كليـــــــة الـطـب
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Contribution of mass spectrometry to bacterial identification

THESIS

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BY

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Born on the 5th of May, 1999

TO OBTAIN THE DEGREE OF DOCTOR OF MEDICINE

Keywords

MALDI-TOF mass spectrometry – Microbial identification – Rare and fastidious microorganisms

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

قَالُوا۟ سُبْحَٰنَكَ لَا عِلْمَ لَنَآ إِلَّا مَا عَلَّمْتَنَآ ۖ إِنَّكَ أَنتَ ٱلْعَلِيمُ ٱلْحَكِيمُ ﴿٣٢﴾

صَدَقَ ٱللَّهُ ٱلْعَظِيمُ

Hippocratic Oath

I swear to fulfill, to the best of my ability and judgment, this covenant :
I will respect the hard-won scientific gains of those physicians in whose steps I walk, and gladly share such knowledge as is mine with those who are to follow.
I will apply, for the benefit of the sick, all measures [that] are required, avoiding those twin traps of overtreatment and therapeutic nihilism.
I will remember that there is art to medicine as well as science, and that warmth, sympathy, and understanding may outweigh the surgeon's knife or the chemist's drug.
I will not be ashamed to say "I know not," nor will I fail to call in my colleagues when the skills of another are needed for a patient's recovery.
I will respect the privacy of my patients, for their problems are not disclosed to me that the world may know. Most especially must I tread with care in matters of life and death. If it is given me to save a life, all thanks. But it may also be within my power to take a life ; this awesome responsibility must be faced with great humbleness and awareness of my own frailty. Above all, I must not play at God.
I will remember that I do not treat a fever chart, a cancerous growth, but a sick human being, whose illness may affect the person's family and economic stability. My responsibility includes these related problems, if I am to care adequately for the sick.
I will prevent disease whenever I can, for prevention is preferable to cure.
I will remember that I remain a member of society, with special obligations to all my fellow human beings, those sound of mind and body as well as the infirm.

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permanant

N°	Nom et Prénom	Cadre	Spécialités
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03	BOUSKRAOUI Mohammed	P.E.S	Pédiatrie
04	KHATOURI Ali	P.E.S	Cardiologie
05	NIAMANE Radouane	P.E.S	Rhumatologie
06	AIT BENALI Said	P.E.S	Neurochirurgie
07	KRATI Khadija	P.E.S	Gastro-entérologie
08	SOUMMANI Abderraouf	P.E.S	Gynécologie-obstétrique
09	RAJI Abdelaziz	P.E.S	Oto-rhino-laryngologie
10	SARF Ismail	P.E.S	Urologie
11	MOUTAOUAKIL Abdeljalil	P.E.S	Ophtalmologie
12	AMAL Said	P.E.S	Dermatologie
13	ESSAADOUNI Lamiaa	P.E.S	Médecine interne
14	MANSOURI Nadia	P.E.S	Stomatologie et chirurgie maxillo faciale
15	MOUTAJ Redouane	P.E.S	Parasitologie
16	AMMAR Haddou	P.E.S	Oto-rhino-laryngologie
17	EL FEZZAZI Redouane	P.E.S	Chirurgie pédiatrique
18	YOUNOUS Said	P.E.S	Anesthésie-réanimation
19	BENELKHAIAT BENOMAR Ridouan	P.E.S	Chirurgie générale

20	ASMOUKI Hamid	P.E.S	Gynécologie-obstétrique
21	CHELLAK Saliha	P.E.S	Biochimie-chimie
22	LOUZI Abdelouahed	P.E.S	Chirurgie-générale
23	AIT-SAB Imane	P.E.S	Pédiatrie
24	GHANNANE Houssine	P.E.S	Neurochirurgie
25	OULAD SAIAD Mohamed	P.E.S	Chirurgie pédiatrique
26	DAHAMI Zakaria	P.E.S	Urologie
27	EL HATTAOUI Mustapha	P.E.S	Cardiologie
28	AMINE Mohamed	P.E.S	Epidémiologie clinique
29	EL ADIB Ahmed Rhassane	P.E.S	Anesthésie-réanimation
30	ELFIKRI Abdelghani	P.E.S	Radiologie
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32	KAMILI El Ouafi El Aouni	P.E.S	Chirurgie pédiatrique
33	MAOULAININE Fadl mrabih rabou	P.E.S	Pédiatrie (Néonatologie)
34	MATRANE Aboubakr	P.E.S	Médecine nucléaire
35	ADMOU Brahim	P.E.S	Immunologie
36	CHERIF IDRISSI EL GANOUNI Najat	P.E.S	Radiologie
37	MANOUDI Fatiha	P.E.S	Psychiatrie
38	BOURROUS Monir	P.E.S	Pédiatrie
39	TASSI Noura	P.E.S	Maladies infectieuses
40	NEJMI Hicham	P.E.S	Anesthésie-réanimation
41	LAOUAD Inass	P.E.S	Néphrologie
42	FOURAIJI Karima	P.E.S	Chirurgie
43	BOUKHIRA Abderrahman	P.E.S	Biochimie-chimie
44	KHALLOUKI Mohammed	P.E.S	Anesthésie-réanimation
45	BSISS Mohammed Aziz	P.E.S	Biophysique
46	EL OMRANI Abdelhamid	P.E.S	Radiothérapie
47	SORAA Nabila	P.E.S	Microbiologie-virologie
48	KHOUCHANI Mouna	P.E.S	Radiothérapie
49	JALAL Hicham	P.E.S	Radiologie
50	EL ANSARI Nawal	P.E.S	Endocrinologie et maladies métaboliques
51	AMRO Lamyae	P.E.S	Pneumo-phtisiologie
52	OUALI IDRISSI Mariem	P.E.S	Radiologie
53	ZAHLANE Mouna	P.E.S	Médecine interne

54	BENJILALI Laila	P.E.S	Médecine interne
55	NARJIS Youssef	P.E.S	Chirurgie générale
56	RABBANI Khalid	P.E.S	Chirurgie générale
57	SAMLANI Zouhour	P.E.S	Gastro-entérologie
58	LAGHMARI Mehdi	P.E.S	Neurochirurgie
59	ABOUSSAIR Nisrine	P.E.S	Génétique
60	BENCHAMKHA Yassine	P.E.S	Chirurgie réparatrice et plastique
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63	EL BOUIHI Mohamed	P.E.S	Stomatologie et chirurgie maxillo faciale
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65	AGHOUTANE El Mouhtadi	P.E.S	Chirurgie pédiatrique
66	HOCAR Ouafa	P.E.S	Dermatologie
67	EL KARIMI Saloua	P.E.S	Cardiologie
68	EL BOUCHTI Imane	P.E.S	Rhumatologie
69	QAMOUSS Youssef	P.E.S	Anésthésie réanimation
70	ZYANI Mohammad	P.E.S	Médecine interne
71	QACIF Hassan	P.E.S	Médecine interne
72	BEN DRISS Laila	P.E.S	Cardiologie
73	MOUFID Kamal	P.E.S	Urologie
74	EL BARNI Rachid	P.E.S	Chirurgie générale
75	ABOUCHADI Abdeljalil	P.E.S	Stomatologie et chirurgie maxillo faciale
76	BASRAOUI Dounia	P.E.S	Radiologie
77	RAIS Hanane	P.E.S	Anatomie Pathologique
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79	ZAOUI Sanaa	P.E.S	Pharmacologie
80	MSOUGAR Yassine	P.E.S	Chirurgie thoracique
81	EL MGHARI TABIB Ghizlane	P.E.S	Endocrinologie et maladies métaboliques
82	DRAISS Ghizlane	P.E.S	Pédiatrie
83	EL IDRISSI SLITINE Nadia	P.E.S	Pédiatrie
84	RADA Noureddine	P.E.S	Pédiatrie
85	BOURRAHOUAT Aicha	P.E.S	Pédiatrie
86	MOUAFFAK Youssef	P.E.S	Anesthésie-réanimation
87	ZIADI Amra	P.E.S	Anesthésie-réanimation

88	ANIBA Khalid	P.E.S	Neurochirurgie
89	TAZI Mohamed Illias	P.E.S	Hématologie clinique
90	ROCHDI Youssef	P.E.S	Oto-rhino-laryngologie
91	FADILI Wafaa	P.E.S	Néphrologie
92	ADALI Imane	P.E.S	Psychiatrie
93	ZAHLANE Kawtar	P.E.S	Microbiologie- virologie
94	LOUHAB Nisrine	P.E.S	Neurologie
95	HAROU Karam	P.E.S	Gynécologie-obstétrique
96	BOUKHANNI Lahcen	P.E.S	Gynécologie-obstétrique
97	FAKHIR Bouchra	P.E.S	Gynécologie-obstétrique
98	BENHIMA Mohamed Amine	P.E.S	Traumatologie-orthopédie
99	HACHIMI Abdelhamid	P.E.S	Réanimation médicale
100	EL KHAYARI Mina	P.E.S	Réanimation médicale
101	AISSAOUI Younes	P.E.S	Anésthésie-réanimation
102	ATMANE El Mehdi	P.E.S	Radiologie
103	EL AMRANI Moulay Driss	P.E.S	Anatomie
104	BELBARAKA Rhizlane	P.E.S	Oncologie médicale
105	ALJ Soumaya	P.E.S	Radiologie
106	OUBAHA Sofia	P.E.S	Physiologie
107	EL HAOUATI Rachid	P.E.S	Chirurgie Cardio-vasculaire
108	BENALI Abdeslam	P.E.S	Psychiatrie
109	MLIHA TOUATI Mohammed	P.E.S	Oto-rhino-laryngologie
110	MARGAD Omar	P.E.S	Traumatologie-orthopédie
111	KADDOURI Said	P.E.S	Médecine interne
112	ZEMRAOUI Nadir	P.E.S	Néphrologie
113	EL KHADER Ahmed	P.E.S	Chirurgie générale
114	DAROUASSI Youssef	P.E.S	Oto-rhino-laryngologie
115	BENJELLOUN HARZIMI Amine	P.E.S	Pneumo-phtisiologie
116	FAKHRI Anass	P.E.S	Histologie-embyologie cytogénétique
117	SALAMA Tarik	P.E.S	Chirurgie pédiatrique
118	CHRAA Mohamed	P.E.S	Physiologie
119	ZARROUKI Youssef	P.E.S	Anesthésie-réanimation
120	AIT BATAHAR Salma	P.E.S	Pneumo-phtisiologie

121	ADARMOUCH Latifa	P.E.S	Médecine communautaire (médecine préventive, santé publique et hygiène)
122	BELBACHIR Anass	P.E.S	Anatomie pathologique
123	HAZMIRI Fatima Ezzahra	P.E.S	Histologie-embyologie cytogénétique
124	EL KAMOUNI Youssef	P.E.S	Microbiologie-virologie
125	EL MEZOUARI El Mostafa	P.E.S	Parasitologie mycologie
126	SERGHINI Issam	P.E.S	Anesthésie-réanimation
127	ABIR Badreddine	P.E.S	Stomatologie et chirurgie maxillo faciale
128	ZIDANE Moulay Abdelfettah	P.E.S	Chirurgie thoracique
129	LAHKIM Mohammed	P.E.S	Chirurgie générale
130	MOUHSINE Abdelilah	P.E.S	Radiologie
131	TOURABI Khalid	P.E.S	Chirurgie réparatrice et plastique
132	BELHADJ Ayoub	P.E.S	Anesthésie-réanimation
133	BOUZERDA Abdelmajid	P.E.S	Cardiologie
134	ABDELFETTAH Youness	P.E.S	Rééducation et réhabilitation fonctionnelle
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136	BENNAOUI Fatiha	P.E.S	Pédiatrie
137	ZOUIZRA Zahira	P.E.S	Chirurgie Cardio-vasculaire
138	SEBBANI Majda	P.E.S	Médecine Communautaire (Médecine préventive, santé publique et hygiene
139	FENANE Hicham	P.E.S	Chirurgie thoracique
140	ABDOU Abdessamad	P.E.S	Chirurgie Cardio-vasculaire
141	HAMMOUNE Nabil	P.E.S	Radiologie
142	ESSADI Ismail	P.E.S	Oncologie médicale
143	ALJALIL Abdelfattah	P.E.S	Oto-rhino-laryngologie
144	LAFFINTI Mahmoud Amine	P.E.S	Psychiatrie
145	RHARRASSI Issam	P.E.S	Anatomie-patologique
146	ASSERRAJI Mohammed	P.E.S	Néphrologie
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148	NASSIM SABAH Taoufik	P.E.S	Chirurgie réparatrice et plastique
149	ELBAZ Meriem	P.E.S	Pédiatrie
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152	BAALLAL Hassan	P.E.S	Neurochirurgie
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222	SBAI Asma	MCHab	Informatique
223	EL AMIRI My Ahmed	MCHab	Chimie de Coordination bio-organnique
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225	SLIOUI Badr	MC	Radiologie
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254	FATH EL KHIR Yassine	MC	Traumato-orthopédie
255	NASSIRI Mohamed	MC	Traumato-orthopédie
256	AIT-DRISS Wiam	MC	Maladies infectieuses
257	AIT YAHYA Abdelkarim	MC	Cardiologie
258	DIANI Abdelwahed	MC	Radiologie
259	AIT BELAID Wafae	MC	Chirurgie générale
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295	CHEHBOUNI Mohamed	MC	Oto-rhino-laryngologie
296	LAIRANI Fatima ezzahra	MC	Gastro-entérologie
297	SAADI Khadija	MC	Pédiatrie
298	TITOU Hicham	MC	Dermatologie
299	EL GHOUL Naoufal	MC	Traumato-orthopédie
300	BAHI Mohammed	MC	Anesthésie-réanimation
301	RAITEB Mohammed	MC	Maladies infectieuses
302	DREF Maria	MC	Anatomie pathologique
303	ENNACIRI Zainab	MC	Psychiatrie
304	BOUSSAIDANE Mohammed	MC	Traumato-orthopédie
305	JENDOUZI Omar	MC	Urologie
306	MANSOURI Maria	MC	Génétique
307	ERRIFAIY Hayate	MC	Anesthésie-réanimation
308	BOUKOUB Naila	MC	Anesthésie-réanimation
309	OUACHAOU Jamal	MC	Anesthésie-réanimation
310	EL FARGANI Rania	MC	Maladies infectieuses
311	IJIM Mohamed	MC	Pneumo-phtisiologie
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316	GHARBI Khalid	MC	Gastro-entérologie

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351	ELKHETTAB Fatimazahra	MC	Anesthésie-réanimation
352	SIDAYNE Mohammed	MC	Anesthésie-réanimation
353	ZAKARIA Yasmina	MC	Neurologie
354	BOUKAIDI Yassine	MC	Chirurgie Cardio-vasculaire
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356	KAAKOUA Mohamed	MC	Oncologie médicale
357	FIQHI Mohammed Kamal	MC	Stomatologie et chirurgie maxillo faciale
358	BEN ELHEND Salah	MC	Radiologie
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360	AWATI El Mehdi	MC	Hématologie
361	HAOUANE Mohamed Amine	MC	Anatomie pathologique
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365	AZRIOUIL Ouahb	MC	Traumato-orthopédie
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368	OUSSAYEH Imane	MC	Anesthésie-réanimation
369	KASSAL Ghizlane	MC	Pédiatrie (Néonatologie)
370	RACHID Chaynez	MC	Pneumo-phtisiologie
371	BALILI Khaoula	MC	Neurologie
372	IGARRAMEN Tariq	MC	Biophysique
373	EL MAGHTOUM Hicham	MC	Anesthésie-réanimation
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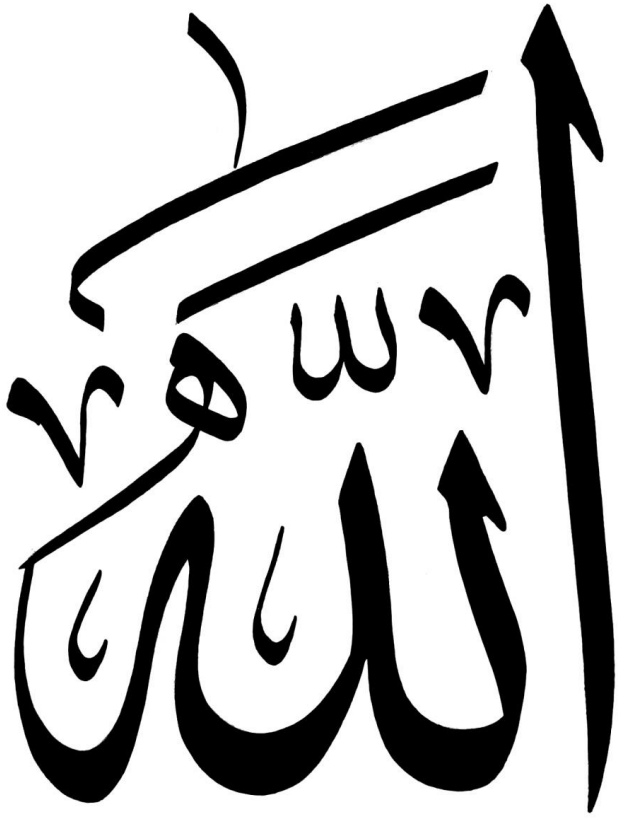

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First and foremost, I thank Allah Almighty for blessing me with the opportunity to be here today and for granting me the honor of influencing people's lives. I am grateful for His answered prayers and for His mercy throughout this hard and rocky journey. Alhamdulillah, now and forever.

To my beloved Mother

No words could ever truly express the depth of my love and gratitude for you. Growing up, I had the profound privilege of watching you seamlessly balance the demanding responsibilities of a devoted teacher with the endless, unconditional care of a loving mother. You taught me my very first lessons in hard work and dedication, showing me through your own daily example what it means to persevere with grace.

Your relentless efforts, the sacrifices you quietly made, and the deeply nurturing environment you created for us have been my greatest sources of inspiration. You cultivated my ambitions and provided the steady, comforting foundation I needed to navigate the demanding years of this academic journey. Every milestone I reach, and the person I have grown into today, is a direct reflection of the love and energy you poured into raising me.

I dedicate this thesis to you as a modest tribute to your unwavering support, your gentle guidance, and your infinite patience. May God bless you with abundant health, happiness, and a long, beautiful life, so you may always remain the guiding light of our family.

To my beloved Father

You have always been the steadfast provider for our family, working tirelessly to give us the best life possible. But beyond that, as a teacher yourself, you understood the profound value of education and always went the extra mile for mine. I will forever cherish the memories of you sitting down to study by my side, generously pouring your time and energy into my success even after a long, exhausting day of work. You did not just provide for my education; you actively built its foundation.

Yet, what truly makes our bond so irreplaceable is the incredible, lighthearted dynamic we share. Amidst the seriousness of life and the heavy demands of this academic journey, our classic Marrakchi humor and the playful jabs we constantly trade have been my greatest refuge. You taught me the importance of relentless hard work, but through our endless banter, you also taught me never to lose my sense of humor.

I dedicate this work to you in profound gratitude. Thank you for being my anchor, my dedicated tutor, and my absolute favorite sparring partner in comedy. May God bless you with enduring health, happiness, and a long life filled with the same joy you bring to mine.

To my dearest eldest sister, Charifa ,Tolopozpoz

Being twelve years older than me, you have always been so much more than just a sister; you are truly the best person in the world to me. Throughout my life, you have cared for me with a depth of kindness and protectiveness that I can never fully repay. When I look back, my most treasured core memories are filled with your presence—from the simple, quiet joy of you taking me to kindergarten on your bike, to the weekends you lovingly spent setting up games just to see me smile.

You have always gone above and beyond to make sure I never lacked anything in this world. Whether it was buying me my very first PC to fuel my early curiosities, or simply being my constant, reliable source of comfort, you have always spoiled me with your endless generosity. As I reach the end of this long academic journey and present this thesis, I want you to know that my success is deeply rooted in the foundation of love and care you provided.

Thank you for your unwavering support, your boundless affection, and for simply being the incredible person you are. I dedicate this work to you, my dear Tolopozpozpoz. May life always treat you with the same immense kindness, warmth, and devotion that you have unconditionally shown me.

To my dear older sister, Sara

Growing up, your remarkable academic journey was a constant source of motivation for me. Watching your dedication and profound skill as an ophthalmologist fills me with immense pride. Seeing you excel in the medical field has been an incredible inspiration, and I am absolutely certain that you will reach the highest pinnacles of success in your career.

Yet, beyond your impressive professional achievements, I am most grateful for the fiercely caring and protective sister you have always been. I will never forget how you were always the very first one to step up and fight off the bigger kids if anyone ever dared to bother me. That unwavering loyalty and fierce protective instinct have made me feel safe my entire life.

Thank you for being my greatest defender, my academic role model, and an endlessly caring sister. I dedicate this thesis to you with all my love and gratitude, wishing you a future as bright, beautiful, and successful as you truly deserve.

To my dearest sister, Hiba

As the youngest of my sisters, being just five years older than me, you are the one I have spent the most time with and the one whose footsteps I have most closely followed. You have always paved the way for me, and I have endlessly benefited from your experiences, your incredible generosity, and the wonderful network of people you bring into our lives. More than anyone, you are the reason I chose to pursue medical biology. Your passion for the specialty inspired my own, and I have absolutely no doubt that you are going to be the greatest biologist to ever exist.

When I was younger, I thought the way you helped me with my medical studies was just a normal part of having an older sister. It is only as I have grown and seen how others interact with their younger siblings that I truly realize how extraordinary your dedication was. The immense amount of time and the unbelievable effort you put into helping me study is the very reason I am in the position I am today. I simply would not have passed my exams or made it to this finish line without you.
You also played a massive role in helping me break out of my shell, gently pushing me to be less introverted and more confident in myself. We always joke that you are my "secretary" because you flawlessly handle all my problems, but the truth is, you are my ultimate problem-solver, my greatest advocate, and my closest confidante.
I dedicate this thesis to you with the most profound gratitude and love. Thank you for being my constant guide and my greatest support.

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We survived medical school together. Through every brutal clinical rotation and endless night shift, you were my lifeline. We didn't just share the stress and the fatigue; we shared the kind of laughs that actually got us through it all. You made these years unforgettable.

To Achraf

We've been around each other since the early days of our studies, but I'm so glad our bond actually deepened over time. You're this crazy mix of being completely serious and studious, yet hilarious and always up for an adventure. I'm still amazed by how much random knowledge you hold on literally every subject. But beyond all that, you're just a genuinely good guy who is always there for anyone in need. Watching you and Kenza together brings me so much joy.

To Chaimae

You are hands down one of the funniest people I know, and the absolute definition of chill. You were one of the first people I really clicked with in med school. I love your open mind and how incredibly smart you are. I know you value your personal space, but I still consider you as close a friend as anyone could ever be. Seriously though, please take it easy on the medical supplements!

To Kenza

You were probably my very first friend in med school, and I'll never forget the massive effort you made to pull me out of my bubble. You are so incredibly sweet and beautifully real with your emotions. We've survived so many night shifts together, spending hours just poking fun at everyone around us. You've got a heart of gold, but let's be honest—you're just as crazy as I am, and I know exactly how much you love making me uncomfortable!

To Souha

Getting to know you has been one of the best surprises of these past few years. You have such a beautiful, rich soul. I'll never forget you acting as our group driver, taking us everywhere while turning car rides into concerts with your amazing voice and introducing me to so many great Arabic songs. From your talent on the piano to your weird obsession with Tabasco and your love for reading, you are truly one of a kind. You're also one of the toughest people I've ever met—you can face an absolute storm and still keep a smile on your face. Your free, generous spirit is a constant inspiration to me.

To Taoufik

You've really become like an older brother to me. I know you come off as shy at first, but once you get comfortable, the absolute demon inside comes out! Even so, you are the most sensible and ridiculously altruistic person I know. You're the first one I go to whenever I have a problem. You're also a wizard at the pool table (top 10 in the world, easily), and even though your cooking is famously terrible, the fact that you still make the effort to cook for us on every trip shows exactly how big your heart is. Thanks for always taking our jokes in stride.

To Zakaria

To the 190cm handsome guy of our group: you're basically our little brother. We give you a hard time constantly, but you know it's just because we love you. I'm going to miss all our FIFA tournaments and the real football matches where you somehow always saved us as our goalkeeper. More than anything, thank you for being the kind of friend who will literally drive over to pick me up from home whenever I need you.

To my incredible Internat family: Ayoub, Bouchra, Kenza, Mahdi, and Yasmine

Our internat days would not have been the same without you. We managed to build our own little family amidst the absolute chaos of the hospital, and I am so grateful for every laugh, every shared meal, and every brutal shift we survived together.

To Ayoub

My internat brother. We have spent countless hours on endless calls playing Dofus, and those gaming sessions are genuinely some of my favorite memories. You have such a pure, outgoing soul, and you are truly one of the nicest people I know. Plus, you never fail to make me laugh until my sides hurt—especially when you randomly invent a brand-new allergy for yourself out of thin air.

To Bouchra

The one in the group who actually has real allergies! You are an incredibly resilient person, beautifully proud of your Fassi roots, and honestly one of the coolest girls I've ever met. Bonding over our shared love for anime and gaming has been amazing. I am so incredibly happy for you and Mahdi on your recent engagement; you two are an absolutely perfect match.

To Kenza

It honestly pains me to admit this, but you are probably the funniest person I know, and the worst part is you don't even have to try. Beyond the effortless humor, you are exceptionally smart, and I know for a fact you are going to make a phenomenal anesthesiologist. Maybe once you're officially at the top of your field, you'll finally be able to feed your dog, Snow, a little bit more!

To Mahdi

Who knew that surviving six grueling months of neonatology together would forge such a deep bond? You are an absolute legend. From our shared obsession with anime and video games to your undeniable skills as an amazing cook, you naturally became the ultimate "father figure" of our group. I am incredibly lucky to have you as a friend.

To Yasmine

It is funny how a few coincidental night shifts can bring someone so incredibly special into your life. You are such a sweet, thoughtful, and timid soul at first glance, but I know firsthand just how hilarious you can be when you want to. I love our shared obsession with cats, our endless coffee runs, and our constant hunts for good pastries. You approach your work with such impressive seriousness, yet you always bring so much warmth to the people around you—not to mention how you completely destroy all of us at bowling. You are someone I deeply cherish, and I look forward to sharing a lifetime of coffees, pastries, and quiet moments with you.

To my brothers for life: Ali, Ayoub el Ame, Ayoub Haoufadi, Mehdi Benlarabi, Saad el Fadil, Tariq, Moad, Ibrahim and Youssef

There are friends you make during your internat years who quickly become like family, and then there are those who become something much deeper. You are some of the absolute best people I have ever had the privilege of knowing, each extraordinary in your own unique way.

What started as just another routine football match turned into a day that changed my perspective on everything. When things took a sudden and critical turn on that pitch, it was your immediate reactions, your sharp instincts, and your quick thinking that made all the difference in the world. I am only able to stand here today, writing these words and celebrating this milestone, because you were there for me when it mattered most.

It is a rare thing to find people you can genuinely trust with your life, but I found exactly that in all of you. Words will never be enough to repay that kind of debt. You are my brothers forever, and I want you to know that just as you stepped up for me in my most vulnerable moment, I will always be in your corner, without hesitation, for the rest of our lives.

To the Mcdwich Gang: Assaad, Ayoub Bindar, and Marouan

I will always cherish the chicken we eat at Mcdwich together. It has become a ritual that I simply cannot give up, and the brotherhood we share around that table is something I truly value.

To Ayoub Bindar

You are quite simply the nicest guy around. I am grateful for the countless times you handed over your notes so we could prep for our exams, and for the hours we spent bonding in our services. You are a great football player on the pitch, and honestly, I am just glad Arsenal finally won the league for you.

To Assaad

The crazy anesthesiologist of our group. You walk through life with such a carefree energy, yet the second someone actually needs you, you drop everything to be incredibly helpful. It is a rare balance, and it makes you an amazing friend to have in my corner.

To Marouan

The absolute chad of the crew. You have somehow managed to build a literal Greek god physique, but unfortunately, we all know your gaming skills do not quite follow suit. Despite your struggles with a controller, you are an incredible friend, and I wouldn't trade our group traditions for anything.

To my neonatology group: Mahdi, Mehdi, Nada, and Salma

We spent an amazing six months together in the neonatology department, and I would not trade that time for anything. Sharing such a demanding experience allowed us to grow incredibly close in a relatively short amount of time. We formed a bond that extends far beyond the hospital walls, and it brings me a lot of comfort knowing that I can always count on each of you. Thank you for making those six months so memorable and for always having my back.

To my friend Hakim

You are quite simply a great guy. I really value the fact that you are always down to hang out, no matter what is going on. Your easygoing nature and reliable presence have brought a lot of good times and necessary breaks into my routine.

To my friend Assya

You are genuinely one of the people I appreciate the most. I love the bond we share, whether we are talking about our mutual passion for nature or just relentlessly making fun of each other. Our constant banter is something very special to me, and it has always been the perfect escape from the stress of our studies.

To my friend Mohamed

You were one of the very first people I crossed paths with in medical school, and I am so glad our friendship started right from day one. I have always admired your brilliant mind and how you consistently managed to stay at the top of our class. Your sharp intellect and steady performance were always something I deeply respected.

To my friends from Group 4: Anas, Bouchra, Oumaima, and Soukaina

Thank you for the memorable times we shared throughout our formation. Navigating the demands of our training was so much easier with you all by my side. Beyond the hospital walls and the serious moments, I will always appreciate the great times and the solid camaraderie we built as a group.

To my new friends: Faiçal, Hamza, and Sara

I am incredibly glad that Hiba brought you into my life. Getting to know the three of you has been a genuine privilege, and I am grateful for the impact you have had on my journey.

To Hamza

As an immunology professor, you have this rare ability to completely break down the barrier between teacher and student, making it feel like it doesn't even exist. You are an incredibly cultured and genuinely kind person. You always go out of your way to be helpful in any way you can, and I deeply appreciate the humble guidance you have shown me.

To Sara

You are simply the greatest ORL in town. But beyond your professional talent, I love that you are just as much of a big nerd as I am. Being able to bond over our shared love for anime is something I truly enjoy. More importantly, you possess a moral compass unlike anything I have ever seen, and I have profound respect for the integrity you show in everything you do.

To Faiselles

I need to state clearly that you are the absolute craziest and funniest person I know. But behind all that energy is genuinely one of the nicest people around. You consistently go above and beyond for anyone in need, always stepping up to help in whatever way you can. Still, I have to reinstate it—you are completely crazy, and I value that about you immensely.

To my biology mates: Ayoub el Ame, Houssam the rapper, Jerbocho, and Othmane Zika

You guys make every single day I come in something I genuinely look forward to. The mornings we spend wolfing down breakfasts together and our routine coffee breaks at the avenue are truly some of the best parts of my routine. We have built a very specific kind of humor that nobody else will ever quite get, and I value that shared bond immensely. Thank you for making the daily grind so enjoyable and for being such great company.

To my lifelong friends, Diyaa and Naja

You are the only friends from my childhood that I have kept in contact with, and there is a profound reason for that. As we grew up and our lives evolved, our bond proved to be truly unbreakable. Having friends who have known me for this long and still stand by my side today is a rare privilege that I do not take for granted.

To Naja

We share so much in common, and that connection has always been a cornerstone of our friendship. You have consistently been the one to introduce me to things I ended up getting completely hooked on, from countless games to the entire world of Wuxia. I am incredibly glad to know you. Having someone who completely understands my interests and seamlessly shares in my passions is a rare gift, and I will always cherish the countless hours we have spent diving into those different worlds together.

To Diyaa

You are such a remarkably special character. You are a genuinely bright person, and honestly, your name fits you perfectly. You radiate a certain light and warmth, constantly doing great things for the people around you with such natural ease. I have absolute certainty that you are going to achieve truly great things in the future, and I will proudly be cheering you on every step of the way.

To the incredible staff of the Biology Lab: Teachers, Residents, and Secretaries

I want to express my deepest gratitude to all of you. You are, without a doubt, some of the most genuinely caring and supportive people I have had the pleasure of working with. Your constant benevolence creates a warm environment that makes coming to work every single day something I truly appreciate and look forward to.

To the teachers and residents, thank you for your guidance, your patience, and for sharing your expertise so generously. And to the secretaries and the rest of the lab staff, I want to specifically acknowledge the immense amount of hard work you put in every single day. So much of what you do happens behind the scenes and often goes unnoticed, but it is the very foundation that keeps everything running smoothly.

Thank you all for your kindness, your dedication, and for making this lab such an exceptional place to work and grow.

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PROFESSOR OF HIGHER EDUCATION IN BIOLOGY HEAD OF THE BIOLOGY
Department at the CHU Mohammed VI of Marrakech

It is a profound honor and an immense privilege to have you preside over my thesis jury. As an extraordinary educator and a true master of your craft, you have left an indelible mark on my academic journey.

My time spent in your department was truly transformative; it was under your exceptional leadership that a genuine love for microbiology was instilled within me. You are deeply inspiring, and your impressive ability to effortlessly find clear, elegant solutions to even the most complex problems is something I have always marveled at.

I look up to you immensely and sincerely aspire to become a professional of your caliber one day. Please accept this thesis as a humble expression of my profound respect, my highest admiration, and my deepest gratitude for the example you set.

TO MY ESTEEMED PROFESSOR AND THESIS DIRECTOR, PROFESSOR ASMAE LAMRANI HANCHI

It is an immense honor to have you evaluate this thesis. As an amazing teacher, your vast knowledge and impeccable scientific rigor have always commanded my deepest respect. Observing your daily dedication—consistently giving one hundred and ten percent of your effort and constantly going above and beyond to ensure the absolute best results for your patients—has been a profound lesson in what it means to be a truly devoted biologist.

Yet, beyond your remarkable professional standards, you possess the absolute kindest soul. Your deep sensitivity, warmth, and genuine kindness make every interaction with you a privilege. You are not only an extraordinary mentor whose guidance has been invaluable, but also a truly wonderful human being.

Please accept this dedication as a sincere testament to my profound gratitude, my highest regard, and my enduring admiration.

TO MY ESTEEMED PROFESSOR AND MEMBER OF THE JURY, PROFESSOR FATIHA BENNAOUI PROFESSOR OF NEONATOLOGY

It is a great honor to have you sit on my thesis jury. Spending six months as an intern in your neonatology department was a deeply rewarding experience that I will always carry with me.

I will always remember the consultations we shared, where your remarkably calm demeanor and genuine kindness truly stood out. You are an excellent tutor who consistently took the time to patiently teach and guide us, all while providing the utmost, compassionate care for your fragile patients.

Your steady guidance and dedication to teaching made my passage through neonatology an incredibly memorable part of my medical training. Please accept this dedication as a sincere token of my gratitude, my high regard, and my profound respect for the wonderful educator and physician that you are.

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Your expertise in the field of infectiology is highly respected, and having a professional of your standing participate in my defense brings immense value to this milestone in my academic journey.

Please accept this dedication as a sincere expression of my profound gratitude and my highest respect.

<u>TO MY ESTEEMED PROFESSOR AND MEMBER OF THE JURY, PROFESSOR MANAL RHEZALI</u>
<u>PROFESSOR OF ANESTHESIOLOGY AND INTENSIVE CARE</u>

It is an immense honor to have you on my thesis jury, but your presence here today is profoundly special to me for reasons that go far beyond this academic milestone.

There are simply no words that can adequately express the depth of my gratitude toward you. During the most critical and vulnerable moment of my life, your exceptional expertise, swift action, and unwavering care gave me the most precious gift imaginable. The very fact that I am able to stand here today, completing this journey and presenting this thesis, is a direct testament to your incredible skill and dedication as a physician.

I owe you a debt of gratitude that transcends the professional realm. To have the person who protected my future now sitting on this jury to witness this accomplishment holds a meaning I cannot easily put into words.

Please accept this dedication as a symbol of my eternal gratitude, my deepest respect, and my highest admiration.

وَمَنْ أَحْيَاهَا فَكَأَنَّمَا أَحْيَا النَّاسَ جَمِيعًا

ABREVIATIONS

LIST OF ABBREVIATIONS

API	Analytical Profile Index (bioMérieux miniaturised biochemical gallery system)
ASTRA	Antibiotic Susceptibility Test Rapid Assay
BCID	Blood Culture Identification
BLAST	Basic Local Alignment Search Tool
CFU	Colony-Forming Unit
CHCA	α-Cyano-4-hydroxycinnamic acid (MALDI-TOF matrix compound)
CLSI	Clinical and Laboratory Standards Institute
CoNS	Coagulase-Negative Staphylococci
COVID-19	Coronavirus Disease 2019
DNA	Deoxyribonucleic Acid
GNB	Gram-Negative Bacilli
GPB	Gram-Positive Bacilli
GPC	Gram-Positive Cocci
HACEK	Haemophilus, Aggregatibacter, Cardiobacterium, Eikenella, Kingella
ITS	Internal Transcribed Spacer
IVD	In Vitro Diagnostic
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight
MBT	MALDI Biotyper (Bruker Daltonik platform)
MBT-ASTRA	MALDI Biotyper Antibiotic Susceptibility Test Rapid Assay
MDR	Multidrug-Resistant
MM18-A	CLSI Guideline MM18-A (interpretive thresholds for nucleic-acid sequencing-based microbial identification)
MRSA	Methicillin-Resistant Staphylococcus aureus
MS	Mass Spectrometry
MSP	Main Spectrum (BioTyper reference spectrum)
MSSA	Methicillin-Susceptible *Staphylococcus aureus*
NMIC	BD Phoenix combined identification-and-susceptibility panel for Gram-negative organisms
ONPG	ortho-Nitrophenyl-β-galactopyranoside (β-galactosidase test substrate)
PCR	Polymerase Chain Reaction
PDP	Prélèvement distal protégé (protected distal sampling; respiratory specimen code)
PMIC	BD Phoenix combined identification-and-susceptibility panel for Gram-positive organisms
PNA-FISH	Peptide Nucleic Acid Fluorescence In Situ Hybridisation
RNA	Ribonucleic Acid

STAR-BL	Selective Testing of Antibiotic Resistance — β-Lactamase (Bruker MBT STAR-BL assay)
TOF	Time-of-Flight
USD	United States Dollar
VITEK	VITEK 2 automated identification and susceptibility system (bioMérieux)

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OUTLINE

INTRODUCTION

I. Clinical Context and Objectives of the Study

The identification of microorganisms isolated from clinical specimens constitutes one of the central functions of any medical microbiology laboratory. Far from being a purely technical exercise, accurate microbial identification directly conditions the quality of patient care: it informs the selection of appropriate antimicrobial therapy, guides infection control measures, contributes to epidemiological surveillance, and enables the detection of emerging or unusual pathogens before they propagate within hospital environments. In the context of a university hospital serving as a national referral centre, these imperatives are amplified by the complexity and diversity of the patient population, which encompasses immunocompromised individuals, patients with chronic diseases, surgical and intensive care recipients, and cases referred from peripheral institutions precisely because standard diagnostic approaches have proven insufficient.

Historically, the identification of clinically significant microorganisms has relied upon a sequential application of phenotypic and biochemical methods — a paradigm that served clinical microbiology for several decades and whose foundations remain in use today. These methods, reviewed in Section I of the Discussion, encompass macroscopic examination of colony characteristics, microscopic analysis including Gram staining, and a range of biochemical investigations from simple enzymatic tests to commercial multi-test gallery systems such as the API range and automated platforms such as the VITEK II system. While this approach performs reliably for the most commonly encountered and metabolically active species, it is recognised to carry fundamental limitations in the context of rare, slow-growing, or phenotypically atypical organisms — categories that are disproportionately represented in the diagnostic workload of referral-level institutions.

The advent of molecular biology, and in particular 16S ribosomal RNA gene sequencing, introduced a more discriminatory alternative for organisms that resist reliable phenotypic characterisation. However, as discussed in Section II of the Discussion, the technical demands, cost, and operational complexity of molecular sequencing have prevented its adoption as a first-line routine identification method in the majority of clinical laboratories. It is against this

backdrop that Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS), examined in detail in Section III of the Discussion, emerged over the past two decades as a transformative first-line identification technology. By generating unique mass spectral fingerprints from intact microbial cells and comparing them against reference databases within minutes, MALDI-TOF MS has substantially altered the landscape of routine microbial identification, offering accuracy, speed, and cost-effectiveness unmatched by prior methods.

Of particular relevance to the present study is the application of MALDI-TOF MS to rare and fastidious microorganisms — organisms whose low prevalence, special growth requirements, or atypical metabolic profiles rendered them consistently difficult or impossible to identify reliably by conventional phenotypic means. These organisms, which are discussed in Section IV of the Discussion according to their major taxonomic groupings, represent a clinically significant subset of the isolates processed in any high-complexity laboratory. Their accurate identification carries direct implications for therapeutic management, infection control classification, and epidemiological understanding.

The present work reports a retrospective descriptive study conducted in the microbiology laboratory of a university hospital with a national referral function, covering a six-year period from 2018 to 2023.

II. Study Rationale and Objectives

1. Rationale

Three convergent lines of evidence define the rationale for the present study.

The first concerns the limitations of conventional phenotypic identification for rare and fastidious organisms, discussed in Section I. Both manual biochemical galleries and automated platforms such as the BD Phoenix deliver reliable results for common pathogens, but their discriminating power degrades for organisms that are rare, slow-growing, or poorly represented in commercial databases. In the absence of in-house 16S rRNA gene sequencing during the study period, such isolates frequently remained unresolved or reported at an insufficient level of certainty for clinical use.

The second concerns the contribution of MALDI-TOF mass spectrometry to this category of identification failure, reviewed in Section III. The Bruker MALDI Biotyper has been shown to provide species-level identification for a substantial proportion of organisms — including HACEK group members, rare non-fermenters, nutritionally variant streptococci, anaerobes, and clinically important yeasts — that previously required molecular methods or remained uncharacterised. This added value is nevertheless heterogeneous, being highest for yeasts and common Gram-negative bacilli and more variable for taxa with overlapping spectral profiles or limited database representation.

The third concerns the institutional trajectory. The BD Phoenix 100, in use since 2015, was complemented by a BD Phoenix M50 in 2019. The Bruker MALDI Biotyper was introduced in 2020 as the primary identification platform, subsequently alongside a bioMérieux VITEK MS. This creates a structured before-and-after comparison across the study period 2018–2023, providing a direct framework for evaluating the diagnostic value added by MALDI-TOF MS in a national referral laboratory setting.

2. Objectives

The present study pursues the following objectives:

- To characterise the spectrum and taxonomic distribution of the microorganisms isolated at Mohamed VI University Hospital over the period 2018–2023.
- To evaluate the diagnostic contribution of MALDI-TOF mass spectrometry by comparing species-level identification rates achieved by the Bruker MALDI Biotyper against those of conventional phenotypic methods, and to identify the organism categories for which MALDI-TOF provided identification not achievable by conventional means.
- To document the residual identification gap and discuss the underlying technical and database-related factors that account for isolates remaining unresolved despite MALDI-TOF analysis.

METHODS

I. Study Design

This was a retrospective, single-centre, observational study conducted in the Microbiology Laboratory of Mohamed VI University Hospital. Laboratory records covering the six-year period from January 2018 to December 2023 were reviewed with the objective of describing the spectrum of rare and fastidious microorganisms identified over this interval, and of evaluating the diagnostic contribution of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) relative to the conventional phenotypic methods used in the laboratory during the same period.

The study is descriptive in nature and contextually comparative. The comparison between the conventional and the mass-spectrometric approaches is structured around the institutional timeline of instrument acquisition, which defines two successive diagnostic phases within the study period. These are described in detail in Section 3.

II. Laboratory Setting

The Microbiology Laboratory of Mohamed VI University Hospital serves a university hospital fulfilling a national referral function and processes clinical specimens originating from all hospital departments — medical, surgical, intensive care, neonatology, haematology-oncology, solid organ transplantation and ambulatory consultation units — as well as from referred external institutions. Over the six-year study period, the laboratory received and processed in excess of 40.000 microbial isolates, representing the broad clinical and microbiological scope characteristic of a tertiary referral centre.

The laboratory operates routine workflows for aerobic and anaerobic bacterial culture, yeast identification, and antimicrobial susceptibility testing, with specialised diagnostic services for blood-culture-positive episodes, sterile-site infections, and immunocompromised host microbiology. In addition to bacteriology, the laboratory encompasses a viral serology unit responsible for the serological diagnosis of viral infections, and a molecular biology unit providing nucleic acid amplification-based diagnostics including PCR and GeneXpert platforms for the detection of respiratory, gastrointestinal, and sexually transmitted pathogens.

Mycobacteriology is conducted under a dedicated biosafety-compliant workflow distinct from the routine bacteriology section. All three of these units — viral serology, molecular biology, and mycobacteriology — fall outside the scope of the present analysis, which is confined to the bacteriology and mycology workflows where MALDI-TOF mass spectrometry was implemented..

III. Diagnostic Phases and Institutional Timeline

The study period encompasses a clearly dated methodological transition, reflecting the progressive introduction of advanced identification platforms into the laboratory. Two diagnostic phases are defined.

- **Phase I — Conventional phenotypic identification period (January 2018 – December 2019).** During this phase, microbial identification relied exclusively on conventional methods: Gram staining, colony morphology assessment, individual enzymatic tests (catalase, oxidase, coagulase, urease and others), manual API biochemical galleriesapplied to selected isolates, and the BD Phoenix automated identification and antimicrobial susceptibility testing platform (Becton Dickinson Diagnostic Systems, Sparks, MD, USA). The BD Phoenix 100 instrument, which had been installed in the laboratory in 2015, served as the principal automated identification platform throughout this phase. In 2019, a BD Phoenix M50, a higher-throughput successor sharing the same panel technology, the same identification and susceptibility databases, and the same EpiCenter data-management, replaced the BD Phoenix 100.
- **Phase II — MALDI-TOF mass spectrometry period (January 2020 – December 2023).** In 2020, a Bruker MALDI Biotyper mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) was introduced into the laboratory and progressively assumed the first-line identification role previously held by the BD Phoenix system, which thereafter continued to be used primarily for antimicrobial susceptibility testing following MALDI-TOF identification. The Bruker Biotyper platform was operated with the MBT Compass IVD software — the in-vitro diagnostic configuration of the Biotyper software certified for routine clinical use — together with the associated MBT IVD reference library.

IV. Microorganisms Included in the Study

The population of interest comprises all bacterial and yeast isolates recovered from clinical specimens in the Microbiology Laboratory over the study period that fulfilled one or both of the following criteria: (i) the organism belonged to a species considered rare in routine clinical practice, defined operationally as species encountered at low frequency in the laboratory and/or insufficiently represented in the conventional phenotypic identification databases in use during Phase I; (ii) the organism exhibited fastidious biological properties — slow growth, special nutritional or atmospheric requirements, enriched or specific culture media — that rendered its routine identification by conventional phenotypic methods difficult, unreliable, or incomplete.

Isolates were grouped for analysis into four taxonomic categories reflecting the major divisions encountered in routine clinical microbiology: Gram-negative bacilli, Gram-positive cocci, obligate anaerobes, and yeasts. Mycobacteria were managed through a distinct laboratory workflow and are not included in the primary analysis; they are discussed conceptually in the Discussion (Section IV.6) only insofar as they relate to the broader context of fastidious organism identification.

Isolates considered to represent routine commensal contamination of the specimen, repeated isolates of the same species from the same patient within a clinical episode (retained as a single entry), and quality-control or reference strains were excluded from the analysis.

V. Conventional Phenotypic Identification (Phase I and Continued Use in Phase II)

Conventional phenotypic identification during Phase I — and, in selected cases, during Phase II as a confirmatory or complementary approach — followed a standard stepwise workflow. Primary isolation was performed on appropriate culture media according to specimen type (Columbia blood agar, chocolate agar, MacConkey agar, Sabouraud dextrose agar, Schaedler anaerobic agar and selective media as clinically indicated), with incubation conditions adapted to the organism class (aerobic at 36 ± 1 °C, CO_2-enriched atmosphere for fastidious organisms, anaerobic jars for obligate anaerobes). Primary orientation was based on colony morphology,

haemolytic pattern, Gram staining and rapid enzymatic tests (catalase, oxidase, coagulase, urease).

Species-level identification was then pursued by one of two complementary pathways. Manual API biochemical galleries were used for isolates whose morphology and preliminary tests suggested a specific family or genus: API 20 E for Enterobacterales, API 20 NE for non-fermenting Gram-negative bacilli, API Staph for staphylococci, API Strep for streptococci and related genera, API NH for Neisseria and Haemophilus species API 20 A for anaerobes, read according to the manufacturer's instructions and interpreted via the APILAB Plus database. Automated identification was performed on the BD Phoenix 100 (2015–2019) or BD Phoenix M50 (from 2019 onwards) using the appropriate NMIC/ID or PMIC/ID combined identification-and-susceptibility panels, with bacterial suspensions prepared at 0.5–0.6 McFarland turbidity in Phoenix ID broth and analysed according to the manufacturer's protocols. Antimicrobial susceptibility testing was performed on the same Phoenix panels in parallel with identification during Phase I, and retained throughout Phase II following MALDI-TOF identification.

No molecular identification — in particular no 16S rRNA gene sequencing — was performed in-house during the study period. For the limited cases in which molecular confirmation was deemed clinically indispensable, isolates were referred to external reference laboratories; these referrals are not included within the primary dataset of the present study.

VI. MALDI-TOF MS Identification (Phase II)

From 2020 onwards, MALDI-TOF MS identification became the first-line method for routine identification of bacterial and yeast isolates recovered from clinical specimens. The principal platform used in our laboratory was the Bruker MALDI Biotyper, operated with the MBT Compass IVD software (Bruker Daltonik, Bremen, Germany) and the associated MBT IVD reference library. The bioMérieux VITEK MS platform was introduced during the latter part of Phase II and used in parallel for a subset of isolates.

Identification was performed on fresh isolated colonies obtained from pure primary or subcultured plates. Sample preparation followed the manufacturer's recommendations applicable

during Phase II. For the majority of routine isolates, the direct transfer protocol was applied, consisting of the deposit of a small amount of colony material on a polished steel target plate, followed by overlay with matrix solution (α-cyano-4-hydroxycinnamic acid, CHCA) and air-drying before spectrum acquisition. For Gram-positive cocci, for yeasts, and for isolates yielding low-confidence scores on direct transfer, the direct transfer with on-target formic acid lysis protocol was used, with addition of 1 µL of 70 % aqueous formic acid to the colony deposit prior to matrix overlay. Full ethanol/formic acid extraction was reserved as a rescue protocol for isolates yielding scores below the manufacturer's genus-level reliability threshold after the first two approaches, and for selected fastidious organisms requiring enhanced protein extraction.

Spectral acquisition, automated database comparison and result reporting were performed through the MBT Compass IVD software using the default linear positive mode parameters and the manufacturer's recommended log score thresholds: a score of ≥ 2.0 was accepted as reliable species-level identification, a score of 1.7 to < 2.0 was accepted as genus-level identification only, and a score of < 1.7 was considered unreliable. Daily instrument calibration was performed using the Bruker Bacterial Test Standard (BTS) in accordance with the manufacturer's quality-control protocol.

It must be acknowledged that the precise version numbers of the MBT IVD reference library and the MBT Compass IVD software in use at each point during Phase II were not fully retrievable from the laboratory records available for this retrospective review. Sequential database updates delivered by the manufacturer throughout Phase II progressively expanded the reference library, with consequent improvement in identification coverage particularly for rare organisms; however, the exact mapping between individual isolate identifications and specific library versions was not systematically archived. This constitutes a recognised methodological limitation inherent to the retrospective design and is discussed further in the Discussion. Notwithstanding this constraint, the three broad categories of identification outcome — species-level, genus-level and unidentified — remain applicable across the entire study period and constitute the basis of the analysis presented in the Results.

VII. Data Collection

Data were extracted retrospectively from the laboratory information system and from archived culture records maintained by the Microbiology Laboratory over the study period. For each isolate included in the analysis, the following information was collected when available: the date of isolation; the type of clinical specimen from which the organism was recovered (blood culture, sterile-site aspirate, respiratory tract specimen, urine, deep-seated pus, intra-abdominal fluid, prosthetic device-associated sample or other); the clinical department of origin; the taxonomic group of the organism (Gram-negative bacilli, Gram-positive cocci, anaerobes, yeasts); the final identification assigned; the level of taxonomic resolution achieved (species level, genus level, unidentified); and the principal identification method used (conventional phenotypic methods including API galleries and BD Phoenix, MALDI-TOF MS, or a combination).

Turnaround time from sample reception to identification result was not systematically measured during the study period. Qualitative comparisons between the two phases in terms of speed of identification are therefore discussed only in general terms, with reference to the published literature and to the operational experience of the laboratory; no quantitative turnaround time data are presented.

The clinical impact of identification on patient management — in particular, instances in which the identification obtained prompted a modification of antimicrobial therapy or further clinical investigation — was noted when explicitly documented in the available laboratory records, but was not systematically assessed. Cases in which clinical impact is discussed in the Results section are presented as illustrative examples rather than as a systematically analysed outcome.

VIII. Data Analysis

Data analysis was descriptive throughout, consistent with the retrospective and observational design of the study. The identification outcomes were tabulated according to taxonomic group and diagnostic phase. Within each group, the distribution of species identified, the proportion of isolates reaching species-level identification, the proportion of genus-level-

only identifications, and the proportion of isolates that remained unidentified were reported in absolute numbers and as percentages of the relevant subgroup. Where direct comparison between Phase I and Phase II was informative — notably for organism categories encountered in both phases — the proportional contribution of MALDI-TOF MS to species-level identification relative to conventional methods was described in narrative and categorical terms.

No inferential statistical analysis was performed. The absence of formal hypothesis testing reflects both the descriptive design of the study and the structural differences between the two phases — in particular, the absence of parallel identification of the same isolates by both methods, which would be required for a formal agreement analysis. Rates and proportions are therefore presented as descriptive summaries and not as statistically compared quantities.

Cautious phrasing is used throughout the Results section to reflect these methodological choices: terms such as the majority, a limited number, several cases and a substantial proportion are preferred over numerical precision in contexts where retrospective record completeness cannot be formally verified.

IX. Ethical Considerations

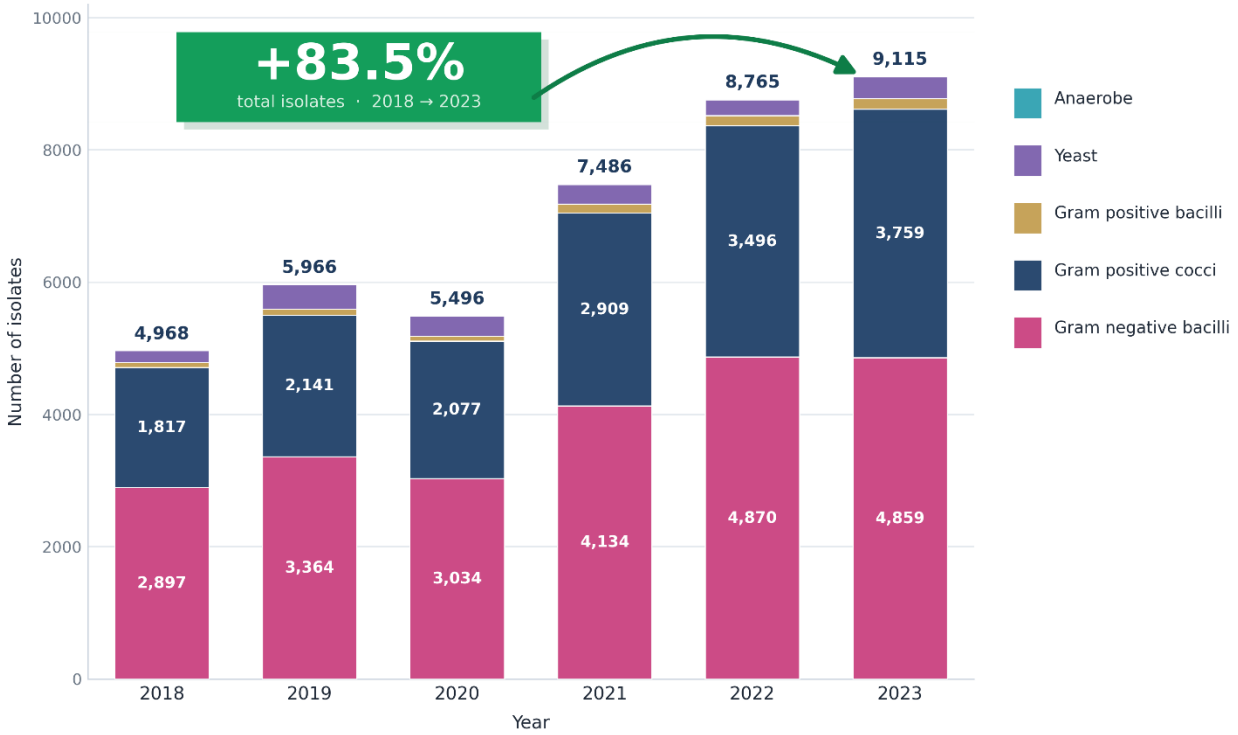
The study was conducted exclusively using anonymised microbiological data collected as part of routine diagnostic activity in the Microbiology Laboratory. No additional biological sampling, patient contact or experimental intervention was undertaken. No individual-level clinical data are reported; where clinical context is mentioned in the Results or Discussion for illustrative purposes, it is limited to the type of specimen, the clinical department of origin and the general clinical scenario, with no information that could allow patient identification.

Given the retrospective design, the use of pre-existing anonymised laboratory records and the absence of any intervention affecting patient care, formal ethical committee approval was not required under the institutional framework applicable to the laboratory. All data were handled in accordance with the confidentiality standards and institutional regulations governing the use of retrospective laboratory data for academic and research purposes.

RESULTS

I. Overview of the Study Collection

Over the six-year study period (2018–2023), a total of 42,070 microbial isolates were processed by the laboratory and retained for analysis after exclusion of duplicate isolates from the same patient and episode, quality-control strains, and reference cultures. The annual workload increased substantially across the study window, from 4,923 isolates in 2018 to 9,165 isolates in 2023, representing a 1.84-fold expansion of the laboratory's identification activity. The intermediate years showed a progressive growth pattern (5,992 in 2019; 5,570 in 2020; 7,565 in 2021; 8,855 in 2022), with 2020 standing out as the only year in which total isolate volume declined relative to the preceding year — a feature attributable to the operational disruption of the early COVID-19 pandemic period and the partial deployment of MALDI-TOF mass spectrometry, which coincided in time



(Figure 1 — Annual isolate volume by organism group, 2018–2023).

Eleven specimen categories were tracked across the period: blood cultures, urine cultures , pus and wound specimens, cerebrospinal fluid, serous body fluids, deep respiratory specimens , catheter tips, expectorated sputum, genital specimens, joint fluid, and stool cultures. The corpus was dominated by three specimen types: pus and wound specimens (13,884 isolates, approximately 33.0% of the total), blood cultures (11,637 isolates, 27.7%), and urine cultures (10,226 isolates, 24.3%). Together these three categories accounted for approximately 85% of the entire dataset. The remaining specimen types each contributed less than 5% of the total, but several of them showed disproportionately strong growth across the study window. In particular, expectorated sputum increased 6.5-fold (18 → 117 isolates), deep respiratory specimens increased 3.96-fold (179 → 709), and serous body fluids increased 3.78-fold (104 → 393), consistent with the laboratory's expanding role in deep-respiratory and serous-fluid investigations during the latter part of the period

Table 1. Distribution of isolates by specimen type and year, 2018–2023.

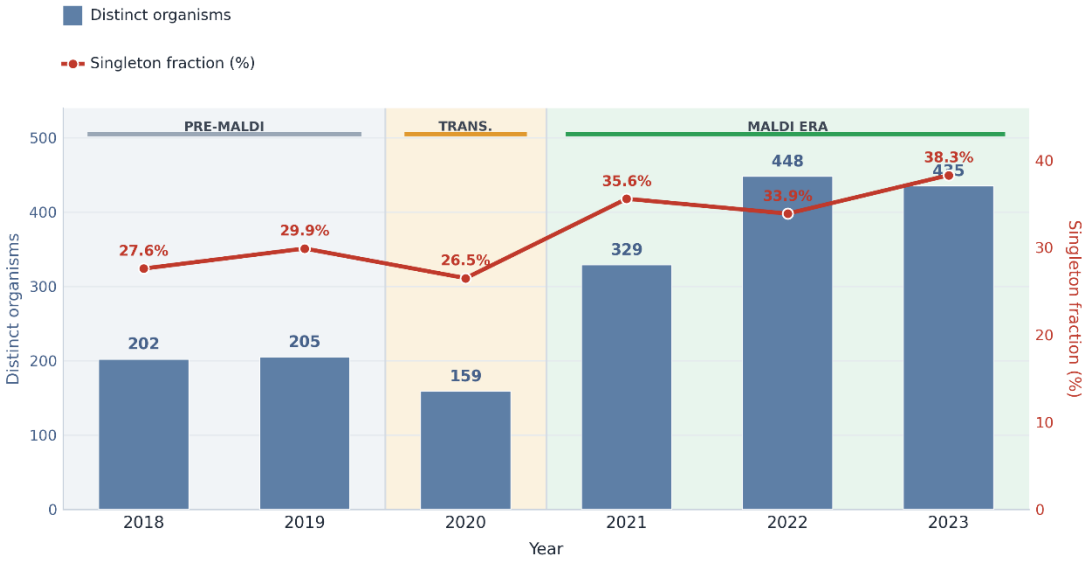
Specimen type	2018	2019	2020	2021	2022	2023	6-yr total
	PRE-MALDI		TRANS.	MALDI ERA			
Pus and wound specimens	1,431	1,796	1,804	2,560	3,003	3,290	**13,884**
Blood cultures	1,550	1,570	1,817	2,011	2,303	2,386	**11,637**
Urine cultures	1,363	1,825	1,266	1,797	2,224	1,751	**10,226**
Deep respiratory (PDP/LBA/aspirates)	179	186	182	314	387	709	**1,957**
Serous body fluids	104	179	165	329	386	393	**1,556**
Catheter tips	129	168	152	198	211	265	**1,123**
Cerebrospinal fluid	87	103	102	201	187	156	**836**
Genital specimens	42	94	30	70	56	80	**372**
Expectorated sputum	18	27	31	64	80	117	**337**
Joint fluid	13	11	8	18	17	14	**81**
Stool cultures	7	33	13	3	1	4	**61**
TOTAL	**4,923**	**5,992**	**5,570**	**7,565**	**8,855**	**9,165**	**42,070**

Specimen types are presented in descending order of cumulative six-year volume. Cell shading ∝ each specimen's own year-to-year magnitude (per-row scale).

Following the canonicalization of organism nomenclature, the dataset comprised 406 distinct canonical organisms. These were distributed across five taxonomic groups: 187 Gram-negative bacilli (GNB), 112 Gram-positive cocci (GPC), 69 Gram-positive bacilli (GPB), 22 yeasts, and 16 anaerobes.

The number of distinct organisms detected per year evolved markedly across the study window: 202 in 2018, 205 in 2019, 159 in 2020, 329 in 2021, 448 in 2022, and 435 in 2023. The pre-MALDI floor of approximately 200 distinct organisms in 2018 and 2019 contrasted with a **sustained two-fold expansion of measurable diversity from 2021 onwards**, once MALDI-TOF mass spectrometry had become routinely embedded in the workflow.

The 2020 trough — 159 distinct organisms — does not reflect a true reduction in microbial diversity but rather the operational profile of a transitional year, during which the platform was only partially deployed and identification practices were uneven



(Figure 2 — Distinct organisms recovered per year, 2018–2023).

A complementary indicator of identification depth is the singleton fraction, defined as the proportion of distinct organisms encountered only once during a given year. This fraction rose from 27.6% in 2018 and 29.9% in 2019 to 35.6% in 2021, 33.9% in 2022, and 38.3% in 2023, with the transitional 2020 value (26.5%) again deviating from the broader pattern. The increase reflects an enrichment of the rare-organism tail of the inventory, consistent with the diagnostic

detection of species that conventional phenotypic workflows would have left at the genus level or misidentified. A related metric, the distinct-organism-per-1,000-isolates ratio, increased from 19.6–24.8 in the pre-MALDI years to a stable 24.9–26.7 across 2021–2023, indicating that the diversity gain was not a mechanical consequence of higher isolate throughput but a true broadening of the species inventory recoverable from clinical specimens

Table 2. Diversity metrics by year, 2018–2023.

Metric	2018	2019	2020	2021	2022	2023
Total isolates	4,923	5,992	5,570	7,565	8,855	9,165
Distinct organisms (canonical)	202	205	159	329	448	435
Singletons (n = 1 organisms)	34	35	26	69	74	93
Singleton fraction (%)	27.6%	29.9%	26.5%	35.6%	33.9%	38.3%
Distinct organisms per 1,000 isolates	24.8	19.6	17.8	25.9	24.9	26.7
Diagnostic phase	Pre-MALDI	Pre-MALDI	Transitional	MALDI era	MALDI era	MALDI era

Singleton fraction = singletons / distinct organisms. Distinct/total ratio expressed per 1,000 isolates. Diagnostic phase as defined in Materials and Methods (Pre-MALDI: 2018–2019, conventional phenotypic methods only; Transitional: 2020, partial MALDI-TOF deployment; MALDI era: 2021–2023, MALDI-TOF first-line).

The overall pattern of these findings is one of stable workload composition by specimen type, a near-doubling of measurable organism diversity from 2021 onwards, and an enrichment of rare-tail species — three features that constitute the macroscopic signature of MALDI-TOF integration in the laboratory and that the subsequent subsections (III.2 to III.5) examine at the level of individual organism groups.

II. Gram-Negative Bacilli

Gram-negative bacilli (GNB) constituted the largest taxonomic group in the study collection, comprising 187 distinct species and the majority of high-volume isolates across the six-year period. The five most frequently recovered GNB species were Escherichia coli (7,798 isolates), Klebsiella pneumoniae (5,070), Pseudomonas aeruginosa (2,424), Acinetobacter baumannii (1,971), and Enterobacter cloacae (1,756). Together these five species accounted for the bulk of the GNB workload, with E. coli alone representing approximately 18.5% of the entire study corpus across all groups. The remaining GNB diversity — 182 additional species — was

distributed across a long tail of less common Enterobacterales, non-fermenting bacilli, and rare or fastidious organisms whose recovery profile shifted markedly between the two diagnostic phases.

For Acinetobacter, the pre-MALDI inventory was effectively limited to A. baumannii and a smaller number of A. lwoffii isolates (228 and 11 in 2018 respectively). From 2021 onwards, the dataset records a substantially broadened Acinetobacter species inventory, including A. pittii (5 isolates total), A. nosocomialis (29), A. ursingii (3), A. johnsonii (8), A. bereziniae (10), A. variabilis (7), A. soli (4), A. lactucae (6), A. junii (3), A. haemolyticus (1), A. radioresistens (1), A. schindleri (1), A. gyllenbergii (3), and A. towneri (1). All of these species except A. baumannii and A. lwoffii were either entirely absent from the 2018–2019 inventory or recorded as occasional isolates without species-level resolution.

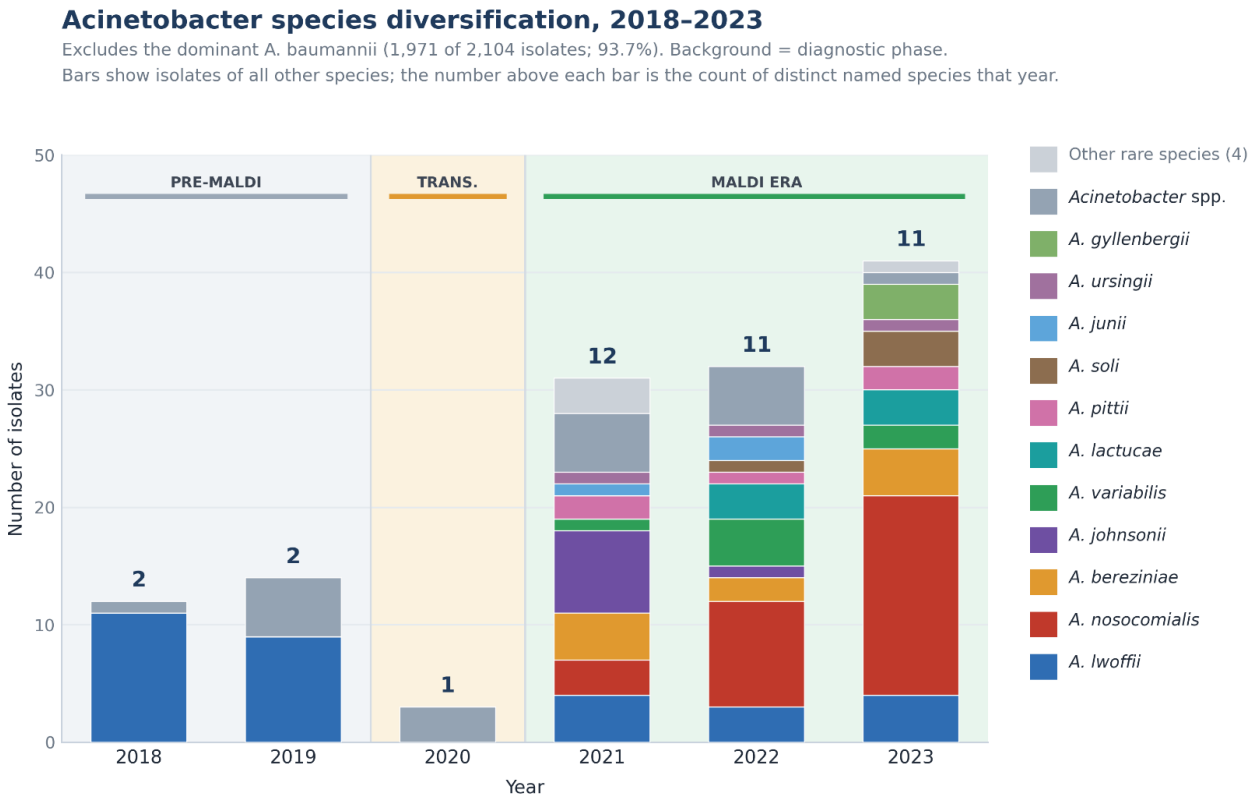


Figure 3 — Acinetobacter species detected per year, 2018–2023

Within the genus Pseudomonas, the pre-MALDI inventory recorded P. aeruginosa as the only routinely speciated organism, accompanied by an unspeciated Pseudomonas spp. bucket (39 isolates across the period) and a small number of P. putida and P. oryzihabitans isolates. From 2021 onwards, the species-level inventory expanded to include P. monteilii (28 isolates total), P. stutzeri (13), P. fluorescens (3), P. luteola (7), P. fulva (11), P. mosselii (6), P. mendocina (3), P. alcaligenes (1), P. cichorii (1), P. rhodesiae (1), P. citronellolis (1), P. tolaasii (1), P. protegens (2), P. synxantha (1), P. otitidis (1), P. gessardii (1), P. resinovorans (1), P. guariconensis (1), P. alcaliphila (1), and P. thivervalensis (1). The majority of these non-aeruginosa pseudomonads were recorded as singleton or near-singleton detections.

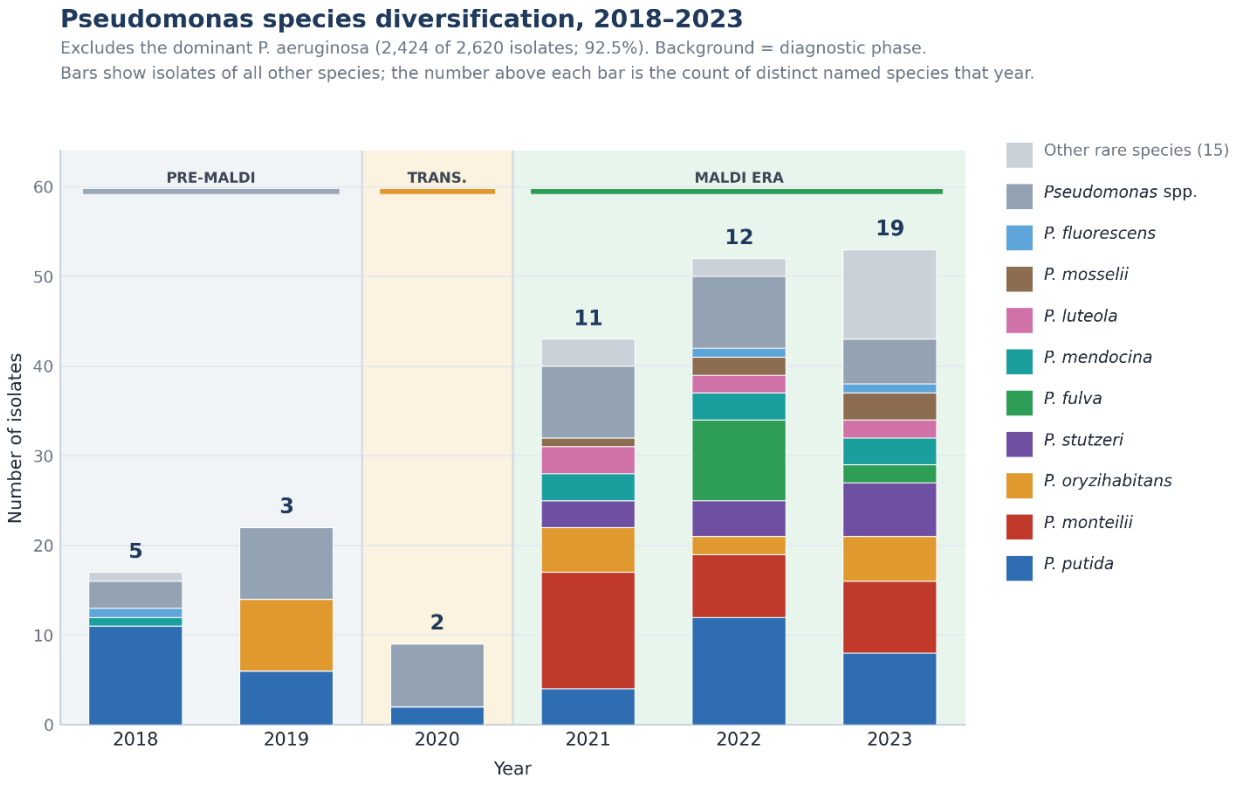


Figure 4 — Pseudomonas species detected per year, 2018–2023

Enterobacter hormaechei rose from 9 isolates in 2018 to 330 in 2023, with the bulk of the increase concentrated in 2021–2023.

The HACEK group — Haemophilus, Aggregatibacter, Cardiobacterium, Eikenella, and Kingella — showed both a quantitative and a qualitative expansion across the study window. The annual HACEK total rose from 6 isolates in 2018 to 52 in 2023, with a cumulative total of 139

isolates over the six years. Haemophilus influenzae dominated the group with 119 isolates (86% of the HACEK total) and showed a progressive increase from 5 isolates in 2018 to 45 in 2023. Eikenella corrodens and Kingella kingae were each recorded for the first time in the dataset in 2023; Aggregatibacter segnis appeared in 2022; and Haemophilus parahaemolyticus and Haemophilus pittmaniae — both rare members of the genus — were recorded only in the MALDI era

Table 3. HACEK group recovery and first-time detections by year, 2018–2023.

Organism	2018	2019	2020	2021	2022	2023	Total	Pre-MALDI presence	First detected
	PRE-MALDI		TRANS.	MALDI ERA					
Haemophilus influenzae	5	15	11	20	23	45	**119**	Yes	2018
Haemophilus parainfluenzae	1	5	—	1	2	4	**13**	Yes	2018
Haemophilus parahaemolyticus	—	—	—	—	—	1	**1**	No	**2023**
Haemophilus pittmaniae	—	—	—	—	1	—	**1**	No	**2022**
Haemophilus spp. (genus-only)	—	1	1	—	—	—	**2**	Yes	2019
Aggregatibacter segnis	—	—	—	—	1	—	**1**	No	**2022**
Eikenella corrodens	—	—	—	—	—	1	**1**	No	**2023**
Kingella kingae	—	—	—	—	—	1	**1**	No	**2023**
HACEK total	**6**	**21**	**12**	**21**	**27**	**52**	**139**	—	—

No = organism first recovered only after MALDI-TOF deployment

HACEK = Haemophilus, Aggregatibacter, Cardiobacterium, Eikenella, Kingella. "—" indicates no isolate recorded in the given year. Pre-MALDI presence indicates whether the species was recorded in 2018 or 2019. First detected indicates the first year in which the species appears in the laboratory's inventory across the study window. Cardiobacterium hominis and Aggregatibacter actinomycetemcomitans were not recovered in the present dataset and are therefore not listed.

Stenotrophomonas maltophilia was steadily recovered throughout the study period, with 147 isolates in total and an annual count rising from 19 in 2018 to 47 in 2023. The species was therefore recovered in both diagnostic phases.

A heterogeneous group of less common Gram-negative organisms was recovered exclusively or predominantly in the MALDI era. Achromobacter xylosoxidans recorded 14

isolates, all between 2021 and 2023. Burkholderia cepacia was recorded across both eras (14 isolates), with a single isolate of Burkholderia gladioli in 2019. Comamonas kerstersii (13 isolates) and Shewanella algae (3 isolates) appeared exclusively from 2021. The dataset also records single or very low-frequency detections of Wohlfahrtiimonas chitiniclastica, Sphingomonas paucimobilis, Sphingobium yanoikuyae, Brevundimonas diminuta, Brevundimonas nasdae, Cupriavidus pauculus, Delftia acidovorans, Chryseobacterium indologenes, Chryseobacterium tructae, Ochrobactrum anthropi, Rhizobium radiobacter, Herbaspirillum huttiense, Herbaspirillum aquaticum, Kerstersia gyiorum, and Kalamiella piersonii

Within the Enterobacterales, a number of less commonly encountered species were also recovered predominantly or exclusively in the MALDI era. Enterobacter kobei (44 isolates, all from 2021), E. bugandensis (26), E. roggenkampii (4), E. ludwigii (3), Klebsiella variicola (26), Providencia rettgeri (19), Mixta calida (9 isolates of a recently described species), Kosakonia cowanii (1), Edwardsiella hoshinae (1), and Pantoea septica / Pantoea eucrina (1 each) all appeared in the post-2020 inventory. The Citrobacter, Proteus, and Serratia genera also showed expanded species-level resolution, with Serratia ureilytica (10), Serratia liquefaciens (4), Serratia nematodiphila (4), Proteus hauseri (12), and Proteus penneri (4) all recorded post-2020.

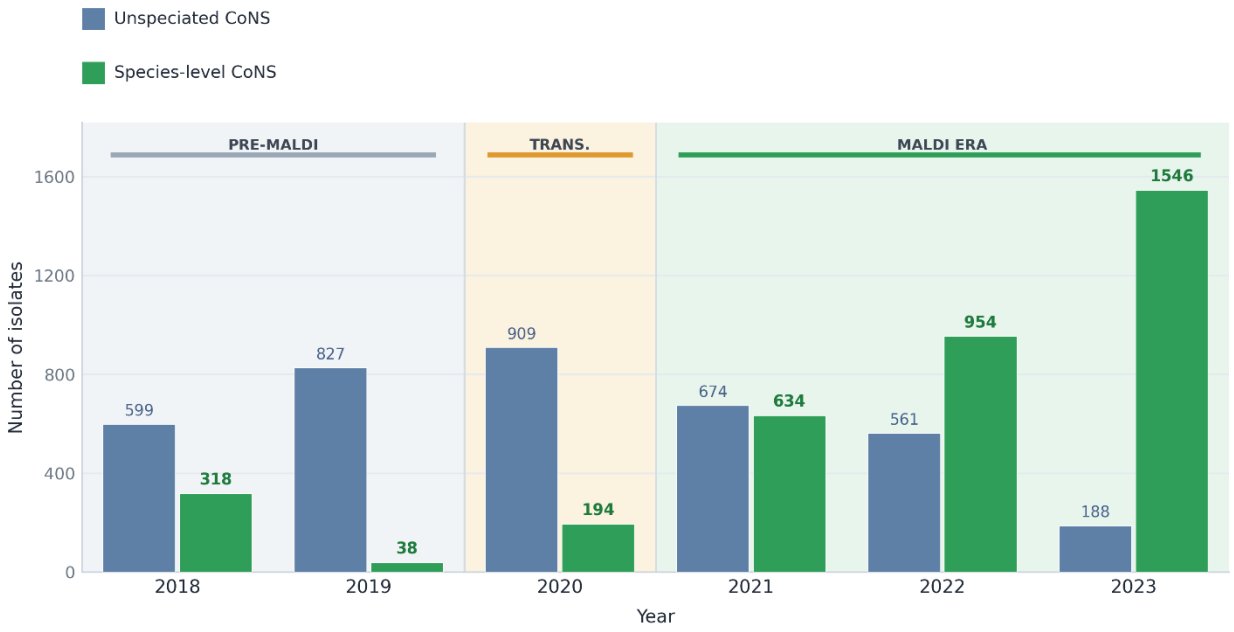
A small number of GNB entries were not resolved beyond the genus level despite the deployment of MALDI-TOF: Pseudomonas spp. (39 cumulative isolates), Salmonella spp. (73), Aeromonas spp. (3), Pantoea spp. (12), Kluyvera spp. (11), Chryseobacterium spp. (2), and Vibrio spp. (2). The persistence of these genus-only entries in the MALDI era likely reflects either the boundaries of database coverage for less commonly encountered species, the limits of spectral discrimination between closely related taxa within these genera.

III. Gram-Positive Bacteria

Gram-positive cocci (GPC) constituted the second-largest taxonomic group in the dataset, comprising 112 distinct species. The most frequently recovered GPC entries were the unspeciated coagulase-negative staphylococci (CoNS) bucket (3,758 isolates), Staphylococcus aureus methicillin-susceptible (MSSA) (2,891), S. epidermidis (1,462), Enterococcus faecalis (1,071), S. haemolyticus (1,012), S. hominis (948), Enterococcus faecium (866), and S. aureus methicillin-resistant (MRSA) (406). The internal composition of the GPC inventory underwent the most pronounced transformation observed in the entire dataset between the two diagnostic phases, primarily through the progressive resolution of two unspeciated diagnostic buckets — the CoNS bucket and the alpha-haemolytic / viridans-group streptococci bucket — and through the species-level identification of nutritionally variant and otherwise fastidious GPC.

The unspeciated CoNS bucket recorded 599 isolates in 2018 and rose to 827 in 2019 and 909 in 2020, before declining sharply to 674 in 2021, 561 in 2022, and 188 in 2023 — a roughly threefold reduction between the 2020 peak and 2023. Over the same window, the number of CoNS isolates resolved to a named species rose from 318 in 2018 to 1,546 in 2023, with a marked acceleration from 2021 onwards (194 in 2020 → 634 in 2021 → 954 in 2022 → 1,546 in 2023). The number of distinct CoNS species detected each year rose modestly from approximately 13–15 in the pre-MALDI period to a stable 14 in the MALDI era, but the substantive change was not in the breadth of the species list — it was in the proportion of CoNS isolates assigned to a named species rather than left in the genus-level bucket.

Within the CoNS species resolved by MALDI-TOF, the dataset records S. epidermidis (1,462 cumulative isolates, with a marked rise from 105 in 2018 to 656 in 2023), S. haemolyticus (1,012, rising from 141 to 368), S. hominis (948, rising from 38 to 445), S. capitis (62), S. caprae (6), S. arlettae (2), S. auricularis (2), S. delphini (1), S. xylosus (1), S. carnosus (2), S. felis (1), S. chromogenes (1), S. schleiferi (1), S. lentus (1), S. kloosii (2), S. gallinarum (2), and Staphylococcus borealis (17 isolates, all in 2023). Staphylococcus argenteus (1 isolate in 2021), a member of the S. aureus complex, was also recorded.



(Figure 5 — Coagulase-negative staphylococci: unspeciated bucket versus species-level identifications by year, 2018–2023;

The viridans-group streptococci showed an analogous and equally striking transition. The unspeciated alpha-haemolytic / viridans bucket recorded 85 isolates in 2018, peaked at 144 in 2019, and then declined progressively across the MALDI era (63 in 2020; 97 in 2021; 90 in 2022; **5 in 2023**). Over the same period, the number of isolates resolved to a named viridans species rose from 56 in 2018 to 231 in 2023, and the number of distinct viridans species detected each year increased from approximately 13 in 2018 to 16 in 2022 and 2023. By the final year of the study, the laboratory's viridans inventory included S. mitis, S. oralis, S. anginosus, S. constellatus, S. intermedius, S. gordonii, S. salivarius, S. sanguinis, S. parasanguinis, S. mutans, S. vestibularis, S. gallolyticus, and rarer entries such as S. lutetiensis, S. peroris, S. australis, S. infantis, and S. sinensis. The Streptococcus anginosus group itself, recorded as a distinct entry, registered 88 isolates in 2022 and 2 in 2023, having been entirely absent from the 2018–2020 inventory.

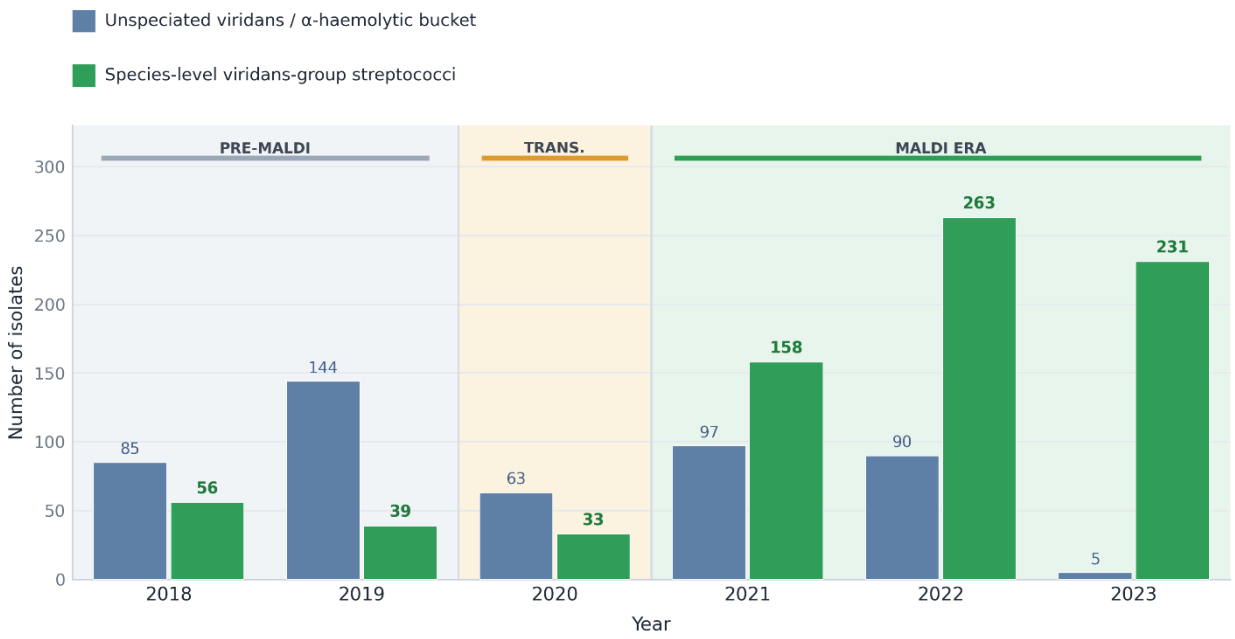


Figure 6 — Viridans-group streptococci: unspeciated bucket versus species-level identifications by year, 2018–2023).

The enterococcal inventory showed a related but distinct pattern. E. faecalis (1,071 isolates) and E. faecium (866) dominated, with E. casseliflavus (41) and E. gallinarum (25) recorded across both diagnostic eras. The MALDI era introduced E. avium (48 isolates, of which 47 were post-2020), E. hirae (11), E. durans (6), E. raffinosus (6), into the routine inventory. An unspeciated Enterococcus spp. bucket (513 cumulative isolates) persisted across all years, declining from 119 in 2019 to 26 in 2023.

A heterogeneous group of fastidious and rare GPC was recovered exclusively or predominantly during the MALDI era. The nutritionally variant streptococci recorded Granulicatella elegans (1 isolate, 2022), Granulicatella spp. (3, post-2020), and Abiotrophia defectiva (1, in 2023). Aerococcus viridans (16 isolates, predominantly 2021–2022) and the Aerococcus spp. bucket (15, post-2020) emerged as a distinct entry during the MALDI era. Rothia mucilaginosa recorded 15 cumulative isolates with a marked concentration in 2023 (11 isolates), and Rothia dentocariosa (1 isolate, 2018) is recorded as a single pre-MALDI entry. Six distinct Gemella species were recorded across the dataset — G. morbillorum (4 isolates), G. haemolysans (2), G. sanguinis (1), G. bergeri (1), and the unspeciated Gemella spp. (2) —

predominantly in the 2021–2023 window. Globicatella sanguinis (3 isolates, 2021–2022), Globicatella spp. (1, 2022), Helcococcus kunzii (1, 2023), and Lactococcus lactis (5, post-2020) also appeared

Table 4. Rare and fastidious Gram-positive cocci recovered by year, 2018–2023.

Organism	2018	2019	2020	2021	2022	2023	Total	First detected
	PRE-MALDI		TRANS.	MALDI ERA				
Granulicatella elegans	—	—	—	—	1	—	**1**	**2022**
Granulicatella spp.	—	—	1	—	—	2	**3**	**2020**
Abiotrophia defectiva	—	—	—	—	—	1	**1**	**2023**
Aerococcus viridans	—	—	—	4	11	1	**16**	**2021**
Aerococcus spp.	—	—	—	1	—	14	**15**	**2021**
Rothia mucilaginosa	—	—	—	1	3	11	**15**	**2021**
Rothia dentocariosa	1	—	—	—	—	—	**1**	2018
Gemella morbillorum	—	—	—	1	—	3	**4**	**2021**
Gemella haemolysans	—	—	—	—	—	2	**2**	**2023**
Gemella sanguinis	—	—	—	—	1	—	**1**	**2022**
Gemella bergeri	—	—	—	—	1	—	**1**	**2022**
Gemella spp.	1	—	1	—	—	—	**2**	2018
Globicatella sanguinis	—	—	—	1	2	—	**3**	**2021**
Globicatella spp.	—	—	—	—	1	—	**1**	**2022**
Helcococcus kunzii	—	—	—	—	—	1	**1**	**2023**
Lactococcus lactis	—	—	—	—	—	5	**5**	**2023**

Green = organism first detected in the MALDI era (2021–2023)

Includes the nutritionally variant streptococci (*Granulicatella*, *Abiotrophia*), *Aerococcus*, *Rothia*, *Gemella*, *Globicatella*, *Helcococcus*, and *Lactococcus*.
"—" indicates no isolate recorded. "spp." denotes a genus-only entry not resolved to species.

A small number of GPC entries persisted as genus-level identifications throughout the dataset: Streptococcus spp. (135 cumulative isolates), Staphylococcus spp. (61), Enterococcus spp. (513), the alpha-haemolytic / viridans bucket (484), the beta-haemolytic bucket (78), and the non-haemolytic bucket (98). The progressive shrinkage of these buckets across the MALDI era — viridans from 144 to 5, enterococci from 119 to 26, beta-haemolytic from 26 to 0, and non-haemolytic from 28 to 0 — represents one of the concrete laboratory-level signatures of MALDI-TOF integration in the present dataset.

The Gram-positive bacilli (GPB) group, which comprised 69 distinct species in the dataset, showed a transition pattern that paralleled the CoNS and viridans-group transitions described in

III.3 but at a smaller absolute scale. The unspeciated Corynebacterium spp. bucket declined from approximately 40 cumulative isolates in the pre-MALDI period to approximately 20 in the MALDI era. Over the same window, Corynebacterium striatum — entirely absent from the 2018–2019 inventory — rose to 120 cumulative isolates between 2021 and 2023, with annual counts of 31, 39, and 50 across those three years. Corynebacterium amycolatum recorded 34 cumulative isolates between 2021 and 2023, and Corynebacterium urealyticum 8 isolates over the same period. A further 10 named Corynebacterium species — C. afermentans, C. aurimucosum, C. phoceense, C. mucifaciens, C. argentoratense, C. propinquum, C. xerosis, C. riegelii, C. coyleae, and C. diphtheriae — were recorded exclusively in the MALDI era, with no detections in 2018 or 2019. Cutibacterium acnes and Cutibacterium avidum, taxonomically reclassified from Propionibacterium, also entered the routine inventory in the post-2020 window, as noted in III.4.

IV. Anaerobic Bacteria

Anaerobic bacteria constituted the smallest taxonomic group in the dataset, comprising 16 distinct canonical species across the six-year period. The total volume of anaerobic isolates recovered was modest in absolute terms relative to the other organism groups, and this scarcity is itself a defining feature of the laboratory's anaerobe inventory. It reflects an upstream methodological feature of the laboratory's routine culture practice, in which aerobic culture is the standard workflow and anaerobic culture is performed selectively, and is therefore not attributable to limitations of the MALDI-TOF identification step.

No anaerobic genera were recorded in the 2018–2019 dataset. All anaerobic microorganisms identified in this study were detected exclusively during the post-MALDI-TOF period (2020–2023), reflecting a marked expansion in the diversity of anaerobic taxa recovered after 2020.

The MALDI era introduced a broader anaerobic species inventory, the bulk of which was concentrated in 2021–2023. Cutibacterium acnes and Cutibacterium avidum, both clinically relevant in prosthetic-joint and implant-associated infections, were recorded post-2020. Three Actinomyces species — A. oris, A. odontolyticus, and A. naeslundii — appeared in the inventory

exclusively in the MALDI era. The closely related genus Schaalia — taxonomically reclassified from Actinomyces — recorded Schaalia turicensis and Schaalia radingae during the same period. Clostridium perfringens and Clostridium tertium were recorded post-2020 as discrete species-level entries, distinct from the Clostridium spp. bucket. Fusobacterium necrophorum, the recognised agent of Lemierre syndrome, was recorded as a single isolate in 2023. Prevotella bivia (post-2020), Bifidobacterium breve (post-2020), and Actinotignum schaalii (post-2020) further illustrate the breadth of anaerobic species accessible at species level in the post-2020 inventory.

A small number of anaerobic entries remained at genus level throughout the dataset, including Clostridium spp. and Bacteroides spp., for which species-level resolution was either unavailable or not pursued in the routine workflow.

Table 5. Anaerobic bacterial species recovered by year, 2018–2023.

Organism	2018	2019	2020	2021	2022	2023	Total	First detected
	PRE-MALDI		TRANS.	MALDI ERA				
Bacteroides fragilis	—	—	1	1	1	—	**3**	**2020**
Cutibacterium acnes	—	—	—	—	2	1	**3**	**2022**
Cutibacterium avidum	—	—	—	—	—	3	**3**	**2023**
Prevotella bivia	—	—	—	1	—	—	**1**	**2021**
Actinomyces oris	—	—	—	—	—	1	**1**	**2023**
Actinomyces odontolyticus	—	—	—	1	1	1	**3**	**2021**
Actinomyces naeslundii	—	—	—	—	—	1	**1**	**2023**
Schaalia turicensis	—	—	—	—	—	2	**2**	**2023**
Schaalia radingae	—	—	—	1	—	—	**1**	**2021**
Clostridium perfringens	—	—	—	1	—	—	**1**	**2021**
Clostridium tertium	—	—	—	—	1	—	**1**	**2022**
Fusobacterium necrophorum	—	—	—	—	—	1	**1**	**2023**
Bifidobacterium breve	—	—	—	—	—	1	**1**	**2023**
Anaerobes (genus-only)	—	1	2	—	—	—	**3**	2019

Green = organism first detected in the MALDI era (2021–2023)

"—" indicates no isolate recorded. The "Anaerobes (genus-only)" entry corresponds to isolates reported at higher taxonomic levels in the laboratory's pre-MALDI inventory. The total number of anaerobic isolates is modest in absolute terms relative to the other organism groups, reflecting the laboratory's predominantly aerobic culture workflow rather than a limitation of MALDI-TOF identification.

V. Yeasts

Yeasts constituted a smaller but distinct taxonomic group in the dataset, comprising 22 canonical species across the six-year period. The yeast inventory was dominated by Candida albicans (766 cumulative isolates), which was recovered consistently across both diagnostic phases. The remainder of the inventory underwent a substantial transition between the pre-MALDI and MALDI eras, with the most pronounced shift occurring in the resolution of the unspeciated Candida bucket and the introduction of cryptic and rare species into the routine identification workflow.

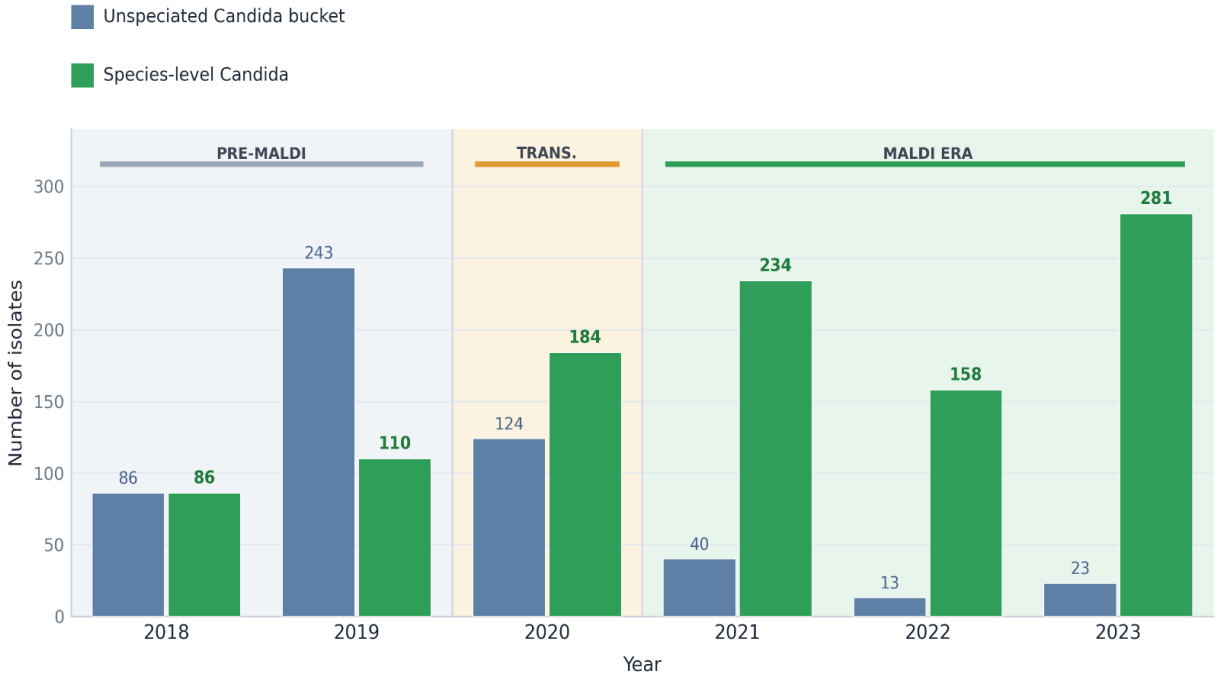
The unspeciated Candida spp. bucket recorded 86 isolates in 2018, peaked at 243 in 2019, and then declined progressively across the MALDI era (124 in 2020; 40 in 2021; 13 in 2022; 23 in 2023). Over the same window, the number of Candida isolates resolved to a named species rose substantially from 2021 onwards, with the speciated workload concentrated in C. albicans (continuous across all years), Candida tropicalis (133 cumulative isolates, all recorded in the MALDI era), Candida glabrata (67 isolates, MALDI era only), Candida parapsilosis (57 isolates, MALDI era only), Candida krusei (recorded post-2020), Candida dubliniensis (recorded post-2020), and Candida lusitaniae / Clavispora lusitaniae (15 isolates, post-2020).

The Candida parapsilosis complex, which conventional phenotypic methods do not resolve into its constituent species, was further differentiated in the MALDI era, with Candida orthopsilosis (3 isolates, recorded in 2021) appearing as a distinct entry alongside C. parapsilosis sensu stricto. Candida metapsilosis, the third member of the complex, was not recorded in the present dataset.

Several genera of yeasts beyond Candida were recovered exclusively or predominantly during the MALDI era. Cryptococcus neoformans (47 cumulative isolates) was the most frequently recovered, with eight isolates recorded as early as 2018 — indicating that this organism was identifiable in the pre-MALDI period through complementary phenotypic methods including capsule staining — and the remaining 39 distributed across the MALDI era. Magnusiomyces capitatus, taxonomically reclassified from Geotrichum capitatum, recorded 9 isolates between

2021 and 2023. Trichosporon asahii recorded 3 isolates, all post-2020. Cyberlindnera fabianii, Saccharomyces cerevisiae, and Geotrichum candidum each appeared in the post-2020 inventory as low-frequency entries. Rhodotorula mucilaginosa and Rhodotorula glutinis were recorded sporadically across the period.

Filamentous fungi were not the primary focus of the present analysis, but the dataset records 11 cumulative isolates of Aspergillus spp., with 7 isolates concentrated in 2023, identified through the laboratory's routine workflow when these organisms were recovered alongside yeasts on standard media.



(Figure 7 — Candida speciation transition: unspeciated bucket versus species-level identifications by year, 2018–2023)

Table 6. Yeast species recovered by year, 2018–2023.

Organism	2018	2019	2020	2021	2022	2023	Total	First detected
	PRE-MALDI		TRANS.	MALDI ERA				
Candida albicans	86	110	121	161	100	188	**766**	2018
Candida spp. (unspeciated)	86	243	124	40	13	23	**529**	2018
Candida tropicalis	—	—	13	29	44	47	**133**	2020
Candida glabrata (*C. glabrata* + *Nakaseomyces glabrata*)	—	—	30	24	39	13	**106**	2020
Candida parapsilosis	—	—	11	11	8	27	**57**	2020
Cryptococcus neoformans	8	6	—	14	11	8	**47**	2018
Clavispora lusitaniae	—	—	—	6	6	3	**15**	2021
Aspergillus spp.	—	—	2	—	2	7	**11**	2020
Candida dubliniensis	—	—	1	2	4	3	**10**	2020
Candida krusei (*Pichia kudriavzevii*)	—	—	5	3	—	1	**9**	2020
Magnusiomyces capitatus	—	—	—	1	6	2	**9**	2021
Candida lusitaniae	—	—	4	—	—	2	**6**	2020
Candida kefyr (*Kluyveromyces marxianus*)	—	—	1	1	2	2	**6**	2020
Saccharomyces cerevisiae	—	—	1	1	2	—	**4**	2020
Candida orthopsilosis	—	—	—	3	—	—	**3**	2021
Trichosporon asahii	—	—	1	1	1	—	**3**	2020
Cyberlindnera fabianii	—	—	—	—	1	—	**1**	2022
Geotrichum candidum	—	—	—	—	—	1	**1**	2023
Rhodotorula spp.	—	—	1	—	—	—	**1**	2020

First-detection year by phase: Pre-MALDI (2018–19) Transitional (2020) MALDI era (2021–23)

"—" indicates no isolate recorded. The *Candida glabrata* entry combines isolates reported under both the historical name (*C. glabrata*) and the current taxonomic designation (*Nakaseomyces glabrata*) following recent reclassification. Comparable synonyms are noted in parentheses for *C. krusei* (current: *Pichia kudriavzevii*) and *C. kefyr* (current: *Kluyveromyces marxianus*). *Aspergillus* spp. is included as an opportunistic mould category recovered alongside yeasts on standard media; filamentous fungi were not the primary focus of the present analysis.

VI. Cross-Cutting Findings

Several findings emerged from the dataset that did not fit neatly within a single organism group and that describe the diagnostic capacity of the laboratory as a whole across the six-year period.

Diversity metrics across the full dataset. The singleton fraction — defined in III.1 as the proportion of distinct organisms encountered only once during a given year — rose from 27.6% in 2018 to 38.3% in 2023, with the transitional 2020 value (26.5%) deviating from the broader pattern. The increase represents an enrichment of the rare-organism tail of the inventory, with progressively more species being recorded as singletons as the diagnostic depth of the laboratory expanded. Across the full study period, more than 250 species were recorded

exclusively in the MALDI era (defined as 2020 onwards) and were not recovered at all in the 2018–2019 dataset. The most numerically prominent of these MALDI-era-only entries were Candida tropicalis (133 cumulative isolates), Corynebacterium striatum (120), the Streptococcus anginosus group (90 cumulative isolates), and Aerococcus viridans (16). The full list extends across all five organism groups and includes both abundant entries — such as Candida glabrata (67), Candida parapsilosis (57), and Enterobacter kobei (44) — and a long tail of singleton or near-singleton detections enumerated in subsections III.2 to III.5.

Specimen-type signature. The diversity of distinct organisms recovered varied considerably across specimen types and across the study window

Table 7. Distinct organisms recovered per specimen type per year, 2018–2023

Specimen type	2018	2019	2020	2021	2022	2023	2018→2023 fold
	PRE-MALDI		TRANS.	MALDI ERA			
Pus and wound specimens	106	103	93	174	246	210	1.98×
Blood cultures	122	95	101	148	177	190	1.56×
Urine cultures	50	55	51	90	87	75	1.50×
Deep respiratory (PDP/LBA/aspirates)	28	33	29	50	64	65	2.32×
Serous body fluids	38	38	38	60	82	91	2.39×
Catheter tips	21	26	32	39	42	51	2.43×
Cerebrospinal fluid	28	33	29	48	42	40	1.43×
Genital specimens	16	22	13	27	24	24	1.50×
Expectorated sputum	15	16	14	24	24	33	2.20×
Joint fluid	6	5	3	13	9	9	1.50×
Stool cultures	6	7	5	2	1	4	0.67×
TRUE DISTINCT (across all specimen types)	**202**	**205**	**159**	**329**	**448**	**435**	**2.15×**

Cell shading ∝ distinct-organism count. Fold = 2023 ÷ 2018: ≥1× <1×

Specimen types are presented in descending order of cumulative six-year isolate volume (matching Table 1). Each cell reports the number of distinct canonical organisms detected in that specimen type during the corresponding year. The bottom row reports the true distinct organism count across all specimen types combined (after deduplication of organisms recorded in more than one specimen type). The aggregate sum across specimen-type rows therefore exceeds the true distinct count, as the same organism may appear in multiple specimen categories. Local laboratory codes for the specimen types are listed in the footnote of Table 1.

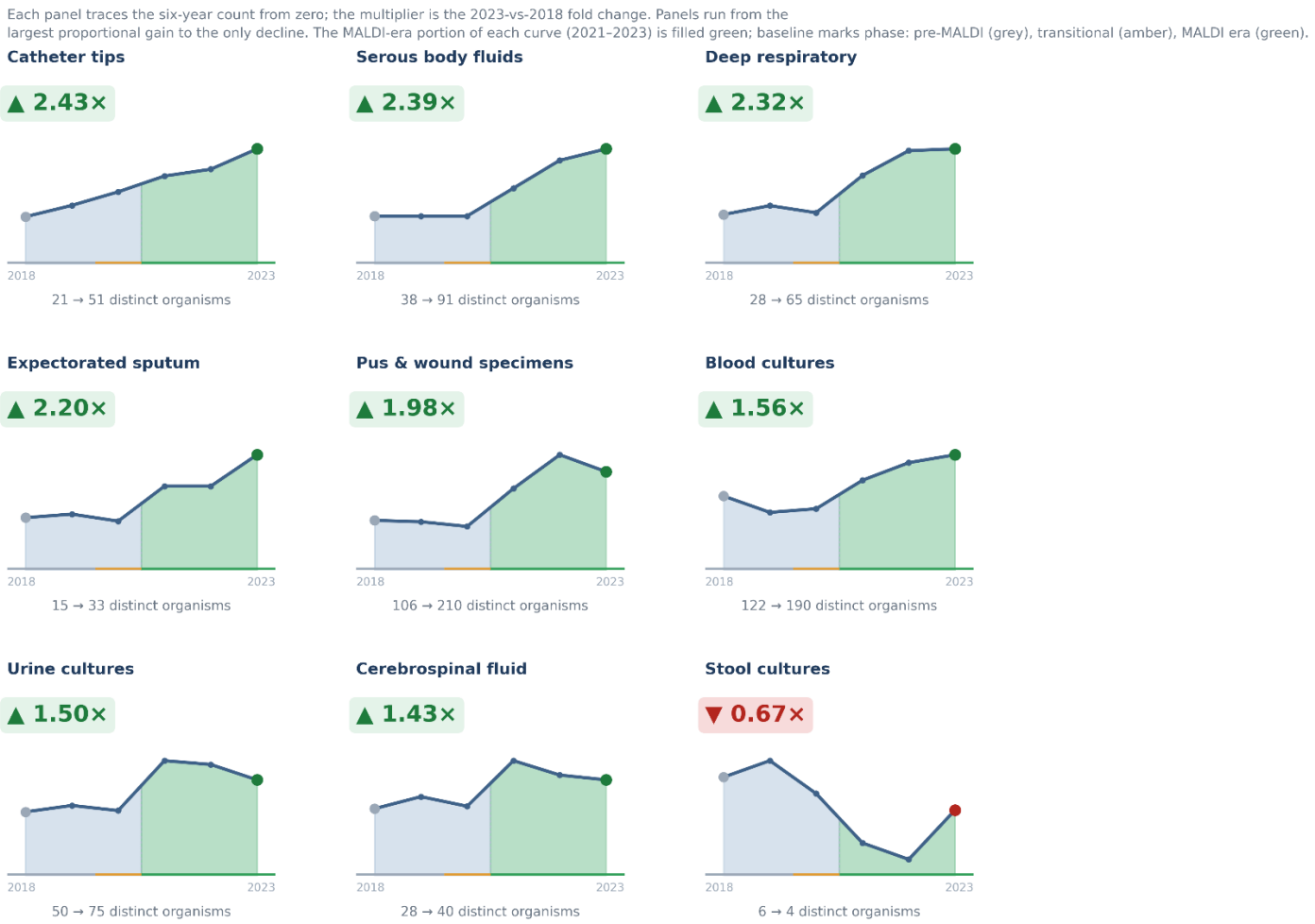


Figure 8 — Distinct organisms recovered per specimen type per year, 2018–2023

Pus and wound specimens recorded the highest absolute distinct-organism count in every year, peaking at 246 organisms in 2022. Blood cultures recorded the second-highest absolute counts, rising from 122 distinct organisms in 2018 to 190 in 2023.

Several lower-volume specimen categories showed the largest proportional expansions: catheter tips recorded a 2.43-fold increase in distinct organisms (21 in 2018 to 51 in 2023), serous body fluids a 2.39-fold increase (38 to 91), deep respiratory specimens a 2.32-fold increase (28 to 65), and expectorated sputum a 2.20-fold increase (15 to 33).

Stool cultures recorded a 0.67-fold change, corresponding to a decrease in distinct organism diversity across the study period. This reduction is likely attributable to the progressive implementation of gastrointestinal multiplex PCR assays, which enabled direct and more

sensitive detection of enteric pathogens, thereby reducing reliance on conventional stool culture for pathogen identification..

Persistent genus-only entries. A small number of organism categories remained at genus level throughout the dataset despite the deployment of MALDI-TOF mass spectrometry. The genus-only entries enumerated in III.2 (Pseudomonas spp., 39 isolates; Salmonella spp., 73; Aeromonas spp., 3; Pantoea spp., 12; Kluyvera spp., 11; Chryseobacterium spp., 2; Vibrio spp., 2) and in III.3 (Streptococcus spp., 135; Staphylococcus spp., 61; Enterococcus spp., 513; alpha-haemolytic / viridans bucket, 484; beta-haemolytic bucket, 78; non-haemolytic bucket, 98) together represent a residual fraction of the dataset that was not resolved to species level. The shrinkage trajectories for several of these buckets across the MALDI era — viridans from 144 to 5, Enterococcus spp. from 119 to 26, beta-haemolytic from 26 to 0, and non-haemolytic from 28 to 0 — indicate progressive resolution rather than static persistence. The Salmonella genus-only category, by contrast, reflects the laboratory's deliberate workflow of reporting at the genus level pending serotyping.

Selected first-time detections. A subset of organisms recorded for the first time in the MALDI era warrants enumeration as cross-cutting observations. Eikenella corrodens and Kingella kingae (HACEK members, first detected in 2023); Aggregatibacter segnis (first detected in 2022); Haemophilus parahaemolyticus and Haemophilus pittmaniae (MALDI era only); Fusobacterium necrophorum (single isolate, 2023); Granulicatella elegans (2022); Abiotrophia defectiva (2023); Helcococcus kunzii (2023); Globicatella sanguinis (2021–2022); Wohlfahrtiimonas chitiniclastica (singleton); Kalamiella piersonii (singleton); Sphingobium yanoikuyae (singleton); Staphylococcus borealis (17 isolates in 2023, of a species formally named in 2020); Magnusiomyces capitatus (2021–2023); Trichosporon asahii (post-2020); and Candida orthopsilosis (2021) constitute a non-exhaustive set of first-time detections accessible to the routine workflow once MALDI-TOF was deployed. Each represents a discrete identification event in the present dataset rather than a cumulative epidemiological observation.

VII. Summary of MALDI-TOF Added Diagnostic Value

Reading across subsections III.1 to III.6, three convergent descriptive features characterise the laboratory's diagnostic inventory across the six-year study period.

The first is a near-doubling of measurable organism diversity between the pre-MALDI years (2018–2019, approximately 200 distinct organisms per year) and the MALDI era (2021–2023, 329 to 448 distinct organisms per year), under conditions of a more modest 1.84-fold increase in total isolate volume. This expansion is therefore not attributable to throughput alone, as confirmed by the distinct-organism-per-1,000-isolates ratio, which rose from 19.6–24.8 in the pre-MALDI period to a stable 24.9–26.7 across 2021–2023.

The second is a progressive resolution of unspeciated genus-level diagnostic buckets across all five organism groups. The coagulase-negative staphylococci bucket declined from a 2020 peak of 909 isolates to 188 isolates in 2023, while species-level CoNS identifications rose from 318 in 2018 to 1,546 in 2023. The viridans-group streptococci bucket declined from 144 isolates in 2019 to 5 isolates in 2023. The unspeciated Candida bucket declined from a 2019 peak of 243 isolates to 23 in 2023. The unspeciated Corynebacterium bucket declined from approximately 40 to approximately 20 cumulative isolates between the two phases. In each case, the shrinkage of the genus-level bucket was accompanied by a corresponding expansion of named species in the inventory.

The third is the appearance, in the post-2020 inventory, of more than 250 species that were not recorded at all in the 2018–2019 dataset. These MALDI-era-only entries spanned the full taxonomic breadth of the study scope: Gram-negative bacilli (including expanded Acinetobacter, Pseudomonas, and Enterobacter speciation, and rare non-fermenters such as Achromobacter xylosoxidans, Comamonas kerstersii, and Wohlfahrtiimonas chitiniclastica), Gram-positive cocci (including Staphylococcus borealis, multiple Gemella species, and the nutritionally variant streptococci), Gram-positive bacilli (including Corynebacterium striatum and the named coryneform species enumerated in III.6), anaerobes (including the Cutibacterium,

Schaalia, and Actinotignum entries described in III.4), and yeasts (including Magnusiomyces capitatus, Trichosporon asahii, Clavispora lusitaniae, and Candida orthopsilosis).

These three descriptive features characterise a laboratory whose identification depth widened, whose unspeciated reporting categories shrank, and whose accessible species inventory expanded substantially across the diagnostic transition. The interpretation of these findings, including comparison with published rates of MALDI-TOF identification performance and discussion of the clinical relevance of the species recovered, are addressed in section IV (Discussion).

DISCUSSION

I. Conventional Methods of Microbial Identification

The identification of microorganisms in clinical microbiology laboratories has long been grounded in a systematic, sequential approach that moves from macroscopic observation to increasingly specific analytical techniques. This hierarchical methodology — beginning at the colony level and progressing through microscopy, biochemical characterisation, and where necessary molecular analysis — reflects both the historical development of the discipline and the practical organisation of routine laboratory workflow. Understanding the scope, performance, and limitations of each level of this approach is essential to appreciating the diagnostic context into which MALDI-TOF MS was subsequently introduced. The following subsections describe each stage of conventional microbial identification in turn.

1. Macroscopic Examination: Colony Morphology

The first and most immediate step in the identification of a microorganism is the macroscopic examination of its growth characteristics on solid culture media. Following inoculation of a clinical specimen onto appropriate agar plates and incubation under defined atmospheric conditions and temperature — typically 35–37°C for the majority of clinically relevant bacteria — the resulting colonies are examined visually and systematically.(12) Although macroscopic examination alone is rarely sufficient for definitive species-level identification, it provides critical preliminary information that guides the subsequent stages of the identification process and, in many cases, allows experienced microbiologists to generate an initial working hypothesis regarding the likely genus or group of the isolate.(13)

Colony morphology is described according to a standardised set of parameters that capture the physical appearance of bacterial growth. These parameters include the overall **size** of the colony, typically expressed in millimetres after a defined incubation period; the **form** or shape of the colony when viewed from above, which may be circular, irregular, filamentous, rhizoid, or spindle-shaped; the **margin** or edge of the colony, which may be entire (smooth and well-defined), undulate (wavy), lobate, erose (irregularly toothed), or filiform; and the **elevation** of the colony above the agar surface, which ranges from flat to raised, convex, umbonate (centrally

raised), or crateriform.(12,13) The **surface texture** may be described as smooth, rough, granular, wrinkled, or mucoid — the last characteristic being particularly associated with encapsulated organisms such as Klebsiella pneumoniae or Streptococcus pneumoniae.(14) **Opacity refers** to the degree of light transmission through the colony, ranging from transparent to translucent to opaque, whilst **pigmentation** — when present — constitutes one of the most immediately distinctive macroscopic features: the blue-green pyocyanin pigment of Pseudomonas aeruginosa, the golden-yellow carotenoid pigment of Staphylococcus aureus, and the red pigment of Serratia marcescens are among the most clinically recognisable examples.(13,15)

When colonies are grown on blood-containing media such as Columbia blood agar or tryptic soy agar supplemented with 5% sheep blood, the pattern of **haemolysis** observed around the colony constitutes an additional parameter of considerable diagnostic value. Alpha-haemolysis, characterised by a zone of greenish discoloration surrounding the colony resulting from the partial oxidation of haemoglobin to methaemoglobin, is classically associated with viridans streptococci and Streptococcus pneumoniae. Beta-haemolysis, defined by a clear, sharply defined zone of complete red cell lysis, is a hallmark of Streptococcus pyogenes (Group A), Streptococcus agalactiae (Group B), and certain staphylococci. Gamma-haemolysis, or the absence of any haemolytic activity, is observed with enterococci and many other organisms.(12,16)

The use of selective and differential culture media further enhances the macroscopic identification process by restricting growth to certain bacterial groups whilst simultaneously generating colorimetric or morphological changes that assist in their presumptive identification. MacConkey agar, for example, inhibits the growth of Gram-positive organisms whilst differentiating lactose-fermenting Enterobacteriaceae — which produce pink to red colonies due to acid production — from non-lactose fermenters, which appear colourless or pale.(14) Chromogenic media, such as chromID MRSA or chromID CPS, exploit enzymatic reactions linked to chromogenic substrates to produce colony colours directly associated with specific bacterial species or resistance phenotypes, offering an additional layer of macroscopic discriminatory power.(15)

Figure 9. Schematic reference diagram of the standard descriptive parameters used in the macroscopic characterisation of bacterial colonies in clinical microbiology. Parameters are organised by category: colony form (view from above), margin morphology, elevation profile (side view), haemolysis pattern on blood agar, surface texture and opacity, and clinically significant pigmentation examples.

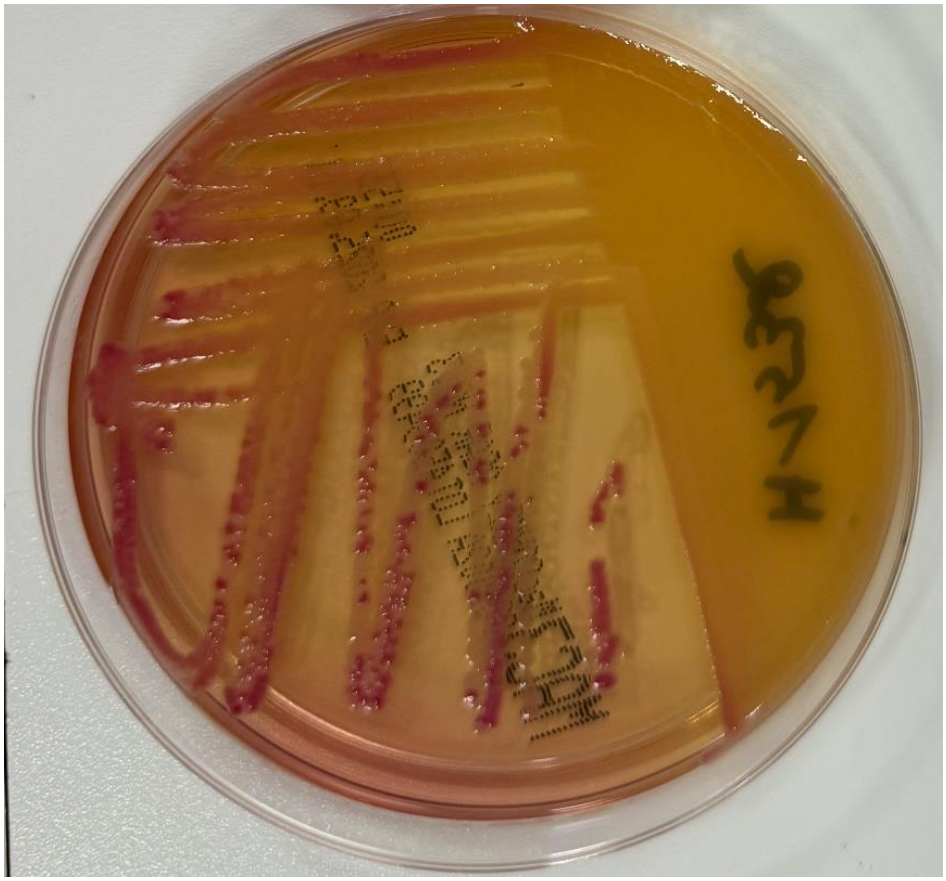


Figure 10. MacConkey agar plate illustrating differential colony morphology based on lactose fermentation.

Lactose-fermenting colonies (pink to red colouration) are visible alongside non-lactose-fermenting colonies (pale, colourless appearance), demonstrating the medium's capacity to simultaneously inhibit Gram-positive organisms and differentiate Enterobacteriaceae on the basis of their metabolic activity. The bile salts and crystal violet in the medium suppress Gram-positive growth, whilst the neutral red pH indicator produces the characteristic colour change in acid-producing lactose fermenters. [Original photograph — Microbiology Laboratory, Mohamed VI University Hospital.]

Figure 11. MacConkey agar plate showing growth of Serratia marcescens, demonstrating the characteristic red pigmentation attributable to the production of prodigiosin, a tripyrrole secondary metabolite synthesized optimally at temperatures below 37°C. The pigment production constitutes a distinctive macroscopic identification cue readily observable on colony inspection. Note the lactose-negative phenotype of this organism, consistent with its classification among the non-lactose-fermenting Enterobacteriaceae. [Original photograph — Microbiology Laboratory, Mohamed VI University Hospital.]

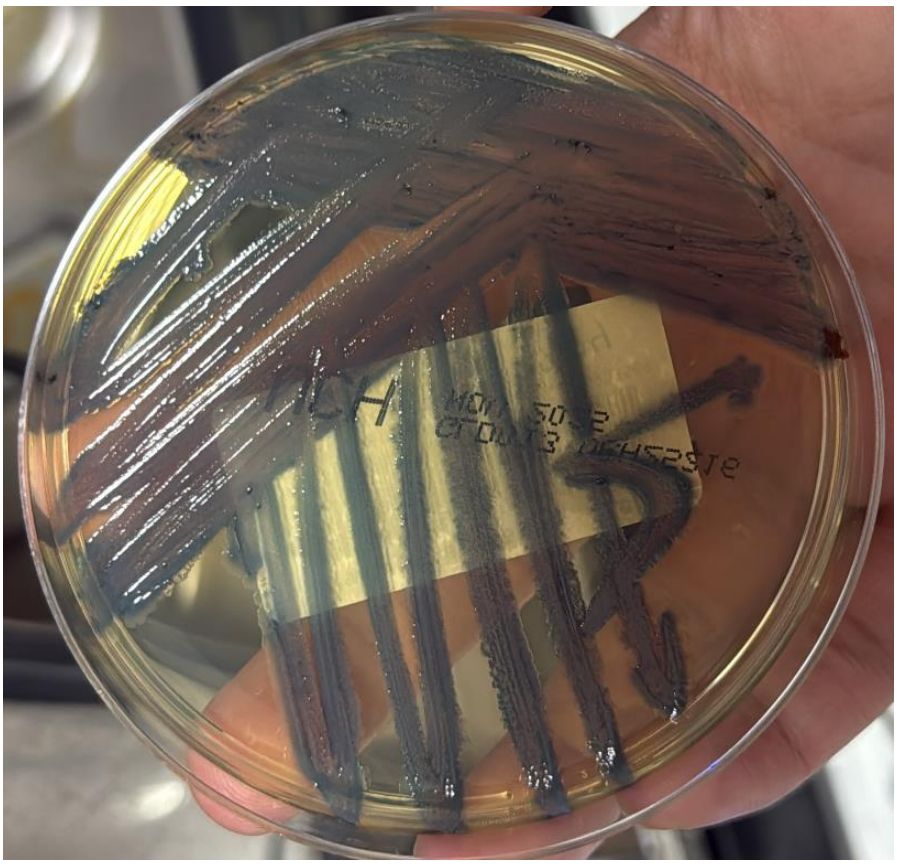


Figure 12. Chromogenic UHC (Urine Hardy CHROMagar) agar plate showing growth of Enterobacter hormaechei,

a clinically significant member of the Enterobacter cloacae complex increasingly recognised as an independent species of nosocomial importance. Chromogenic media exploit the reaction between species-specific bacterial enzymes and chromogenic substrates incorporated into the agar, generating colony colorations that allow presumptive organism identification directly from primary culture without additional biochemical testing. The colony colouration observed is attributable to enzymatic cleavage of the chromogenic substrate by this organism, producing a visually distinctive result that distinguishes it from other Enterobacteriaceae growing on the same medium. This illustrates the enhanced discriminatory power of chromogenic media over conventional non-selective agars in the presumptive identification of clinically relevant Gram-negative bacilli. [Original photograph — Microbiology Laboratory, Mohamed VI University Hospital.]

Figure 13. Columbia blood agar plate showing growth of S. aureus with characteristic beta-haemolysis. The clear,

sharply demarcated zone of complete erythrocyte lysis surrounding each colony results from the activity of staphylococcal haemolysins — principally alpha-toxin — which disrupt red blood cell membranes, producing a zone of complete haemoglobin clearance readily visible to the naked eye. The golden-yellow pigmentation of the colonies, attributable to carotenoid synthesis, constitutes an additional macroscopic identification cue characteristic of this species. Together, the beta-haemolytic pattern and the golden pigmentation represent two of the most diagnostically significant macroscopic features in clinical bacteriology, providing an immediate presumptive identification of S. aureus prior to any formal biochemical or mass spectrometric testing. [Original photograph — Microbiology Laboratory, Mohamed VI University Hospital.]

2. Odour-Based Characteristics

Whilst macroscopic visual examination remains the primary component of colony assessment, olfactory characteristics have historically constituted an informal but practically useful supplement to colony identification in experienced microbiologists. Certain bacterial species produce volatile metabolic by-products during growth that generate characteristic and reproducible odours detectable upon opening culture plates, and awareness of these associations can provide immediate and cost-free diagnostic cues prior to any formal testing.(13)

Among the most clinically recognised examples, Pseudomonas aeruginosa produces a distinctive grape-like or fruity odour attributable to the volatile compound 2-aminoacetophenone, a metabolite of tryptophan degradation.(13) Proteus species, particularly Proteus mirabilis, generate a pungent, putrid odour associated with the production of indole and other sulphur-containing volatile compounds during the degradation of amino acids. Haemophilus influenzae is classically described as producing a musty or bleach-like odour on chocolate agar. Anaerobic bacteria, discussed further in Section IV, frequently produce strongly offensive odours attributable to short-chain fatty acids, indole, and hydrogen sulphide generated during fermentation of peptides and amino acids — odours that can serve as an immediate alert to the presence of anaerobic growth when anaerobic culture plates are opened.(13,16)

It must be emphasised, however, that odour-based identification carries significant limitations and can under no circumstances serve as a standalone identification criterion. Olfactory perception is inherently subjective and varies between individuals; odour production is influenced by culture media composition, incubation duration, and atmospheric conditions; and the volatile compounds responsible for characteristic odours may be shared by multiple species or absent in atypical strains. In contemporary laboratory practice, odour observation is best regarded as a supplementary, hypothesis-generating cue that contributes to the preliminary assessment of a culture rather than as a formal identification step.(13) Its role has diminished further with the adoption of rapid mass spectrometric identification methods, which deliver unambiguous species-level results independently of any sensory assessment.

3. Microscopic Examination

Following macroscopic assessment of colony characteristics, the next fundamental step in conventional microbial identification is microscopic examination.

3.1 Fresh State Examination and Motility Assessment

Prior to preparing a fixed and stained smear, examination of living, unstained bacteria in the fresh state constitutes a rapid and informative preliminary step in microbial identification. Performed by placing a small quantity of bacterial suspension between a glass slide and coverslip — either as a simple wet mount or as a hanging drop preparation using a depression slide — fresh state examination allows direct observation of living cells under bright-field or phase-contrast microscopy without the distortion that fixation and staining may introduce.(12,13) Beyond the assessment of motility, which represents its primary diagnostic application, fresh state examination permits evaluation of cell morphology, approximate size, cellular arrangement, and — when combined with negative staining techniques such as India ink or nigrosin — the presence of a capsule, a feature of particular relevance for encapsulated organisms such as Cryptococcus neoformans and Klebsiella pneumoniae.(14)

The principal diagnostic value of fresh state examination lies in the assessment of bacterial motility. The ability of a bacterium to move actively and directionally through a liquid medium is a fundamental phenotypic characteristic that contributes to its preliminary identification and, in certain clinical contexts, provides a rapid presumptive result of immediate practical value. Bacterial motility is almost exclusively mediated by flagella — complex proteinaceous appendages anchored in the cytoplasmic membrane and cell wall, whose rotational movement propels the cell through its environment. The arrangement of flagella on the bacterial cell surface varies systematically between species and constitutes an additional taxonomic character of significance.(13,16)

Five principal flagellar arrangements are recognised in clinical bacteriology. Peritrichous bacteria possess flagella distributed over the entire cell surface and characteristically display a slow, tumbling motility pattern; this arrangement is exemplified by Escherichia coli, Salmonella species, and Listeria monocytogenes.(12) Monotrichous bacteria carry a single polar flagellum at

one end of the cell and exhibit rapid, darting motility; Pseudomonas aeruginosa and Vibrio cholerae are among the most clinically relevant examples. Lophotrichous organisms bear a tuft of several flagella emerging from a single pole, producing vigorous directional movement; Helicobacter pylori is the most diagnostically significant representative of this group. Amphitrichous bacteria possess flagella at both poles and display bidirectional motility; Campylobacter species, whose characteristic corkscrew motility is directly observable in fresh stool preparations, fall within this category. Finally, atrichous organisms carry no flagella and are entirely non-motile; Klebsiella pneumoniae, Shigella species, and all staphylococci are representative examples.(13,14)

Of particular clinical significance are several species whose motility characteristics constitute a rapid presumptive identification cue. Listeria monocytogenes produces characteristic tumbling motility at room temperature (20–25°C) but is non-motile or poorly motile at 37°C, a temperature-dependent phenomenon attributable to flagellin gene expression that is diagnostically exploited in the motility test and constitutes a cardinal feature of its identification.(12) Vibrio cholerae exhibits a markedly rapid, polar darting motility that has historically provided an immediate presumptive diagnosis of cholera when fresh stool preparations are examined directly under the microscope during outbreak investigations. Campylobacter species produce a distinctive shooting-star or corkscrew motility in fresh stool preparations that allows experienced microbiologists to generate a rapid presumptive identification before culture results are available.(16)

A critical interpretive precaution in fresh state motility assessment is the rigorous distinction between **true motility** — active, directional, purposeful movement driven by flagellar rotation — and **Brownian motion** — the passive, random, vibratory displacement exhibited by all microscopic particles suspended in liquid due to molecular collisions.(13) Brownian motion produces a quivering or jiggling movement that may superficially resemble bacterial motility to the inexperienced observer, but differs fundamentally in its directionality and character: Brownian motion does not produce net displacement of a cell through the medium, whilst true motility does. This distinction requires experience and constitutes a recognised source of

interpretive error, particularly among trainees. All non-motile organisms exhibit Brownian motion, and an erroneous attribution of motility to a non-motile species on this basis represents a significant preliminary misidentification.(14)

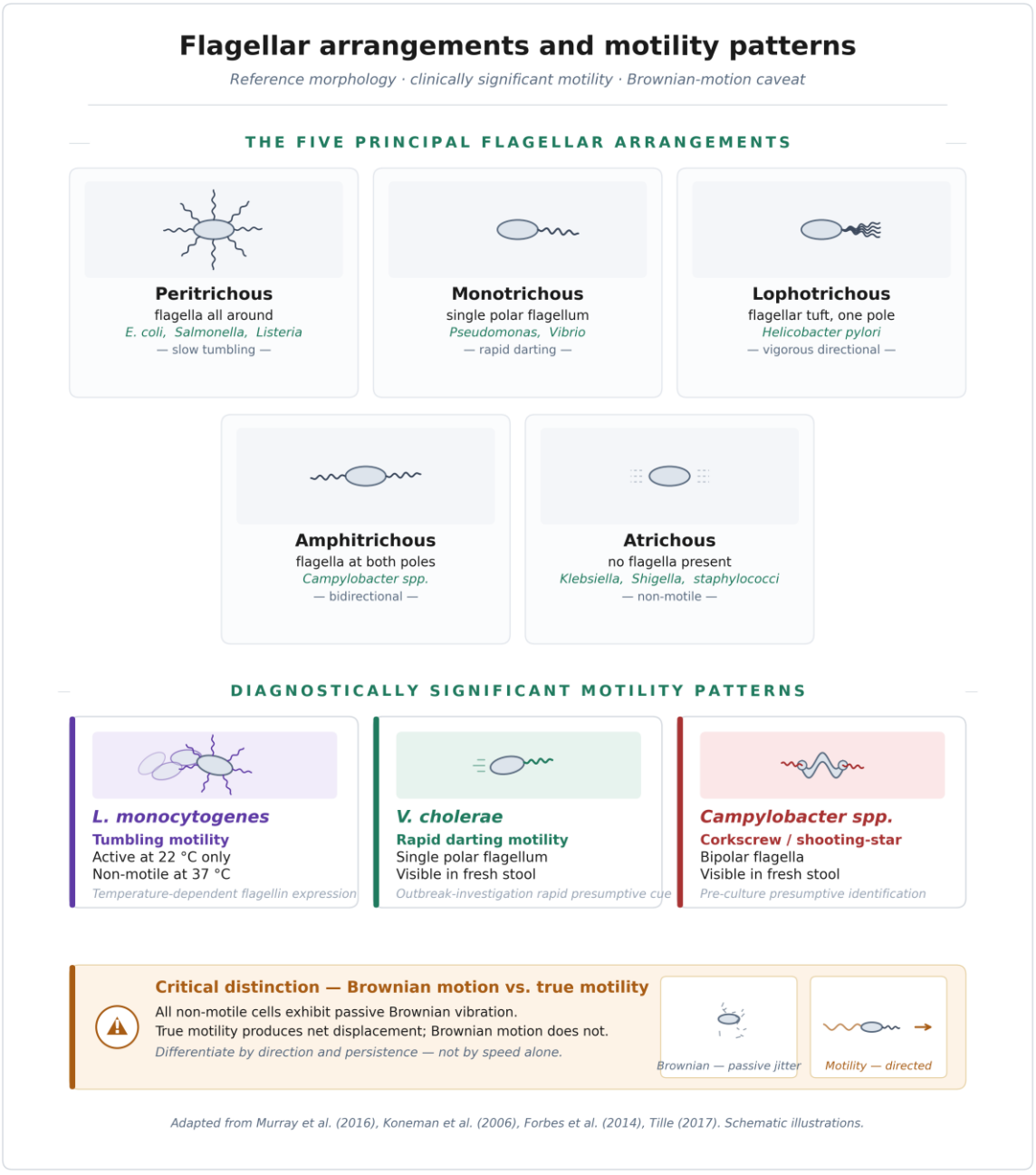


Figure 14. Schematic diagram of the five principal flagellar arrangements in bacteria and their associated motility patterns.

Upper panel: peritrichous (flagella uniformly distributed, slow tumbling); monotrichous (single polar flagellum, rapid darting); lophotrichous (polar tuft, vigorous directional). Lower panel: amphitrichous (flagella at both poles, bidirectional); atrichous (no flagella, non-motile, indicated by crossed lines). Central section: three organisms of particular clinical diagnostic significance whose motility characteristics constitute a rapid presumptive identification cue. The warning panel emphasizes the critical distinction between active flagellar locomotion and passive Brownian motion.

3.2 Gram Staining

Whilst fresh state examination provides immediate information on motility and gross cellular morphology, it does not permit reliable characterisation of cell wall architecture, precise morphological detail, or the discrimination of bacterial groups based on staining properties. For this purpose, examination of a fixed and stained preparation is required. Among the various staining techniques employed in clinical bacteriology, the Gram stain — developed by the Danish bacteriologist Hans Christian Gram in 1884 and subsequently refined for routine laboratory use — remains the single most important and universally applied preparatory step in microbial identification.(12) It divides the bacterial world into two fundamental categories that reflect deep differences in cell wall architecture, guides the selection of subsequent biochemical and culture-based investigations, and in many clinical contexts provides the first actionable microbiological result available to the treating physician.(12,13)

3.2-1 Principle

The differential staining property of the Gram technique rests upon fundamental differences in the structure of the bacterial cell wall. Gram-positive bacteria possess a thick peptidoglycan layer — typically 20 to 80 nanometres in depth — situated directly exterior to the cytoplasmic membrane, with no outer lipid membrane.(12,14) This dense meshwork of cross-linked murein retains the crystal violet-iodine complex during the decolourisation step, resulting in a purple colouration. Gram-negative bacteria, by contrast, possess a considerably thinner peptidoglycan layer — typically 2 to 7 nanometres — sandwiched between the cytoplasmic membrane and an outer membrane composed of phospholipids, lipopolysaccharide, and various proteins.(12) The outer membrane is efficiently dissolved by the organic solvent used during decolourisation, causing the loss of the crystal violet-iodine complex and leaving the cell colourless until it is counterstained with safranin, which imparts a pink to red colouration.(13,16)

This fundamental dichotomy, Gram-positive purple versus Gram-negative pink, is not merely a technical convenience. It reflects profound biological differences in membrane permeability, susceptibility to antimicrobial agents, response to host immune defences, and clinical behaviour. The lipopolysaccharide of the Gram-negative outer membrane, for example,

acts as an endotoxin capable of triggering systemic inflammatory responses, whilst the thick peptidoglycan of Gram-positive organisms is a target for beta-lactam antibiotics including penicillins and cephalosporins.(12)

Figure 15. Schematic diagram of the Gram staining procedure and its mechanistic basis. Upper panel: the four sequential steps of the procedure — crystal violet (primary stain), Gram's iodine (mordant), acetone–ethanol decolourisation (critical step), and safranin (counterstain). Central panel: comparative cell wall architecture of Gram-positive organisms (thick peptidoglycan, no outer membrane) and Gram-negative organisms (thin peptidoglycan, lipopolysaccharide outer membrane), illustrating the structural basis of the differential staining outcome.

3.2-2 Procedure

The Gram staining procedure is performed on a heat-fixed or methanol-fixed smear of the bacterial isolate prepared on a clean glass slide. It consists of four sequential steps, each of which must be carried out with attention to timing and reagent quality to ensure reliable and reproducible results.(15,16)

In the **first step**, crystal violet is applied to the fixed smear and allowed to act for approximately one minute. Crystal violet is a basic dye that penetrates the cell wall of all bacteria, staining both Gram-positive and Gram-negative organisms purple at this stage.

In the **second step**, Gram's iodine solution — the mordant — is applied for one minute. Iodine forms large insoluble complexes with crystal violet within the bacterial cell, trapping the dye and increasing the resistance of the stain to subsequent removal. At this stage, all bacteria remain purple.

The **third step** — decolourisation — is the most critical and technically demanding stage of the procedure. An organic solvent, typically a mixture of acetone and ethanol or ethanol alone, is applied briefly — for approximately 10 to 30 seconds — and immediately rinsed with water. In Gram-negative bacteria, the solvent dissolves the lipid-rich outer membrane and extracts the crystal violet-iodine complex through the thin peptidoglycan layer, rendering the cells colourless. In Gram-positive bacteria, the solvent dehydrates the thick peptidoglycan layer, causing it to contract and tighten its pores, thereby retaining the crystal violet-iodine complex and preserving the purple colour. Over-decolourisation is a common technical error that causes false Gram-negative results for Gram-positive organisms; under-decolourisation produces false Gram-positive results for Gram-negative organisms.(13,15)

In the **fourth step**, safranin — the counterstain — is applied for one minute. Safranin stains the now-colourless Gram-negative cells pink to red, whilst Gram-positive cells, already purple, are unaffected. After rinsing and drying, the slide is examined under oil immersion at 1000× magnification.(12)

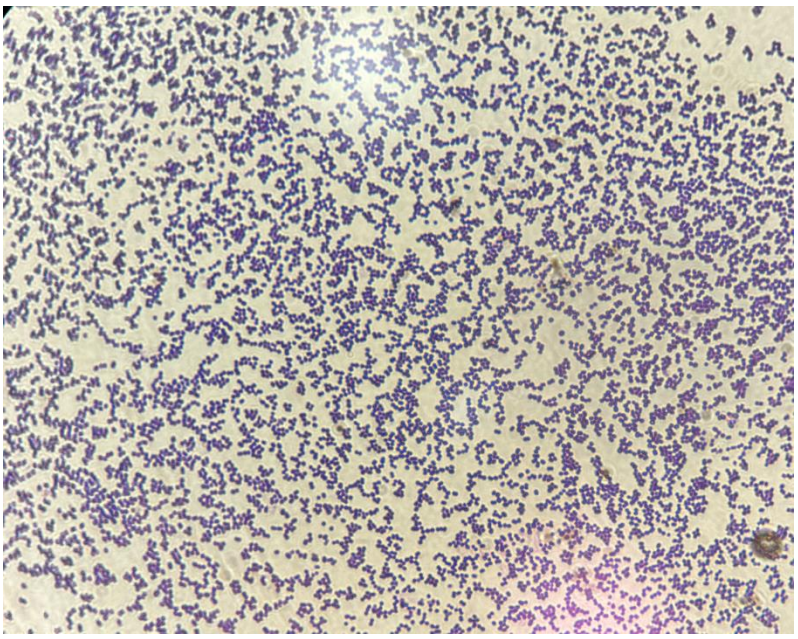


Figure 16. Gram stain of S. aureus (1000×, oil immersion). Gram-positive cocci (purple) arranged in irregular grape-like clusters characteristic of staphylococci. The thick peptidoglycan cell wall retains the crystal violet-iodine complex following decolourisation,

3.2-3 Interpretation: Cell Morphology and Arrangement

Beyond the Gram reaction itself, microscopic examination of the stained smear provides critical additional information through the assessment of **cell morphology** and **cellular arrangement**, both of which carry significant discriminatory value in preliminary identification.(13,14)

With respect to cell morphology, bacteria are broadly classified as **cocci** (spherical cells), **bacilli** (rod-shaped cells), **coccobacilli** (intermediate ovoid forms), **spirochetes** (helical cells), and **vibrios** (comma-shaped curved rods). Within each morphological category, cell size, regularity of shape, and the presence of particular structural features such as spores, capsules, or bipolar staining may further narrow the differential.(12)

Cellular arrangement — the spatial organisation of cells relative to one another following division — provides an additional layer of discriminatory information. Among cocci, clustering in irregular grape-like aggregates is characteristic of staphylococci; pairs (diplococci) or chains are associated with streptococci and enterococci; and pairs of flattened, coffee-bean shaped diplococci are characteristic of Neisseria species. Among bacilli, palisade arrangements or

letters-of-the-alphabet formations are associated with corynebacteria; single rods with rounded ends are typical of most Enterobacteriaceae; and long, thin, poorly staining rods may suggest mycobacteria or nocardiae, which require alternative staining techniques for reliable visualisation.(17)

The combination of Gram reaction, cell morphology, and arrangement — interpreted in the context of the clinical specimen type, the culture media from which the organism was isolated, and the macroscopic colony characteristics already observed — constitutes the preliminary identification framework upon which subsequent biochemical and mass spectrometric investigations are built.(1,3)

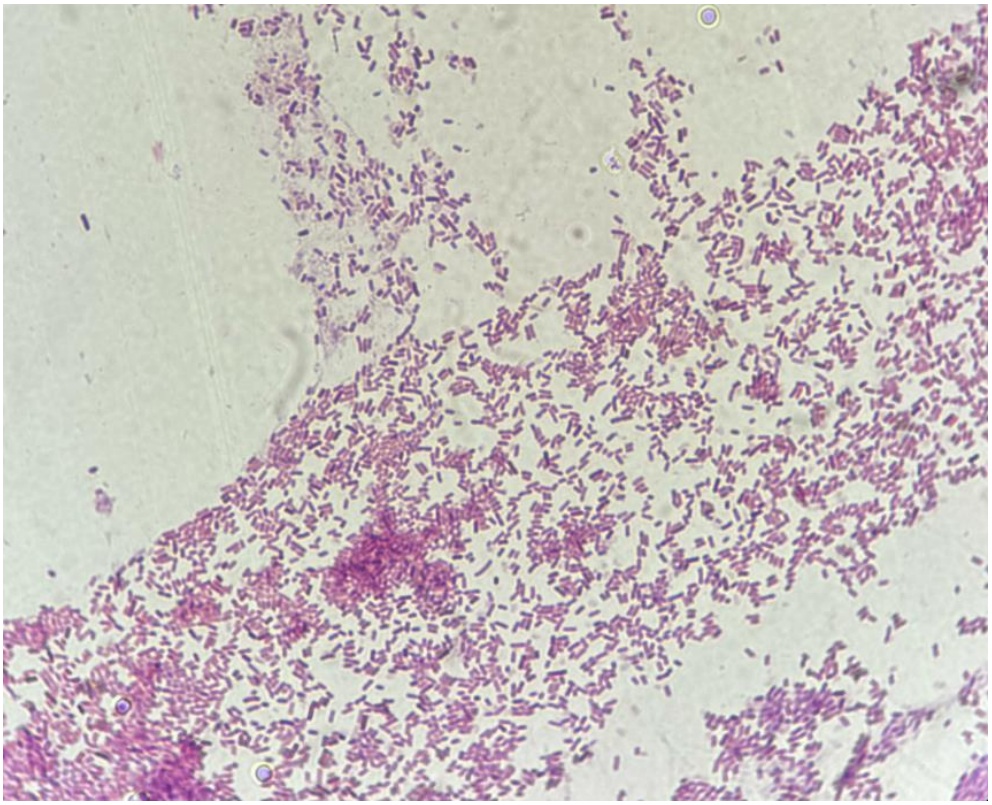


Figure 17. Gram stain of Escherichia coli, 1000× oil immersion.

Gram-negative bacilli (pink) with characteristic rod morphology, demonstrating loss of crystal violet-iodine complex following dissolution of the lipid-rich outer membrane during decolourisation, with subsequent counterstaining by safranin. [Original photograph — Microbiology Laboratory]

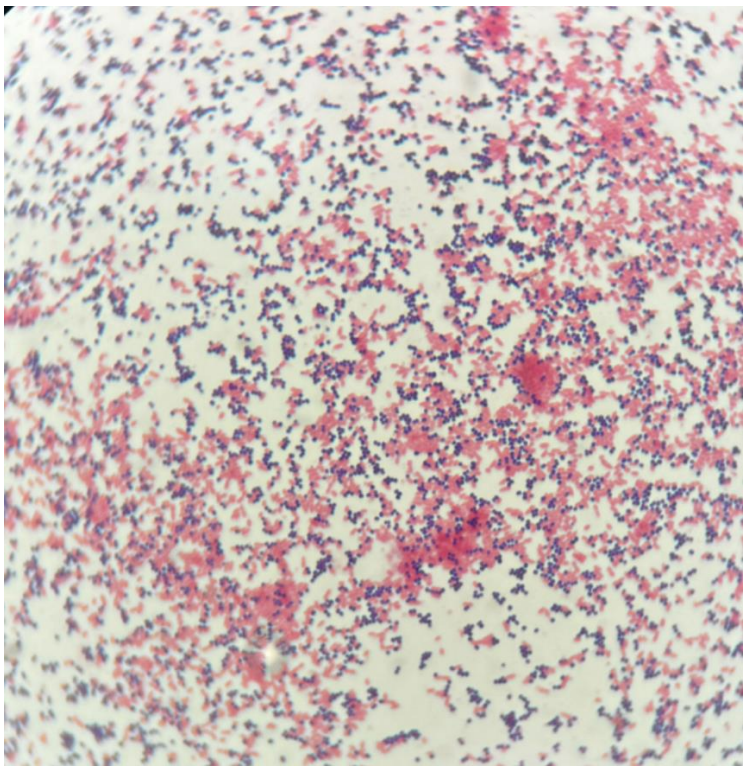


Figure 18. Gram stain of a polymicrobial specimen, 1000× oil immersion.
The differential colouration clearly distinguishes Gram-positive organisms (purple) from Gram-negative organisms (pink) within the same microscopic field, illustrating the practical discriminatory capacity of the Gram staining technique in the identification of mixed bacterial populations. [Original photograph — Microbiology Laboratory

4. Biochemical Identification Methods

Although Gram staining orients the identification process by assigning an isolate to a broad morphological and tinctorial category, it rarely permits identification at the species level. More discriminating phenotypic information is traditionally obtained through biochemical testing, a family of methods founded on the principle that the enzymatic equipment of a bacterium and its ability to metabolise defined substrates together constitute a reproducible signature that is, in most cases, specific enough to identify the organism within its genus or family. Whereas morphological criteria describe only the structural organisation of cells and colonies, biochemical tests explore functional metabolic properties: the hydrolysis of peptides and sugars, the utilisation of specific carbon sources, the production of characteristic enzymes, the reduction of nitrates, and the fermentative versus oxidative use of carbohydrates. The

combined pattern of positive and negative reactions across a panel of carefully chosen substrates is then interpreted as a code or profile characteristic of a given taxon (14,17).

Biochemical identification has evolved considerably since its introduction. From the individual tube reactions of the classical school of Enterobacteriaceae taxonomy, which required prolonged incubation, extensive reagent preparation and manual interpretation, the field has progressively moved towards miniaturised commercial galleries, then towards fully automated platforms combining identification and antimicrobial susceptibility testing within the same instrument (13,17). Before the widespread implementation of mass spectrometry and nucleic acid-based methods, biochemical identification represented the cornerstone of routine bacterial identification in clinical microbiology. In our own laboratory, it constituted the principal comparator during the initial part of the study period, and individual enzymatic tests continue to occupy a central place in daily practice as rapid orientation tools used in conjunction with Gram staining.

4.1 Individual Enzymatic Tests

At the most elementary level, biochemical identification relies on a small number of rapid single tests that explore key enzymatic activities. These tests are performed directly on a fresh isolated colony, using reagent strips, commercial reagent drops, or simple liquid or solid media. They yield a result within seconds to a few hours and serve as first-line orientation tests, applied in sequence or in parallel according to the Gram reaction and colony morphology (12–14,17).

The catalase test detects the enzymatic decomposition of hydrogen peroxide (H_2O_2) into water and molecular oxygen, a reaction catalysed by catalase, an iron-containing haemoprotein present in most aerobic and facultatively anaerobic bacteria. A small inoculum is placed on a glass slide and mixed with a drop of 3 % hydrogen peroxide; the immediate appearance of effervescence indicates a positive reaction(12,17). In routine practice, catalase is the principal test used to distinguish Staphylococcus (catalase-positive) from Streptococcus and Enterococcus(catalase-negative) within Gram-positive cocci, and it also contributes to the differentiation of aerobic Gram-positive rods such as Corynebacterium and Listeria from

anaerobes(13,14). Care must be taken not to use colonies growing on blood-containing media, since erythrocyte catalase may generate false-positive reactions.

The oxidase test detects cytochrome c oxidase, the terminal enzyme of the respiratory electron transport chain, through the ability of the organism to oxidise an artificial electron donor — typically N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride — to a coloured product. A fresh colony is smeared on a filter-paper strip impregnated with the reagent, and the appearance of a deep purple to black colouration within ten to thirty seconds denotes a positive result (13,17). In Gram-negative bacilli, the oxidase reaction is of major taxonomic value: Enterobacterales are, with rare exceptions, oxidase-negative, whereas Pseudomonas, Vibrio, Aeromonas, Neisseria, Moraxella, Pasteurella and HACEK organisms are oxidase-positive. In the context of rare and fastidious isolates, the oxidase reaction is often the first orientation test performed on a suspected non-fermenting Gram-negative bacillus or on a fastidious Gram-negative coccobacillus(12,14).

The coagulase test specifically addresses the identification of Staphylococcus spp. and, in particular, the distinction between S. aureus and the coagulase-negative staphylococci. Two forms are routinely performed. The slide coagulase test detects clumping factor, a cell-wall-associated protein that binds fibrinogen and causes visible agglutination when a colony is emulsified in plasma on a glass slide. The tube coagulase test detects both clumping factor and free coagulase, an enzyme secreted into the culture medium that activates prothrombin and leads to fibrin clot formation in a plasma tube incubated at 37 °C(12,13). The tube test remains the reference method, since a small proportion of clumping-factor-negative S. aureus strains may yield a falsely negative slide test. Latex agglutination kits combining the detection of clumping factor, protein A and, in more recent formulations, capsular polysaccharides, have progressively replaced the tube test in most routine settings (14).

The urease test detects the enzymatic hydrolysis of urea into ammonia and carbon dioxide. The reaction is read on Christensen urea agar or in urea broth containing phenol red as pH indicator; alkalinisation of the medium by ammonia shifts the indicator to a bright pink-red colour (13,17). Urease activity is characteristic of Proteus, Morganella and Providencia among

Enterobacterales, where it is typically intense and rapid, and of several fastidious or slow-growing organisms including Helicobacter pylori, Brucella, Ureaplasma and, more weakly, Klebsiella and some Yersinia species. Urease is also a useful orientation test for yeasts: Cryptococcus neoformans is strongly urease-positive, a feature historically exploited for its presumptive identification before the advent of mass spectrometry(12,14).

The indole test measures the ability of the organism to degrade tryptophan into indole, pyruvate and ammonia through the action of tryptophanase. Indole is then revealed by reaction with a benzaldehyde-based reagent — most commonly Kovac's reagent or, more recently, the spot DMACA reagent — yielding a red colouration (Kovac's) or a blue-green colouration (DMACA) (13,17). Indole is a classical component of the IMViC series used to characterise Enterobacteriaceae, and it remains decisive in the identification of Escherichia coli, which is typically indole-positive, and in its distinction from Klebsiella pneumoniae, which is usually indole-negative (12,14). Rapid spot tests performed directly on colonies growing on tryptophan-rich media now allow a result within seconds and are widely used as screening tests in urinary microbiology.

The ONPG (β-galactosidase) test detects the enzyme β-galactosidase, which hydrolyses o-nitrophenyl-β-D-galactopyranoside into galactose and the yellow chromogen o-nitrophenol. It is particularly useful for distinguishing late lactose-fermenting organisms, which possess β-galactosidase but lack the permease required for lactose utilisation on selective media, from true lactose-negative organisms(13,17). Within Enterobacterales, ONPG positivity supports the identification of genera such as Citrobacter and some Salmonella species, and contributes to the resolution of atypical late-fermenting E. coli or Shigella strains(12,14).

The esculin hydrolysis test assesses the ability of the organism to hydrolyse esculin, a β-glucoside, into esculetin and glucose. Esculetin reacts with the ferric ions present in bile-esculin agar to form a blackish-brown complex, which is the readout of the test(12,13). Because the medium also contains bile salts, it simultaneously evaluates two properties: tolerance to bile and hydrolysis of esculin. The bile-esculin reaction is a cornerstone of the presumptive identification of Enterococcus and group D streptococci, and an analogous principle is exploited in the

identification of certain anaerobes, notably members of the Bacteroides fragilis group, which are bile-resistant and esculin-positive (14,17).

Applied in sequence to a Gram-characterised isolate, these individual tests allow a rapid and economical orientation towards a genus or group of genera. They remain routinely used in our laboratory, in combination with Gram staining, as the first layer of bacterial identification, particularly when colonies suggest a common pathogen for which no further characterisation is required. Their discriminating power, however, becomes rapidly insufficient when species-level identification is required within a large family such as Enterobacteriaceae or the non-fermenting Gram-negative bacilli. This limitation historically motivated the development of the multi-test commercial systems described below (1,2).

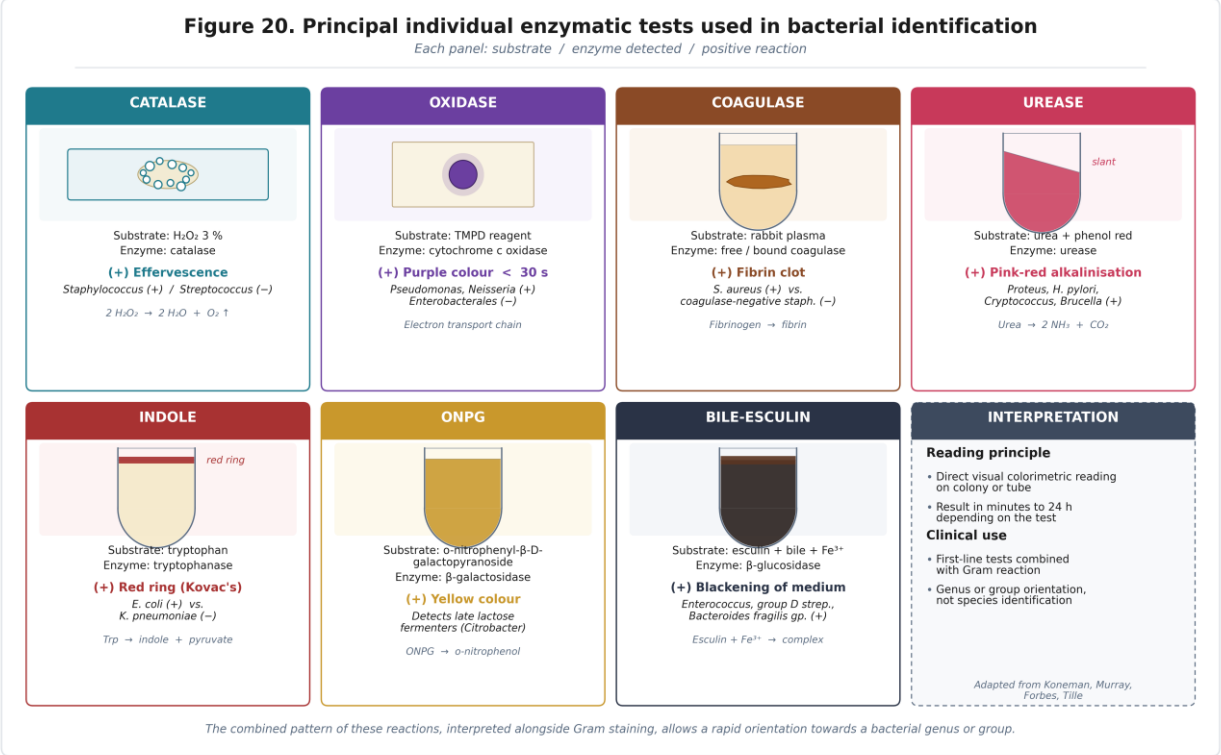


Figure 19. **Principal individual enzymatic tests used in bacterial identification,** showing for each test the substrate, the enzyme detected and the interpretation of a positive reaction. The combined pattern of these reactions, interpreted alongside Gram staining, allows a rapid orientation towards a bacterial genus or group.(12–14,17) (adapted from Koneman et al. (2006), Murray et al. (2016), Forbes et al. (2014), Tille (2017).)

4.2 API Gallery Systems (Miniaturised Biochemical Galleries)

The limitations of individual biochemical tests and tube-based identification schemes — high reagent and media consumption, extensive technician time, and variable inter-laboratory reproducibility — progressively motivated the development of miniaturised, standardised multi-test galleries from the late 1960s onwards. The API (Analytical Profile Index) system, manufactured by bioMérieux (Marcy-l'Étoile, France), rapidly became the most widely adopted of these systems in clinical microbiology and has remained, for several decades, the reference commercial manual gallery in laboratories throughout Europe, including our own(1,2,13).

All API kits share a common principle of construction. Each kit consists of a plastic strip bearing a series of cupules, moulded in line, into which dehydrated substrates corresponding to defined biochemical tests have been lyophilised at the factory(1). The strip is rehydrated and inoculated by distributing, into each cupule, a standardised bacterial suspension prepared in sterile saline or demineralised water. Depending on the test, certain cupules are overlaid with mineral oil to create anaerobic conditions, for instance to assess lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase or urease activity. The strip is then incubated, usually at 36 ± 1 °C for 18 to 24 h, within a humid chamber to prevent desiccation. At the end of incubation, reagents such as Kovac's, Voges-Proskauer reagents (α-naphthol and potassium hydroxide) or ferric chloride are added to specific cupules to reveal colour reactions. Each cupule is read as positive or negative according to a colour key provided by the manufacturer(1,2,13).

The central innovation of the API system lies in the conversion of this pattern of reactions into a numerical profile code. The tests are grouped into consecutive triplets, each triplet being assigned the values 1, 2 and 4 for the first, second and third test respectively. The arithmetic sum of the values of the positive tests within a triplet yields a digit between 0 and 7. The concatenation of these digits along the whole strip generates a numerical profile — seven digits for the API 20E, for example — which is then entered into a database (originally the printed Profile Register and later the Analytical Profile Index and its computerised derivatives) to retrieve the most likely identification together with a discrimination index and, when relevant, a list of additional tests to confirm the answer (1,2).

Several gallery types are used in the routine identification of clinical isolates, and the four most relevant to the present study are briefly described below.

The API 20E is historically the archetype of the system and remains the most widely used biochemical gallery for the identification of Enterobacteriaceae. It comprises twenty tests, including β-galactosidase (ONPG), arginine dihydrolase, lysine and ornithine decarboxylases, citrate utilisation, H_2S production, urease, tryptophan deaminase, indole, acetoin production (Voges-Proskauer), gelatin liquefaction and fermentation of nine carbohydrates. Additional tests such as oxidase and nitrate reduction may be performed in parallel. In their foundational evaluation of the kit on 206 strains of Enterobacteriaceae belonging to 34 taxa, Holmes and colleagues reported that the Analytical Profile Index correctly identified 88 % of the strains, with 2 % misidentified, and that most subsequent authors reported identification rates between 92 % and 100 % for routine Enterobacteriaceae(1). The API 20E has also been adapted, with additional tests, to the identification of non-fermenting Gram-negative bacilli and of some rare Gram-negative organisms (2).

The API 20 NE is specifically designed for non-fermenting or slowly fermenting Gram-negative bacilli, such as Pseudomonas, Acinetobacter, Achromobacter, Burkholderia, Stenotrophomonas, Bordetella and Moraxella, and for a number of fastidious Gram-negative organisms not adequately covered by the 20E format. It combines conventional tests (reduction of nitrates, indole, fermentation of glucose) with assimilation tests in which the organism is incubated in a minimal medium with a single carbon source (2,13). Overall agreement with conventional identification in evaluation studies has ranged from roughly 75 % to more than 97 %, with the lowest rates observed for the less common Pseudomonas, Acinetobacter and Burkholderia species, and with documented risks of misidentification — notably of Brucella melitensis as Ochrobactrum anthropi or Moraxella phenylpyruvica, a pattern that remains pertinent to the identification of rare pathogens in routine practice(2).

The API Staph is intended for the identification of Staphylococcus and Micrococcus species. It includes a series of sugar fermentation tests, arginine dihydrolase, urease, alkaline phosphatase and the Voges-Proskauer reaction, and is particularly used for the identification of

coagulase-negative staphylococci recovered from clinically relevant specimens such as blood cultures or prosthetic material. The API NH (Neisseria-Haemophilus), the API Coryne for coryneform Gram-positive rods, and the API 20 Strep for streptococci constitute complementary galleries adapted to more specialised identification needs(13,17).

The API 20 A (also referred to as the API Anaerobe gallery in older literature) is dedicated to the identification of obligate anaerobes, mostly Bacteroides, Prevotella, Porphyromonas, Fusobacterium, Clostridium and anaerobic Gram-positive cocci. Its interpretation is more delicate than that of the aerobic galleries because of the slow growth and metabolic heterogeneity of anaerobic taxa; in practice, the gallery must often be complemented by analysis of short-chain fatty acids by gas chromatography or by specific morphological criteria, and its discriminating power at the species level remains limited for many clinically important anaerobes(14,17).

Across all these formats, the API system offers several well-established advantages: standardised reagents, reproducible reading, a compact format adapted to benchtop work, a large commercial database regularly updated by the manufacturer, and a cost per identification that remains lower than that of automated systems for laboratories with moderate throughput (1,2). In our own laboratory, the API 20E has historically been the workhorse of phenotypic identification of Gram-negative bacilli, complemented by API 20 NE, API Staph and API 20 A for the taxonomic groups indicated above. These galleries defined the pre-MALDI reference against which the added value of mass spectrometry is analysed in the present work.

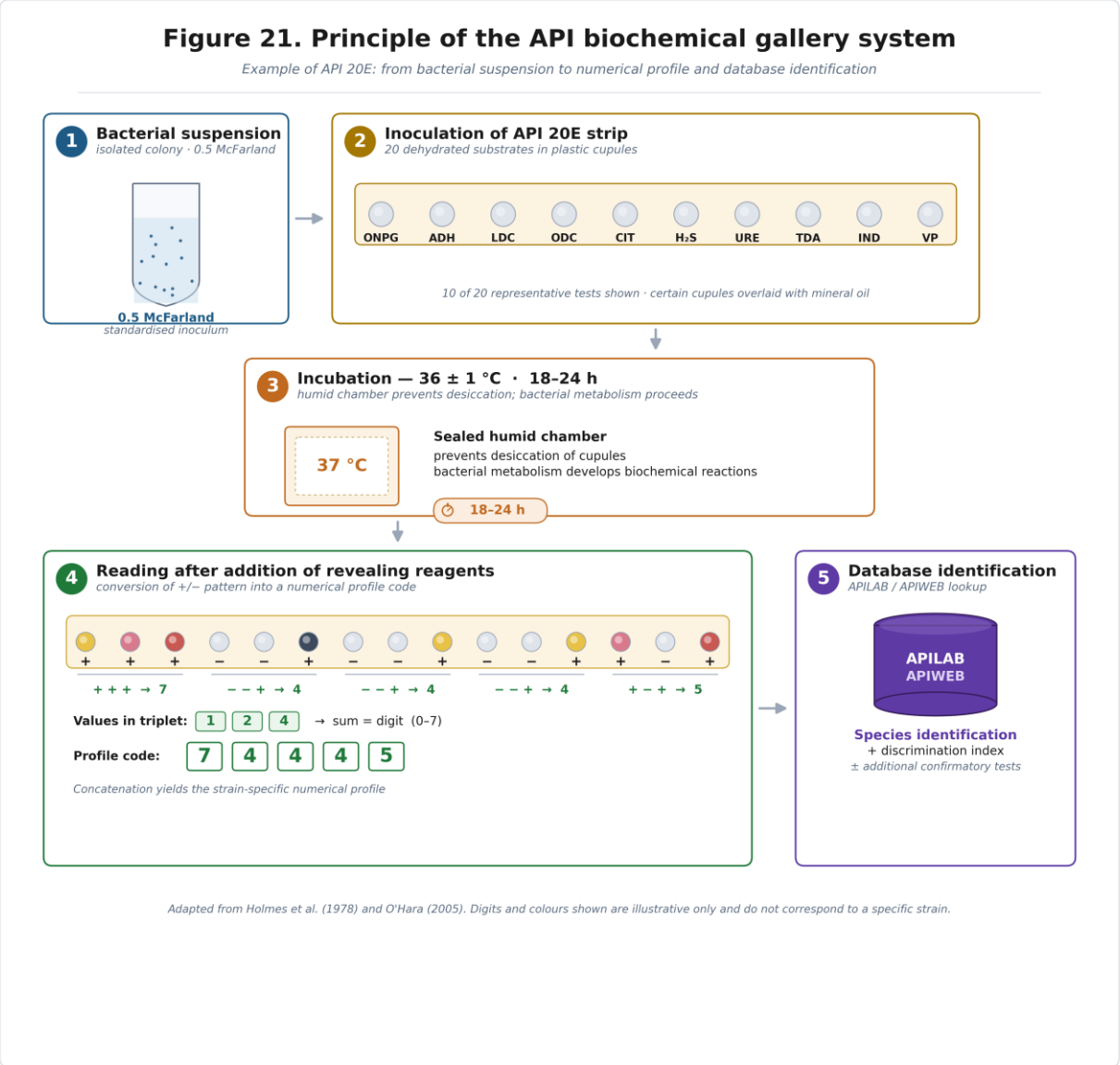


Figure 20. Principle of the API biochemical gallery system, illustrated on the API 20E format.
A standardised bacterial suspension is inoculated into a strip of dehydrated-substrate cupules, incubated for 18–24 h, and read colorimetrically after addition of revealing reagents. The pattern of positive and negative reactions is converted into a numerical profile code (digits 0–7 per triplet) that is compared against the APILAB database to yield a species identification with an associated discrimination index. Digit values and colours shown are illustrative only and do not correspond to a specific strain. (adapted from Holmes et al. (1978) and O'Hara (2005).)

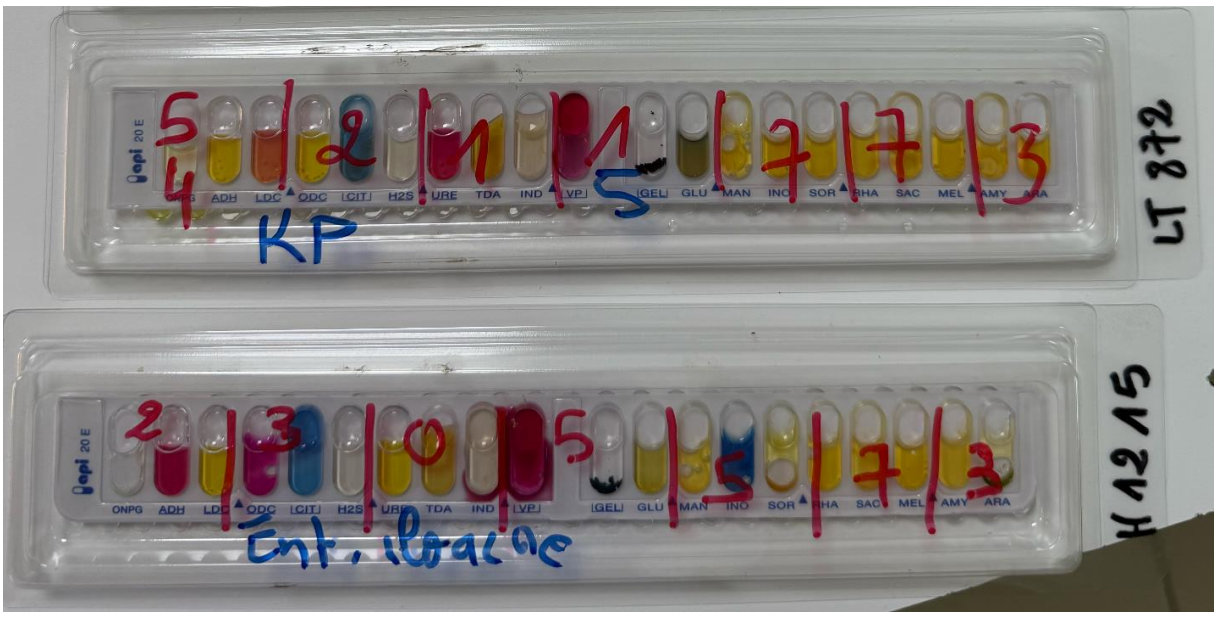


Figure 21. API 20E gallery inoculated and read in the laboratory, showing the colorimetric pattern obtained after 18–24 h of incubation at 36 ± 1 °C. Original photograph — Microbiology Laboratory, Mohamed VI Hospital

4.3 The BD Phoenix Automated Identification System

The accumulated limitations of manual biochemical galleries — overnight incubation, operator-dependent reading, time-consuming profile entry and the need to run an additional automated susceptibility platform in parallel — led, from the mid-2000s onwards, to the progressive introduction of fully automated benchtop platforms combining identification and antimicrobial susceptibility testing within a single instrument and a single reagent panel (2,14,17). In our own laboratory, this transition took place in two successive steps, both of which frame the pre-MALDI comparator period covered by the present study. A BD Phoenix 100 instrument (Becton Dickinson Diagnostic Systems, Sparks, Maryland, USA) was acquired in 2015 and became the main automated identification platform for routinely isolated Gram-negative bacilli and Gram-positive cocci; a BD Phoenix M50instrument was subsequently added in 2019, extending the instrument park and allowing a higher daily throughput of combined identification and susceptibility tests.

Principle of operation. The BD Phoenix platform relies on single-use, closed, moulded plastic panels comprising two wings. One wing is devoted to bacterial identification and contains

multiple miniaturised reaction wells with dehydrated biochemical substrates — a combination of chromogenic and fluorogenic compounds — that together explore enzymatic activities, carbohydrate utilisation, and a number of additional metabolic properties (2)(O'Hara, 2005). The second wing accommodates the antimicrobial susceptibility test (AST) in broth microdilution format, such that a single calibrated bacterial suspension inoculates both wings through a fluid-transfer step performed manually in the cabinet. Two families of identification panels are used: the NID panel (Negative IDentification) for Gram-negative organisms and the PID panel (Positive IDentification) for Gram-positive organisms; combination ID/AST panels (NMIC, PMIC) are also available and are the format used in routine practice, as they couple identification and susceptibility within a single workflow. The NID panel described by O'Hara contains 45 wells of dried biochemical substrates together with two fluorescence control wells, allowing the instrument to monitor both reaction kinetics and internal quality criteria(2).

Once inoculated and sealed, the panels are loaded into the instrument, which is capable of handling up to 99 panels simultaneously in the Phoenix 100 configuration, with one panel position reserved for the internal thermometer(8). From this point, all operations — incubation at 35 ± 1 °C, periodic optical reading of each well, kinetic interpretation of the chromogenic and fluorogenic signals and computation of an identification with an associated confidence score — are fully automated. Results are stored in the BD EpiCenter data management software, from which they are transmitted to the laboratory information system. Turnaround times from panel loading to identification are typically of the order of a few hours, shorter for fermentative organisms than for non-fermenters, and most panels are reported within a working day(2) (O'Hara, 2005).

The Phoenix M50. The BD Phoenix M50 is a more compact successor developed on the same core technology as the Phoenix 100, with a reduced panel capacity adapted to laboratories with moderate-throughput needs and to workflow segmentation within larger laboratories. In our own setting, its introduction in 2019 allowed the concomitant processing of additional panels during periods of high routine activity and provided a redundancy of the automated identification platform during maintenance of the Phoenix 100. The operating principle, reagent panels and

database are shared with the Phoenix 100 line, which preserves the consistency of the identification comparator across the pre-MALDI portion of the study period.

Performance and scope of the system. The operational value of the BD Phoenix platform for routine clinical practice has been documented in evaluations conducted under conditions closely comparable to those of a university-based hospital laboratory. In their comparative study of 305 consecutive clinical isolates — including Enterobacteriaceae, non-fermenting Gram-negative bacilli, staphylococci, enterococci and streptococci — analysed in parallel by the Phoenix system and by manual reference methods, with discrepancies resolved by additional biochemical testing and 16S rRNA gene sequencing at a national reference centre, Donay and colleagues reported an overall concordance at the species level of 92.5 %, with 94.6 % for Enterobacteriaceae, 89.4 % for non-fermenting Gram-negative bacilli, 91.8 % for staphylococci, 86.2 % for enterococci and 84.6 % for streptococci (3). Frequently isolated species such as Escherichia coli, Pseudomonas aeruginosa and S. aureus were identified with concordance rates of 95.6 %, 100 % and 100 % respectively, and the median time to identification was of the order of three hours, with combined identification and susceptibility results available within approximately ten to eleven hours for most organisms (3). Consistent findings were subsequently summarised in the review of commercial identification systems by O'Hara, which positioned the Phoenix line among the reliable automated platforms for routinely encountered pathogens (2). In a dedicated evaluation of 260 clinical isolates — encompassing Gram-positive cocci, Enterobacteriaceae and non-fermenting Gram-negative bacilli collected from Polish hospitals — Stefaniuk and colleagues, using the NMIC/ID-5 and PMIC/ID-4 combination panels, reported an overall identification agreement of 95.8 % against API reference systems, with 100 % agreement for Gram-positive cocci, 96.0 % for non-fermenting Gram-negative bacilli and 92.5 % for Enterobacteriaceae; the eleven discrepant cases observed were predominantly at the species level, with only three discrepancies at the genus level, all involving Enterobacteriaceae(4). Within our own laboratory, the Phoenix line therefore served as the main automated phenotypic comparator against which the contribution of MALDI-TOF mass spectrometry, introduced from 2020 onwards, is analysed in the present study.

Known limitations. The limitations inherent to automated biochemical platforms apply to the BD Phoenix as they do to the other systems of the same family. The discriminating power of the system depends on the breadth and currency of the proprietary database, which may lag behind ongoing taxonomic revisions — in particular for recently described species or for taxa reclassified on the basis of molecular methods (2,14). In the evaluation by Donay and colleagues, identification performances were significantly lower for several clinically relevant organisms represented by smaller numbers of isolates, including Klebsiella pneumoniae (92.8 %), Enterobacter cloacae (91.6 %), Pseudomonas fluorescens(50 %), Alcaligenes xylosoxidans (50 %), Acinetobacter baumannii (40 %) and A. lwoffi (50 %), and misidentifications were particularly frequent within non-fermenting Gram-negative bacilli, where three strains of Acinetobacter were reported by the Phoenix as Moraxella and one strain of Alcaligenes xylosoxidans as Pseudomonas oryzihabitans(3). Within Gram-positive cocci, the same study documented recurrent confusion between Enterococcus faecalis, E. faecium and E. casseliflavus-gallinarum, and a reciprocal misidentification between Streptococcus pneumoniae and Streptococcus mitis — a pattern of viridans-group mis-assignment that is well known across phenotypic platforms(3,18). Fastidious organisms with special nutritional requirements — Haemophilus, Brucella, HACEK group, Campylobacter, Helicobacter — are either outside the panel's scope or poorly covered by the database; anaerobes are not addressed by the standard NID/PID panels and must be identified through dedicated methods. These limitations, which are common to all biochemical platforms and are summarised in Section I.5 below, are precisely those for which MALDI-TOF mass spectrometry has offered the most significant added value in our experience and in the published literature(19,20).

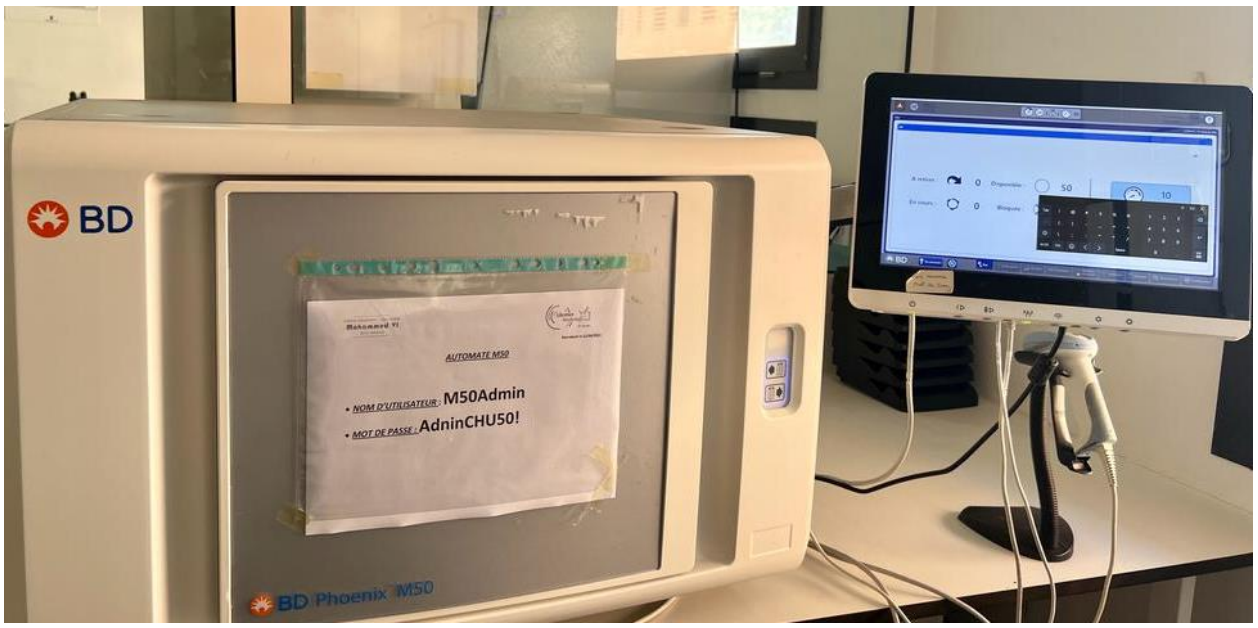


Figure 22. The BD Phoenix automated identification platform used in the laboratory,

showing the reader-incubator module, the inoculated NMIC/PMIC combined identification-and-susceptibility panels and the EpiCenter data-management workstation. [Original photograph — Microbiology Laboratory, Mohamed VI University Hospital]

4.4 Other Automated Systems

Two additional automated platforms, although not in routine use in our laboratory, warrant brief mention as they occupy a significant place in the comparative literature used as background for the present work. The VITEK 2 system (bioMérieux, Marcy-l'Étoile, France) is historically the main European competitor of the Phoenix line; it operates on reagent cards containing between 41 and 64 biochemical and enzymatic tests, read kinetically every fifteen minutes, and delivers an identification with a confidence score within approximately 3 to 10 hours depending on the card and the organism(2,5,18) . In their evaluation of the new VITEK 2 colorimetric GN card on 655 Gram-negative rods, Funke and Funke-Kissling reported that 97.3 % of isolates were correctly identified to the species level, with approximately 92 % of identifications obtained within seven hours of incubation(5) . A parallel evaluation by Wallet and colleagues, performed on 580 clinical and stock isolates, reported 97 % correct identifications for Gram-negative bacilli and 94.4 % for Gram-positive cocci with the colorimetric cards, against 89.1 % and 87.5 % respectively for the older fluorimetric format(18). The MicroScan WalkAway

system (Beckman Coulter, formerly Dade Behring) is based on microdilution panels combining dehydrated chromogenic and fluorogenic substrates and, in various configurations, provides identification and susceptibility results within 2 to 20 hours depending on the panel type (2). Both systems are relevant here only as comparators cited in the external literature; neither has been used within the present study.

5. Limitations of Conventional Identification Methods

Although individual enzymatic tests, commercial biochemical galleries and automated phenotypic platforms have long constituted the backbone of bacterial identification in clinical microbiology, several intrinsic limitations of these methods have become increasingly apparent as the spectrum of organisms recovered from human specimens has broadened. These limitations, which concern turnaround time, discriminating power, dependence on bacterial growth and on database quality, and the handling of atypical or rare isolates, jointly constitute the scientific rationale for the development and routine implementation of mass-spectrometry-based identification (12–14,17,19).

A first limitation is the time required for identification. Conventional methods depend on prior isolation of the organism in pure culture, typically requiring 18 to 24 h of primary incubation, and then on an additional 18 to 48 h of metabolic incubation in biochemical media before a reliable identification can be released (19). Individual enzymatic tests such as catalase, oxidase or indole can be performed within minutes, but they only allow orientation to a genus or group of genera; species-level identification typically requires an overnight gallery or an automated card reading, so that the definitive result is seldom available before 48 to 72 h after the initial specimen reception. For fastidious or slow-growing organisms — for example Brucella, Haemophilus, HACEK organisms or anaerobes — this delay may extend to several additional days. In a comparative routine study, Cherkaoui et al. (2010) showed that replacing conventional biochemical identification with MALDI-TOF MS shortened the average turnaround time by more than one eight-hour laboratory shift, even in a setting where automated identification was already in use. While the present work does not formally measure turnaround times, this conceptual gain is pertinent to the interpretation of our observations.

A second limitation concerns the discriminating power of biochemical methods and, more specifically, their ability to identify rare or atypical organisms. Commercial galleries and automated cards are optimised for the species most frequently encountered in routine practice, which are also those best represented in the manufacturer's database. When confronted with organisms that are uncommon, phenotypically atypical, or belonging to a taxonomic group with overlapping biochemical profiles, the system may yield several of the following unfavourable outcomes: a low discrimination result requiring additional tests, an erroneous identification, or a complete absence of identification (1,2,5,18). Already in the founding evaluation of the API 20E, Holmes and colleagues showed that only 88 % of 206 strains of Enterobacteriaceae could be correctly identified with the Analytical Profile Index, with 2 % of misidentifications, and that many atypical strains required additional conventional tests to resolve the profile (1). In the later, larger evaluation of the VITEK 2 colorimetric cards, Wallet and colleagues reported 97–98 % correct identifications on frequent taxa but markedly lower performances for rare or non-fermenting organisms, with documented misidentifications of non-fermenting Gram-negative bacilli and of viridans streptococci (18). A similar pattern is described in the API 20 NE literature, in which misidentifications of Brucella melitensis as Ochrobactrum anthropi or Moraxella phenylpyruvica, and the unreliable resolution of Acinetobacter genomospecies, have been reported repeatedly (2). These observations illustrate a general point: biochemical identification is at its most powerful precisely where clinical microbiology needs it least — for the routine identification of common species — and at its weakest where it would be most needed, namely for the identification of rare and fastidious pathogens.

A third limitation arises from the comparison of biochemical methods with mass spectrometry performed under real routine conditions. In their comparative study of 720 consecutive isolates identified in parallel by two MALDI-TOF MS platforms and by conventional biochemical methods, with discordant results resolved by 16S rRNA gene sequencing, Cherkaoui and colleagues found that MALDI-TOF MS correctly identified 99.1 % (Bruker) and 99.4 % (Shimadzu) of the isolates to the species level, against substantially lower performances for the conventional biochemical approach on the same taxa, and estimated that implementing mass

spectrometry as the first-line test would reduce marginal reagent costs by approximately USD 5 per isolate without loss of accuracy(19). The same study also showed that the biochemical approach performed particularly poorly on Gram-positive aerobic rods, on viridans streptococci and on Gram-negative anaerobes, confirming that the discriminating power of phenotypic testing remains markedly group-dependent (19).

A fourth limitation is the dependence on bacterial growth and on a pure culture. All biochemical methods require the organism to grow reliably on standard or selective media, under either aerobic or anaerobic conditions, up to a sufficient density to prepare a calibrated suspension. For fastidious organisms with special nutritional requirements (Haemophilus, Capnocytophaga, Brucella), for slow-growing bacteria (Nocardia, certain anaerobes, mycobacteria), or for organisms that do not grow at all on routine media, conventional methods are either severely delayed or inapplicable(12,14,17). Mixed cultures further complicate the picture, since biochemical tests inoculated from a non-pure inoculum will produce unreliable or contradictory patterns and must be re-cultured before identification can be attempted.

A fifth limitation concerns the interpretation and subjectivity of reading. Reading of individual tests, of API cupules and, to a lesser extent, of automated cards remains partly subjective, particularly when colour reactions are weak, delayed or atypical. Inter-observer variability has been documented in the literature for tests such as indole, Voges-Proskauer and oxidase, and the quality of the reading also depends on the freshness of the reagents and on strict adherence to the incubation times recommended by the manufacturer (13,17). Automated platforms reduce this subjectivity but do not eliminate the underlying problem, since the database interpretation of a given biochemical pattern remains probabilistic and assigns a likelihood, not a certainty, to the proposed identification.

A sixth limitation is of a taxonomic and database nature. Bacterial taxonomy has undergone continuous and far-reaching reorganisation since the 1990s, driven largely by molecular methods: genera and species have been split, merged or renamed, and entirely new species have been described on the basis of 16S rRNA gene sequencing, multilocus sequence analysis and whole-genome comparisons (12,14). Biochemical databases, by their nature, lag

behind this taxonomic evolution: a newly described species may not appear in the commercial database for several years, and previously recognised entities may remain listed under outdated names. Consequently, organisms such as members of the Enterobacter cloacae complex, Raoultella species, Acinetobacter genomospecies, coagulase-negative staphylococci or Nocardia species — all of clinical significance and all repeatedly identified as rare or fastidious in the literature and in our own practice — are not always reliably distinguished by phenotypic methods(2,12).

Finally, and as a consequence of the preceding points, conventional identification imposes a significant hidden cost in terms of technician time, reagent consumption and, most importantly, diagnostic delay for the individual patient. The repeated need for additional tests, subcultures and specialist review of atypical profiles translates into extra workload for the laboratory and potentially into delayed or inadequate antimicrobial therapy for the patient (19,20). It is precisely this convergence of constraints — slow, costly, operator-dependent, database-bound, and poorly adapted to rare and fastidious organisms — that has created the scientific and operational niche into which mass spectrometry has been introduced, and within which the added value of MALDI-TOF MS in our own laboratory is evaluated in the present study.

II. Molecular Identification by 16S rRNA Gene Sequencing

1. Rationale for a Genotypic Approach

The limitations of conventional biochemical methods and of automated phenotypic platforms, detailed in Section I.5, delimit a well-defined clinical niche in which phenotypic identification is either unreliable or simply inapplicable: rare organisms, fastidious organisms with special nutritional requirements, isolates with atypical or ambiguous metabolic profiles, closely related species within taxonomically complex genera, and newly described taxa that have not yet been incorporated into commercial databases(6,12,14,17). These situations share a common denominator: the information required to distinguish one species from another is no longer present in the organism's metabolic phenotype as explored by growth-based or

enzymatic tests. Either the discriminating biochemical characters do not exist, or they cannot be reliably elicited under the standardised conditions imposed by commercial systems.

Molecular identification approaches this problem from a different angle. Rather than inferring species identity from the reactions produced by live organisms in a set of substrates, molecular methods read identity directly from the sequence of a taxonomically informative genetic marker(12,14,17). Among these markers, the 16S ribosomal RNA gene has acquired a unique status and, for more than two decades, has represented the reference molecular target for bacterial identification in clinical microbiology and in bacterial taxonomy more generally(6,7,19). The present section describes the structural and methodological bases of 16S rRNA sequencing, its performance as a reference method against which phenotypic and mass-spectrometric approaches are benchmarked, and its intrinsic limitations. It closes on a methodological note that defines the place — or rather, the absence — of 16S rRNA sequencing within the present retrospective study.

2. The 16S rRNA Gene: Structure and Taxonomic Value

Ribosomal RNA molecules are essential structural and functional components of the ribosome and, as such, are among the most ancient, ubiquitous and functionally conserved macromolecules found in living organisms (12,14). In bacteria, the ribosome comprises three rRNA species — the 5S, 16S and 23S rRNA — encoded by genes typically organised into one or several ribosomal operons within the chromosome. The 16S rRNA gene, approximately 1,500 base pairs long in most bacterial species, has proven particularly well suited to the identification and phylogenetic classification of bacteria for three reasons(6,17).

First, the gene is universally present in all known bacteria. This universality allows a single analytical approach to be applied to virtually any cultivable bacterial isolate, regardless of its taxonomic position, provided that sufficient genomic material can be extracted. Second, the 16S gene shows a mosaic structure alternating highly conserved regions — where the sequence is almost identical across distant taxa, a consequence of strong functional constraints on the three-dimensional structure of the ribosome — and nine hypervariable regions, traditionally designated V1 to V9, in which sequence variation accumulates progressively with evolutionary

distance (14,17,21). The conserved regions serve as anchor points for universal amplification primers, while the variable regions provide the taxonomic signal used to distinguish between genera and species. Third, the gene is present at sufficient copy numbers in the bacterial genome — typically one to fifteen copies, depending on the species — to allow reliable direct amplification from purified genomic DNA without preliminary enrichment.

These structural properties explain the dual use of the 16S gene in clinical microbiology. At the phylogenetic level, global sequence comparison provides the backbone of modern bacterial taxonomy: the conservation of the 16S rRNA sequence across bacterial lineages was first noted in the 1960s in Bacillus species, and the systematic use of this gene as a universal phylogenetic marker followed the pioneering body of work by Woese and colleagues, who established that the 16S gene behaves as a molecular chronometer — a gene whose degree of conservation reflects its essential role in ribosomal function and whose residual variation therefore marks the evolutionary distance between organisms(12,22). On this basis, the reorganisation of bacterial classification from the 1980s onwards has been driven to a large extent by 16S rRNA-based trees, and newly proposed species are customarily defined with reference to their 16S sequence relative to the closest validly published neighbour (14,22). At the diagnostic level, sequencing a fragment of the gene from a clinical isolate and comparing it to an annotated reference database provides an identification that is often accurate to the species level, and at least to the genus level in almost all cases where a reasonable-quality sequence can be obtained(6,19,22).

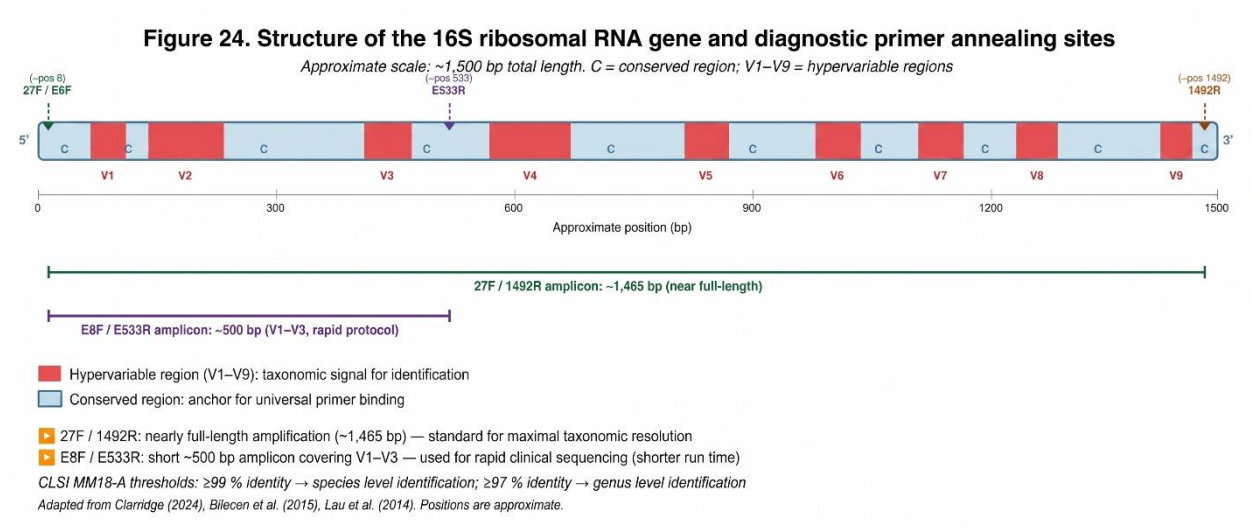


Figure 23. Structure of the 16S ribosomal RNA gene (~1,500 bp), showing the alternation of conserved regions (blue) and nine hypervariable regions V1–V9 (red), together with the annealing sites of the two primer pairs most commonly used in clinical microbiology: the near-full-length 27F/1492R pair (~1,465 bp amplicon, covering all variable regions) and the short E8F/E533R pair (~500 bp amplicon, covering V1–V3 for rapid protocols). CLSI MM18-A interpretive thresholds (≥99 % identity = species; ≥97 % = genus) are indicated. Positions are approximate. (adapted from Clarridge (2004), Bilecen et al. (2015), Lau et al. (2014).)

3. Methodology

The 16S rRNA-based identification of a bacterial isolate follows a reproducible four-step workflow: extraction of genomic DNA, amplification of a 16S fragment by the polymerase chain reaction, determination of the nucleotide sequence of the amplicon, and comparison of the sequence obtained against a reference database(6,8,21).

Genomic DNA extraction is performed from a fresh bacterial subculture in pure form. For Gram-negative bacteria, commercial silica-column kits such as QIAamp (Qiagen) or bacterial genomic DNA kits are widely used and provide DNA of sufficient purity for PCR within one to two hours. For Gram-positive bacteria, the thicker peptidoglycan cell wall typically requires an enzymatic pre-treatment — for example incubation with lysozyme at 37 °C — followed by chemical lysis with SDS and either column-based purification or a classical phenol-chloroform extraction(21). Extracted DNA is quantified by spectrophotometry at 260 nm and its integrity verified on an agarose gel before amplification.

PCR amplification uses universal or broad-range primers that anneal to conserved regions flanking the taxonomically informative variable regions. Two primer sets are particularly well represented in the clinical microbiology literature used as background in this work. The classical pair combines a forward primer designated S-D-Bact-0008-a-S-20 or 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), binding at position 8 of the gene, with a reverse primer rP2 (5'-ACGGCTACCTTGTTACGACTT-3') close to the 3' end, yielding a nearly full-length amplicon of approximately 1,500 bp. A shorter configuration, often used when turnaround time is critical, combines primers E8F (5'-AGAGTTTGATCCTGGCTCAG-3') and E533R (5'-TTACCGCGGCTGCTGGCA-3') to amplify the first approximately 500 bp of the gene, which encompass the V1-V3 hypervariable regions(8). Amplification is performed on a thermocycler with a thermostable DNA polymerase, and the specificity of the reaction is verified by agarose gel electrophoresis of the PCR product.

Sequencing of the amplicon is routinely performed by the Sanger dideoxy method on a capillary automated sequencer, for example an Applied Biosystems 3130 or 3500 Genetic Analyzer(21). Reading in both forward and reverse directions allows the generation of a consensus sequence with a high per-base accuracy over the length of the amplicon. Short fragments covering approximately 500 bp can usually be resolved in a single sequencing run, whereas nearly full-length sequencing of the entire 1,500-bp gene requires two to four internal sequencing primers and the assembly of overlapping reads (6,8).

Database comparison is the final and most interpretive step. The consensus sequence is submitted to a public reference database through a similarity search algorithm — typically BLAST (Basic Local Alignment Search Tool) against GenBank (National Center for Biotechnology Information, United States), or a curated comparison against specialised databases such as SILVA, EzBioCloud or the Greengenes 16S rDNA repository. The interpretation of the result relies on the identity score (percentage of identical nucleotides over the aligned region) relative to the best-matching reference sequence, and on the distance to the next-best match. The 2008 guideline of the Clinical and Laboratory Standards Institute (CLSI) MM18-A provides interpretive thresholds that have been widely adopted in the clinical literature: species-level identification is generally

accepted when the best match is at ≥ 99 % sequence identity and the separation from the next species is ≥ 0.8 %, genus-level identification when the identity is ≥ 97 %, and lower identities are treated cautiously and often indicate either a novel taxon or a suboptimal sequence quality (6).

The overall workflow, as summarised in the comprehensive review by Clarridge, has become considerably more accessible since the early years of the technique. The progressive decrease in sequencing costs, the availability of commercial kits for DNA extraction and amplification, the development of user-friendly comparative sequence analysis software, and the expansion and partial curation of public reference databases have jointly reduced 16S rRNA sequencing from a specialised research technique to a routine component of reference clinical microbiology laboratories, although it remains beyond the technical and economic reach of smaller laboratories(22).

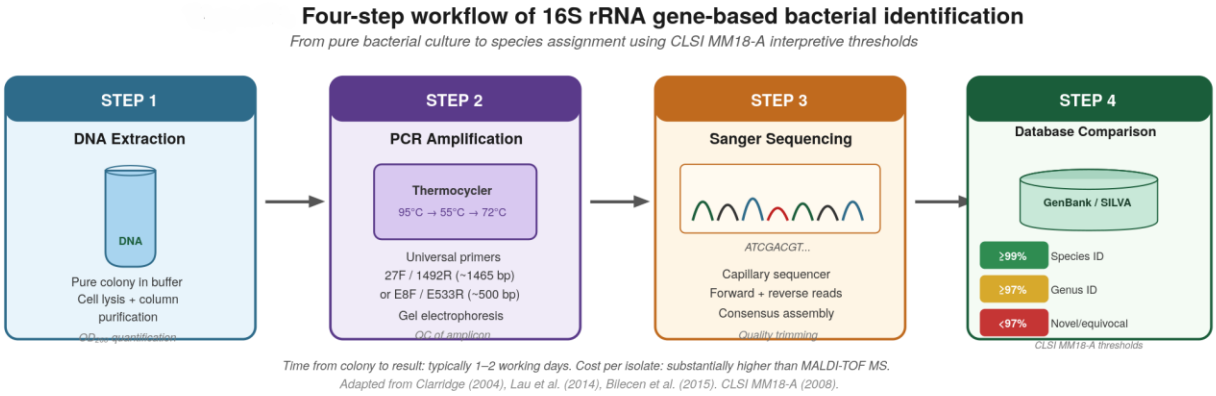


Figure 24. Four-step workflow of 16S rRNA gene-based bacterial identification, from pure culture DNA extraction through PCR amplification with universal primers, Sanger capillary sequencing, to database comparison and taxonomic assignment applying the CLSI MM18-A interpretive thresholds (≥99 % identity for species-level, ≥97 % for genus-level identification). Average time from colony to result: 1–2 working days. (adapted from Clarridge (2004), Lau et al. (2014), Bilecen et al. (2015).)

4. Applications and Position as Reference Method

In contemporary clinical microbiology, 16S rRNA gene sequencing occupies a well-defined position, captured by the phrase "molecular gold standard" that recurs throughout the comparative literature(6,19). In his foundational review of the impact of 16S rRNA gene sequence analysis on clinical microbiology, Clarridge emphasised that the most significant contribution of the technique has been the possibility of identifying bacteria by sequence rather than by phenotype — that is, of bypassing the need for the organism to display a reproducible metabolic profile in order to be named, and of thereby reaching taxa that had either escaped phenotypic identification or had been consistently misidentified by conventional means (22). This conceptual shift, which underpins the three practical roles described below, also explains why 16S rRNA sequencing has remained, for two decades, the reference against which every new bacterial identification technology — including MALDI-TOF mass spectrometry — has been benchmarked.

The first and most consistent role is the arbitration of discordant or inconclusive phenotypic results. In virtually every comparative evaluation of an automated biochemical platform or of a MALDI-TOF mass spectrometry system against conventional methods, isolates yielding discrepant, low-discrimination or no-identification results are submitted to 16S rRNA sequencing at a reference centre for definitive taxonomic assignment(3,4,18,19). This arbitration function is precisely why 16S sequencing appears as a recurrent methodological component of the studies summarised in Sections I.4 and I.5, even though it was not used in our laboratory during the period considered.

The second role is the identification of rare, fastidious or novel organisms for which phenotypic tools are either uninformative or unavailable. This is the scenario in which 16S rRNA sequencing has had its most substantial clinical impact. The extensive series of Hong Kong-based publications by Lau, Woo and colleagues, summarised and extended in their 2014 review, has shown that 16S rRNA gene sequencing has made it possible to characterise and report a large number of previously unrecognised pathogens or unusual clinical presentations, including Arcobacter butzleri, Eggerthella species, Catabacter hongkongensis, Solobacterium moorei, Granulicatella adiacens and Abiotrophia defectiva bacteraemias, and the identification of

fastidious anaerobic Gram-positive bacilli from blood cultures(6). In their study, sixty-seven isolates belonging to sixty-seven different difficult-to-identify species from forty-four genera were used as a benchmark panel, with 16S rRNA sequencing explicitly designated as the reference method(6) .

The third role is benchmarking of newer identification technologies. The positioning of MALDI-TOF mass spectrometry as an alternative to 16S rRNA sequencing for difficult-to-identify organisms has been the subject of a dedicated body of literature, within which the work of Bizzini and colleagues is particularly informative. Starting from a collection of 1,405 clinical isolates that had required 16S rRNA sequencing over an eight-year period because they could not be satisfactorily identified by conventional methods (VITEK 2 or API), they selected 433 isolates representing 207 different species from 84 genera; 410 were eventually analysed by MALDI-TOF MS on a Bruker Microflex LT instrument, with the BioTyper database updated in September 2008(7). MALDI-TOF MS yielded a high-confidence score (≥ 2.0) for 204 of 410 isolates (49.8 %), of which 188 (92.2 %) were concordant with the 16S identification at the species level; an additional 73 isolates (17.8 %) gave a genus-level score (1.7–2.0), and no reliable identification was obtained for 133 isolates (32.4 %) (7). The authors concluded that MALDI-TOF MS, coupled with progressive expansion of the reference spectra library, could progressively assume the first-line role previously reserved to 16S sequencing for difficult-to-identify isolates, while 16S sequencing would remain the final reference for organisms that escape the MALDI-TOF database. This conclusion — that MALDI-TOF MS does not replace 16S sequencing but reduces the proportion of isolates for which it remains indispensable — directly underpins the scientific rationale for the present thesis.

5. Limitations of 16S rRNA Sequencing

Despite its status as reference method, 16S rRNA gene sequencing is not a universal solution, and a lucid appraisal of its limitations is essential in any thesis that situates itself, as the present one does, at the interface between phenotypic identification and mass spectrometry.

The first limitation is practical and economic. 16S sequencing requires a molecular biology platform — thermocyclers, a capillary sequencer, bioinformatic pipelines, dedicated

reagents and trained personnel — that is not available in every microbiology laboratory, in particular outside tertiary or reference centres (6,7). Even where the platform exists, the turnaround time per isolate remains of the order of one to two working days in routine practice, considerably longer than MALDI-TOF mass spectrometry (a few minutes after colony selection) and longer than most automated biochemical platforms, and the per-isolate cost is substantially higher, of the order of several tens of euros per identification(7,19). These constraints explain why, in most clinical laboratories, 16S sequencing is reserved for isolates that cannot be identified by routine methods rather than used as a first-line tool.

A second limitation is intrinsic to the discriminating power of the gene itself. The 16S rRNA gene evolves too slowly to reliably separate certain pairs or groups of very closely related species, for which intragenomic sequence identity approaches or exceeds 99 % (6,17). Well-documented examples include the differentiation between Streptococcus mitis, S. oralis and S. pneumoniae within the viridans group, the resolution of Bacillus cereus, B. anthracis and B. thuringiensis within the B. cereus group, the separation of species within the Enterobacter cloacaecomplex or within the Acinetobacter calcoaceticus-baumannii complex, and various members of the Mycobacteriumgenus. In these situations, supplementary targets such as rpoB, tuf, gyrB or internal transcribed spacer regions, or whole-genome approaches, are required to obtain reliable species-level assignments(6,7).

A third limitation is database-dependent and is shared, in a different form, with MALDI-TOF mass spectrometry. The taxonomic reliability of a 16S identification is only as good as the reference sequences deposited in public databases. Misannotated, low-quality or unverified entries are well documented in GenBank, and even curated databases are never exhaustive for recently described or rarely isolated species. A 16S sequence that matches only distantly to existing records cannot, by itself, establish whether the isolate belongs to a known but under-sampled species, to a novel species, or to a sequencing artefact — a question that can only be resolved by additional phenotypic and genomic investigations(6).

A fourth limitation is pre-analytical. 16S sequencing requires a pure bacterial culture from which high-quality DNA can be extracted; mixed cultures, contaminated specimens or organisms

present in very low biomass can yield unreadable or chimeric sequences. Direct 16S amplification from clinical specimens — for example cerebrospinal fluid in suspected meningitis, heart valve tissue in endocarditis, or joint fluid in septic arthritis — is possible and has been used for selected indications, but it introduces further complexity related to the detection of environmental and laboratory contaminants, particularly in specimens with low bacterial load(6).

A fifth, more conceptual limitation, highlighted by Clarridge, concerns the phenomenon of microheterogeneity within the 16S rRNA gene itself. Because most bacterial genomes carry several copies of the 16S operon (typically one to fifteen), and because these copies are not always strictly identical, intragenomic sequence variation may generate ambiguous chromatograms, complicate consensus reading and, in some taxa, blur the species-level signal(22). More broadly, the relationship between sequence similarity and species identity is not uniform across the bacterial kingdom: a given threshold of 97 % or 99 % identity does not carry the same taxonomic meaning within a slowly evolving genus such as Mycobacterium as within a fast-evolving genus such as Neisseria, and the interpretation of any 16S-based identification must therefore take into account the specific taxonomic context of the organism considered(6,22). This point, although technical, has direct clinical implications, since it defines the limits within which even a molecular gold standard can reliably name an organism.

6. Place of Molecular Identification in the Present Study

In the present retrospective study, no molecular identification — neither 16S rRNA gene sequencing nor any alternative sequencing-based approach — was performed in-house on the isolates collected between 2017 and 2023. Molecular identification is therefore not a methodological component of this work and does not appear in the Materials and Methods section. It has nevertheless a threefold bearing on the interpretation of the results and on the discussion.

First, 16S rRNA sequencing constitutes the conceptual reference standard against which the performance of both conventional phenotypic identification and MALDI-TOF mass spectrometry has been evaluated in the international literature that frames the present study(6,7,19). In the absence of in-house sequencing, the identifications obtained by MALDI-TOF

during the study period cannot be formally confirmed against a molecular gold standard in this thesis, and this constraint is stated explicitly in the Discussion as a limitation of the study.

Second, the literature on 16S rRNA sequencing defines the contours of the clinical problem that MALDI-TOF is expected to address, namely the identification of rare and fastidious organisms recovered in routine practice. The proportion of isolates that historically required 16S sequencing — between 30 % and 50 % of difficult-to-identify strains in reference-laboratory series(6,7) — provides an indicative upper bound on the fraction of our own collection for which phenotypic methods alone would have been insufficient, and therefore on the theoretical maximum contribution that MALDI-TOF can deliver in terms of new species-level identifications.

Third, the limitations of 16S sequencing — in particular its inability to reliably resolve closely related species within certain genera — set a principled limit to the expectations that can be placed on any identification method, whether phenotypic, mass-spectrometric or molecular. When MALDI-TOF yields an ambiguous identification at the species level within the S. mitis-pneumoniae group, within the A. calcoaceticus-baumannii complex, or within the E. cloacae complex, this ambiguity is not specific to the technology but reflects the intrinsic difficulty of species delimitation within these taxa (6). This distinction is essential for a fair interpretation of the MALDI-TOF results reported in Section 6 of the Results and revisited in the Discussion.

III. MALDI-TOF Mass Spectrometry in Clinical Microbiology

1. Historical Background and Conceptual Emergence

The application of mass spectrometry to the analysis of biological macromolecules has a history extending more than four decades, but the specific method designated matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry emerged as a defined technique in 1987 and 1988, when Karas and Hillenkamp, and independently Tanaka and colleagues, independently demonstrated that large, non-volatile biomolecules could be ionised intact and analysed in the gas phase by co-crystallising them with a small organic matrix compound and irradiating the mixture with a pulsed laser(9). This work was recognised with a share of the Nobel Prize in Chemistry in 2002. For roughly a decade, the technique remained largely confined to the

analytical chemistry and proteomics communities; its low intra- and inter-laboratory reproducibility on whole bacterial cells precluded its adoption for diagnostic purposes. The first proof-of-concept demonstrations that intact bacterial cells could be characterised by their mass spectral profiles appeared in the mid-1990s, but the decisive transition to clinical utility required the development of standardised reference databases, robust bioinformatic matching algorithms, and user-friendly commercial platforms (9,23,24).

The decisive breakthrough into clinical microbiology occurred between 2008 and 2012, driven by the simultaneous arrival of two competing commercial platforms — the Bruker MALDI Biotyper and the bioMérieux VITEK MS — and by the publication of a series of large prospective studies demonstrating that whole-cell MALDI-TOF MS could identify bacteria from routine clinical isolates with a speed, accuracy and cost profile that surpassed those of conventional phenotypic methods(10,19,20,23,24) . Since then, the technology has been adopted by the majority of clinical microbiology laboratories in high-income countries, and it has fundamentally reshaped the workflow of bacterial identification, reducing the time from colony to reported identification from the 24 to 48 hours characteristic of biochemical methods to a matter of minutes (20,24,25). The present study was conducted over the period when this transition occurred in our own laboratory: the Bruker MALDI Biotyper was introduced in 2020, initially as a first-line identification platform, and subsequently supplemented by a bioMérieux VITEK MS instrument.

2. Physical and Chemical Principles

2.1 The Ionisation Mechanism

MALDI-TOF mass spectrometry is a so-called soft ionisation technique, meaning that the energy imparted to the analyte molecules during the ionisation process is sufficient to lift them into the gas phase as intact, charged species, without fragmenting their covalent bonds (9,24,26). This property is essential in clinical microbiology, where the objective is to detect large, intact proteins rather than peptide fragments.

The ionisation process proceeds as follows. A small quantity of bacterial material — the equivalent of a fraction of a single colony — is deposited on one of the circular spots of a polished stainless steel target plate. A saturated solution of the matrix compound, most

commonly α-cyano-4-hydroxycinnamic acid (CHCA), is then applied and allowed to co-crystallise with the bacterial material by evaporation of the solvent at room temperature. The matrix serves three indispensable roles: it absorbs the energy of the laser beam (thereby protecting the larger analyte molecules from direct photodegradation), it facilitates the vaporisation of the sample, and it acts as a proton donor that promotes the ionisation of the analyte molecules (9,24,26). The loaded target plate is placed inside the mass spectrometer under high vacuum, and each spot is sequentially irradiated by brief, focused pulses of a UV nitrogen laser (most commonly at 337 nm wavelength in the Bruker Biotyper). The energy of the laser pulse causes the matrix-analyte co-crystal to sublime and desorb into a gas-phase plume, carrying with it analyte molecules that have acquired a single positive charge (z = +1) through proton transfer from the matrix (9,24).

Figure 25 illustrates the complete physical process from target plate to spectrum.

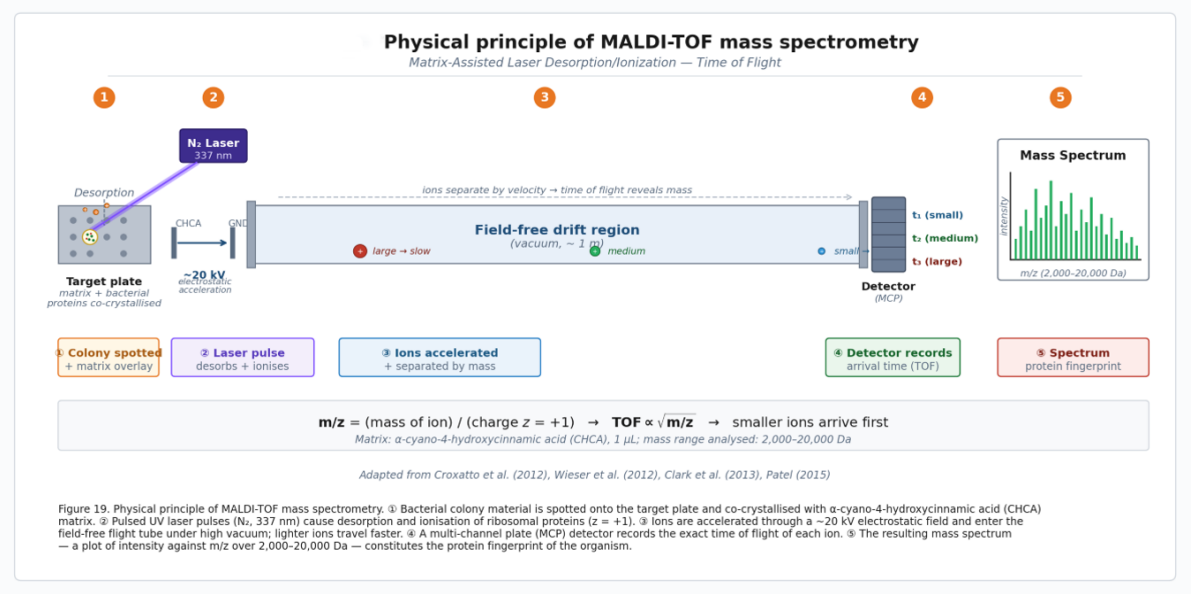


Figure 19. Physical principle of MALDI-TOF mass spectrometry. ① Bacterial colony material is spotted onto the target plate and co-crystallised with α-cyano-4-hydroxycinnamic acid (CHCA) matrix. ② Pulsed UV laser pulses (N_2, 337 nm) cause desorption and ionisation of ribosomal proteins (z = +1). ③ Ions are accelerated through a ~20 kV electrostatic field and enter the field-free flight tube under high vacuum; lighter ions travel faster. ④ A multi-channel plate (MCP) detector records the exact time of flight of each ion. ⑤ The resulting mass spectrum — a plot of intensity against m/z over 2,000–20,000 Da — constitutes the protein fingerprint of the organism.

Figure 26. Physical principle of MALDI-TOF mass spectrometry.

① Bacterial colony material is spotted onto the target plate and co-crystallised with α-cyano-4-hydroxycinnamic acid (CHCA) matrix. ② Pulsed UV laser pulses (N_2, 337 nm) cause desorption and ionisation of ribosomal proteins (z = +1). ③ Ions are accelerated through a ~20 kV electrostatic field and enter the field-free flight tube under high vacuum; lighter ions travel faster. ④ A multi-channel plate (MCP) detector records the exact time of flight of each ion. ⑤ The resulting mass spectrum — a plot of intensity against m/z over 2,000–20,000 Da — constitutes the protein fingerprint of the organism. (Adapted from Croxatto et al. (2012), Wieser et al. (2012), Clark et al. (2013), Patel (2015).)

2.2 The Time-of-Flight Mass Analyser

Once generated, the charged ions are accelerated through an electrostatic field created between two electrodes subjected to a potential of approximately 20 kV, thereby acquiring kinetic energies that are, in the linear TOF configuration, approximately equal across all ions regardless of their mass — since all ions receive the same electrostatic push(9,26). This means that smaller, lighter ions accelerate to higher velocities than heavier ones, and therefore travel through the subsequent field-free drift region — the flight tube — more rapidly. The flight tube, which is maintained under high vacuum to prevent collisions with residual gas molecules, typically measures of the order of 1 metre in length. At the far end of the tube, a detector — most commonly a multi-channel plate (MCP) detector — records the exact time at which each ion arrives. This measured time of flight, combined with knowledge of the applied acceleration potential and the tube length, allows the instrument's software to calculate the mass-to-charge ratio (m/z) of each ion with high precision. Since $z = +1$ for all practical purposes in this application, the m/z value is simply equal to the molecular mass of the ion in daltons (9,24). The result, accumulated from approximately 240 laser shots applied to different positions of the same sample spot, is a mass spectrum: a two-dimensional plot of ion intensity against m/z over the mass range typically analysed, which extends from 2,000 to 20,000 Da in routine bacterial identification(9,26,27).

2.3 The Biological Basis: Ribosomal Proteins as Biomarkers

A fundamental property of the MALDI-TOF MS approach, and one that explains both its high reproducibility and its remarkable discriminating power, is that the protein signals dominating the mass spectrum of whole bacterial cells are not random but correspond to a reproducible subset of the cellular proteome. Detailed characterisation of the biomolecules detected in MALDI-TOF spectra has established that the majority of signals above 4 kDa correspond to intact proteins from the inside of the bacterial cell — specifically to proteins that are abundant, basic in charge (isoelectric point generally > 9) and of medium hydrophobicity: a set of properties that favour efficient ionisation under MALDI conditions (24,28). Foremost among these are the ribosomal proteins, which collectively account for more than 20 % of total

cellular protein mass and which generate the most intense and reproducible peaks in the spectrum (24,28). Additional contributions come from nucleic acid-binding proteins such as the DNA-binding protein HU, cold-shock proteins and ribosome-associated factors(24). The practical consequence is that the resulting mass spectrum constitutes a ribosomal protein fingerprint — a pattern of peak positions and relative intensities that is determined by the species-specific sequences of these highly conserved yet taxon-discriminating proteins, and that is sufficiently stable across different culture conditions, media compositions and growth phases to serve as a reproducible species signature(24,28,29).

Figure 27 illustrates the species-specificity of these fingerprints by showing aligned representative mass spectra for six clinically important organisms.

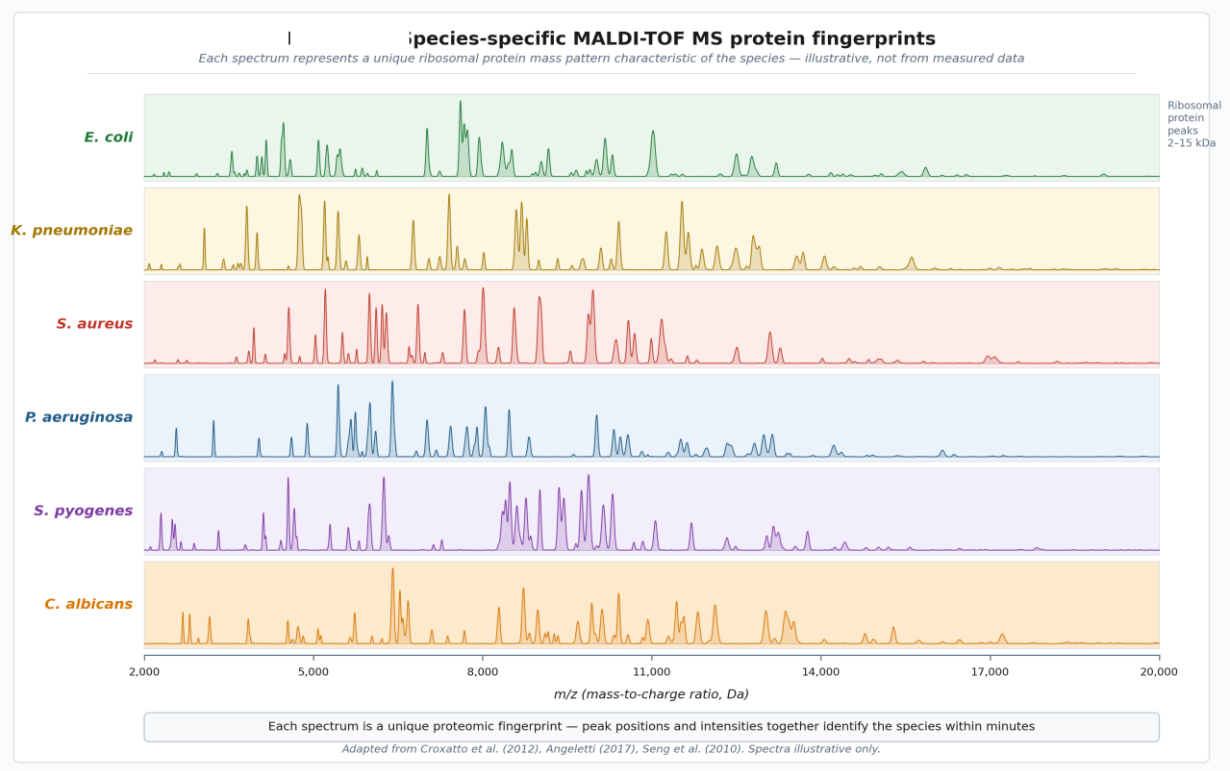


Figure 27. Species-specific MALDI-TOF MS protein fingerprints for six clinically important organisms (E. coli, K. pneumoniae, S. aureus, P. aeruginosa, S. pyogenes, C. albicans), shown as aligned intensity plots against m/z (2,000–20,000 Da).

Each spectrum represents a unique pattern of ribosomal protein peaks that is reproducible across growth conditions and serves as the species identification signature compared against the reference database. Peak positions and intensities are illustrative; they are not derived from measured experimental data. (Adapted from Croxatto et al. (2012), Angeletti (2017), Seng et al. (2010).)

3. The Bruker MALDI Biotyper Platform

The Bruker MALDI Biotyper system (Bruker Daltonik GmbH, Bremen, Germany) is the platform used in our laboratory from 2020 onwards, and one of the two commercially dominant systems used across the clinical microbiology literature that constitutes the background for the present study. It comprises three integrated components: the MALDI-TOF mass spectrometer itself (in the Microflex LT or Microflex LT/SH benchtop configuration used in clinical routine), the **MBT Compass IVD software** — the in-vitro diagnostic configuration of the Biotyper software package certified for routine clinical use — and the associated IVD reference database [19,27,25]. (9,27,29).

The **mass spectrometer** operates in linear positive mode, using a pulsed nitrogen laser at 337 nm. The standard polished steel MSP 96 target plate accommodates 96 individual sample spots, allowing batch processing of up to 94 clinical isolates per run alongside a calibration standard and a quality control sample. Daily calibration is performed using the Bruker Bacterial Test Standard, a lyophilised preparation of Escherichia coli extract spiked with myoglobin and RNase A, which provides a set of reference m/z values covering the analytical range(24). Each clinical isolate is typically deposited in duplicate on adjacent spots, and for each spot, 240 laser shots are accumulated in automated mode (40-shot steps from different positions within the spot) to generate a composite spectrum with optimal signal-to-noise ratio(27).

The **MBT Compass IVD software** performs the spectral comparison and generates the identification output. For each acquired spectrum, the software applies background subtraction, peak detection and normalisation before comparing the processed spectrum against all reference spectra contained in the database. The comparison is based on a pattern-matching algorithm that takes into account both the m/z positions and the relative intensities of the detected peaks. The degree of agreement between the query spectrum and each reference spectrum is expressed as a log score ranging from 0.000 to 3.000, the formula of which was developed and empirically calibrated by Bruker on the basis of the entire BioTyper database contents(9,29). The software then presents a ranked hit list of the top ten matching database entries.

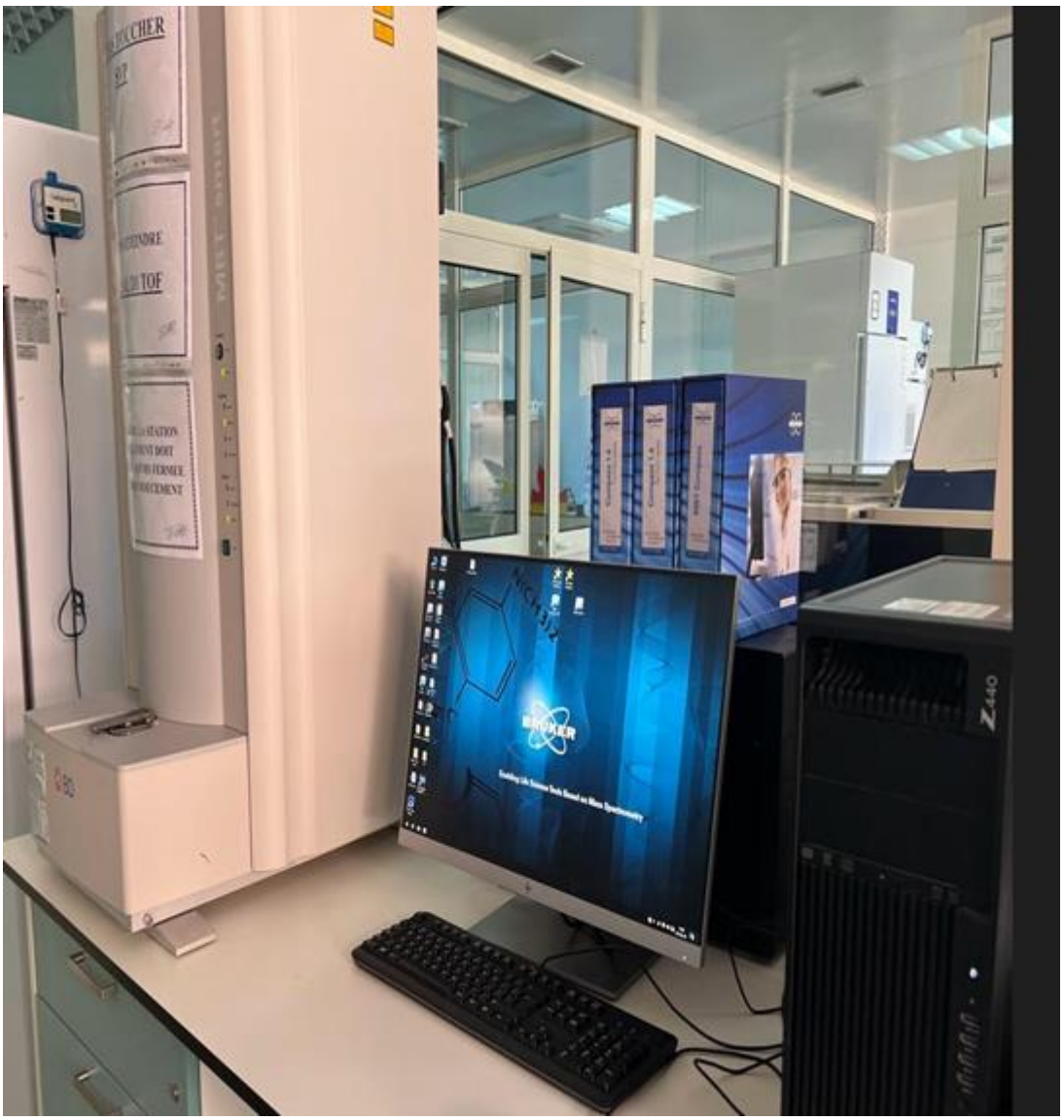


Figure 28. The Bruker MALDI Biotyper instrument as deployed in the Microbiology Laboratory, Mohamed VI University Hospital. [Original photograph]

The MBT IVD reference library — integrated with the MBT Compass IVD software — is the repository of reference mass spectra against which all unknown spectra are compared. In the versions used across the studies surveyed in the present work, the database contained in excess of 5,000 reference spectra at the time of Biotyper version 3.0, with more recent versions exceeding 8,000 entries and covering more than 2,600 validated bacterial and fungal species (21). Each database entry is a main spectrum (MSP) computed from the averaged spectra of multiple measurements of a single strain, selected as representative of the species after quality control steps. Multiple MSPs from different strains of the same species are included for many

entries, improving the representativeness of the database for intraspecific variability(9,24). Critically, the database is an open platform: individual laboratories can generate their own reference spectra for organisms not represented in the commercial database — a feature that has been exploited extensively for rare, fastidious, and newly described species, and that is directly relevant to the study reported in this thesis(24,27).

Figure 29 illustrates the complete database comparison and identification workflow.

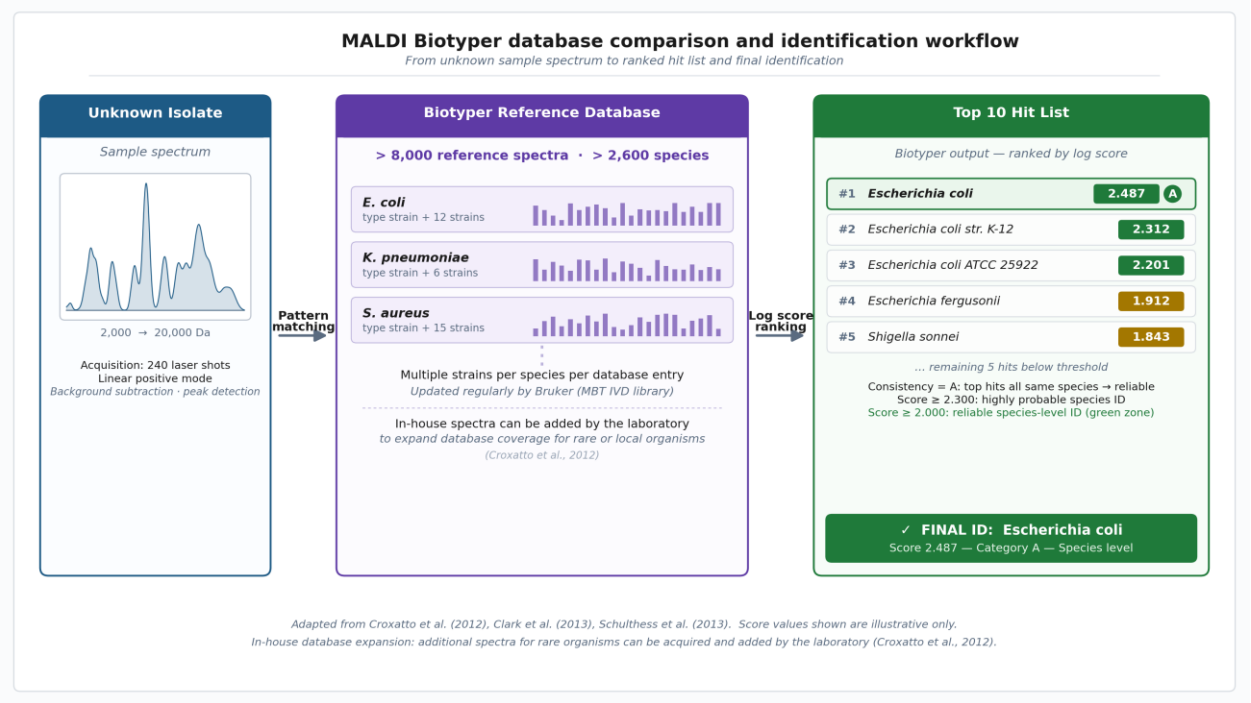


Figure 29. MALDI Biotyper database comparison and identification workflow.

An unknown sample spectrum is compared by pattern-matching algorithm against all reference spectra (main spectra, MSPs) in the BioTyper database (> 8,000 entries, > 2,600 species). The software generates a ranked hit list of the top 10 matching entries, each assigned a log score (0–3.0). The combination of log score and consistency category (A = species consistent; B = genus consistent; C = no consistency) determines the final reported identification. Score values shown in the example are illustrative only. (Adapted from Croxatto et al. (2012), Clark et al. (2013), Schulthess et al. (2013).

4. Sample Preparation Methods

The quality and reproducibility of the MALDI-TOF MS identification is critically dependent on the sample preparation method used to present the bacterial material to the target plate. Three protocols are in widespread use for routine bacterial isolates, ranging from the simplest and most rapid to the most technically demanding and time-consuming(9,24,27).

Figure 30 shows the three methods side by side.

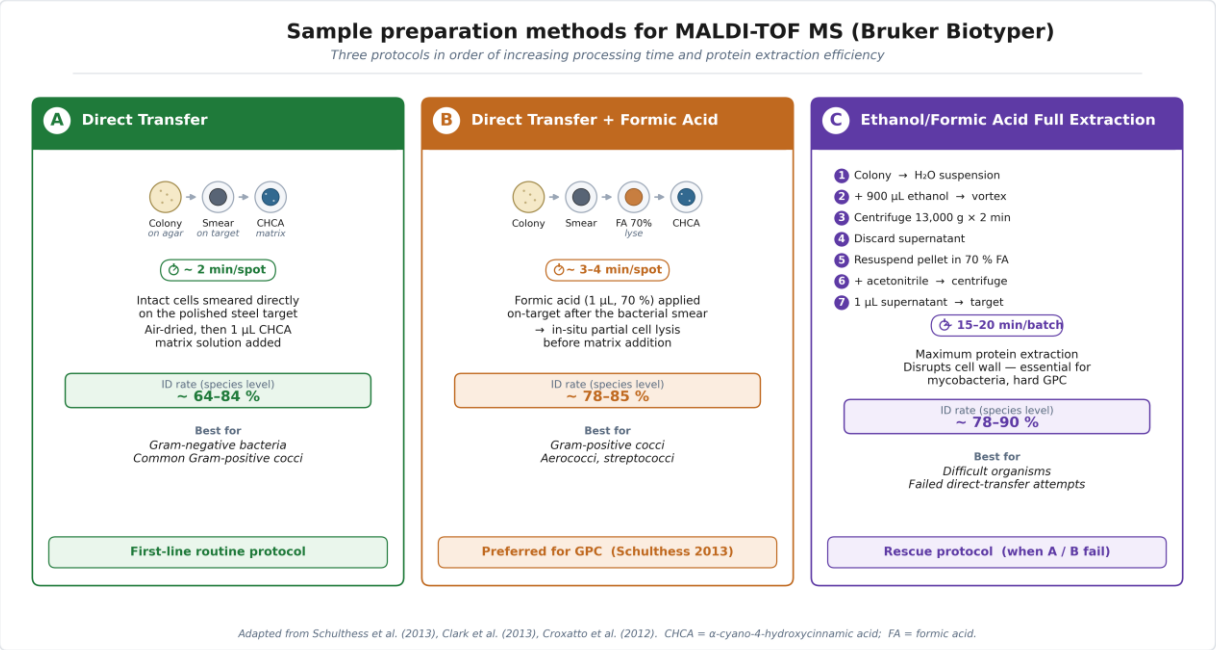


Figure 30. The three sample preparation protocols for MALDI-TOF MS (Bruker Biotyper), shown in order of increasing processing time and protein extraction efficiency.

A — Direct transfer (~2 min/spot): colony smeared directly on target, overlaid with CHCA matrix; species-level identification rate ~64–84 %. B — Direct transfer with formic acid (~3–4 min/spot): 1 µL of 70 % formic acid added on-target before matrix for in-situ cell lysis; rate ~78–85 %; preferred protocol for Gram-positive cocci. C — Ethanol/formic acid full extraction (~15–20 min/batch): complete cell disruption followed by protein extraction; reserved for failed A/B results and difficult organisms. (adapted from Schulthess et al. (2013), Clark et al. (2013), Croxatto et al. (2012).)

The **direct transfer method** is the standard first-line approach for the majority of routine clinical isolates. A small quantity of fresh colony material is smeared directly onto a spot of the polished steel target using a toothpick or a loop, allowed to dry for a few seconds, and then overlaid with 1 µL of saturated CHCA matrix solution. The protocol requires no dedicated equipment beyond the target plate, adds only one to two minutes of hands-on time per spot, and is fully compatible with high-throughput batch processing of 96 samples per run (24,27). For the majority of Gram-negative bacteria and many Gram-positive cocci, the direct transfer method yields species-level identification scores at a rate that renders it a satisfactory first-line tool. However, for organisms with thick or chemically complex cell walls — in particular Gram-positive cocci such as streptococci and aerococci, and for mycobacteria — the direct transfer alone may not suffice to release adequate quantities of soluble ribosomal proteins, resulting in identification rates at the species level of approximately 64–84 % in different published series(27).

The **direct transfer with formic acid (on-target lysis) method** adds a single additional step to the basic protocol: after the colony material has dried on the target, 1 µL of 70 % aqueous formic acid is applied and allowed to dry before the matrix is added. The formic acid causes in-situ partial cell lysis, disrupting the cell wall and releasing a larger quantity of soluble proteins into the co-crystallisation matrix(9,27). In the systematic comparison published by Schulthess and colleagues, the direct transfer-formic acid method increased the overall species-level identification rate for 156 Gram-positive cocci from 64.1 % (direct transfer) to 77.6 %, with particularly marked improvements for aerococci and streptococci; this method also proved superior for the prospective study arm, in which 95.6 % of 1,619 clinical Gram-positive cocci showed congruence between MALDI-TOF MS and conventional identification (27). This method adds only 22.6 % to the total preparation time when 12 samples are processed, and this overhead decreases proportionally to 8.5 % when 96-well targets are used at full capacity (27). It is the preferred first-line method for Gram-positive cocci in many reference clinical laboratories.

The **ethanol/formic acid full extraction method** is the most technically demanding protocol and is reserved for organisms that yield unreliable identification scores with both preceding

methods(9,24,27). A loopful of colony material is suspended in 300 µL of distilled water; 900 µL of absolute ethanol is added, and the suspension is centrifuged at 13,000 g for 2 minutes; the supernatant is discarded; the pellet is resuspended in 50 µL of 70 % formic acid; 50 µL of acetonitrile is added; after a second brief centrifugation, 1 µL of the clarified supernatant is deposited on the target and overlaid with matrix(24,27). This protocol disrupts the cell wall completely and removes lipid and polysaccharide contaminants, resulting in the maximal protein extraction yield and the highest identification rates for difficult organisms. It is the protocol of choice for mycobacteria (following a preliminary heat-inactivation step at 95 °C for safety), actinomycetes, and isolates of any species for which direct transfer methods have failed(9,24).

In our laboratory, the direct transfer and direct transfer-formic acid methods constitute the primary protocols applied to the majority of routine isolates and to Gram-positive cocci respectively.

5. Score Interpretation and Result Reporting

5.1 The Log Score System

The identification output of the Bruker Biotyper is expressed as a log score (also referred to as score value throughout the literature) ranging from 0.000 to 3.000(9,10,29) . This score is not a simple percentage similarity metric but a logarithmically transformed composite index reflecting both the number of shared peaks and their relative intensities between the query spectrum and each reference MSP. The manufacturer established empirical thresholds for interpreting these scores, which have been widely validated in the literature and adopted as the standard reporting framework across clinical microbiology laboratories worldwide(6,10,27,29) .

The standard interpretation thresholds, as used throughout the studies reviewed in this thesis, are as follows. A score of **≥ 2.000** is required for a reliable species-level identification; within this zone, scores between 2.000 and 2.299 are interpreted as secure genus identification and probable species identification, and scores of ≥ 2.300 as highly probable species identification (9). A score of **≥ 1.700 but < 2.000** is interpreted as a probable genus-level identification, which is considered insufficient for species reporting without additional confirmatory testing (9,10,27).

A score of < 1.700 is considered unreliable, and the identification must be repeated using a more extractive sample preparation protocol or declared as unidentified(6,10,27,29) .

In the BioTyper display, these three zones are colour-coded as green (species-level, score ≥ 2.000), yellow (genus-level, 1.700–1.999) and red (unreliable, < 1.700), a colour convention that is universally recognised in the clinical MALDI-TOF MS literature and that is reflected in **Figure 31.**

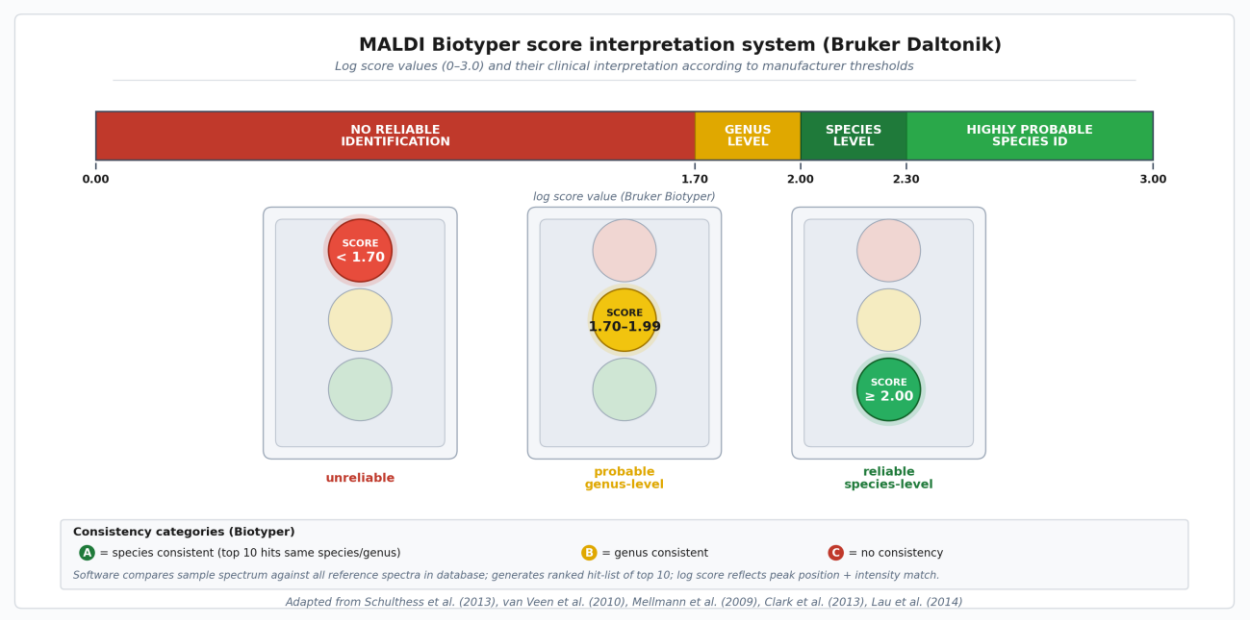


Figure 31. MALDI Biotyper log score interpretation system (Bruker Daltonik).

The continuous score scale (0–3.0) is divided into three clinically interpreted zones colour-coded by the Biotyper software: green (score ≥ 2.000) = reliable species-level identification, reportable; yellow (1.700–1.999) = probable genus-level identification only, additional testing required; red (< 1.700) = unreliable, repeat with alternative sample preparation. Scores ≥ 2.300 within the green zone indicate a highly probable species identification. The traffic-light display shown is illustrative of the Biotyper output conventions. (Adapted from Schulthess et al. (2013), van Veen et al. (2010), Mellmann et al. (2009), Clark et al. (2013), Lau et al. (2014).)

5.2 Consistency Categories

In addition to the numerical log score, the BioTyper software assigns each identification to one of three consistency categories designated A, B and C, which reflect the degree of agreement among the top ten matching hits in the ranked list(9,27). Category A (species consistent) is assigned when the top-scoring hit receives a green (species-level) score and the remaining green

and yellow hits in the top 10 all belong to the same species or genus respectively — a pattern indicating unambiguous identification. Category B (genus consistent) is assigned when the top hit receives a green or yellow score, but the remaining hits do not all converge on the same species, though they share the same genus; this category signals a reliable genus-level result with uncertain species assignment. Category C (no consistency) is assigned when neither species nor genus consistency exists among the top ten hits, typically indicating a poor-quality spectrum, an organism not adequately represented in the database, or a mixed culture (9,27). In practice, the combination of score value and consistency category is used jointly in the reporting algorithm: for example, a score of 2.1 with category A represents a clearly reportable species identification, whereas a score of 2.1 with category B may prompt the laboratory to perform additional identification steps.

(see Figure 29, Section III.3)

6. Routine Performance

6.1 Overall Performance for Common Organisms

The performance of the Bruker MALDI Biotyper in the identification of routinely isolated clinical bacteria has been assessed in a large number of independent prospective and retrospective studies, many of which are included in the reference corpus of the present thesis. The overall species-level identification rates reported across these studies vary between 84 % and approximately 97 %, depending on the sample composition, the database version, the sample preparation protocol and the prevalence of difficult-to-identify organisms in the collection(10,19,20,23,24) .

The pivotal prospective study by Seng and colleagues, which analysed 1,660 clinical isolates representing 45 genera and 109 species using direct deposit on the Biotyper plate, obtained species-level concordance with routine identification methods for 84.1 % of isolates and genus-level concordance for an additional 11.3 %; the misidentification rate was 1.7 % and 2.8 % of isolates remained unidentified, principally because of the absence of reference spectra in the database at that time(20). A subsequent retrospective study by Bizzini and colleagues on 1,371 clinical isolates obtained 93.2 % species-level and 5.3 % genus-level identification rates with the

Biotyper(23) . The large comparative study by Cherkaoui and colleagues, which evaluated 720 consecutive isolates on two MALDI-TOF platforms against conventional methods with 16S rRNA gene sequencing as reference for discordant cases, reported 99.1 % correct species-level identifications with the Bruker system(19). Van Veen and colleagues evaluated 980 consecutive isolates prospectively and obtained an overall species-level rate of 92 %, with particularly high performance for Enterobacteriaceae (97.7 %) and Gram-positive cocci in clusters (94.3 %), contrasting with markedly lower performance for viridans group streptococci and for some miscellaneous groups (10)(van Veen et al., 2010).

A critical element emerging from all these studies is that performance is highly heterogeneous across bacterial groups. Gram-negative fermentative bacilli of the Enterobacteriaceae and the main Gram-positive cocci such as Staphylococcus species are identified with the highest consistency and the fewest misidentifications. Non-fermenting Gram-negative bacilli, viridans streptococci, anaerobes and fastidious organisms with special nutritional requirements present a considerably greater challenge (10,19,24)(van Veen et al., 2010; Cherkaoui et al., 2010; Croxatto et al., 2012). These group-specific differences are directly relevant to the interpretation of the results in the present study, which is specifically focused on rare and fastidious organisms.

6.2 Time to Identification and Cost

A recurrently emphasised advantage of MALDI-TOF MS over both manual and automated phenotypic methods is the radical reduction in time to identification. Seng and colleagues measured the identification delay as approximately 6 to 8.5 minutes from the moment of plate loading to result transmission for the MALDI-TOF approach, compared with 300 to 2,880 minutes for API galleries and 300 to 1,200 minutes for the Phoenix system (20). In their laboratory, bacterial colonies were deposited on the MALDI-TOF plate at 07:00–07:30 and all identifications were available by 09:30 the same morning (20). Cherkaoui and colleagues estimated that implementing MALDI-TOF as the first-line identification test would reduce the average turnaround time by more than one eight-hour laboratory shift relative to the conventional biochemical approach (19). In our own laboratory, this accelerated reporting has

been achieved routinely since the Biotyper was introduced in 2020, though exact turnaround times were not formally measured within the retrospective design of the present study.

From an economic perspective, the MALDI-TOF approach is substantially less expensive per identification than phenotypic methods once the instrument has been acquired. Seng and colleagues estimated the cost of MALDI-TOF identification at approximately €1.43 per isolate — representing only 17 to 32 % of the cost of conventional methods estimated at €4.6 to €8.23 per isolate for automated biochemical platforms (20). This estimate was corroborated by Cherkaoui and colleagues, who reported that the reagents required for phenotypic identification using modern automated platforms cost at least approximately USD 10 per isolate, whereas the reagents required for MALDI-TOF MS did not exceed USD 0.50 per isolate (19,24). These cost savings are particularly significant in high-volume university hospital laboratories such as ours, which process several thousand isolates per year.

6.3 Interlaboratory Reproducibility

An important operational virtue of the MALDI-TOF approach is its high interlaboratory reproducibility, which contrasts with the variable inter-observer and inter-laboratory agreement that has been documented for phenotypic and biochemical identification(29). In the international interlaboratory study by Mellmann and colleagues, 60 blind-coded non-fermenting gram-negative bacteria were distributed to eight different laboratories in six countries, each equipped with a Bruker Biotyper 2.0 instrument. Of 480 total identifications, 474 (98.75 %) were correct at the species level; only six samples were mis-identified, with four attributable to sample interchange, one to laboratory contamination and one to insufficient signal intensity(29) . This level of interlaboratory reproducibility was far superior to what had been achieved with classical phenotypic methods for this particularly challenging bacterial group, and it effectively established the clinical credibility of MALDI-TOF MS as a reference-grade identification tool.

7. Limitations and Unresolved Challenges

Despite its established superiority over conventional phenotypic methods for the vast majority of routine isolates, MALDI-TOF MS has a number of well-characterised limitations that

are pertinent to the scope of the present study and that must be clearly stated in any academic appraisal of the technology.

The most fundamental limitation is **database dependency**. The Biotyper can only identify organisms for which reference spectra exist in its database; an isolate belonging to a species not represented, or poorly represented, in the database will either receive a low score (< 1.7, red) or be misidentified as the nearest-scoring database entry(6,9,24). This is not merely a theoretical concern: in both the Seng (2009) and Bizzini (2011) series, a substantial fraction of the unidentified isolates corresponded to species that were simply absent from the database at the time of analysis. Database expansion — both by the commercial vendor and by in-house addition of laboratory-generated reference spectra — progressively reduces this limitation but does not eliminate it, particularly for newly described taxa(9,24). In the context of the present study, this limitation is especially relevant for rare organisms recovered in the laboratory that had not yet been included in the commercial Biotyper database at the time of analysis: such isolates appear in our results as unidentified or genus-level-only identifications, and their definitive characterisation would in principle require 16S rRNA gene sequencing, which was not available in our laboratory.

A second limitation concerns **organisms with inherently poor spectral discrimination**. Several clinically important taxa share highly similar ribosomal protein compositions, resulting in overlapping mass spectra that the Biotyper cannot reliably resolve to the species level. The most clinically relevant examples include the Streptococcus mitis–oralis–pneumoniae cluster within the viridans group, where the commercial database consistently produces inconsistent or misidentified results; the Acinetobacter calcoaceticus–baumannii complex, whose genomospecies cannot be resolved by MALDI-TOF; the Enterobacter cloacae complex; and coagulase-negative staphylococci, among which certain pairs of closely related species such as S. epidermidis and S. capitis may yield overlapping spectra(10,19,24,27) (van Veen et al., 2010; Cherkaoui et al., 2010; Croxatto et al., 2012; Schulthess et al., 2013). These represent the same taxa that present difficulties for 16S rRNA gene sequencing, underscoring that the resolution boundary is intrinsically biological rather than purely technological.

A third limitation is the inability to provide antimicrobial susceptibility testing within the standard identification workflow. Unlike the BD Phoenix or VITEK 2 platforms, which couple identification to broth microdilution-based susceptibility testing within the same panel, the Biotyper delivers only an identification result in its routine configuration. Antimicrobial susceptibility testing must therefore be performed in parallel on a separate platform. Dedicated MALDI-TOF MS-based resistance detection approaches — including the CE-IVD-certified MBT STAR-BL module for carbapenemase activity and the MBT-ASTRA growth inhibition assay — have been developed and validated as supplementary tools, and are described in Section III.8.2; however, these assays require specific reagents, additional processing time, and separate software modules, and do not yet constitute a comprehensive real-time susceptibility testing solution equivalent to an automated phenotypic platform(9,24,30) . This operational reality means that the MALDI-TOF platform does not replace automated susceptibility platforms in the laboratory but functions in a complementary capacity alongside them — a configuration that characterises our own laboratory throughout Phase II of the study period, where the BD Phoenix continued to provide antimicrobial susceptibility results following MALDI-TOF identification.

A fourth limitation, particularly relevant to the study of fastidious organisms, concerns **culture dependency and minimum biomass requirements**. MALDI-TOF MS requires a cultivable isolate in sufficient quantity — of the order of 10^4 to 10^6 colony-forming units per spot — for spectrum acquisition(26). Fastidious organisms that grow very slowly, require special atmospheric conditions or enriched media, or fail to grow at all on standard routine media, impose additional pre-analytical constraints that may delay identification beyond the nominal few minutes associated with the spectrometric analysis itself. Mixed cultures deposit mixed protein profiles on the target and generate uninterpretable spectra, and prior isolation in pure culture remains mandatory.

Finally, the **quality and maintenance of the database** represent an ongoing operational responsibility for each clinical laboratory. As described in Section III.3, the BioTyper is an open-architecture platform that allows in-house spectral entry; however, the quality of user-generated spectra depends on adherence to validated protocols for spectrum acquisition, quality control

and database curation (9,24). Errors in the database — whether in the form of misidentified reference spectra or of spectra of insufficient quality — propagate directly into misidentifications of clinical isolates, a risk that requires systematic quality control procedures at the laboratory level(24).

These limitations define the operational envelope within which MALDI-TOF MS delivers its added diagnostic value. They are also, precisely, the conditions under which the identification of rare and fastidious microorganisms challenges the system most directly — and which constitute the scientific rationale for the present retrospective study.

8. Extended Applications Beyond Species Identification

The identification of microorganisms from cultured colonies represents the primary and best-established application of MALDI-TOF MS in routine clinical microbiology, and it is the application that forms the focus of the present study. However, the same technology has been extended to two further areas of clinical diagnostic importance — the direct identification of pathogens from positive blood culture broths, and the detection of antimicrobial resistance mechanisms — that deserve mention in this section, both because they illustrate the breadth of the platform's potential and because they have direct relevance to the clinical context of a university hospital with a high-acuity patient population.

9. Direct Identification from Positive Blood Culture Broths

Bloodstream infections and sepsis represent among the most time-critical infectious scenarios in clinical medicine, since the fatality rate of bacteremia treated with inappropriate empirical antibiotic therapy substantially exceeds that of appropriately treated episodes, and the benefit of early targeted therapy is well established(24). In the traditional laboratory workflow, positive blood culture bottles — detected by automated monitoring of CO_2 production in the BACTEC, BacT/ALERT or equivalent systems — are subcultured onto agar plates, from which isolated colonies are then taken for identification and susceptibility testing; this process typically requires a minimum of 24 hours from the moment of positivity detection before a species-level identification is available (24,28).

MALDI-TOF MS offers the possibility of collapsing this interval by applying identification directly to the bacterial pellet obtained from the broth of a positive blood culture bottle, bypassing the subculture step entirely (9,24,28). The principal technical challenge is the separation of the bacterial cells from the host-derived blood components — erythrocytes, leucocytes, plasma proteins and charcoal particles — that would otherwise contaminate the spectrum and produce uninterpretable results. Several approaches have been developed to address this, including centrifugation with erythrocyte lysis using ammonium chloride or saponin, specific separator devices, and the commercial Bruker Sepsityper kit, which provides a standardised reagent set and protocol for processing positive blood culture broths directly on the Biotyper platform (9,24). When a protein extraction step is applied following initial cell separation, species-level identification rates from positive blood cultures have been reported to range from approximately 66 % in early studies to approximately 89–94 % in studies using optimised extraction and modern database versions; performance is consistently higher for Gram-negative bacteria (typically > 90 %) than for Gram-positive cocci (49–75 %), the latter presenting challenges related to cell wall composition and the limited quantity of bacteria recoverable from the broth at the time of positivity(9,24). The turnaround time from blood culture positivity signal to species identification using this approach has been reduced from a conventional 24–48 hours to approximately 1–2 hours in optimised workflows, with documented reductions in time to appropriate antibiotic adjustment and in associated hospital costs(24,28).

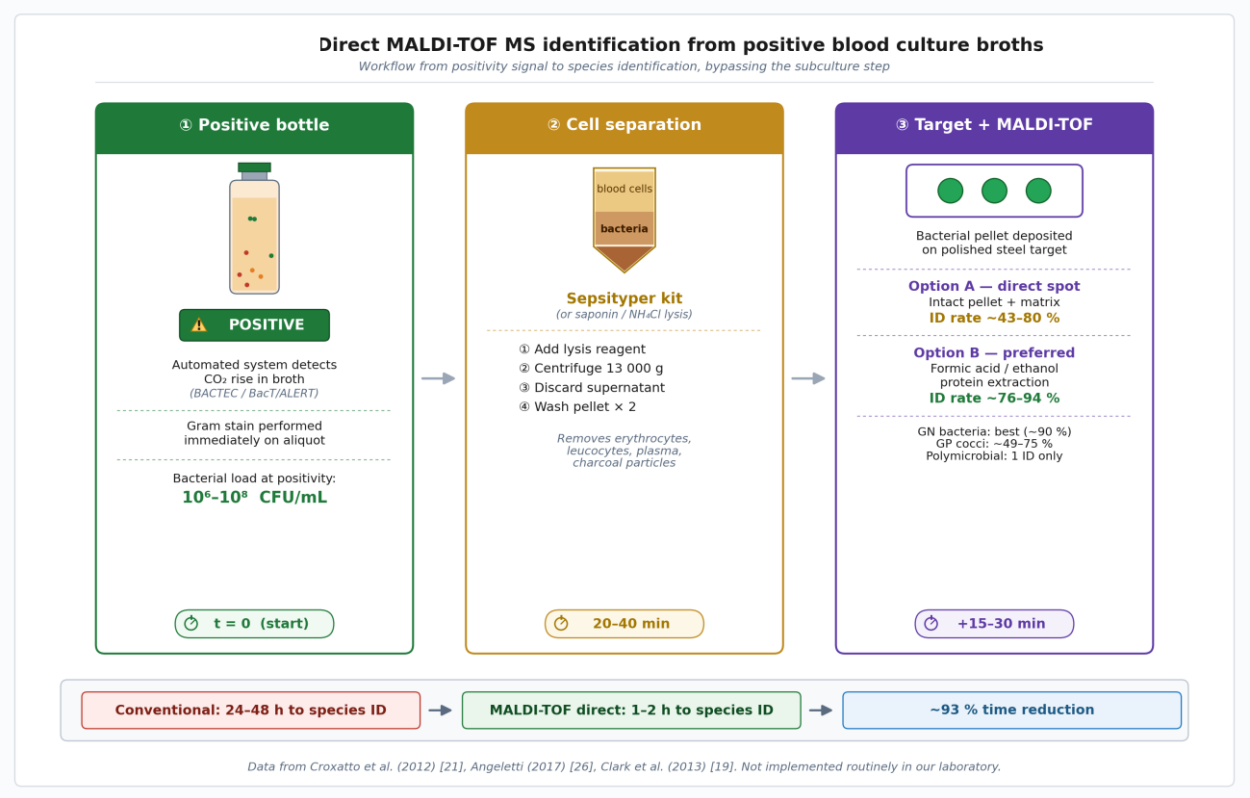


Figure 32. Workflow for direct MALDI-TOF MS identification from positive blood culture broths, bypassing the conventional overnight subculture step.

Following automated detection of culture positivity, a broth aliquot undergoes cell separation using a lysis reagent (Sepsityper kit or equivalent), centrifugation and washing to remove host-derived contaminants (erythrocytes, leucocytes, plasma proteins). The bacterial pellet is then deposited on the MALDI target either as intact cells (Option A) or following ethanol/formic acid protein extraction (Option B, preferred). Species-level identification rates range from ~66–80 % (intact cell method) to ~89–94 % (protein extraction) for Gram-negative bacteria; performance for Gram-positive cocci is lower (~49–75 %). Mean identification time is reduced from 24–48 h (conventional) to approximately 1–2 h. This workflow was not implemented as a routine protocol in our laboratory during the study period. (Data from Croxatto et al. (2012) [21], Angeletti (2017) [26], Clark et al. (2013) [19].)

In our laboratory, the direct blood culture identification approach was not implemented as a formal routine workflow during the study period. It is nevertheless mentioned here as an extension of the platform's capabilities that contextualises the clinical setting in which MALDI-TOF MS is deployed, and which represents a logical future development direction for the laboratory.

9.1 Detection of Antimicrobial Resistance Mechanisms

The standard MALDI-TOF MS workflow for colony identification does not provide direct antimicrobial susceptibility information — a limitation discussed in Section III.7. However, a distinct and growing body of work has explored the capacity of mass spectrometry to detect specific resistance mechanisms through the analysis of molecular signatures in the mass spectrum or through the measurement of antibiotic degradation products(9,30,31) .

The most extensively validated application is the detection of β-lactamase activity through the observation of antibiotic hydrolysis. When a bacterial isolate is incubated with a β-lactam antibiotic and the supernatant is then analysed by MALDI-TOF MS, the presence of an active β-lactamase is revealed by the disappearance of the intact antibiotic peak and the appearance of new peaks corresponding to its hydrolysis products — a mass shift that can be detected within one to four hours(9,31). This principle has been applied to the detection of extended-spectrum β-lactamases (ESBLs) and, most clinically importantly, to the detection of carbapenemase-producing organisms — a category of increasing clinical urgency — with reported sensitivities and specificities approaching 98–100 % in optimised protocols(9,30,31) . The Bruker platform has implemented this functionality through two dedicated software modules: the MBT STAR-BL module, which is CE-IVD certified for the detection of carbapenemase activity, and the MBT-ASTRA (MALDI Biotyper Antibiotic Susceptibility Test Rapid Assay) module, which measures bacterial growth inhibition by quantifying total peak area in the mass spectrum in the presence and absence of an antibiotic, providing a surrogate susceptibility classification in approximately four hours(30,31) .

Beyond β-lactam resistance, MALDI-TOF MS has been applied to the detection of methicillin resistance in S. aureus through the identification of specific biomarker peaks — in particular the PSM-mec peptide at m/z 2,415 Da, which is associated with the mecA gene — allowing rapid MRSA/MSSA discrimination directly from colony material(30,31) . However, not all resistance mechanisms produce detectable spectral signatures, and this remains an area of active investigation and methodological development rather than an established routine tool.

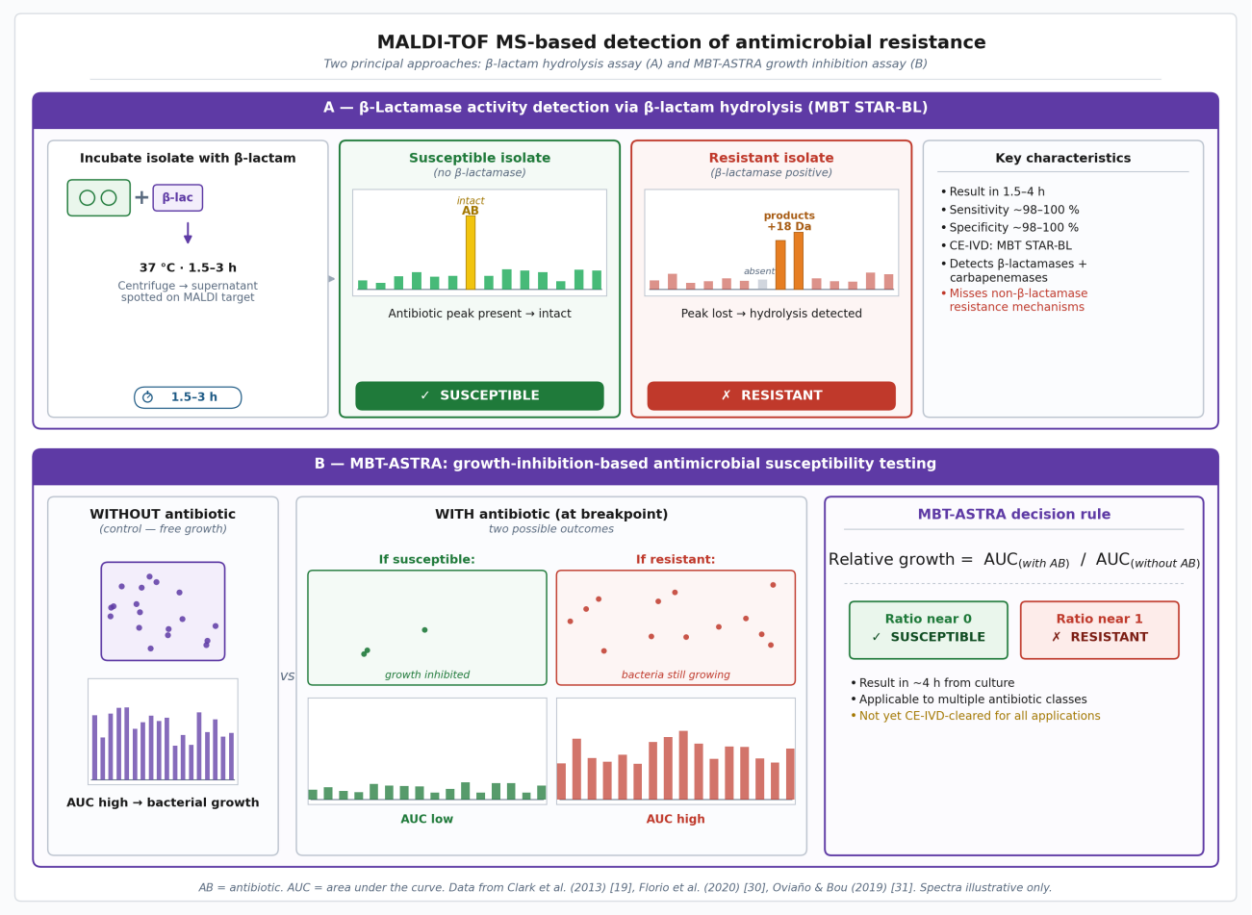


Figure 33. Two principal MALDI-TOF MS-based approaches for antimicrobial resistance detection. Panel A — β-Lactam hydrolysis assay (MBT STAR-BL): a bacterial isolate is incubated with a β-lactam antibiotic; the presence of β-lactamase activity is revealed by disappearance of the intact antibiotic peak and appearance of hydrolysis product peaks shifted by ~+18 Da in the mass spectrum. Sensitivity and specificity for carbapenemase detection approach 98–100 % in optimised protocols; result time 1.5–4 h. Panel B — MBT-ASTRA growth inhibition assay: bacterial suspensions with and without antibiotic at breakpoint concentration are analysed by MALDI-TOF; the ratio of the total spectral AUC (with antibiotic / without antibiotic) constitutes a surrogate growth index — a ratio near zero indicates susceptibility, near 1 indicates resistance; result time ~4 h. Both assays are supplementary to conventional susceptibility testing and were not part of the routine workflow in our laboratory during the study period. (Adapted from Clark et al. (2013) [19], Florio et al. (2020) [30], Oviaño & Bou (2019) [31]. Spectra shown are illustrative only.)

The important conceptual distinction between these resistance detection approaches and the standard identification workflow must be underlined. Whereas colony identification is fully integrated into the Biotyper's routine IVD-certified workflow and is the subject of the present study, resistance detection by MALDI-TOF MS requires separate dedicated assays, additional reagents and specific software modules, and is in most laboratories still supplementary to or confirmatory of established phenotypic susceptibility testing rather than a standalone replacement. In our laboratory during the study period, antimicrobial susceptibility testing was not performed on the BD Phoenix platform, and resistance detection by MALDI-TOF MS was not a component of the routine workflow. These two extended applications are therefore discussed here solely as contextual illustrations of the technology's evolving clinical scope, and do not form part of the primary analysis of this thesis.

IV. Rare and Fastidious Microorganisms in Clinical Microbiology

1. Definitions and Taxonomic Scope

The terms rare and fastidious are both widely used in clinical microbiology, but they are not synonymous and must be clearly distinguished, since they describe different properties that often — though not invariably — co-occur in the organisms of interest to the present study.

A fastidious organism is defined by its biological properties: it grows poorly or not at all on standard non-enriched culture media under routine atmospheric conditions, because it has complex or exacting nutritional or environmental requirements that must be specifically met for replication to occur. These requirements may include the provision of haemin and NAD for haemophilic organisms such as Haemophilus species; supplemental carbon dioxide or strict anaerobiosis; elevated humidity; enriched blood- or chocolate-based media; extended incubation periods; or specific temperature ranges. The term derives from the Latin fastidiosus — exacting, discriminating — and reflects the microorganism's biological tendency to resist cultivation under the standardised conditions applied to the majority of clinical isolates(12,14,17). Fastidiousness is a property of the organism independent of its prevalence: Streptococcus pneumoniae, for instance, is both fastidious in the sense of requiring enriched

media, and at the same time is one of the most frequently isolated pathogen in clinical practice. However, in the context of the present study, the term is applied specifically to organisms whose fastidious properties render their routine identification difficult or unreliable when conventional phenotypic methods are used — particularly those whose slow growth, variable colonial morphology, or reduced metabolic reactivity prevent reliable API or automated panel identification within a standard 18-to-24-hour incubation.

A rare organism is defined by its epidemiological properties: it is encountered infrequently in routine laboratory practice, either because it causes infections only rarely or only in specific clinical contexts, or because it is under-represented in the database of the identification system in use(6,12,13). Rarity has no fixed threshold but is operationally recognised when an organism is sufficiently uncommon that the laboratory technician does not encounter it with the frequency needed to maintain reliable familiarity with its identification characteristics. A corollary is that commercial identification databases — whether biochemical or spectral — tend to include fewer reference profiles for rare organisms, thereby compounding the identification difficulty that their rarity already imposes (6,24).

In practice, the two properties overlap substantially. Many rare organisms are also fastidious because it is precisely their fastidiousness — slow growth, unusual culture requirements — that limits their detection frequency and prevents the accumulation of large numbers of well-characterised strains in reference databases. This overlap is clinically significant: it means that when a laboratory encounters a rare and fastidious organism, it faces a compounded identification challenge in which conventional methods may fail to elicit a reliable phenotypic profile, automated systems may return a low-discrimination or absent result, and the reference database of even a high-performance platform such as the MALDI Biotyper may lack reference spectra for the species in question (6,9,11).

For the purposes of the present study, the population of interest is defined operationally as all bacterial and yeast isolates recovered in our laboratory between 2017 and 2023 for which the identification by conventional phenotypic methods — either the BD Phoenix automated platform or manual API galleries — was absent, unreliable, equivocal or required supplementary testing, or

for which MALDI-TOF MS provided a first-time species-level identification that had not been achievable by phenotypic means. Mycobacteria are acknowledged as an important category of rare and fastidious organisms, but their management in our laboratory during the study period involved dedicated protocols distinct from routine bacteriology and they are discussed in this section from a conceptual perspective only; they do not form part of the primary dataset.

The organisms considered are grouped into four categories corresponding to the major taxonomic divisions encountered in routine clinical microbiology: rare and fastidious Gram-negative bacilli, rare and fastidious Gram-positive cocci, clinically significant anaerobes, and clinically important yeasts. Within each group, the emphasis is on the clinical significance of correct species-level identification and on the contribution of MALDI-TOF MS relative to conventional methods.

2. Rare and Fastidious Gram-Negative Bacilli

Gram-negative bacilli (GNB) collectively represent the most taxonomically diverse group in clinical microbiology, encompassing not only the highly prevalent and biochemically active Enterobacterales but also a broad spectrum of organisms that are either infrequently encountered, poorly reactive in conventional identification systems, or both(9,12,13). Within the present study, several subgroups warrant specific discussion.

2.1 The HACEK Group

The acronym HACEK designates a group of slow-growing, fastidious Gram-negative coccobacilli that share a predilection for the upper respiratory and oropharyngeal tract as their ecological niche and a propensity to cause endovascular infections — particularly culture-negative or delayed-positive endocarditis — as well as bacteraemia and local head-and-neck infections (12,14). The group comprises Haemophilus species (specifically H. parainfluenzae and related non-influenzae species), Aggregatibacter aphrophilus, A. actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens and Kingella species. All require CO_2-enriched atmosphere, blood- or chocolate-enriched media and extended incubation (five to seven days for optimal growth), and several are poorly reactive or entirely non-reactive in conventional biochemical galleries (12,14,17). Van Veen and colleagues specifically highlighted the HACEK

group among the organisms for which MALDI-TOF MS demonstrated superior species-level identification relative to phenotypic methods, reflecting the improvement that spectrometric identification brings precisely where metabolic testing is least reliable(10).

2.2 Non-Fermenting Gram-Negative Bacilli

The non-fermenting Gram-negative bacilli (NFGNB) constitute a taxonomically heterogeneous collection that does not fit within the Enterobacterales and that includes organisms of widely differing clinical significance and epidemiology(12,13). Within this group, Pseudomonas aeruginosa, Acinetobacter baumannii and Stenotrophomonas maltophilia are the species most frequently encountered in clinical practice and present no identification challenges for established platforms. However, a large number of less common non-fermenters — including Achromobacter species, Burkholderia species other than B. cepacia complex, Comamonas, Delftia, Elizabethkingia, Ralstonia, Pandoraea, Chryseobacterium and Sphingomonas species — are much more rarely isolated and are poorly served by the biochemical databases of automated phenotypic platforms(6,8,9).

Elizabethkingia meningoseptica (Chryseobacterium meningosepticum) merits specific mention as it is responsible for neonatal meningitis and healthcare-associated bacteraemia in immunocompromised patients, is intrinsically resistant to the majority of antibiotics used for Gram-negative infections, and is frequently misidentified by automated biochemical systems as other non-fermenters(9,12). Similarly, Achromobacter xylosoxidans and Achromobacter denitrificans — opportunistic pathogens of relevance in cystic fibrosis and in immunocompromised hosts — are prone to misidentification on biochemical platforms, a problem that MALDI-TOF MS has substantially resolved when the organisms are adequately represented in the database(9,11) . Acinetobacter genomospecies within the A. calcoaceticus-baumannii complex represent a special case, as they cannot be resolved to the genomospecies level by any currently available routine method including MALDI-TOF MS — a reflection of their biological proximity rather than a technological limitation (9,10,24)(van Veen et al., 2010; Croxatto et al., 2012; Clark et al., 2013).

2.3 Other Rare Gram-Negative Bacilli and Fastidious Coccobacilli

Beyond the HACEK group and the non-fermenters, a number of additional rare Gram-negative organisms deserve recognition in the present context. Brucella species are obligate intracellular organisms of veterinary origin that cause brucellosis in humans following occupational or alimentary exposure; they grow slowly on standard media and have historically been misidentified as Ochrobactrum anthropi or Moraxella species by the API 20 NE, an error with potentially serious laboratory safety implications(2). MALDI-TOF MS substantially improves the identification of Brucella but requires specific validation and the implementation of inactivation protocols prior to plate loading because of biosafety concerns (9,11). Bartonella species — agents of cat scratch disease, trench fever and bacillary angiomatosis — are among the most fastidious organisms encountered in clinical practice, requiring specific media such as the Bartonella alphaproteobacterial growth medium and extended incubation of up to five weeks for primary isolation; they are generally unidentifiable by standard biochemical systems and represent one of the organisms for which MALDI-TOF MS has demonstrated specific added value when appropriate extraction and database conditions are met (9,11). Miscellaneous rarely isolated Gram-negative coccobacilli encountered in the present study collection include various Capnocytophaga species (from oral flora, particularly in asplenic hosts), Pasteurella multocida (from animal bite wounds), Eikenella corrodens and organisms of the genus Raoultella, whose correct distinction from Klebsiella is clinically relevant but not achievable with certainty by phenotypic methods(6,9,13).

3. Rare and Fastidious Gram-Positive Cocci

Gram-positive cocci represent the second major group of clinical relevance for rare and fastidious organism identification, and the challenge in this group is qualitatively different from that posed by GNB. Whereas the difficulty with GNB often lies in simple failure to grow or react on standard media, the principal challenge for rare Gram-positive cocci lies in the phenotypic similarity of closely related species whose correct identification is nonetheless clinically critical — both for antimicrobial therapy and for the elucidation of pathogenesis (9,12,14,27).

3.1 Viridans Group Streptococci and Related Organisms

The viridans group streptococci are a phylogenetically heterogeneous assemblage that includes five major species groups: mitis, salivarius, bovis, anginosus and mutans(12,13). Within these groups, correct species-level identification is clinically important, since S. pneumoniae must be distinguished from S. mitis and S. oralis for penicillin susceptibility reporting; members of the S. anginosus group (S. anginosus, S. constellatus, S. intermedius) are associated with abscess formation and require specific treatment strategies; and S. bovis group members, particularly S. gallolyticus subsp. gallolyticus (S. bovis biotype I), are associated with colorectal adenoma or carcinoma and their identification should prompt colonic investigation (12,14). Conventional biochemical systems, automated panels and MALDI-TOF MS all struggle to resolve certain pairs within the viridans group, in particular S. pneumoniae versus S. mitis, and members of the S. bovis/equinus complex, principally because of the high degree of ribosomal protein sequence similarity within these closely related taxa(9,10,27) . This limitation is not specific to a particular technology but reflects the underlying biological proximity of these organisms.

3.2 Granulicatella, Abiotrophia and Nutritionally Variant Streptococci

Granulicatella adiacens, G. elegans and Abiotrophia defectiva — the nutritionally variant streptococci formerly designated Streptococcus adjacens and S. defectivus — are rare but clinically important organisms responsible for endocarditis, bacteraemia and central nervous system infections that are disproportionately severe relative to the frequency with which these organisms are encountered(6,12). They grow as satellite colonies around other bacteria on standard blood agar — requiring pyridoxal (vitamin B_6) or cysteine for autonomous growth — a property that makes their primary recognition difficult; they are systematically under-represented in conventional biochemical databases and are routinely misidentified on automated platforms(6,12,14). MALDI-TOF MS significantly improves their identification when adequate reference spectra are present in the database, which has been progressively achieved in the Biotyper database(7,9).

3.3 Coagulase-Negative Staphylococci and Other Rare Gram-Positive Cocci

Coagulase-negative staphylococci (CoNS) represent a paradigmatic example of a group in which species-level identification has become progressively more clinically important as the range of clinically recovered CoNS species has widened and as species-specific differences in virulence and antimicrobial susceptibility have been better characterised (12,13). Correct differentiation of S. lugdunensis from other CoNS is particularly critical, since it is associated with more aggressive endocarditis and skin infections than most other CoNS and may require more aggressive treatment(12,14). S. lugdunensis identification is reliably achieved by MALDI-TOF MS, where it was one of the organisms that showed high performance in the Schulthess series, in contrast to the inconsistency of conventional methods for this species (27). Other rare Gram-positive cocci occasionally encountered in our practice include Aerococcus species, Leuconostoc, Pediococcus, Gemella species and Rothia species — all of which are poorly served by automated biochemical systems and are accurately identified by MALDI-TOF MS when present in the database(7,9,10,27) .

4. Clinically Significant Anaerobic Bacteria

Obligate anaerobes constitute a clinically under-recognised but epidemiologically important component of the microbiological specimen load in a university hospital with referral function, and their correct identification represents one of the most challenging areas of conventional clinical microbiology(12,17). Two properties combine to make anaerobic identification particularly difficult. First, obligate anaerobes require strict anaerobic conditions throughout their cultivation — from specimen reception to subculture, incubation and identification — which is labour-intensive and equipment-demanding. Second, the biochemical reactivity of many clinically relevant anaerobes is limited and variable, making the API 20 A gallery and equivalent systems less discriminating than their aerobic counterparts; the turnaround time for anaerobic biochemical identification may reach 48 to 72 hours beyond the already extended culture period(12–14).

The principal genera encountered in anaerobic clinical microbiology include the Gram-negative bacilli Bacteroides, Prevotella, Porphyromonas, Fusobacterium and Bilophila; the Gram-

positive bacilli Clostridium, Actinomyces, Bifidobacterium, Eggerthella, Propionibacterium (Cutibacterium), Lactobacillus and Peptostreptococcus; and the Gram-positive cocci Finegoldia, Peptoniphilus, Anaerococcus and Parvimonas(9,12,13). The Bacteroides fragilis group remains the most commonly isolated Gram-negative anaerobe in clinical material; Clostridium difficile (Clostridioides difficile) has an entirely separate epidemiological and clinical position as a toxin-producing nosocomial pathogen; and Actinomyces species are responsible for actinomycosis, a chronic granulomatous infection that may mimic malignancy and whose diagnosis depends critically on precise genus- and species-level identification(12,14).

The contribution of MALDI-TOF MS to anaerobic identification has been one of the most actively studied areas in the clinical mass spectrometry literature. Multiple studies reviewed by Biswas and Rolain (2013), Clark et al. (2013) and Croxatto et al. (2012) have collectively demonstrated that MALDI-TOF MS provides substantially more correct identifications at the species level than conventional biochemical methods for most major anaerobic genera, with reported accuracy rates for Bacteroides spp. reaching 97.5 % to 99.5 %, for Clostridium spp. between 84.9 % and 100 %, and for Prevotella and Fusobacterium spp. up to 100 %(9,11) . For Actinomyces species, correct species-level identification by MALDI-TOF MS has been reported for approximately 97 % of isolates when reference spectra are available(11) . However, performance for anaerobic Gram-positive cocci — particularly Finegoldia magna and certain Anaerococcus species — remains more variable, principally because of under-representation in commercial databases(6,9). Anaerobic Gram-negative bacilli other than Bacteroides fragilis group and Fusobacterium are also less well covered, as illustrated by the zero percent species-level identification for anaerobic Gram-negative bacteria in the Lau series(6), attributable almost entirely to absent database entries rather than to inherent spectral limitations.

An additional consideration for anaerobic bacteria is sample preparation: the full ethanol/formic acid extraction protocol significantly improves identification rates compared with direct transfer alone, partly because anaerobic organisms are often collected in small quantities and partly because the polysaccharide capsule of Bacteroides species may interfere with intact-cell spectra(9,11,24) . In our laboratory, the introduction of the Biotyper in 2020 has materially

changed the approach to anaerobic identification, reducing reliance on the API Anaerobe gallery and providing more consistent species-level identifications for organisms recovered from deep-seated infections.

5. Clinically Important Yeasts

Yeast infections — candidaemia, mucocutaneous candidiasis, cryptococcal meningitis and emerging non-albicans species infections — represent a major infectious disease burden particularly in immunocompromised, critically ill and neonatal intensive care unit (NICU) patients, and the correct identification of the causative yeast species is of direct clinical and therapeutic importance(9,12,14). Species-level identification of Candida determines both the intrinsic susceptibility pattern and the appropriate choice of antifungal therapy: C. krusei is intrinsically resistant to fluconazole; C. glabrata has dose-dependent susceptibility to azoles; C. parapsilosis and C. auris have acquired resistance profiles that differ from those of C. albicans; and Cryptococcus neoformans versus C. gattii differentiation carries epidemiological and prognostic implications (9,12,14).

Conventional yeast identification by chromogenic media such as CHROMagar, germ tube production, assimilation tests and API ID 32C provides reliable identification for the most common species — C. albicans, C. glabrata, C. tropicalis, C. parapsilosis and C. krusei — but requires 24 to 72 hours incubation and is less reliable for closely related species pairs and for uncommon Candida species such as C. orthopsilosis and C. metapsilosis, which cannot be distinguished from C. parapsilosis by phenotypic means(9,12).

MALDI-TOF MS has demonstrated exceptionally high performance for yeast identification, generally superior to its performance for bacteria. The large evaluation by Bader and colleagues, which tested 1,148 clinical yeast isolates from 21 species on both the BioTyper and SARAMIS platforms, reported species-level identification rates of 99 % for both systems for species present in their respective databases; crucially, closely related species pairs such as C. orthopsilosis, C. metapsilosis and C. parapsilosis, and C. glabrata, C. nivariensis and C. bracarensis — which are systematically confused by biochemical methods — were correctly resolved by both MALDI-TOF MS systems (9). In a prospective study by van Veen and colleagues,

85 % of 61 yeast isolates representing 12 species were correctly identified at the species level by MALDI-TOF MS on direct transfer, with no misidentifications (10)(van Veen et al., 2010). Bizzini and Greub reported 100 % species-level correct identification for 24 yeast isolates in their routine prospective series(23) . Pre-treatment of yeast cells — either by the formic acid on-target method or by full ethanol/formic acid extraction — is generally recommended to penetrate the thicker yeast cell wall and achieve optimal protein extraction (9,24).

Cryptococcus neoformans identification by MALDI-TOF MS is reliable when appropriate extraction is used, and recent work has demonstrated the ability to distinguish molecular types within the C. neoformans–C. gattii species complex, which carries prognostic significance (9). For rare non-Candida yeasts such as Trichosporon, Geotrichum, Saccharomyces, Pichia and Blastoschizomyces, MALDI-TOF MS has also shown good identification rates when reference spectra are present, as demonstrated in the series by Marklein and colleagues where 92.5 % of 267 clinical yeast isolates across multiple genera were accurately identified(9,24).

In our laboratory, the introduction of MALDI-TOF MS has significantly streamlined yeast identification, reducing the time from colony to reported species from 24–72 hours with conventional methods to a matter of minutes, and has provided species-level identifications for several uncommon yeasts that had previously been reported only at the genus level.

6. Mycobacteria: Conceptual Considerations

Mycobacteria occupy a specific position in the landscape of rare and fastidious organisms. They are obligate aerobes with a lipid-rich cell wall that makes them impermeable to standard staining and resistant to conventional media; primary cultures require specific media (Löwenstein-Jensen, MGIT liquid medium), prolonged incubation of up to eight weeks for slowly growing species, and strict biosafety containment measures for Mycobacterium tuberculosis complex (12,14,17). The identification of non-tuberculous mycobacteria (NTM) — a diverse and growing group of species including M. avium complex, M. abscessus, M. kansasii, M. xenopi, M. gordonae and many others — is particularly challenging by conventional methods and historically required 16S rRNA gene sequencing or hsp65 gene analysis for reliable species assignment.

The application of MALDI-TOF MS to mycobacterial identification presents both promise and specific technical challenges. Because of the thickness and hydrophobicity of the mycobacterial cell wall, standard intact-cell or formic acid protocols yield spectra of insufficient quality; the full extraction protocol must be extended to include a glass bead disruption step followed by heat inactivation at 95 °C for at least one hour to ensure inactivation of viable M. tuberculosis before the plate is removed from the biosafety cabinet (9,11). When this specific protocol is applied, reported accuracy rates for NTM identification by MALDI-TOF MS are encouraging, ranging from 50 % to more than 90 % at the species level depending on the species and the completeness of the database (9,11,32). However, closely related species within the M. avium complex and within the M. abscessus group remain difficult to resolve by mass spectrometry, and 16S rRNA or additional gene sequencing remains the reference method for these cases (9,11).

In our laboratory during the study period (2017–2023), mycobacterial identification was performed by a dedicated protocol distinct from the routine bacteriology workflow. The Biotyper was not routinely applied to mycobacterial isolates within the timeframe of the present study, and mycobacteria therefore do not form part of the primary result dataset. They are discussed here solely to contextualise the scope of MALDI-TOF MS for difficult organisms and to acknowledge their position as a category of fastidious and rare bacteria for which the technology offers significant potential that extends beyond the confines of this work.

DISCUSSION OF RESULTS

I. Principal Findings of the Present Study

The six-year dataset assembled in the present work is best read not as three independent descriptive features but as three facets of one coherent diagnostic transition. The annual identification workload of the laboratory rose by a factor of approximately 1.8 between 2018 and 2023, yet the number of distinct canonical organisms recovered each year rose by a factor of roughly two, from a stable pre-MALDI plateau of approximately 200 distinct entities in 2018 and 2019 to between 329 and 448 across 2021–2023. The corresponding distinct-organism-per-thousand-isolates ratio settled in the 25–27 range across the MALDI era after fluctuating between 17.8 and 24.8 in the years immediately preceding routine spectrometric identification. The expansion of measurable diversity is therefore not a mechanical consequence of higher throughput. Read alongside this, the trajectory of the singleton fraction — from 27.6% in 2018 to 38.3% in 2023 — is best interpreted as a progressive enrichment of the rare tail of the inventory rather than a true rise in microbial heterogeneity within the catchment population. What was altered between the two diagnostic phases was the granularity at which the laboratory could read its own culture output. This single underlying change manifested in the dataset under three closely related guises. The first was the near-doubling of the measurable species inventory just described. The second was the collapse, in the post-2020 window, of the unspeciated genus-level reporting categories that had previously absorbed a substantial fraction of the routine workload — the coagulase-negative staphylococci bucket, the alpha-haemolytic and viridans-group bucket, the unspeciated Candida bucket and the unspeciated Corynebacterium residual — each shrinkage matched in time by an expansion of named species drawn from the same taxonomic territory. The third was the appearance, from 2020 onwards, of more than 250 species that had not been recorded at all in the 2018–2019 dataset, distributed across all five organism groups examined in the present study. These three signals are not additive; they are different views of the same underlying accounting, in which isolates that had been cultured and returned to the clinician under a generic label during the pre-MALDI period now reappear under specific binomials. The substantive question for the remainder of this Discussion is therefore not

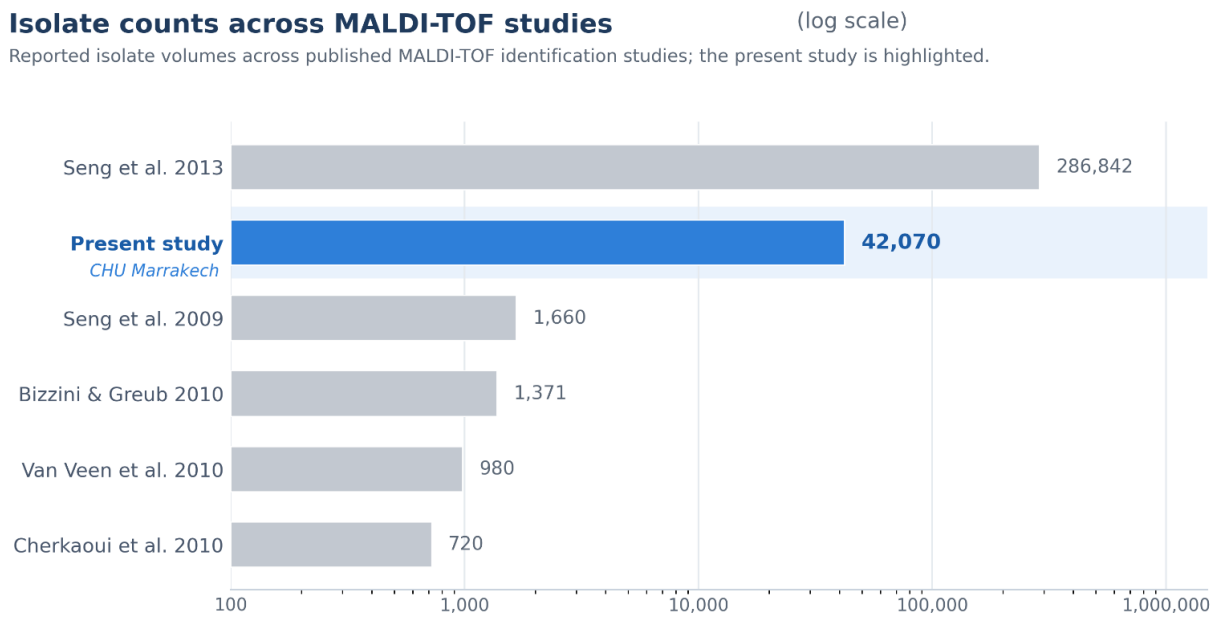
whether the diagnostic capacity of the laboratory expanded — the convergent evidence presented in section III leaves little doubt that it did — but how the magnitude, the shape and the clinical content of that expansion compare to what other published single-centre routine MALDI-TOF series have reported, what it implies for specific organism groups of clinical significance, and what its interpretation owes to, and is constrained by, the methodological choices that shaped the dataset.

II. The Diagnostic Transition Observed in the Laboratory

1. Magnitude and Shape of the Diversity Expansion

The corpus of 42,070 isolates assembled over six continuous years and resolved into 406 distinct canonical organisms places the present study at the larger end of the published single-centre routine MALDI-TOF series, even before any inferential comparison is attempted. The earliest large prospective evaluation of the technology under routine conditions was the Marseille snapshot of Seng and colleagues, in which 1,660 clinical isolates were processed in parallel with conventional phenotypic identification over a defined recruitment window and 95.4% were identified to species level(20). The Lausanne series of Bizzini and Greub examined 1,371 routine clinical isolates retrospectively and reported equivalent species-level performance to that of conventional methods after appropriate score-threshold adjustment(23) ; the Leiden series of van Veen and colleagues prospectively analysed 980 consecutive isolates in a high-throughput diagnostic setting(10) ; and the Geneva series of Cherkaoui and colleagues compared 720 consecutive clinical isolates on two MALDI-TOF platforms against the same conventional reference, with identification rates above 93% on both systems(19). These four series collectively established the species-level performance of the technology under prospective or short-term retrospective single-centre conditions and are appropriately treated in the present work as the reference framework for that performance. The contribution of the present dataset is not, and cannot be, a higher species-level identification rate: no paired conventional-versus-MALDI concordance was measured here, and the absence of in-house 16S rRNA confirmation precludes any claim equivalent to the rates reported in those series. What the present study can offer that

the four cited snapshot series structurally cannot is a longitudinal view of the diagnostic transition itself. Each of those four reports is a snapshot, taken either immediately before or shortly after the integration of MALDI-TOF into routine practice, and none follows a single laboratory across the before-and-after divide over a multi-year window. The only published comparator that does is the eleven-year analysis of the same Marseille laboratory by Seng and colleagues, in which 286,842 clinical isolates were processed across a 91-month conventional-identification phase followed by a 40-month MALDI-TOF phase, and the number of bacterial species annually identified increased from approximately 44 species per year (19 species per 10,000 isolates) under conventional methods to approximately 112 species per year (36 species per 10,000 isolates) under MALDI-TOF, with 128 bacterial species rarely reported as human pathogens being identified during the MALDI period(33). This longitudinal Marseille analysis is, to our knowledge, the only published peer of the present six-year before-and-after design, and its findings — a near-tripling of the annual species count and an approximate doubling of the species-per-isolate ratio after MALDI-TOF integration — are remarkably congruent with the near-doubling of distinct organisms per year and the rise of the distinct-organism-per-thousand-isolates ratio from the 17.8–24.8 range in 2018–2019 to a stable 25–27 range across the MALDI era observed in the present dataset. The case made in the present work is therefore not that our laboratory identifies organisms more accurately than these earlier series did — that comparison cannot be drawn — but that the size and time span of our dataset surface phenomena which the four shorter-window snapshot studies were not designed to capture, and that the patterns observed are quantitatively consistent with the only other published longitudinal series of comparable design. The relative scale and design of the present study against these published reference series are presented in Discussion Figure 1.



Discussion Figure 1. Study-size and study-design context: present study versus published single-centre routine MALDI-TOF series and the longitudinal Marseille comparator (Seng et al., 2013).

2. Collapse of Unspeciated Reporting Categories

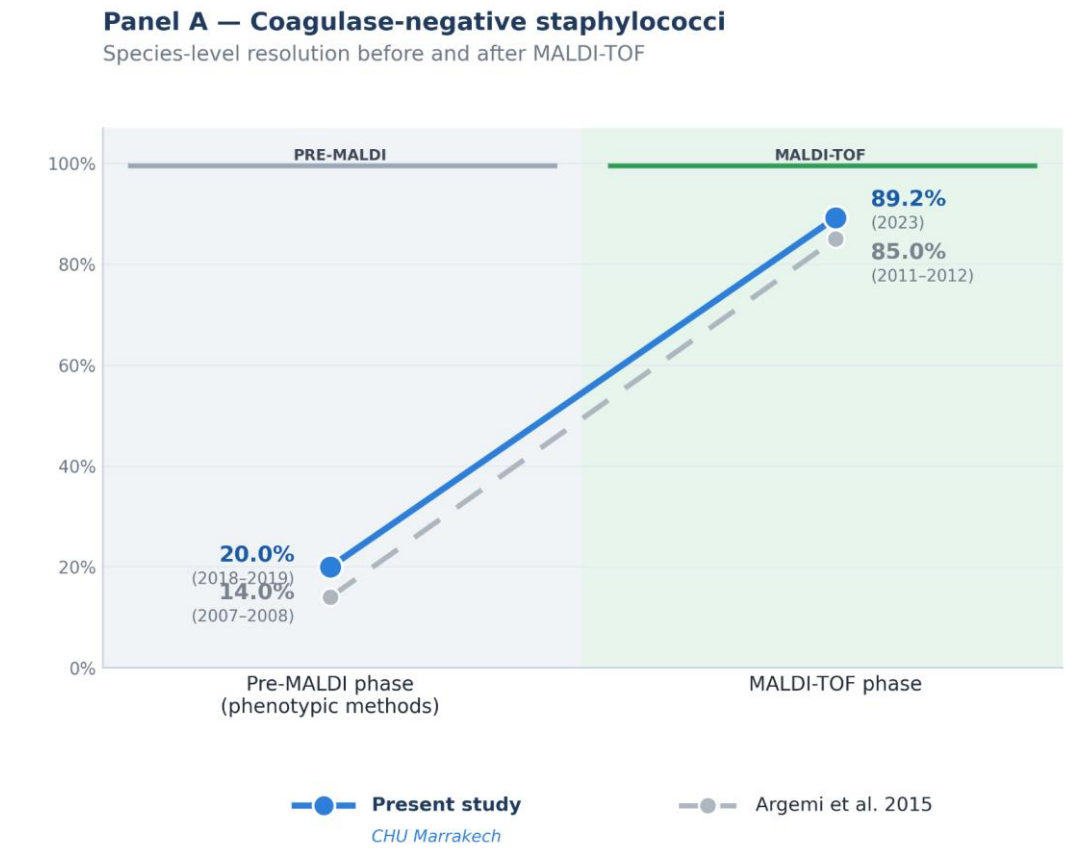
The most distinctive analytical finding of the present study, and the one that least readily admits a direct literature comparator, is the operational collapse of the unspeciated reporting categories in the post-2020 window. The coagulase-negative staphylococci bucket, having grown from 599 isolates in 2018 to a peak of 909 in 2020, contracted to 188 isolates in 2023, while the number of CoNS isolates resolved to a named species over the same interval rose from 318 to 1,546. The alpha-haemolytic and viridans-group bucket fell from a 2019 peak of 144 isolates to five isolates in 2023, with the count of speciated viridans rising over the same window from 56 to 231 across an inventory progressively expanded to include 16 distinct named viridans species. The unspeciated Candida bucket fell from a 2019 peak of 243 isolates to 23 in 2023, with the named-species Candida workload rising in parallel and the inventory enriched by C. tropicalis, C. glabrata, C. parapsilosis, C. krusei, C. dubliniensis and Clavispora lusitaniae, none of which had appeared in the pre-MALDI inventory. The unspeciated Corynebacterium residual was halved between the two phases, against the simultaneous emergence of more than ten named coryneform species drawn from the same taxonomic territory. The argument that

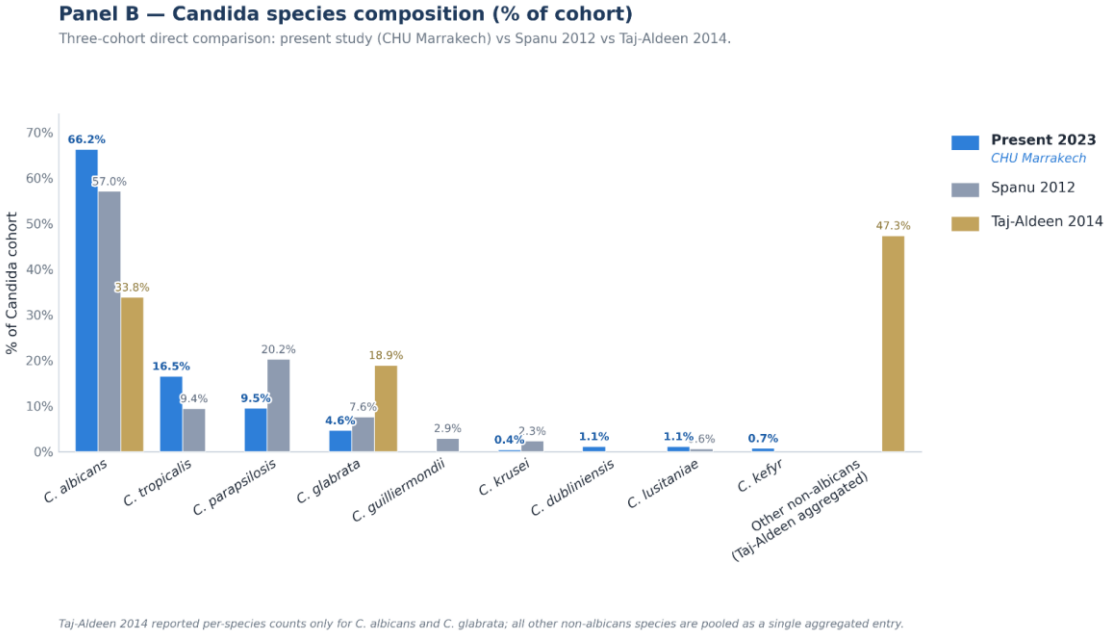
emerges from these four parallel trajectories is that the mass spectrometer and its database did not so much identify organisms that the laboratory had failed to culture as resolve to species level the very same organisms that had hitherto been reported under genus-level labels.

For the coagulase-negative staphylococci component, two published comparators support the proposition that this bucket-collapse is a generalisable signature of MALDI-TOF integration rather than a feature peculiar to the present laboratory. The prospective study of Schulthess and colleagues, in which 1,619 routine clinical Gram-positive cocci were processed by MALDI-TOF in parallel with conventional identification, reported a species-level concordance of 95.6% under controlled conditions and demonstrated that the technical capacity to assign CoNS isolates to species level is reliable when the platform is in routine use(27). The Strasbourg before-and-after analysis of Argemi and colleagues constitutes the closest published peer of the present CoNS observation: 12,479 staphylococci identified by phenotypic methods between January 2007 and August 2008 were compared with 14,913 staphylococci identified by MALDI-TOF MS between January 2011 and August 2012 in the same university hospital, with the proportion of CoNS resolved to species level rising from approximately 14% under the conventional workflow to approximately 85% under MALDI-TOF, and the named-species inventory expanding to include S. epidermidis, S. haemolyticus, S. hominis, S. lugdunensis, S. capitis, S. warneri and several less commonly reported entries(34). The five-fold rise in CoNS species-level reporting that the Strasbourg analysis recorded across roughly comparable workload volumes is structurally consistent with the trajectory observed in the present dataset, in which the CoNS unspeciated bucket fell from 909 to 188 isolates while the named-species CoNS workload rose from 318 to 1,546 over the four-year interval following MALDI-TOF integration.

For the Candida component, the equivalent peer-comparator framework rests on two published series, each anchoring the bucket-collapse argument from a different methodological angle. The observational two-laboratory study of Spanu and colleagues evaluated direct MALDI-TOF identification of Candida species from positive blood-culture broths and reported that 187 of 195 C. albicans isolates and 128 of 148 non-albicans Candida isolates were correctly identified to species level against conventional yeast biochemical identification, demonstrating

that the spectrometric platform reliably resolves the Candida genus to species level even under the demanding conditions of direct-from-broth processing (35). The single-centre seven-year retrospective analysis of Taj-Aldeen and colleagues, conducted at a tertiary hospital in Doha, Qatar, between January 2004 and December 2010, identified 201 candidaemia episodes from 187 patients using MALDI-TOF MS with molecular confirmation by ITS sequencing and reported a species distribution of 33.8% C. albicans and 66.2% non-albicans Candida, with C. glabrata (18.9%) and C. parapsilosis the predominant non-albicans species(36). The Qatari series provides not only methodological validation of the spectrometric workflow but also a regional-comparator species distribution, recovered from a Middle Eastern tertiary-care patient population whose case-mix is geographically closer to the present study's catchment than the Italian and Swiss series of the prior literature. Both peer studies are paired-sample concordance analyses on isolates processed under conditions designed to elicit a like-for-like comparison; neither tracks the operational shrinkage of unspeciated reporting buckets in the routine output of a clinical laboratory across years of accumulating MALDI-TOF use. The bucket-collapse trajectory presented in the present work therefore complements rather than replicates these published series: it is the observable consequence, in routine reporting, of the species-level resolution that the cited validation studies established under controlled conditions, and it is presented in Discussion Figure 2 alongside the species-distribution comparators against which the Candida component of the present inventory is most usefully read.

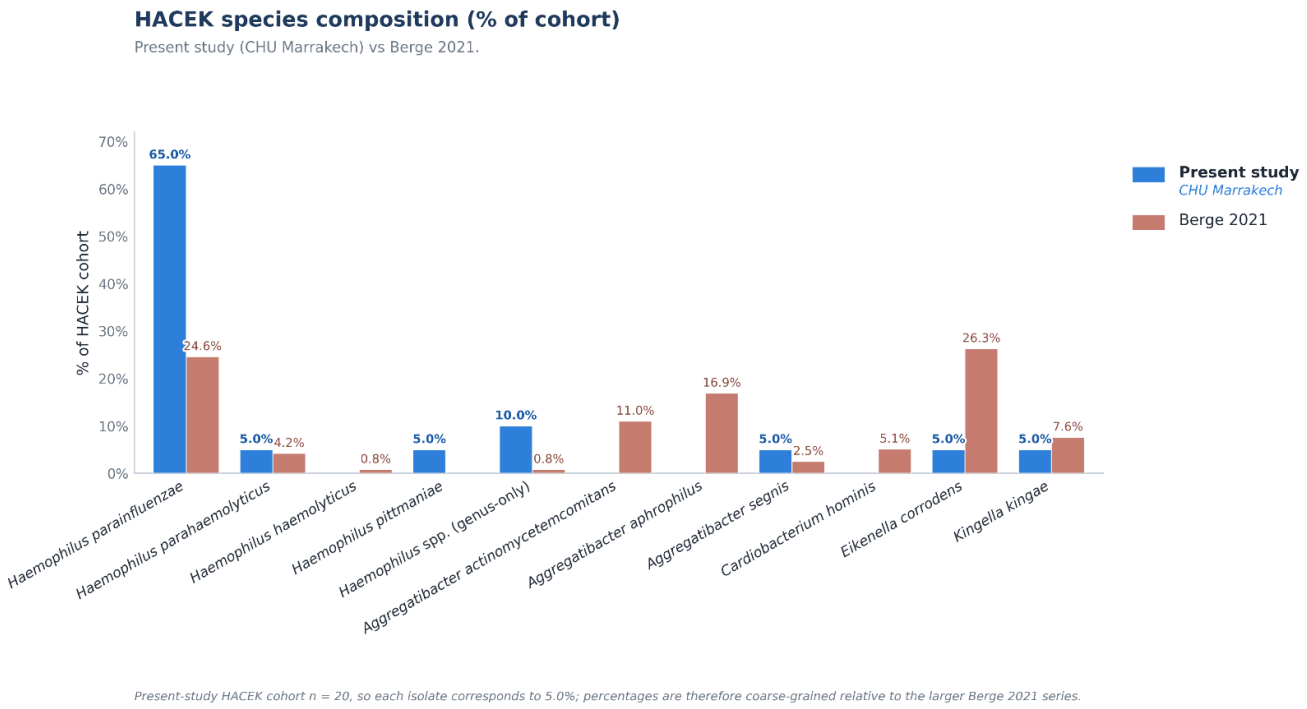




Discussion Figure 2. Bucket-collapse trajectories with literature comparators on a single chart axis: CoNS species-level resolution (present study versus Argemi et al., 2015) and Candida species composition (present study 2023 versus Spanu et al., 2012 versus Taj-Aldeen et al., 2014).

3. Recovery of Rare and Fastidious Organisms

Section IV of the existing text enumerates, on a strictly conceptual basis, the categories of rare and fastidious organisms whose identification has historically constituted the principal limit of conventional phenotypic methods. Read against the dataset assembled in section III, these conceptual categories appear no longer as theoretical illustrations but as concretely populated entries of the laboratory's routine inventory. The HACEK group, conceptually framed in section IV.2.1, expanded from six annual isolates in 2018 to 52 in 2023, with first-time empirical detections of Eikenella corrodens and Kingella kingae in 2023, Aggregatibacter segnis in 2022, and the rare haemophili H. parahaemolyticus and H. pittmaniae recovered exclusively in the MALDI era. The technical capacity of mass spectrometric identification for this organism group has been established under controlled conditions by the multicentre validation of the VITEK MS v2.0 system reported by Branda and colleagues, in which 96% of 226 fastidious Gram-negative isolates including HACEK members were correctly identified to species level against a DNA-sequence reference (37); the present study cannot replicate that performance comparison because no molecular reference was available for paired analysis, but the routine recovery profile of HACEK organisms in our data is consistent with the proposition that the species-level capacity established by Branda has been transferred from a controlled multicentre evaluation into the day-to-day output of a single laboratory. The published comparator that allows the most direct alignment with the present routine recovery is the population-based retrospective study of Berge and colleagues, in which 118 episodes of HACEK bacteraemia were identified by MALDI-TOF MS across two Swedish university hospital catchment areas between 2012 and 2017, with the species-level distribution dominated by Aggregatibacter and Kingella species in proportions broadly similar to those emerging in the present laboratory's post-2020 inventory once the Haemophilus influenzae component is set aside(38). The species composition of HACEK organisms recovered routinely in the present study is presented alongside the Berge cohort in Discussion Figure 3, which uses cohort-normalised percentages so that the two studies — separated by an order of magnitude in case count — can be read on a common scale.



Discussion Figure 3. HACEK species composition: present study (non-influenzae HACEK, 2018–2023) versus Berge et al. (2021) population-based HACEK bacteraemia cohort, plotted on a percent-of-cohort axis.

The remaining categories enumerated in section IV appear in the present dataset as concretely populated entries rather than as theoretical illustrations. The nutritionally variant streptococci, framed in section IV.3.2 as exemplars of conventional phenotypic failure, appear in the present dataset as discrete entries of Granulicatella elegans, Granulicatella spp. and Abiotrophia defectiva; the related rare Gram-positive cocci Aerococcus viridans, Rothia mucilaginosa and several named Gemella species — drawn from the difficult-to-identify universe assembled by Lau and colleagues, whose 1,371-isolate single-centre series specifically validated MALDI-TOF identification of these uncommon GPC categories(6) — populate the same area of the inventory. The yeast inventory described in section IV.5 reappears in the dataset enriched by Magnusiomyces capitatus, Trichosporon asahii, Clavispora lusitaniae, Cyberlindnera fabianii and Saccharomyces cerevisiae as MALDI-era detections, and the anaerobic inventory framed in section IV.4 by Cutibacterium acnes and C. avidum, three named Actinomyces species, Schaalia turicensis and S. radingae, and a 2023 isolate of Fusobacterium necrophorum. Within the non-

fermenting Gram-negative bacilli framed in section IV.2.2, the Acinetobacter speciation expanded from a pre-MALDI inventory effectively limited to A. baumannii and A. lwoffii to a post-2020 inventory of more than 14 named species, and the Pseudomonas non-aeruginosa speciation from three named species in 2018 to 17 in 2023, including P. monteilii, P. stutzeri, P. fluorescens, P. mendocina and a long tail of singletons; both of these expansions are taken up in detail in the group-specific interpretation of section III.1, where they are anchored to the routine-practice comparators of Li, Jeong and others. These observations do not in themselves constitute new performance evidence for MALDI-TOF mass spectrometry, that evidence having been laid down by the validation series cited in sections III.6 and III.7 of the existing text and by the comparators introduced above; what they document is the systematic transfer of the rare and fastidious organism inventory described conceptually in section IV from the published literature into the empirical routine of the laboratory.

III. Group-Specific Interpretation

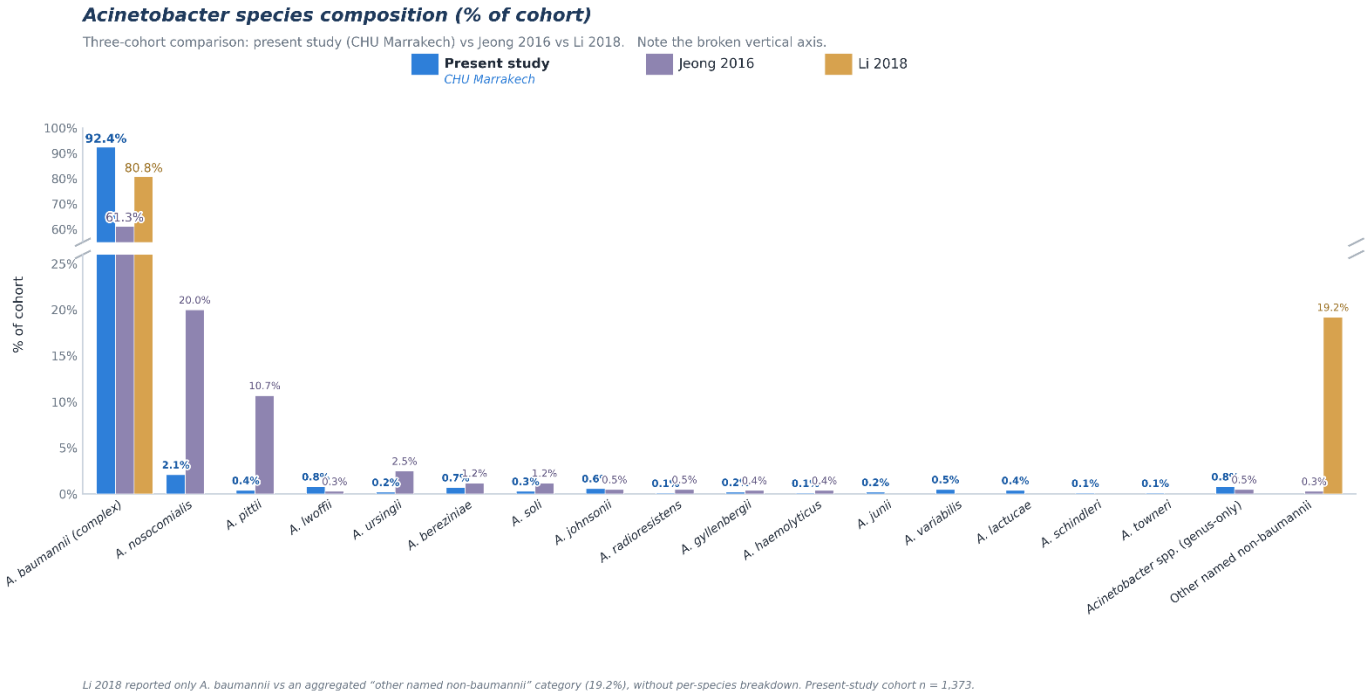
1. Gram-Negative Bacilli

Within the 187 distinct Gram-negative species recorded across the six-year window, the most analytically informative observations relate to the expansion of the Acinetobacter and Pseudomonas non-aeruginosa inventories noted in section II.3. The Acinetobacter speciation expansion deserves an explicit qualification before any comparison is drawn. The genomospecies internal to the A. calcoaceticus-baumannii complex are not reliably resolved by mass spectrometric identification under routine conditions, and the present dataset's A. baumannii entries should therefore be read as designating the complex rather than the genomospecies in the strict sense. This is a biological boundary that has been established successively in the published literature: the A. baumannii complex differentiation by improved MALDI-TOF MS profiling reported by Šedo and colleagues in 2013 introduced spectral processing techniques that separate A. baumannii, A. pittii and A. nosocomialis under controlled conditions but require careful database curation rather than out-of-the-box identification (39)(Šedo et al., 2013); the validation of the same complex differentiation by Espinal and colleagues across 60

epidemiologically unrelated Acb-complex isolates confirmed reliable separation of the three principal members(40); the validation set of Toh and colleagues, comprising 73 isolates across ten Acinetobacter species, demonstrated that all ten species including A. baylyi, A. soli and A. bereziniae are MALDI-distinguishable when the database is appropriately augmented(41); and the Acb-complex revisitation by Marí-Almirall and colleagues subsequently extended the same framework to incorporate the novel A. seifertii and A. dijkshoorniae species through dedicated database augmentation, validated against 78 reference isolates and 780 spectra(42). The same group's later analysis by Šedo and colleagues in 2018, applied to a set of 204 taxonomically well-defined strains across 17 Acinetobacter species, demonstrated that out-of-the-box routine MALDI-TOF identification on commercial Bruker BioTyper databases does not reliably resolve several recently described species and that remedial actions in the form of in-house database expansion are required for routine practice to capture the full diversity of the genus(39) (Šedo et al., 2018). These four methodological studies collectively explain why the present laboratory's pre-2020 Acinetobacter inventory was effectively limited to A. baumannii and A. lwoffii — the database currency available before the routine deployment of MALDI-TOF resolved only the two most common species — and why the post-2020 emergence of more than 14 named Acinetobacter species, including A. lactucae, A. variabilis and A. soli, represents a transfer of the database-augmentation capacity validated in those studies into the laboratory's routine output.

The two large clinical-isolate routine-practice series available as direct comparators for the present Acinetobacter expansion are the Korean multicentre series of Jeong and colleagues, in which 729 routine clinical Acinetobacter isolates from multiple Korean university hospitals were processed by MALDI-TOF MS with explicit per-species counts spanning thirteen named species (447 A. baumannii, 146 A. nosocomialis, 78 A. pittii, 18 A. ursingii, 9 A. bereziniae, 9 A. soli and a long tail of less common entries; (43) Jeong et al., 2016), and the Chinese clinical-laboratory series of Li and colleagues, in which 788 Acinetobacter clinical isolates confirmed by 16S rDNA and rpoB sequencing were resolved by MALDI-TOF MS into nineteen named species, with 637 A. baumannii and a non-baumannii inventory including A. pittii, A. johnsonii, A. junii, A. lwoffii, A. ursingii, A. nosocomialis, A. parvus, A. calcoaceticus, A. haemolyticus, A. tjernbergiae, A.

schindleri, A. radioresistens, A. soli, A. colistiniresistens, A. bereziniae, A. gyllenbergii, A. seifertii and A. variabilis alongside one putative novel species(44). The Korean and Chinese routine-practice inventories are quantitatively dissimilar to the present dataset — both report substantially larger Acinetobacter workloads than this single Moroccan laboratory recovered over six years — but their species-distribution shapes are closely aligned with what the present study recorded post-2020: a dominant A. baumannii component flanked by a long tail of named non-baumannii species drawn from the same taxonomic territory enumerated in the methodological studies cited above. The species-composition alignment between the present 2023 inventory and these two routine-practice series is presented in Discussion Figure 4.



Li 2018 reported only A. baumannii vs an aggregated "other named non-baumannii" category (19.2%), without per-species breakdown. Present-study cohort n = 1,373.

Discussion Figure 4. Acinetobacter species composition: present study post-MALDI inventory (2021–2023) versus Jeong et al. (2016) Korean multicentre cohort versus Li et al. (2018) Chinese clinical-laboratory cohort, plotted on a percent-of-cohort axis.

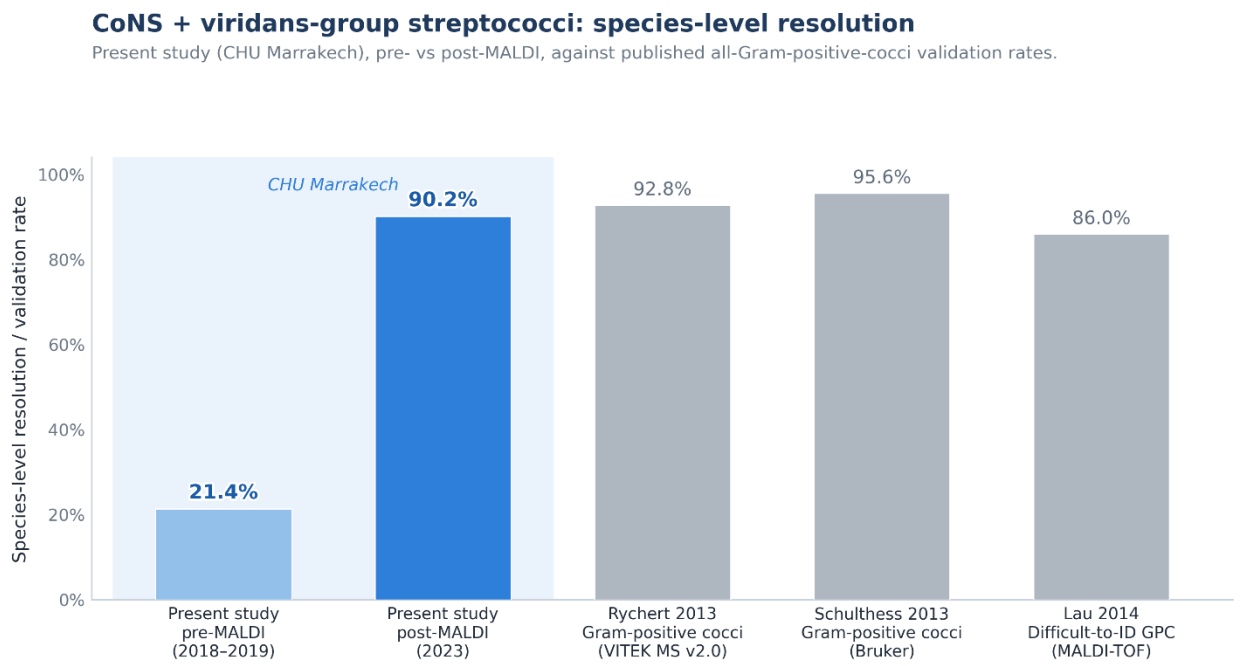
2. Gram-Positive Cocci

The 112 distinct Gram-positive cocci recorded across the six-year window include, beyond the two bucket-collapse trajectories already discussed in VI.2.2, several smaller observations that warrant separate mention. The Streptococcus anginosus group, framed in section IV.3.1 of the existing text as a clinically distinct subset of the viridans aggregate associated with abscess formation, registered 88 isolates in 2022 and is entirely absent from the 2018–2020 inventory — a clean illustration of the principle that the bucket-collapse described in VI.2.2 is not a uniform redistribution but proceeds through the targeted resolution of clinically defined subgroups. The enterococcal inventory, conceptually limited to E. faecalis and E. faecium in the pre-MALDI workflow, expanded post-2020 to include E. avium, E. hirae, E. durans and E. raffinosus, of which only the first appears in any volume. The technical capacity of the spectrometric platform for routine identification of Gram-positive aerobic bacteria, including enterococci, has been established under controlled conditions by two independent multicentre and single-centre validation series cited here as performance peers for the present observation. Rychert and colleagues evaluated the VITEK MS v2.0 platform across multiple US sites for routine clinical Gram-positive aerobic bacteria, with 1,146 isolates spanning 13 genera and 42 species processed in parallel with conventional reference identification, achieving 92.8% species-level identification(45). Schulthess and colleagues prospectively analysed 1,619 routine clinical Gram-positive cocci at the University Hospital Zurich using MALDI-TOF MS in parallel with conventional methods and reported a species-level concordance of 95.6%, with the enterococcal subset reaching above 99% concordance (27). The expanded enterococcal inventory observed in the present laboratory after MALDI-TOF integration is consistent with the species-level resolution that both validation series established for this category under controlled conditions, transferred from those evaluations into the routine output of a single tertiary-care laboratory.

Within the rare and fastidious GPC inventory described in section IV.3.3 of the existing text, the recovery of Aerococcus viridans, Rothia mucilaginosa and several named Gemella species in the present dataset is the empirical correlate of the species-level capacity that the difficult-to-identify Gram-positive cocci series of Lau and colleagues established under

controlled conditions, with 1,371 routine clinical isolates of fastidious or unusual organisms processed by MALDI-TOF MS at a single laboratory and approximately 86% identified to species level(6). The S. lugdunensis identification, framed in section IV.3.3 of the existing text as a clinically critical discrimination within the coagulase-negative staphylococci on account of its distinctive virulence profile, is supported in the present dataset by the post-2020 resolution of the CoNS bucket described in VI.2.2 and is anchored to the same validation literature that supports the broader CoNS species-level resolution argument; the species-level concordance for S. lugdunensis specifically reached 100% in the Schulthess series and is reported in the Argemi before-and-after analysis as one of the named CoNS species whose recovery rose substantially under MALDI-TOF, confirming the clinical relevance of its routine identification (34).

The species-level resolution gain in the two bucket-collapse subgroups of the Gram-positive cocci group (CoNS and viridans-group streptococci) is presented in Discussion Figure 5 alongside the species-level identification rates published by the three GPC validation series cited above; the figure includes an explicit scope qualification given that the present-study rate is restricted to CoNS+viridans whereas the comparator series cover broader GPC scopes

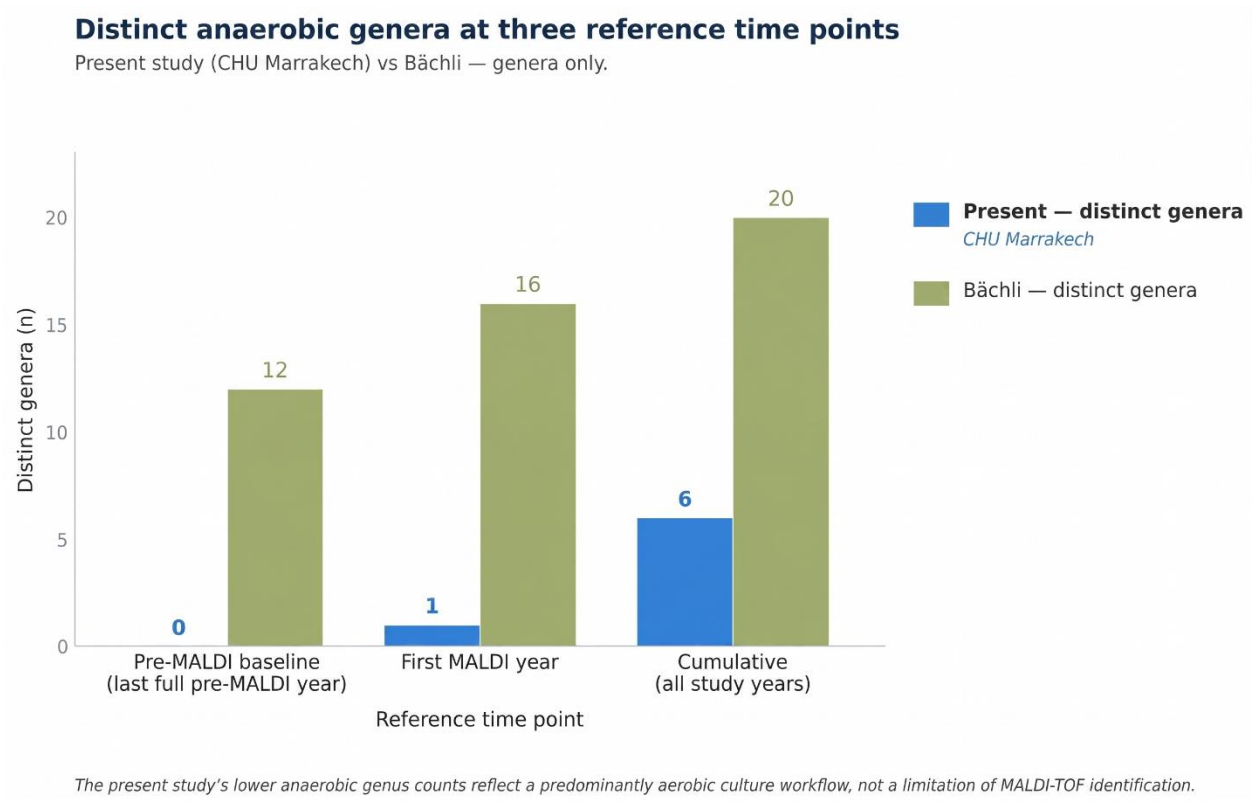


Discussion Figure 5. Species-level resolution rate of CoNS and viridans-group streptococci in the present study (pre-MALDI versus post-MALDI) versus three published Gram-positive cocci validation series (Rychert et al., 2013; Schulthess et al., 2013; Lau et al., 2014).

3. Anaerobic Bacteria

The 16 distinct anaerobic species recorded across the six years constitute the smallest taxonomic component of the dataset, and the interpretive task here is one of scope rather than performance. Bacteroides fragilis is the only anaerobe consistently recovered across both diagnostic phases, and the remaining inventory — Cutibacterium acnes, C. avidum, Actinomyces oris, A. odontolyticus, A. naeslundii, Schaalia turicensis, S. radingae, Clostridium perfringens, C. tertium, Fusobacterium necrophorum, Prevotella bivia, Bifidobacterium breve and Actinotignum schaalii — appears predominantly or exclusively in the post-2020 window, with isolate counts in the single digits for each entry. The principal interpretive point, already framed in section IV.4 of the existing text, is that this scarcity is an upstream methodological feature of the laboratory's predominantly aerobic-only routine culture workflow rather than a measure of the spectrometric platform's capacity to identify anaerobes.

The closest published peer for the anaerobe component of the present diagnostic transition is the longitudinal analysis of Bächli and colleagues at the Institute of Medical Microbiology of the University of Zurich, in which the impact of MALDI-TOF MS integration on anaerobic species and genus diversity was assessed across the laboratory's 2008–2020 routine activity, with the diversity of recorded anaerobes rising from 12 genera and 20 species in 2012 (the year before MALDI-TOF integration at that laboratory) to 16 genera and 31 species in 2013 and to 20 genera and 41 species in 2020 after seven additional years of MALDI-era operation(46). The Zurich trajectory is structurally homologous to the present observation: a baseline anaerobic inventory dominated by a small number of frequently recovered organisms is expanded post-MALDI by the routine identification of species previously absorbed into genus-level reporting categories or referred out to specialised laboratories. The Zurich series differs from the present study in two substantive respects that are worth identifying explicitly. First, the Zurich laboratory operates an active anaerobic culture programme that produces a substantially larger anaerobic isolate volume per year than the present laboratory's predominantly aerobic-only workflow generates, with the result that the post-MALDI anaerobic inventory the Zurich series records (41 species in 2020) substantially exceeds the 16 distinct anaerobic species captured across six years in the present dataset. Second, the Zurich series confirms its MALDI-TOF identifications against partial 16S rRNA gene sequencing for discrepant or low-confidence isolates, a confirmatory step the present study could not perform. The post-2020 emergence of Cutibacterium, Schaalia and Actinotignum in the present inventory — taxonomic categories created or reorganised by molecular work in the recent literature — does, however, indicate that, when anaerobic culture is performed and yields a viable isolate, the laboratory's MALDI-TOF workflow assigns the organism to the current taxonomic nomenclature rather than to the older Propionibacterium and Actinomyces categories that conventional phenotypic galleries would have applied; this is consistent with the Zurich finding that the post-MALDI anaerobic inventory expansion is driven not by new culture recoveries but by the spectrometric resolution of organisms previously reported under outdated or genus-only labels.



Discussion Figure 6. Anaerobe diversity trajectory: present study (2018–2023) versus Bächli et al. (2022) thirteen-year longitudinal anaerobe MALDI-TOF impact analysis at the Institute of Medical Microbiology, University of Zurich.

4. Yeasts

The 22 distinct yeast species recorded over the six-year window display the same overall pattern observed in the bacterial groups — a stable dominant species across both diagnostic phases coexisting with a rapidly expanding rare tail in the MALDI era. Candida albicans, recovered in every year of the study, anchors the inventory. The non-albicans Candida component is taken up in detail as part of the bucket-collapse argument of section II.2 above, where it is read against the species-distribution comparators of Spanu and colleagues for direct-from-broth identification(35) and Taj-Aldeen and colleagues for the regional Middle Eastern candidaemia context(36); the present subsection therefore concentrates on the rare non-Candida yeast component, the Candida parapsilosis-complex resolution, and the contextual scope of the present yeast inventory.

The rare non-Candida yeast component of the present dataset includes Magnusiomyces capitatus, Trichosporon asahii, Cyberlindnera fabianii, Saccharomyces cerevisiae and Geotrichum

candidum, each appearing as a MALDI-era detection with isolate counts in the single digits. The published reference framework for spectrometric identification of these arthroconidial and uncommon yeasts is the dual-platform validation of Kolecka and colleagues, in which 134 reference isolates of medically relevant arthroconidial yeasts spanning the genera Trichosporon, Magnusiomyces, Saprochaete and Geotrichum were processed by both the Bruker Biotyper and the bioMérieux VITEK MS platforms against ITS- and D1/D2-sequencing references, with the augmented in-house spectral databases achieving species-level identification rates above 96% for both systems(47). The same authors documented that out-of-the-box manufacturer databases at the time of that study covered these uncommon yeast genera substantially less reliably than the augmented databases, with several rare species — including Magnusiomyces capitatus and Trichosporon asahii — requiring database augmentation to be identified to species level. The post-2020 emergence of these same rare yeast genera in the present laboratory's routine inventory is the empirical correlate of the database-currency improvement that Kolecka and colleagues identified as the determining factor for routine recognition of these organisms; that the laboratory's MBT IVD library, by 2023, was assigning routine clinical isolates to Magnusiomyces capitatus, Trichosporon asahii, Cyberlindnera fabianii and Saccharomyces cerevisiae directly rather than referring them out as unidentified yeasts indicates that the database augmentation validated by Kolecka has been progressively transferred into the manufacturer's commercial reference library over the intervening decade.

The complementary published comparator for the routine yeast workflow is the two-laboratory series of Sendid and colleagues, conducted at the Dijon and Lille University Hospitals, in which 1,207 routine clinical yeast isolates representing 28 species were processed by MALDI-TOF MS in parallel with the API ID 32C galleries used as the conventional reference with 97.5% of isolates correctly identified to species level by mass spectrometry (1,178 of 1,207 isolates correctly identified, rising to 99% after a second extraction cycle for difficult isolates)(48). The Sendid series is the closest published peer of the present routine yeast workflow because it analyses the full clinical-laboratory yeast workload of two French university hospitals over a defined period rather than restricting itself to a single yeast genus or to a specialised specimen

type, and because its species inventory — C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. lusitaniae, C. kefyr, C. dubliniensis, plus the rare yeasts Magnusiomyces capitatus, Trichosporon spp., Saccharomyces cerevisiae and Cryptococcus neoformans among others — closely overlaps the cumulative yeast inventory of the present study. Two specific points of alignment are worth flagging. First, the Sendid series specifically validated MALDI-TOF resolution of the C. parapsilosis / C. metapsilosis / C. orthopsilosis complex, demonstrating that the spectrometric platform reliably distinguishes the three sister species under routine conditions; the present laboratory recovered three isolates of C. orthopsilosis in 2021 as a discrete entry alongside C. parapsilosis sensu stricto, while C. metapsilosis was not recorded — a partial empirical demonstration of the same complex-level resolution. Second, the Sendid series reported that the principal categories of species-level error under routine MALDI-TOF workflow involved closely related yeast pairs and were resolved by repeat analysis or by ITS sequencing of discrepant isolates. The present study did not perform such confirmatory ITS sequencing in-house, a limitation already acknowledged in section V of this Discussion and pertinent to the interpretation of the rare-yeast detections enumerated above.

Cryptococcus neoformans was recovered across both phases, with the eight pre-MALDI isolates of 2018 reflecting the conventional capsule-staining and biochemical workflow applicable to this organism. Eleven cumulative isolates of Aspergillus spp., concentrated in 2023, are recorded as opportunistic moulds recovered alongside yeasts on standard media; filamentous fungi were not the primary focus of the analysis, and their identification by MALDI-TOF mass spectrometry is conceptually framed in section III.7 of the existing text rather than developed further here. Finally, it should be reported as a feature of dataset scope rather than as a finding that no isolate of Candida auris, the emerging multidrug-resistant species whose global spread has prompted active surveillance recommendations in many jurisdictions, was recovered in the laboratory's six-year inventory; systematic active surveillance for this organism was not part of the routine workflow during the study period, and the absence reported here therefore carries no epidemiological weight.

Yeast species	Cases (n)	Present *CHU Marrakech*	Kolecka 2013	Status
Trichosporon asahii	3	✓	✓	Shared
Magnusiomyces capitatus	9	✓	✓	Shared
Saccharomyces cerevisiae	4	✓	–	Present only
Geotrichum candidum	1	✓	✓	Shared
Cyberlindnera fabianii	1	✓	–	Present only
Clavispora lusitaniae	15	✓	–	Present only
Trichosporon spp. (other)	–	–	✓	Kolecka only
Magnusiomyces clavatus	–	–	✓	Kolecka only
Saprochaete capitata	–	–	✓	Kolecka only
Kalamiella piersonii	1	✓	–	Present only
Rhodotorula spp.	1	✓	–	Present only
Cryptococcus neoformans	47	✓	–	Present only

✓ present in inventory – not detected bar length ∝ present-study case count

Rare non-Candida yeast presence in the present cumulative 2018–2023 inventory compared with the Kolecka et al. (2013) reference inventory of arthroconidial yeasts.

Discussion Figure 7. Rare non-Candida yeast presence in the present cumulative 2018–2023 inventory compared with the Kolecka et al. (2013) reference inventory of arthroconidial yeasts.

IV. Specimen-Type Ecology

The largest proportional gains in distinct-organism diversity between 2018 and 2023 are recorded not in the high-volume specimen categories but in the lower-volume contexts that constitute the device-associated and deep-site ecology of a tertiary-care laboratory. Catheter tips registered a 2.43-fold increase in distinct organisms recovered, serous body fluids a 2.39-fold increase, deep respiratory specimens a 2.32-fold increase and expectorated sputum a 2.20-fold increase across the six-year window (Table 7). The three high-volume categories — pus and wound specimens, blood cultures and urine cultures — recorded smaller fold changes of 1.98, 1.56 and 1.50 respectively, reflecting both the higher pre-MALDI baseline diversity these categories already exhibited and the proportionally smaller scope for additional rare-tail species to be added by the spectrometric workflow. The single category to record a decline in distinct-organism diversity, stool cultures (0.67-fold), likely reflects a shift from conventional stool culture to gastrointestinal multiplex PCR testing for enteric pathogen detection, resulting in

fewer organisms being recovered by culture rather than any change in MALDI-TOF identification capacity for that specimen type.

The interpretive significance of the fold-change pattern is twofold. First, it documents a redistribution of identification depth toward the lower-volume but higher-acuity specimen categories where the rare and fastidious organisms enumerated in section IV of the existing text are most likely to be encountered — device-associated isolates, body-fluid taps and bronchoalveolar specimens. Second, it indicates that the diversity expansion observed in the dataset as a whole, which section II.1 framed as a near-doubling of distinct organisms per year, is not uniformly distributed across specimen types but concentrated in the contexts where conventional phenotypic methods had previously been least productive.

V. Methodological Considerations Specific to the Laboratory's Workflow

The interpretation of the findings reported in section II is conditioned in several specific ways by the methodological configuration of the laboratory across the study period, as set out in detail in section IV of the existing text. The Bruker MALDI Biotyper platform, deployed from 2020 onwards under the MBT Compass IVD software and the associated MBT IVD reference library, served as the first-line identification tool throughout the MALDI era of the dataset. Sample preparation followed the three-protocol hierarchy described in section III.4, with the direct transfer protocol applied to the majority of routine isolates, the direct transfer with on-target formic acid lysis used preferentially for Gram-positive cocci and yeasts, and the full ethanol-formic acid extraction reserved as a rescue protocol and applied selectively to fastidious organisms requiring enhanced protein extraction. Score interpretation followed the manufacturer's standard thresholds described in section III.5, with a log score of $\geq$2.0 accepted as reliable species-level identification, scores of 1.7 to $<$2.0 accepted as genus-level only, and scores below 1.7 considered unreliable. For Streptococcus pneumoniae specifically, the laboratory's confirmatory workflow throughout the study period included optochin sensitivity testing, which provides an independent phenotypic confirmation of the identification independently of the spectrometric workflow.

Two specific constraints follow from this configuration and should be kept in view when reading section II. First, the absence of in-house 16S rRNA gene sequencing — set out in section III of the existing text and acknowledged as a methodological feature of the present study in the Materials and Methods — means that the species-level identifications reported here rest on MALDI-TOF score thresholds and on the laboratory's existing phenotypic confirmatory practices, not on a molecular gold-standard reference. The findings of sections II.3 and III, in particular the first-time empirical detections of Eikenella corrodens, Kingella kingae, Aggregatibacter segnis and the rare haemophili enumerated there, and the post-2020 expansion of the Acinetobacter and Pseudomonas non-aeruginosa inventories, are therefore claims about the spectrometric platform's capacity to resolve organisms that the prior phenotypic workflow had been unable to identify, not claims about a molecularly confirmed taxonomic assignment. The published comparator series cited in section III.1 — the Korean cohort of Jeong and the Chinese cohort of Li for Acinetobacter, both confirmed against 16S rDNA and rpoB sequencing — should be read with this asymmetry in view.

Second, the precise version of the MBT IVD reference library in use at each point during Phase II was not retrievable from the laboratory records available for this retrospective review, as noted in the Materials and Methods. Database expansion delivered by the manufacturer over the course of Phase II progressively increased the reference coverage available to the laboratory; this temporal feature of the library — common to all routine clinical MALDI-TOF deployments and described in IV.7 of the existing text — should be borne in mind when interpreting the appearance of recently described species such as Staphylococcus borealis (formally named in 2020 and recovered in 2023) or the named Acinetobacter species A. lactucae, A. variabilis and A. soli, all of which are demonstrations of database currency rather than of the laboratory's spectrometric workflow having identified an organism that was outside the platform's capacity in the early part of Phase II. The methodological framework articulated in the Acinetobacter literature reviewed in VI.3.1 — the spectral-processing improvements established by Šedo and colleagues in 2013, the Acb-complex differentiation framework of Espinal and colleagues, the validation of all ten species in the Toh series, the database augmentation strategies of Marí-

Almirall and colleagues for A. seifertii and A. dijkshoorniae, and the routine-practice limitations and remedial actions documented by Šedo and colleagues in 2018 — supplies the technical context in which the database-currency observation made above should be read: the manufacturer's commercial reference library has progressively absorbed the validation work of these methodological studies, and the post-2020 inventory expansion observed in the present laboratory is the operational manifestation of that absorption.

A further methodological consideration applies specifically to the bucket-collapse rates presented in Discussion Figure 5. The species-level resolution rate computed for the present study (21.4% pre-MALDI rising to 90.2% post-MALDI) is restricted to the two Gram-positive cocci subgroups for which the source workbook tracks unspeciated reporting buckets against speciated counts — coagulase-negative staphylococci and viridans-group streptococci — whereas the three comparator validation series cited in the same figure(6,27,45) report species-level identification rates across broader Gram-positive cocci scopes. The comparison plotted in Discussion Figure 5 is therefore scope-bounded rather than like-for-like, and the broad alignment of the present-study post-MALDI rate (90.2%) with the rates reported by Rychert (92.8%) and Schulthess (95.6%) and the present-study rate's modest excess over Lau (86.0%) should be read as a coarse confirmation that the laboratory's routine-practice resolution within the two bucket-collapse subgroups falls within the range of validation-set performance reported in the literature, not as an equivalent or superior performance metric. Turnaround time from sample reception to identification result, an additional methodological dimension along which the present laboratory's workflow could be characterised, was not measured during the study period and is acknowledged as a limitation of the present analysis in section VI.

VI. Strengths and Limitations

The principal strengths of the present study lie in the structural features of the dataset assembled rather than in any single analytical claim made from it. The six-year continuous coverage of the laboratory's identification activity, distributed across both diagnostic phases under the institutional timeline set out in the Materials and Methods, provides a clean before-and-after split rather than the snapshot view that is typical of single-centre MALDI-TOF evaluations; the 41,796 isolates resolved into the canonical inventory place the study at the larger end of the published single-centre routine MALDI-TOF series, as visualised in Discussion Figure 1, and align in design with the only published longitudinal peer of comparable scope, the eleven-year Marseille analysis of Seng and colleagues. The taxonomic breadth of the dataset — Gram-negative bacilli, Gram-positive cocci, Gram-positive bacilli, yeasts and obligate anaerobes — and the eleven specimen categories tracked together describe the diagnostic activity of a national-referral tertiary-care laboratory whose case-mix is broad rather than artificially narrowed, lending external validity to the descriptive observations reported. The canonical normalisation pipeline, applied uniformly to the data of all six years before any analysis, ensures that the year-on-year comparisons drawn in section II are not artefacts of nomenclatural drift across the period. The dataset is, by design, a record of routine laboratory output rather than a curated validation set, and the bucket-collapse phenomenon described in VI.2.2 and visualised in Discussion Figure 2 is precisely the kind of operational signature that only routine data can surface; the parallel rise in CoNS species-level resolution observed here (20.0% to 89.2%) and at Strasbourg University Hospital under the before-and-after analysis of Argemi and colleagues (14.0% to 85.0%) provides external corroboration that this signature is generalisable rather than local. The detection of recently described species — Staphylococcus borealis, formally named in 2020 and recovered in 2023, and the named Acinetobacter species A. lactucae, A. variabilis and A. soli, alongside Mixta calida and other recent reclassifications — provides direct empirical evidence that the laboratory's MBT IVD reference library was current with the contemporary taxonomic literature throughout the latter part of the study period.

The limitations are best stated explicitly. The retrospective design of the study, while necessary for the assembly of a six-year continuous dataset, prevents formal verification of record completeness, and the cautious phrasing applied throughout section III of the Results reflects this constraint. No molecular confirmation of identifications was performed in-house, in particular no 16S rRNA gene sequencing, with the consequence that the identification reliability rests on MALDI-TOF score thresholds and on the laboratory's existing phenotypic confirmatory practices rather than on a molecular gold-standard reference, as discussed in section V; this is a sharper constraint when the present-study findings are read alongside the Acinetobacter and HACEK comparator series cited in VI.3, several of which (37,38,43,44,48) used 16S rDNA, rpoB, ITS or DNA-sequence references to confirm their MALDI-TOF identifications, and the same level of molecular corroboration cannot be claimed for the present dataset. The exact version of the MBT IVD reference library in use at each point during Phase II was not retrievable from the laboratory records, and the database currency observations made in section II and reiterated as a strength above are therefore inferences from species-level recoveries rather than from a documented version mapping. Turnaround times from sample reception to identification result were not measured during the study period; the qualitative claims made in passing about the speed of identification under the MALDI-TOF workflow are therefore consistent with the published comparator literature reviewed in section III.6 of the existing text but cannot be supported by any quantitative measure internal to the present dataset. The year 2020, treated as a transitional partial-rollout year throughout the analysis, coincided in time with the early COVID-19 pandemic period and its operational disruption to the laboratory; the difficulty of disentangling the partial-rollout effect from the pandemic effect on the 2020 figures has been noted at every relevant point in section III and constitutes an irreducible interpretive ambiguity for that single year. The 2019 multidrug-resistant bacteria rate anomaly, identified during data preparation and noted in the Results, remains methodologically unresolved.

Several further limitations apply to specific subsections of this Discussion. The anaerobic inventory described in section III.3 is shaped by the laboratory's predominantly aerobic-only routine culture practice and is therefore a measure of the upstream specimen-handling

workflow, not of the spectrometric platform's anaerobe identification capacity; the comparison with Bächli and colleagues at the Institute of Medical Microbiology of the University of Zurich, presented in Discussion Figure 6, makes this constraint visible rather than concealing it, since the Zurich laboratory operates an active anaerobic culture programme that the present laboratory does not. The Sendid Dijon–Lille comparator used in VI.3.4 reports species-level concordance against API ID32C as the conventional reference rather than against ITS sequencing for the entire cohort, and the present-study yeast inventory is therefore read against a comparator whose own gold-standard reference is itself a phenotypic method for the majority of isolates, with molecular confirmation reserved for discrepant cases. The Pseudomonas non-aeruginosa speciation expansion described in VI.3.1 is reported without a routine-practice peer-comparator because no published series of comparable design was identified during the literature search underlying this Discussion; the expansion is therefore documented as a feature of the present laboratory's database currency rather than as a finding referenced to an external benchmark, and this asymmetry between the Acinetobacter and Pseudomonas threads of VI.3.1 should be acknowledged. The Candida species composition comparator analysis presented in Discussion Figure 2 Panel B reveals a notable divergence between the present-study C. glabrata proportion (4.6% of the speciated Candida cohort) and the proportions reported by Spanu and colleagues (7.6%) and by Taj-Aldeen and colleagues (18.9%); the present study cannot determine whether this divergence reflects a true regional epidemiological feature of the catchment population, a difference in selective antifungal pressure, or a residual database-currency or specimen-handling effect, and it is reported here as an observation requiring further investigation rather than as a finding with a settled interpretation. Mycobacteria are excluded from the dataset, as set out in the Materials and Methods, and no quantitative claim about mycobacterial identification can be drawn from the present work; conceptual mention of this category is confined to section IV.6 of the existing text. Clinical impact on patient management — instances in which a MALDI-TOF identification prompted a modification of antimicrobial therapy or further clinical investigation — was not systematically analysed, as set out in section 7 of the Materials and Methods, and any clinical illustrations elsewhere in the thesis are therefore presented as

illustrative cases rather than as a formally analysed outcome. Finally, the data assembled here are those of a single laboratory operating under a specific national-referral case-mix, and the generalisability of the descriptive findings to other settings is not established by the present design.

CONCLUSION

The methodological priority for the laboratory going forward, identified in the present discussion, is the introduction of in-house 16S rRNA gene sequencing as a confirmatory tool for the discordant, low-discrimination, and unidentified isolates that the spectrometric workflow currently refers, when necessary, to external reference laboratories.

The second priority is the systematic measurement of turnaround times in any future audit of the laboratory's identification activity. The qualitative claims made throughout this study about the speed of MALDI-TOF identification cannot be substantiated by the present dataset, and warrant direct empirical verification under the laboratory's own conditions.

Beyond these two methodological priorities, the extended applications of the platform—such as direct identification from positive blood-culture broths, and the detection of antimicrobial resistance mechanisms through hydrolysis assays and growth-inhibition modules—represent the natural development trajectory for the laboratory's MALDI-TOF deployment. While neither formed part of the routine workflow during the study period, both are well-placed to contribute to the diagnostic turnaround for bloodstream infections and to the rapid resistance characterisation of high-acuity isolates in the years ahead.

Read alongside the laboratory's empirical findings, the present work supports the proposition that mass spectrometric identification, as a routine first-line tool, transforms not only the accuracy of the most common identifications and the overall variety of species detected, but also the diagnostic visibility of the rare and fastidious organisms whose role in clinical microbiology has been progressively acknowledged, but whose routine identification has historically been the limiting step.

ABSTRACT

Abstract

Background. Accurate identification of rare and fastidious microorganisms remains a persistent challenge for conventional phenotypic methods, whose limited databases and dependence on metabolic reactivity frequently leave such organisms unidentified or resolved only to genus level. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) has been proposed as a faster and more discriminating alternative. This work evaluates its diagnostic contribution within the routine microbiology laboratory of a tertiary referral hospital.

Methods. We conducted a retrospective, single-centre, observational study in the Microbiology Laboratory of Mohammed VI University Hospital, Marrakesh, over the six-year period from 2018 to 2023. After exclusion of duplicate, quality-control and reference isolates, 42,070 microbial isolates were retained. The study period spans the institutional transition from conventional phenotypic identification (2018–2019), through a transitional year of partial deployment (2020), to routine first-line use of MALDI-TOF MS (2021–2023). The spectrum of organisms recovered and the depth of identification were compared across these diagnostic phases.

Results. The retained isolates resolved to 406 distinct canonical organisms — 187 Gram-negative bacilli, 112 Gram-positive cocci, 69 Gram-positive bacilli, 22 yeasts and 16 anaerobes. Whilst total isolate volume increased 1.84-fold across the period, the number of distinct organisms recovered per year almost doubled, rising from about 200 in 2018–2019 to between 329 and 448 in 2021–2023; this diversity gain was independent of throughput, as the distinct-organism-per-1,000-isolates ratio rose from 19.6–24.8 to a stable 24.9–26.7. The singleton fraction rose from 27.6% to 38.3%, and more than 250 species were recorded exclusively from 2020 onwards. Genus-level diagnostic categories contracted markedly as species-level resolution improved: the unspeciated coagulase-negative staphylococci, *viridans*-group streptococci and *Candida* buckets all declined as the corresponding named species expanded.

Conclusion. Across the six-year period, the introduction of MALDI-TOF MS was associated with a substantial broadening of the laboratory's diagnostic inventory and a marked improvement in the species-level resolution of rare and fastidious organisms that conventional pheno-

typic methods had previously left unresolved. These findings support MALDI-TOF MS as a transformative first-line identification tool in a tertiary referral setting.

Keywords: MALDI-TOF mass spectrometry; rare microorganisms; fastidious microorganisms; bacterial identification; clinical microbiology; species-level identification.

Résumé

Introduction. L'identification précise des micro-organismes rares et exigeants demeure un défi persistant pour les méthodes phénotypiques conventionnelles, dont les bases de données limitées et la dépendance à la réactivité métabolique laissent fréquemment ces organismes non identifiés ou identifiés au seul niveau du genre. La spectrométrie de masse de type MALDI-TOF (désorption-ionisation laser assistée par matrice, couplée à un analyseur à temps de vol) a été proposée comme une alternative plus rapide et plus discriminante. Ce travail évalue sa contribution diagnostique au sein du laboratoire de microbiologie de routine d'un centre hospitalier universitaire de référence.

Méthodes. Nous avons mené une étude rétrospective, monocentrique et observationnelle au Laboratoire de Microbiologie du Centre Hospitalier Universitaire Mohammed VI de Marrakech, sur une période de six ans, de 2018 à 2023. Après exclusion des isolats dupliqués, des souches de contrôle qualité et des souches de référence, 42 070 isolats microbiens ont été retenus. La période d'étude couvre la transition institutionnelle de l'identification phénotypique conventionnelle (2018–2019), à travers une année de déploiement partiel (2020), jusqu'à l'utilisation de routine en première intention du MALDI-TOF MS (2021–2023). Le spectre des organismes isolés et la profondeur de l'identification ont été comparés entre ces phases diagnostiques.

Résultats. Les isolats retenus correspondaient à 406 organismes canoniques distincts — 187 bacilles à Gram négatif, 112 cocci à Gram positif, 69 bacilles à Gram positif, 22 levures et 16 anaérobies. Alors que le volume total d'isolats augmentait d'un facteur 1,84 sur la période, le nombre d'organismes distincts identifiés par an a presque doublé, passant d'environ 200 en 2018–2019 à un intervalle de 329 à 448 en 2021–2023 ; ce gain de diversité était indépendant du débit, le ratio d'organismes distincts pour 1 000 isolats passant de 19,6–24,8 à une valeur stable de 24,9–26,7. La fraction de singletons est passée de 27,6 % à 38,3 %, et plus de 250 espèces n'ont été recensées qu'à partir de 2020. Les catégories diagnostiques au niveau du genre

se sont nettement réduites à mesure que la résolution au niveau de l'espèce s'améliorait : les regroupements non spéciés de staphylocoques à coagulase négative, de streptocoques du groupe *viridans* et de *Candida* ont tous diminué tandis que les espèces nommées correspondantes augmentaient.

Conclusion. Sur l'ensemble de la période de six ans, l'introduction du MALDI-TOF MS s'est accompagnée d'un élargissement substantiel de l'inventaire diagnostique du laboratoire et d'une amélioration marquée de la résolution au niveau de l'espèce des organismes rares et exigeants que les méthodes phénotypiques conventionnelles laissaient auparavant non résolus. Ces résultats confortent le rôle du MALDI-TOF MS comme outil d'identification de première intention à fort impact dans un centre hospitalier universitaire de référence.

Mots-clés : spectrométrie de masse MALDI-TOF ; micro-organismes rares ; micro-organismes exigeants ; identification bactérienne ; microbiologie clinique ; identification au niveau de l'espèce.

ملخص

المقدمة: لا يزال التعرّف الدقيق على الكائنات الدقيقة النادرة والصعبة الاستنبات يمثّل تحدياً مستمراً أمام الطرق المظهرية التقليدية، إذ كثيراً ما تؤدي محدوديةُ قواعد بياناتها واعتمادُها على التفاعل الأيضي إلى ترك هذه الكائنات دون تحديد أو تحديدها على مستوى الجنس فقط. وقد طُرحت مطيافيةُ الكتلة من نوع ***MALDI-TOF*** (التأيُّن بالامتزاز الليزري بمساعدة المادة الحاملة، المقترن بمحلِّل زمن التحليق) كبديلٍ أسرع وأكثر قدرةً على التمييز. يهدف هذا العمل إلى تقييم إسهامها التشخيصي داخل مختبر الميكروبيولوجيا الروتيني لمركزٍ استشفائي جامعي مرجعي.

المنهجية: أجرينا دراسةً استعاديةً أحاديةَ المركز ورصديةً في مختبر الميكروبيولوجيا بالمركز الاستشفائي الجامعي محمد السادس بمراكش، على مدى ست سنوات من 2018 إلى 2023. وبعد استبعاد العزلات المكرَّرة وسلالات ضبط الجودة والسلالات المرجعية، تم الاحتفاظ بـ 42 070 عزلةً ميكروبية. تغطّي فترةُ الدراسة الانتقالَ المؤسسي من التحديد المظهري التقليدي (2018-2019)، مروراً بسنةٍ انتقالية من النشر الجزئي (2020)، وصولاً إلى الاستخدام الروتيني كخطٍّ أول لتقنية ***MALDI-TOF (2021-2023).*** وقد قُورن طيفُ الكائنات المعزولة وعمقُ التحديد عبر هذه المراحل التشخيصية.

النتائج : توزّعت العزلاتُ المحتفظ بها على 406 كائنات متمايزة — 187 عصيّةً سلبيةَ الغرام، و112 مكوّراً إيجابيَّ الغرام، و69 عصيّةً إيجابيةَ الغرام، و22 خميرة، و16 كائناً لاهوائياً. وبينما ارتفع الحجم الإجمالي للعزلات بمعامل 1.84 خلال الفترة، فإن عدد الكائنات المتمايزة المحدَّدة سنوياً تضاعف تقريباً، إذ ارتفع من نحو 200 في 2018-2019 إلى ما بين 329 و448 في 2021-2023؛ وكان هذا المكسبُ في التنوّع مستقلاً عن حجم العمل، حيث ارتفع معدّلُ الكائنات المتمايزة لكل 1000 عزلة من 19.6-24.8 إلى قيمةٍ مستقرة تتراوح بين 24.9 و26.7. وارتفعت نسبةُ الكائنات الفردية ***(singletons)*** من 27.6 % إلى 38.3 %، وسُجِّل أكثر من 250 نوعاً حصرياً ابتداءً من سنة 2020. وتقلّصت بوضوحٍ الفئاتُ التشخيصية على مستوى الجنس مع تحسُّن الدقة على مستوى النوع: إذ تراجعت المجموعاتُ غير المحدَّدة نوعياً من المكورات العنقودية السلبية للتخثُّر، والمكورات العقدية من مجموعة ***viridans*** ، وفطريات ***Candida*** ، في مقابل تزايد الأنواع المسمَّاة المقابلة لها.

الخلاصة: على امتداد فترة الست سنوات، ارتبط إدخالُ تقنية ***MALDI-TOF*** باتساعٍ ملموسٍ في الحصيلة التشخيصية للمختبر وبتحسُّنٍ واضحٍ في الدقة على مستوى النوع للكائنات النادرة والصعبة الاستنبات التي كانت الطرق المظهرية التقليدية تتركها دون تحديد. وتدعم هذه النتائجُ اعتمادَ تقنية ***MALDI-TOF*** كأداة تحديدٍ من الخط الأول ذات أثرٍ تحويلي في سياق مركزٍ استشفائي جامعي مرجعي.

الكلمات المفتاحية: مطيافية الكتلة ***MALDI-TOF*** ؛ الكائنات الدقيقة النادرة؛ الكائنات الدقيقة الصعبة الاستنبات؛ التحديد البكتيري؛ الميكروبيولوجيا السريرية؛ التحديد على مستوى النوع.

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قسم الطبيب

أقْسِم بِالله العَظِيم

أن أراقبَ الله في مِهْنَتِي.

وأن أصُونَ حياة الإنسان في كآفّةِ أطوَارهَا في كل الظروف

والأحَوال باذلا وسْعِي في انقاذها مِن الهَلاكِ والمرَضِ

والألَم والقَلَق.

وأن أحفَظَ لِلنَاسِ كرَامَتهُم، وأسْتر عَوْرَتهُم، وأكتمَ سِرَّهُمْ.

وأن أكونَ عَلى الدوَام من وسائِل رحمة الله، باذلا رعايتي الطبية للقريب والبعيد،

للصالح والطالح، والصديق والعدو.

وأن أثابر على طلب العلم، وأسَخِّرَه لِنَفْعِ الإنْسَان لا لأذَاه.

وأن أُوَقّرَ مَن عَلَّمَني، وأُعَلّمَ مَن يَصْغرَني، وأكون أخا لِكُلِّ زَميلٍ في المِهنَةِ الطِّبِّيَة

مُتعَاونِينَ عَلى البرِّ والتقوى.

وأن تكون حياتي مِصْدَاق إيمَاني في سِرّي وَعَلانيَتي،

نَقِيَّة مِمّا يُشينهَا تجَاهَ الله وَرَسُولِهِ وَالمؤمِنين.

والله على ما أقول شهيد

كليـــــــة الطب
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MARRAKECH
جامعة القاضي عياض
UNIVERSITÉ CADI AYYAD

السنة 2026 الاطروحة رقم 186

مساهمة قياس طيف الكتلة في التعرف على البكتيريا

الأطروحة

قُدمت ونوقشت علانية يوم 23 يونيو 2026

من طرف

السيد بومعزي عادل

طبيب داخلي سابق بالمركز الاستشفائي الجامعي بمراكش

المزداد في 5 ماي 1999

لنيل شهادة الدكتوراه في الطب

الكلمات المفتاحية

التعرف على الميكروبات – الكائنات الحية – MALDI-TOF قياس طيف الكتلة الدقيقة النادرة والصعبة النمو

اللجنة

السيدة	ن .صراع أستاذة فيعلم الأحياء الدقيقة وعلم الفيروسات.	الرئيس
السيدة	أ .العمراني حنشي أستاذة في علم الأحياء الدقيقة وعلم الفيروسات .	المشرف
السيدة	ف .بناوي أستاذة في طب الأطفال	الحكام
السيدة	م .الغزالي أستاذة فيالتخدير والإنعاش	
السيدة	م .إدالين أستاذة في الأمراض التعفنية	