOURNIS

Réactifs

- API NaCl 0,85 % Medium, 2 ml (Réf. 20 070)
- Réactifs : JAMES (Réf. 70 542)
  NIT 1 + NIT 2 (Réf. 70 442)
  Zn (Réf. 70 380)
- Oxydase (Réf. 56 635*)
  * référence non commercialisée dans certains pays : utiliser un réactif équivalent.
- Huile de paraffine (Réf. 70 100)
- McFarland Standard (Réf. 70 900) point 0,5
- Catalogue Analytique API 20 NE (Réf. 20 090) ou logiciel d'identification **apiweb™** (Réf. 40 011) (consulter bioMérieux)

Matériel

- Pipettes ou PSIpettes
- Protège-ampoule
- Portoir pour ampoules
- Equipement général de laboratoire de bactériologie

PRECAUTIONS D'UTILISATION

- **Pour diagnostic *in vitro* et pour contrôle microbiologique.**
- **Pour usage professionnel uniquement.**
- Ce coffret contient des composants d'origine animale. La maîtrise de l'origine et/ou de l'état sanitaire des animaux ne pouvant garantir de façon absolue que ces produits ne contiennent aucun agent pathogène transmissible, il est recommandé de les manipuler avec les précautions d'usage relatives aux produits potentiellement infectieux (ne pas ingérer; ne pas inhaler).
- Les prélèvements, cultures bactériennes et produits ensemencés doivent être considérés comme potentiellement infectieux et doivent être manipulés de façon appropriée. Les techniques aseptiques et les précautions usuelles de manipulation pour le groupe bactérien étudié doivent être respectées tout au long de la manipulation ; se référer à "CLSI M29-A, *Protection of Laboratory Workers from Occupationally Acquired Infections ; Approved Guideline* - Révision en vigueur". Pour informations complémentaires sur les précautions de manipulation, se référer à "Biosafety in Microbiological and Biomedical Laboratories - CDC/NIH - Dernière édition", ou à la réglementation en vigueur dans le pays d'utilisation.
- Ne pas utiliser les réactifs après la date de péremption.
- Avant utilisation, s'assurer de l'intégrité de l'emballage et des composants.
- Ne pas utiliser de galeries ayant subi une altération physique : cupule déformée, ...
- Ouvrir les ampoules délicatement comme suit :
  - Placer l'ampoule dans le protège-ampoule.
  - Tenir l'ensemble verticalement dans une main (bouchon blanc vers le haut).
  - Bien enfoncer le bouchon.
  - Exercer une pression horizontale avec le pouce sur la partie striée du bouchon de façon à casser l'extrémité de l'ampoule.
  - Retirer l'ampoule du protège-ampoule et conserver le protège-ampoule pour une utilisation ultérieure.
  - Enlever délicatement le bouchon.

- Les performances présentées sont obtenues avec la méthodologie indiquée dans cette notice. Toute déviation de méthodologie peut altérer les résultats.
- L'interprétation des résultats du test doit être faite en tenant compte du contexte clinique ou autre, de l'origine du prélèvement, des aspects macro et microscopiques de la souche et éventuellement des résultats d'autres tests, en particulier de l'antibiogramme.

CONDITIONS DE STOCKAGE

Les galeries et milieux se conservent à 2-8°C jusqu'à la date limite d'utilisation indiquée sur l'emballage.

ECHANTILLONS (PRELEVEMENT ET PREPARATION)

API 20 NE ne doit pas être utilisé directement à partir des prélèvements cliniques ou autres.
Les microorganismes à identifier doivent dans un premier temps être isolés sur un milieu de culture adapté (ex. gélose Trypcase Soja) selon les techniques usuelles de bactériologie.

MODE OPERATOIRE

Test Oxydase

Le test oxydase doit être réalisé selon les instructions du fabricant. Il constitue le 21ème test d'identification à noter sur la fiche de résultats.

Sélection des colonies

API 20 NE doit être utilisé avec des bacilles à Gram négatif non entérobactéries et non fastidieux.

NOTE 1 : certaines espèces de bacilles à Gram négatif non entérobactéries qui sont oxydase négative (*S. maltophilia*, *Acinetobacter*...) sont parfaitement identifiées avec API 20 NE. On s'aidera du contexte clinique ou bactériologique pour utiliser cette galerie.

NOTE 2 : Les microorganismes fastidieux, exigeants et nécessitant des précautions de manipulation particulières (ex. *Brucella* et *Francisella*) ne font pas partie de la base de données API 20 NE. Il convient d'utiliser d'autres techniques pour exclure ou confirmer leur présence.

Préparation de la galerie

- Réunir fond et couvercle d'une boîte d'incubation et répartir environ 5 ml d'eau distillée ou déminéralisée [ou toute eau sans additif ou dérivée susceptibles de libérer des gaz (Ex : Cl_2, CO_2 ...)] dans les alvéoles pour créer une atmosphère humide.
- Inscrire la référence de la souche sur la languette latérale de la boîte. (Ne pas inscrire la référence sur le couvercle, celui-ci pouvant être déplacé lors de la manipulation).
- Sortir la galerie de son emballage individuel.
- Placer la galerie dans la boîte d'incubation.

Préparation de l'inoculum

- Ouvrir une ampoule d'API NaCl 0.85 % Medium (2 ml) comme indiqué au paragraphe "Précautions d'utilisation" de la notice du produit ou utiliser un tube contenant 2 ml de solution saline à 0.85 %, sans additif.
- A l'aide d'une pipette ou d'une PSIpette, prélever 1 à 4 colonies de morphologie identique, par aspirations ou par touches successives. Utiliser préférentiellement des cultures jeunes (18-24 heures).
- Réaliser une suspension d'opacité égale à 0.5 de McFarland. Cette suspension doit être utilisée extemporanément.

  NOTE : Pour le bon fonctionnement des tests de la galerie API 20 NE, il est très important d'ajuster la densité de l'inoculum au point 0.5 de McFarland. En particulier, une turbidité plus faible conduit à des résultats faussement négatifs. Ne pas toucher les cupules lors des manipulations et veiller à ne pas laisser la galerie exposée à l'air longtemps après inoculation.

Inoculation de la galerie

- Remplir les tubes (et non les cupules) des tests NO_3 à PNPG avec la suspension précédente en utilisant la pipette ayant servi au prélèvement. Pour éviter la formation de bulles au fond des tubes, poser la pointe de la pipette ou de la PSIpette sur le côté de la cupule, en inclinant légèrement la boîte d'incubation vers l'avant.
- Ouvrir une ampoule d'API AUX Medium comme indiqué au paragraphe "Précautions d'utilisation" et y transférer environ 200 µl de la suspension précédente. Homogénéiser avec la pipette en évitant la formation de bulles.
- Remplir tubes et cupules des tests [GLU] à [PAC] en veillant à créer un niveau horizontal ou légèrement convexe, mais jamais concave. Des cupules incomplètement remplies ou trop remplies peuvent entraîner des résultats incorrects.
- Remplir d'huile de paraffine les cupules des trois tests soulignés (GLU, ADH, URE) pour former un ménisque convexe.
- Refermer la boîte d'incubation et incuber à 29°C ± 2°C pendant 24 heures (± 2 heures).

LECTURE ET INTERPRETATION

Lecture de la galerie

- Après incubation, la lecture de la galerie doit se faire en se référant au Tableau de Lecture.
- Noter sur la fiche de résultats toutes les réactions spontanées (GLU, ADH, URE, ESC, GEL, PNPG).
- La révélation des deux tests NO_3 et TRP doit se faire en mettant les tests d'assimilation à l'abri d'une contamination par l'air ; pour cela, placer le couvercle de la boîte d'incubation au dessus de ces tests, pendant la période de révélation des tests NO_3 et TRP.
- **Test NO_3 :**
  - Ajouter une goutte de réactifs NIT 1 et NIT 2 dans la cupule NO_3.
  - Après 5 mn, une couleur **rouge** indique une réaction **positive**, à noter sur la fiche de résultats.
  - Une réaction négative peut être due à la production d'azote (éventuellement signalée par la présence de microbulles) ; ajouter 2-3 mg de réactif Zn dans la cupule NO_3.
  - Après 5 mn, une cupule restée **incolore** indique une réaction **positive** à noter sur la fiche de résultats. Si la cupule devient **rose-rouge**, la réaction est **négative** car les nitrates encore présents dans le tube ont alors été réduits en nitrites par le zinc.

  La réaction utilisée pour l'identification de la bactérie est la réduction des nitrates ; elle est positive si l'une ou l'autre des deux réactions précédentes (production de NO_2 ou de N_2) est positive.

  La production de N_2 peut cependant être utilisée seule comme test complémentaire dans le Catalogue Analytique.
- **Test TRP :**
  Ajouter une goutte de réactif JAMES. Une couleur **rose** diffusant dans toute la cupule indique une réaction **positive**.

• **Tests d'assimilation :**
Observer la pousse bactérienne. Une cupule **trouble** indique une réaction **positive.**
Des pousses d'intensité intermédiaire peuvent être observées et notées ∓ ou ±.
Une fois cette lecture effectuée, l'identification doit être pratiquée comme indiqué au paragraphe "Interprétation".
Une réincubation est nécessaire dans les cas suivants :
- faible discrimination ;
- profil inacceptable ou profil douteux ;
- si la note suivante est indiquée pour le profil obtenu :

IDENTIFICATION NON VALIDE
AVANT 48 H D'INCUBATION

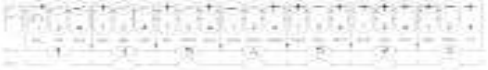
Alors, éliminer, à l'aide d'une pipette ou d'une PSIpette, les réactifs NIT 1, NIT 2 et JAMES par aspiration, recouvrir immédiatement les tests NO_3 et TRP d'huile de paraffine en formant un ménisque convexe, incuber à nouveau à 29°C ± 2°C puis lire 24 h plus tard, sauf les trois premiers tests : NO_3, TRP, GLU qui doivent être lus uniquement à 24 h.

Interprétation

L'identification est obtenue à partir du **profil numérique.**

• Détermination du profil numérique :
Sur la fiche de résultats, les tests sont séparés par groupes de trois et une valeur 1, 2 ou 4 est indiquée pour chacun. En additionnant à l'intérieur de chaque groupe les valeurs correspondant à des réactions positives, on obtient 7 chiffres ; la réaction de l'oxydase qui constitue le 21° test est affectée de la valeur 4 lorsqu'elle est positive.

• Identification :
Elle est réalisée à partir de la base de données (V7.0)
* à l'aide du Catalogue Analytique :
- Rechercher le profil numérique dans la liste des profils.
* à l'aide du logiciel d'identification **apiweb™** :
- Entrer manuellement au clavier le profil numérique à 7 chiffres.



1 154 575 ***Pseudomonas aeruginosa***

CONTROLE DE QUALITE

Les galeries et milieux font l'objet de contrôles de qualité systématiques aux différentes étapes de leur fabrication.

Le **Contrôle de Qualité Minimum** peut être utilisé pour vérifier que les conditions de stockage et de transports n'ont pas d'impact sur les performances de la galerie API 20 NE. Ce contrôle peut être réalisé en suivant les instructions et critères attendues ci-dessus en lien avec le référenciel CLSI M50-A Quality Control for Commercial Microbial Identification Systems.

Comme aucun substrat de la galerie n'est sensible aux conditions de stockage et de transports, le Contrôle de Qualité Minimum peut être réalisé en testant deux souches : ***Aeromonas hydrophila* ATCC® 35654** qui présente des tests principalement positifs et ***Alcaligenes faecalis* ATCC 35655**, qui présente des tests principalement négatifs avec API 20 NE.

Dans le cas où un **contrôle de Qualité Complet** est exigé pour cette galerie, les quatre souches suivantes devront être testées pour vérifier les réactions positives et négatives de la plupart des tests de la galerie API 20 NE.

1. *Aeromonas hydrophila* ATCC 35654
2. *Alcaligenes faecalis* ATCC 35655
3. *Sphingobacterium multivorum* ATCC 35656
4. *Pseudomonas aeruginosa* ATCC 27853

ATCC : American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, USA.

	NO3	TRP	GLU	ADH	URE	ESC	GEL	PNPG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	OX
1.	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	+	−*	−	+
2.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	+	+
3.	−	−	−	−	+	+	−	+	+	+	+	−	+	+	−	−	−	−	−	−	+
4.	+	−	−	V	V	−	+	−	+	−	−	+	+	−	+	+	+	+	+	−	+

* Des réactions faiblement positives peuvent être observées.

Profils obtenus à partir de colonies cultivées sur gélose Trypcase Soja et après 48 heures d'incubation pour les tests ADH à [PAC].

Il est de la responsabilité de l'utilisateur de s'assurer que le contrôle de qualité est mis en oeuvre conformément à la législation locale en vigueur.

LIMITES DU TEST

- Le système API 20 NE est destiné à l'identification des bacilles à Gram négatif non entérobactéries et non fastidieux présents dans la base de données (voir Tableau d'Identification en fin de notice) et à eux seuls. Il ne peut être utilisé pour identifier d'autres micro-organismes ou exclure leur présence.
- Les bacilles à Gram négatif non fermentants, isolés de patients atteints de mucoviscidose, peuvent générer des profils biochimiques atypiques susceptibles d'altérer leur identification.
- Seules des cultures pures contenant un seul type de microorganisme doivent être utilisées.

RESULTATS ATTENDUS

Se référer au Tableau d'Identification en fin de cette notice pour les résultats attendus des différentes réactions biochimiques.

PERFORMANCES

5728 souches de diverses origines et souches de collection appartenant aux espèces de la base de données ont été testées :

- 92,53 % des souches ont été correctement identifiées (avec ou sans tests complémentaires).
- 3,13 % des souches n'ont pas été identifiées.
- 4,34 % des souches ont été mal identifiées.

ELIMINATION DES DECHETS

Les ampoules d'API AUX Medium non utilisées peuvent être éliminées comme déchets non dangereux.
Eliminer tous les réactifs utilisés ou non utilisés (autre que les ampoules d'API AUX Medium) ainsi que les matériels à usage unique contaminés en suivant les procédures relatives aux produits infectieux ou potentiellement infectieux.
Il incombe à chaque laboratoire de gérer les déchets et les effluents qu'il produit selon leur nature et leur dangerosité, et d'en assurer (ou faire assurer) le traitement et l'élimination selon les réglementations applicables.

TABLEAU DE LECTURE

TESTS	COMPOSANTS ACTIFS	QTE (mg/cup.)	REACTIONS/ENZYMES	RESULTATS	
				NEGATIF	POSITIF
NO3	potassium nitrate	0.136	réduction des Nitrates en nitrites	NIT 1 + NIT 2 / 5 min incolore	rose-rouge
			réduction desNitrates en azote	Zn / 5 min rose	incolore
TRP	L-tryptophane	0.2	formation d'indole (TRyptoPhane)	JAMES / immédiat incolore vert pâle / jaune	rose
GLU	D-glucose	1.92	fermentation (GLUcose)	bleu à vert	jaune
ADH	L-arginine	1.92	Arginine DiHydrolase	jaune	orange / rose / rouge
URE	urée	0.76	UREase	jaune	orange / rose / rouge
ESC	esculine citrate de fer	0.66 0.072	hydrolyse (ß-glucosidase) (ESCuline)	jaune	gris / marron / noir
GEL	gélatine (origine bovine)	0.6	hydrolyse (protéase) (GELatine)	pas de diffusion du pigment	diffusion du pigment noir
PNPG	4-nitrophényl-(D-galactopyranoside	0.22	(ß-galactosidase (Para-NitroPhényl-ßD-Galactopyranosidase)	incolore	jaune
GLU	D-glucose	1.56	assimilation (GLUcose)	transparence	trouble
ARA	L-arabinose	1.4	assimilation (ARAbinose)	transparence	trouble
MNE	D-mannose	1.4	assimilation (MaNosE)	transparence	trouble
MAN	D-mannitol	1.36	assimilation (MANnitol)	transparence	trouble
NAG	N-acétyl-glucosamine	1.28	assimilation (N-Acétyl-Glucosamine)	transparence	trouble
MAL	D-maltose	1.4	assimilation (MALtose)	transparence	trouble
GNT	potassium gluconate	1.84	assimilation (potassium GluconaTe)	transparence	trouble
CAP	acide caprique	0.78	assimilation (acide CAPrique)	transparence	trouble
ADI	acide adipique	1.12	assimilation (acide ADipique)	transparence	trouble
MLT	acide malique	1.56	assimilation (MaLaTe)	transparence	trouble
CIT	trisodium citrate	2.28	assimilation (trisodium CITrate)	transparence	trouble
PAC	acide phénylacétique	0.6	assimilation (acide PhénylACétique)	transparence	trouble
OX	(voir notice du test oxydase)	-	cytochrome-oxydase	(voir notice du test oxydase)	

• Les quantités indiquées peuvent être ajustées en fonction des titres des matières premières.
• Certaines cupules contiennent des composants d'origine animale notamment peptone bovine/porcine.

BIOMÉRIEUX

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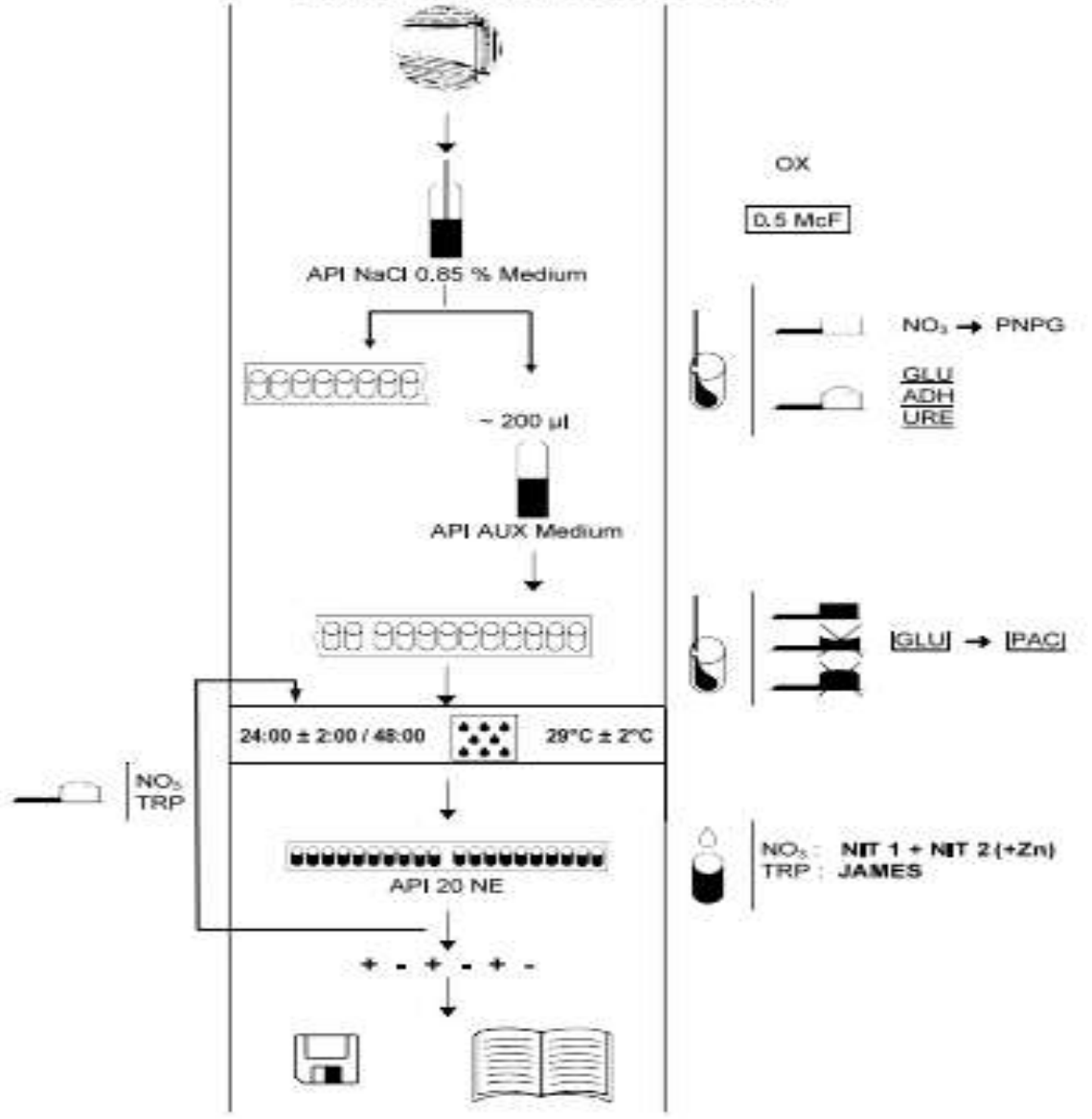
Imprimé en France

CE

METHODOLOGIE / PROCEDURE / METHODIK / TECNICA / PROCEDIMENTO / ΔΙΑΔΙΚΑΣΙΑ / METOD / METODE / METODYKA



Appendix 2: List of tests for GN card

Well	Test	Mnemonic	Amount/Well
2	Ala-Phe-Pro-ARYLAMIDASE	APPA	0.0384 mg
3	ADONITOL	ADO	0.1875 mg
4	L-Pyrrolydonyl-ARYLAMIDASE	PyrA	0.018 mg
5	L-ARABITOL	lARL	0.3 mg
7	D-CELLOBIOSE	dCEL	0.3 mg
9	BETA-GALACTOSIDASE	BGAL	0.036 mg
10	H2S PRODUCTION	H2S	0.0024 mg
11	BETA-N-ACETYL-GLUCOSAMINIDASE	BNAG	0.0408 mg
12	Glutamyl Arylamidase pNA	AGLTp	0.0324 mg
13	D-GLUCOSE	dGLU	0.3 mg
14	GAMMA-GLUTAMYL-TRANSFERASE	GGT	0.0228 mg
15	FERMENTATION/ GLUCOSE	OFF	0.45 mg
17	BETA-GLUCOSIDASE	BGLU	0.036 mg
18	D-MALTOSE	dMAL	0.3 mg
19	D-MANNITOL	dMAN	0.1875 mg
20	D-MANNOSE	dMNE	0.3 mg
21	BETA-XYLOSIDASE	BXYL	0.0324 mg
22	BETA-Alanine arylamidase pNA	BAlap	0.0174 mg
23	L-Proline ARYLAMIDASE	ProA	0.0234 mg
26	LIPASE	LIP	0.0192 mg
27	PALATINOSE	PLE	0.3 mg
29	Tyrosine ARYLAMIDASE	TyrA	0.0276 mg
31	UREASE	URE	0.15 mg
32	D-SORBITOL	dSOR	0.1875 mg
33	SACCHAROSE/SUCROSE	SAC	0.3 mg
34	D-TAGATOSE	dTAG	0.3 mg
35	D-TREHALOSE	dTRE	0.3 mg
36	CITRATE (SODIUM)	CIT	0.054 mg
37	MALONATE	MNT	0.15 mg
39	5-KETO-D-GLUCONATE	5KG	0.3 mg
40	L-LACTATE alkalinisation	lLATk	0.15 mg
41	ALPHA-GLUCOSIDASE	AGLU	0.036 mg
42	SUCCINATE alkalinisation	SUCT	0.15 mg
43	Beta-N-ACETYL-GALACTOSAMINIDASE	NAGA	0.0306 mg
44	ALPHA-GALACTOSIDASE	AGAL	0.036 mg
45	PHOSPHATASE	PHOS	0.0504 mg
46	Glycine ARYLAMIDASE	GlyA	0.012 mg
47	ORNITHINE DECARBOXYLASE	ODC	0.3 mg
48	LYSINE DECARBOXYLASE	LDC	0.15 mg
52	DECARBOXYLASE BASE	0DEC	N/A
53	L-HISTIDINE assimilation	lHISa	0.087 mg
56	COUMARATE	CMT	0.126 mg
57	BETA-GLUCORONIDASE	BGUR	0.0378 mg
58	O/129 RESISTANCE (comp.vibrio.)	O129R	0.0105 mg
59	Glu-Gly-Arg-ARYLAMIDASE	GGAA	0.0576 mg
61	L-MALATE assimilation	lMLTa	0.042 mg
62	ELLMAN	ELLM	0.03 mg
64	L-LACTATE assimilation	lLATa	0.186 mg

Appendix 3: List of antibiotic discs with their respective charge and their critical diameter

		critical Diameter (mm)			critical Diameter (mm)	
Antibiotics	***Disc charge in µg***	**S ≥**	**R <**	**Disc charge in µg**	**R S ≥<R <**	**R <**
	Pseudomonas aeruginosa			***Acinetobacter baumannii***		
			Penicillins			
piperacillin	*30*	18	18	100	21	16
piperacillin-tazobactam	*30/6*	18	18	100/10	21	16
Ticarcillin	*75*	18	18	75	20	15
Ticarcillin-clavulanic acid	*75/10*	18	18	75/10	20	15
			Cephalosporins			
Cefepime	*30*	19	19	30	18	15
Cefotaxime	-			30	23	15
Ceftazidime	*10*	16	16	30	18	15
Ceftriaxone	-			30	21	14
			Carbapenems			
Imipenem	*10*	20	17	10	23	17
Méropenem	*10*	24	18	10	21	15
			Monobactam			
Aztreonam[1]	*30*	50	16	30	-	-
			Fluoroquinolone			
Ciprofloxacin	*5*	25	22	5	21	21
Levofloxacin	*5*	20	17	5	21	18
			Aminoglycoside			
Amikacin	*30*	18	15	30	18	15
Gentamycin	*10*	15	15	10	17	17
Netilmicin	*10*	12	12	10	16	16
Tobramycin	*10*	16	16	10	17	17
			Tetracycline			
Doxycycline	-	-	-	30	13	10
Minoclycline	-	-	-	30	16	13
Tetracycline	-	-	-	30	15	12
			Others			
Trimethoprim-sulfaméthoxazole	-	-	-	1,25-23,75	16	13

Appendix 4: Ethical clearance

REPUBLIQUE DU CAMEROUN
Paix – Travail - Patrie

MINSTERE DE LA SANTE PUBLIQUE

SECRETARIAT GENERAL

COMITE REGIONAL D'ETHIQUE DE LA RECHERCHE POUR LA SANTE HUMAINE DU CENTRE

Tél : 222 21 20 87/ 677 94 48 89/ 677 75 73 30
Mail : crersh_centre@yahoo.com

REPUBLIC OF CAMEROON
Peace – Work – Fatherland

MINISTRY OF PUBLIC HEALTH

SECRETARIAT GENERAL

CENTRE REGIONALETHICS COMMITTEE FOR HUMAN HEALTH RESEARCH

CE N° 0493 /CRERSHC/2019

Yaoundé, le 18 FEV 2019

CLAIRANCE ETHIQUE

Le Comité Régional d'Ethique de la Recherche pour la Santé Humaine du Centre **(CRERSH/C)** a reçu la demande de clairance éthique pour le projet de recherche intitulé : **« Developement of diagnostic against resistant nosocomial bacteria»** soumis par Mademoiselle **MADAHA LONGLA Estelle.**

Après son évaluation, il ressort que le sujet est digne d'intérêt, les objectifs sont bien définis et la procédure de recherche ne comporte pas de méthodes invasives préjudiciables aux participants. Par ailleurs, le formulaire de consentement éclairé destiné aux participants est acceptable.

Pour ces raisons, le Comité Régional d'éthique approuve pour une période de six (06) mois, la mise en œuvre de la présente version du protocole.

L'intéressée est responsable du respect scrupuleux du protocole et ne devra y apporter aucun amendement aussi mineur soit-il sans l'avis favorable du Comité Régional d'Ethique. En outre, elle est tenue de:

- collaborer pour toute descente du Comité Régional d'éthique pour le suivi de la mise en œuvre du protocole approuvé ;
- et soumettre le rapport final de l'étude au Comité Régional d'éthique et aux autorités compétentes concernées par l'étude.

La présente clairance peut être retirée en cas de non-respect de la réglementation en vigueur et des directives sus mentionnées.

En foi de quoi la présente Clairance Ethique est délivrée pour servir et valoir ce que de droit.

LE PRESIDENT

REPUBLIQUE DU CAMEROUN * Comité Régional d'Ethique de la Recherche pour la Santé Humaine du Centre * MINISTERE DE LA SANTE PUBLIQUE
CRERSH/C
CENTRE

DOBO BÊYE Casimir
Pharmacien

Ampliation:
- CNERSH

Appendix 5: Concern form

English Version

REPUBLIC OF CAMEROON
UNIVERSITY OF YAOUNDE I
DEPARTMENT OF MICROBIOLOGY

REPUBLIQUE DU CAMEROUN
UNIVERSITE DE YAOUNDE I
DEPARTEMENT DE MICROBIOLOGIE

The Laboratory for Public Health Research Biotechnologies
LAPHER BIOTECH - The Biotechnology Centre, Nkolbisson
Director: Dr Wilfred F. [illegible]; Tel: (237) 677579180; Fax: 222317429;
wfmbuchum@yahoo.com

CONSENT FORM

I, the undersigned, Mr / Ms / Miss (Name (s) and Given Name (s)

.....

To have been invited to participate in the research work entitled **Development of a diagnosis test against resistant nosocomial bacteria** whose main investigator is called MADAHA LONGLA Estelle.

- I understood the information note that was given to me about this study
- Or I have been read and explained the information notice for this study
- I understood the purpose and objectives of this study
- I received all the answers to the questions I asked
- The risks and benefits were presented to me and explained
- I understand that I am free to accept or refuse to participate
- My consent does not relieve the investigators of the search of their responsibilities, I retain all my rights guaranteed by law

I freely agree to participate in this study under the conditions specified in the information leaflet, that is to say:

1. To answer survey questions
2. To communicate the medical information
3. To allow the use of any bacterial isolates from potentially infected samples taken from me.

I agree that Bacterial samples isolated in this study can be used for further studies.

Made in Yaounde

Principal Investigator: MADAHA LONGLA Estelle

Participant (Names and Address).....

Appendix 6: Information Notice

REPUBLIC OF CAMEROON
UNIVERSITY OF YAOUNDE I
DEPARTMENT OF MICROBIOLOGY

REPUBLIQUE DU CAMEROUN
UNIVERSITE DE YAOUNDE I
DEPARTEMENT DE MICROBIOLOGIE

The Laboratory for Public Health Research Biotechnologies
LAPHER BIOTECH - The Biotechnology Centre, Nkolbisson
Director: Dr Wilfred F. [illegible]; Tel: (237) 6775791803; Fax: 22237429;
wfmbacham@yahoo.com

INFORMATION NOTICE

Invitation to participate in the research on bacterial virulence and resistance

RESEARCH TOPIC

Development of a diagnostic test against resistant nosocomial bacteria

PRINCIPAL INVESTIGATOR: Mrs. MADAHA LONGLA Estelle

THESIS SUPERVISOR: Prof. Wilfred FON MBACHAM

1. RESEARCH OBJECTIVES

General objective:

" To identify the virulence and the resistance molecular markers of bacteria mostly incriminated in nosocomial infections to better understand the mechanisms involved in their pathogenicity and resistance to antimicrobial agent in Cameroon. After that, I will design a simple procedure to identify those genes.

Specific objectives:

1. Determination of the phenotypes of resistance of *Acinetobacter baumannii* and *Pseudomonas aeruginosa*;
2. Molecular identification of isolates
3. Identification of virulence and resistance genes by PCR and sequencing.
4. Elaboration of a multiplex PCR protocol for identification of several resistance genes of

2. STUDY DURATION:

This study will last 3 years

3. TARGET POPULATION:

The target population is any person potentially infected by bacteria

4. PARTICIPANT INVOLVEMENT IN THIS RESEARCH:

- Participants will be asked to give the authorization for the use of potential bacteria isolated on his/her Samples to fulfil the purpose of this study.
- Bacterial isolated in this study can be used for further studies.
- Participants also has a right to withdraw from this research without any explanation at any time in the research process and will not been blamed for this withdrawal.
-

5. FINANCIAL COMPASATION

Participation in the study is free of charge and participant will not be paid for participating.

6. ETHICAL CONSIDERATIONS:

1. This study is completetly safe and without any risk for participant.
2. By the end of this study we will better understand the mecanisms developed by bacteria to persist in those who are infected. Consequently, this will help for the policy regarding the treatment of such infections.
3. All personal informations concerning participant will be kept confidentially by every member of this research team.

7. PERSON TO BE CONTACT IN CASE OF ANY REQUEST

1. **Principal investigator**: Mrs MADAHA LONGLA Estelle
   Tel: 674 38 47 48 / 690 23 50 81
   Email: madahaestelle@gmail.com

2. **Pr. MBACHAM FON Wilfred**
   Tel: 677579180
   Email: wfinbacham@yahoo.com

3. **National Ethical Committee of Research for Human Health**
   Tel: 243674339
   Email: cnethique_minsante@yahoo.fr

Appendix 7: Questionnaire

Version française

REPUBLIC OF CAMEROON
UNIVERSITY OF YAOUNDE I
DEPARTMENT OF MICROBIOLOGY

REPUBLIQUE DU CAMEROUN
UNIVERSITE DE YAOUNDE I
DEPARTEMENT DE MICROBIOLOGIE

The Laboratory for Public Health Research Biotechnologies
LAPHER BIOTECH - The Biotechnology Centre, Nkolbisson
Director: Dr. Wilfred F. [redacted]; Tel: (237) 677570180; Fax 22215418;
wfaleh[redacted]@yahoo.com

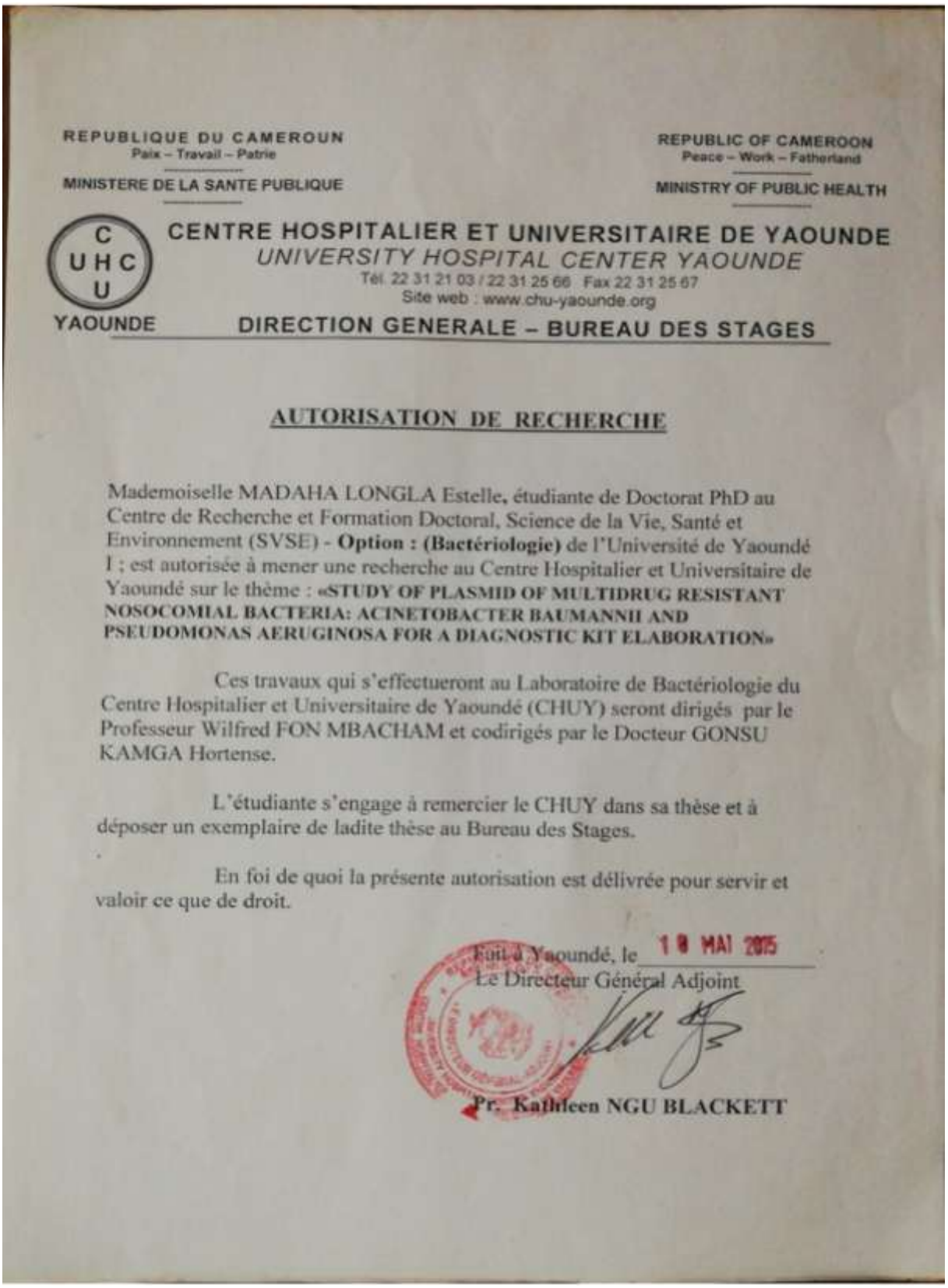
QUESTIONNAIRE

Titre de la recherche: *Development of a diagnostic test against resistant nosocomial bacteria*

Données personnelles du participant

1. Code du participant ……
2. Quel âge avez-vous?.....
3. Sexe: FEMININ ……MASCULIN ……
4. Produit pathologique……

Appendix 8: Research authorization of Yaounde University Teaching Hospital



REPUBLIQUE DU CAMEROUN
Paix – Travail – Patrie

MINISTERE DE LA SANTE PUBLIQUE

REPUBLIC OF CAMEROON
Peace – Work – Fatherland

MINISTRY OF PUBLIC HEALTH

CHUY
YAOUNDE

CENTRE HOSPITALIER ET UNIVERSITAIRE DE YAOUNDE
UNIVERSITY HOSPITAL CENTER YAOUNDE
Tél. 22 31 21 03 / 22 31 25 66 Fax 22 31 25 67
Site web : www.chu-yaounde.org

DIRECTION GENERALE – BUREAU DES STAGES

AUTORISATION DE RECHERCHE

Mademoiselle MADAHA LONGLA Estelle, étudiante de Doctorat PhD au Centre de Recherche et Formation Doctoral, Science de la Vie, Santé et Environnement (SVSE) - **Option : (Bactériologie)** de l'Université de Yaoundé I ; est autorisée à mener une recherche au Centre Hospitalier et Universitaire de Yaoundé sur le thème : **«STUDY OF PLASMID OF MULTIDRUG RESISTANT NOSOCOMIAL BACTERIA: ACINETOBACTER BAUMANNII AND PSEUDOMONAS AERUGINOSA FOR A DIAGNOSTIC KIT ELABORATION»**

Ces travaux qui s'effectueront au Laboratoire de Bactériologie du Centre Hospitalier et Universitaire de Yaoundé (CHUY) seront dirigés par le Professeur Wilfred FON MBACHAM et codirigés par le Docteur GONSU KAMGA Hortense.

L'étudiante s'engage à remercier le CHUY dans sa thèse et à déposer un exemplaire de ladite thèse au Bureau des Stages.

En foi de quoi la présente autorisation est délivrée pour servir et valoir ce que de droit.

Fait à Yaoundé, le 10 MAI 2015
Le Directeur Général Adjoint

Pr. Kathleen NGU BLACKETT

Appendix 9: Research authorization of Yaounde Central Hospital

REPUBLIQUE DU CAMEROUN
Paix-Travail-Patrie
MINISTERE DE LA SANTE PUBLIQUE
SECRETARIAT GENERAL
DIRECTION DE L'HOPITAL CENTRAL DE YAOUNDE

REPUBLIC OF CAMEROON
Peace-Work-Fatherland
MINISTRY OF PUBLIC HEALTH
SECRETARIAT GENERAL
DIRECTORATE OF CENTRAL HOSPITAL

Yaoundé, le 08 AVR. 2015

N° 200 /15 /L/DHCY/SG/AG

Réf : V/L du 30/03/2015

LE DIRECTEUR
A
Madame MADAHA LONGLA Estelle
Yaoundé.

Objet: Autorisation de collecte de données.

Monsieur,

En réponse à votre lettre sus référenciée dont l'objet est repris en marge,

J'ai l'honneur de vous informer que je marque mon accord pour la collecte des données de votre étudiante la nommée **MADAHA LONGLA Estelle**, à l'Hôpital Central de Yaoundé, du **13 Avril au 31 Décembre 2015.** L'intéressée retirera l'autorisation de stage dans mes services.

Il est exigé qu'un enseignant de l'hôpital soit associé à cette collecte.

Veuillez croire, **Monsieur**, à l'expression de ma parfaite considération.

LE DIRECTEUR

Appendix 10: Research authorization of Yaounde University Teaching Hospital

REPUBLIQUE DU CAMEROUN
Paix - Travail - Patrie

MINSANTE

REPUBLIC OF CAMEROON
Peace - Work - Fatherland

CENTRE PASTEUR DU CAMEROUN

Yaoundé, le 16 Janvier 2015

Le Chef de Service bactériologie

Autorisation de collecte des souches bactériennes

Je soussignée, **Dr Marie-Christine FONKOUA,** Chef de service bactériologie au Centre Pasteur du Cameroun, autorise Mlle **MADAHA Estelle**, étudiante en thèse de Doctorat Ph.D à l'université de Yaoundé I, à collecter les souches de *Pseudomonas aeruginosa* et *Acinetobacter baumannii* isolées au Laboratoire de bactériologie entre Janvier 2015 et mars 2016. Ces souches seront exclusivement utilisées pour la réalisation de ses travaux de thèse dont le thème est «Development of diagnostic test against resistant nosocomial bacteria», sous la supervision du **Pr Wilfred MBACHAM**.

En foi de quoi la présente autorisation lui est délivrée pour servir et valoir ce que de droit.

Dr Marie-Christine FONKOUA

CENTRE PASTEUR DU CAMEROUN Laboratoire National de Référence et de Santé Publique
Membre du Réseau International des Instituts Pasteur
BP 1274 YAOUNDE, 451, Rue 2005, Yaoundé 2 - CAMEROUN – site Internet: www.pasteur-yaounde.org
Tel : (237) 222 23 10 15 / 222 23 18 03 - Télécopie : (237) 22 23 15 64 – email : cpc@pasteur-yaounde.org

Appendix 11. Articles published: Occurrence of *bla$_{TEM}$* and *bla$_{CTXM}$* Genes and Biofilm Forming Ability among Clinical Isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in Yaoundé, Cameroon

Appendix 12 Articles published: Whole-genome sequence of multi-drug resistant *Pseudomonas aeruginosa* strains UY1PSABAL and UY1PSABAL2 isolated from human broncho-alveolar lavage, Yaoundé, Cameroon