Culturomics; back to basics in microbiology in the genomic era

Student: Veranja Liyanapathirana
Supervisor: Prof M. Ip
Joint Graduate Seminar Dec 2013
Department of Microbiology,
Faculty of Medicine
The Chinese University of Hong Kong

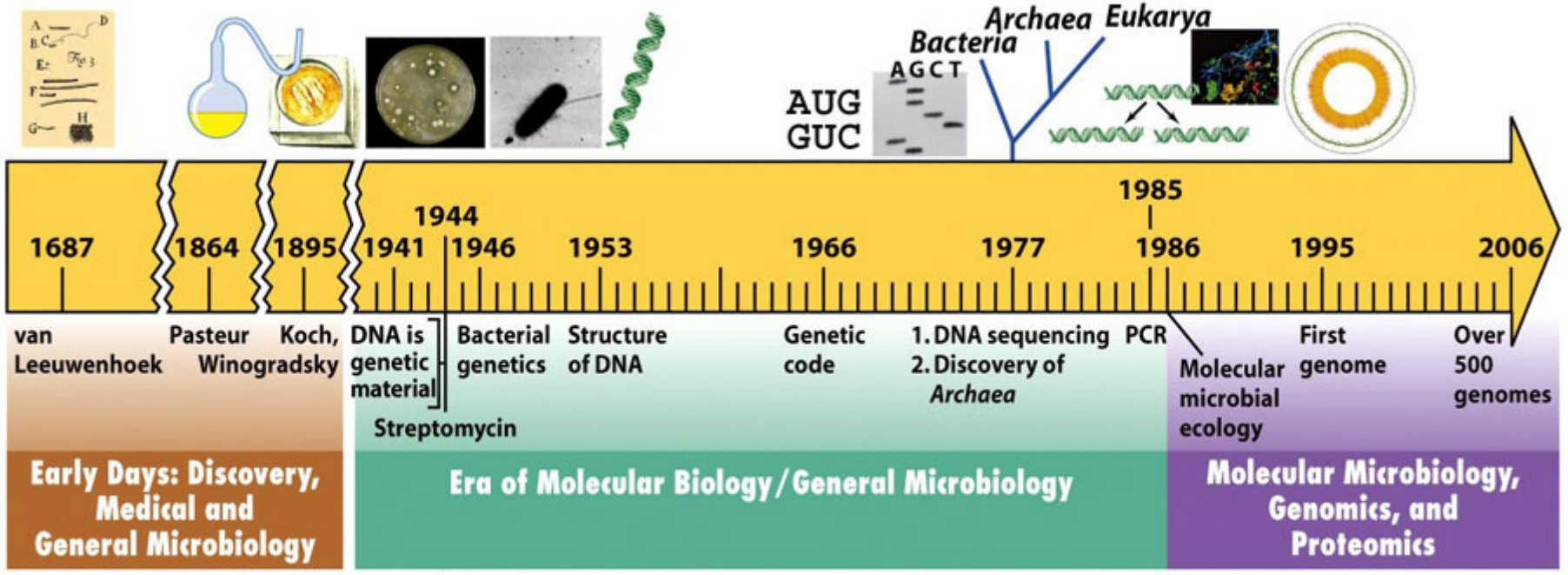
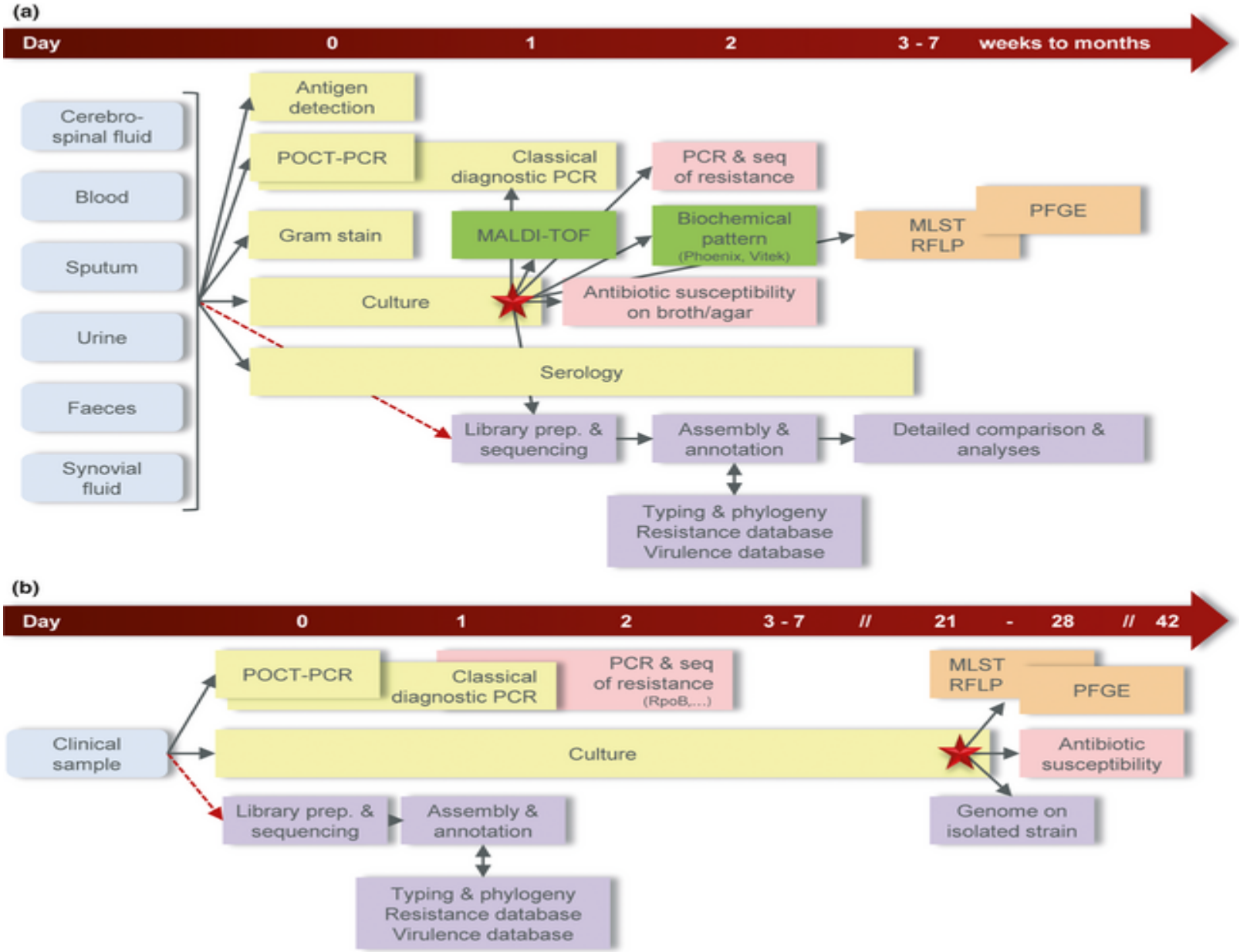


Figure 1-17 Brock Biology of Microorganisms 11/e

- NGS and MALDI TOF
- High throughput and laboratory automation
- Characterization of single microorganisms to characterization of microbial communities in different physiological and disease conditions

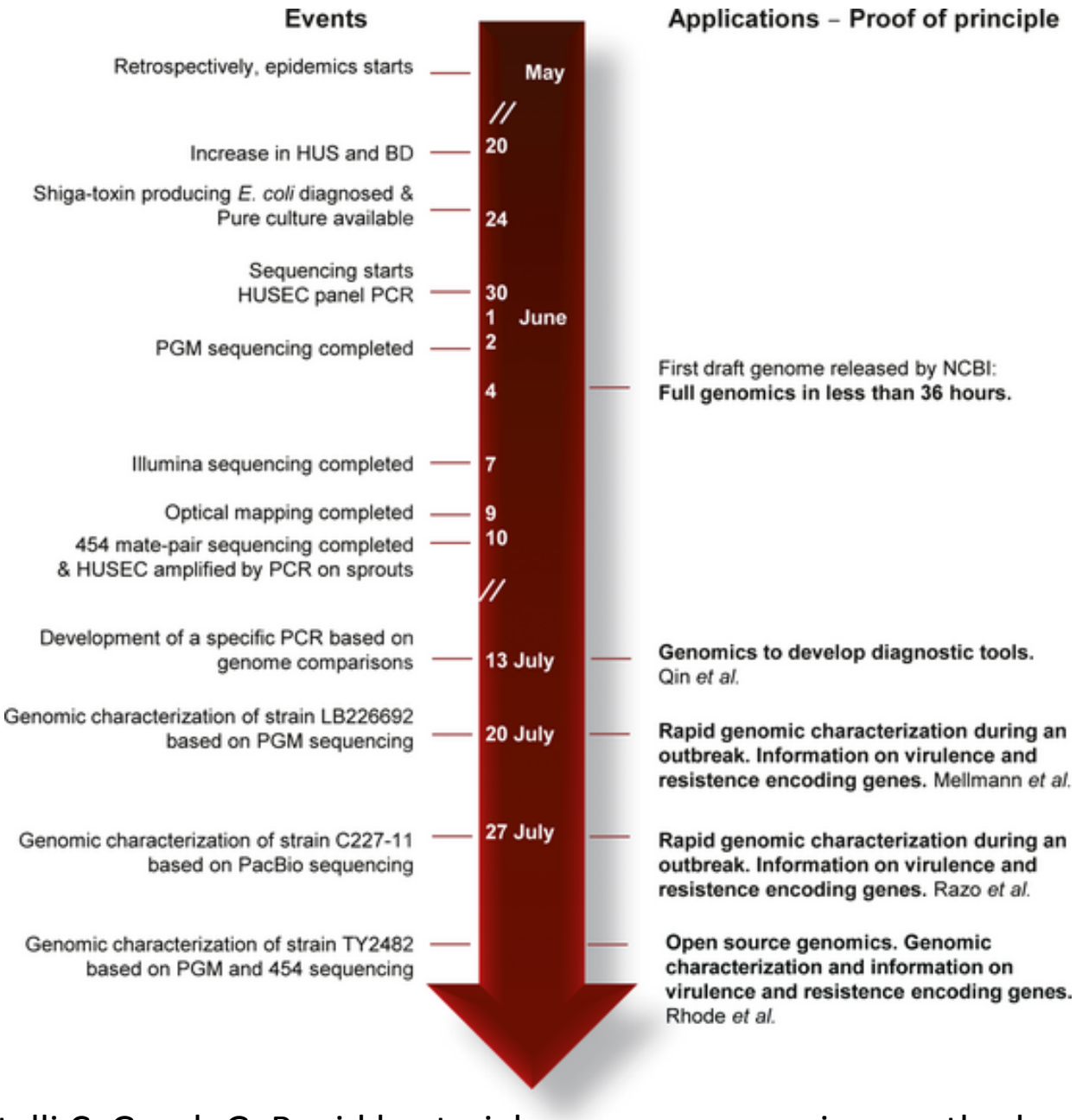
NGS



Bertelli C, Greub G. Rapid bacterial genome sequencing: methods and applications in clinical microbiology. Clin Microbiol Infect. 2013 Sep;19(9):803-13.

A genomic day in the life of a clinical microbiology laboratory. Long SW, et al. Journal of Clinical Microbiology. 2013 April 01;51(4):1272-7.

- Tested the feasibility of using WGS in routine clinical microbiology workflow
- 130 isolates from 116 patient samples were tested with WGS either from pure colonies or primary plates
- Genomic DNA extracted using DNeasy 96 blood and tissue kit (Qiagen), libraries constructed using NexteraXT DNA (Illumina) (12h) sequenced using Miseq (Illumina) bench top sequencer (2 x 250 protocol 39 h) and assembled de novo using the on instrument software (Miseq reporter) (2 to 4 h).
- Contigs analyzed by BLAST to identify high homology matches in the NT database and Ref seq database.
- Even lower read counts give accurate ID, ability to identify individual bacteria from mixed cultures,
- Shortcomings identified – need to improve extraction methods, improve the databases,
- ?susceptibility profiles, expenses, time taken



Bertelli C, Greub G. Rapid bacterial genome sequencing: methods and applications in clinical microbiology. Clin Microbiol Infect. 2013 Sep;19(9):803-13.

Metagenomics

- Characterizes microbial communities from the nucleic acid that are directly extracted from the samples
- By passes the need to isolate or culture bacteria
- Marked improvement with the emergence of next generation sequencing technologies
- In addition to profiling the organisms, transcriptome and network analysis reveal the active networks functional at the given environmental conditions

Deep amplicon sequencing: variable regions in 16s RNA, rpoB, cpn 60 and viral RNA polymerase

Advantages

- Higher sensitivity due to target enrichment
- Comparatively cheaper

Disadvantages

- Targeted gene may not be truly universal
- Primer bias
- Variant gene copy numbers

Metagenomic shot gun sequencing

Advantages

- Sequences of all microorganisms can be potentially recovered
- No need of previous knowledge on what microorganisms may be encountered

Disadvantages

- Decreased sensitivity ?
- Library preparation and bioinformatics analysis is complex
- Relatively more expensive
- Un accounted for sequences and host sequences

Adapted from: Miller RR, Montoya V, Gardy JL, Patrick DM, Tang P. Metagenomics for pathogen detection in public health. Genome Med. 2013 Sep 20;5(9):81

The need for an isolate

- Despite the emergence of molecular methods, and rapid automation, culture based diagnosis still remains the corner stone in diagnosing infectious diseases
- The need for an isolate
    - Complete whole genome sequences
    - Further characterizations of pathogenic, metabolic and antimicrobial susceptibility profiles
    - Development of diagnostic tests
    - Proving causation
    - “compare with gold standard”

Re-emergence of culture based methods

- 1. Provision of the natural environment
- 2. Use the available annotated sequences to identify components that are essential for microbial growth in novel media
- 3. Change the incubation conditions and time

- Cultivation of previously uncultivated bacteria
- Cultivation of species thought as being un cultivable axenically

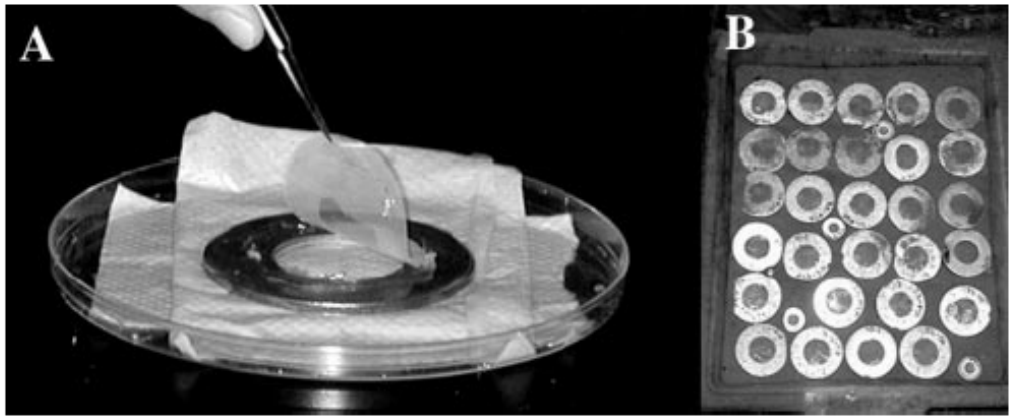
Science
AAAS

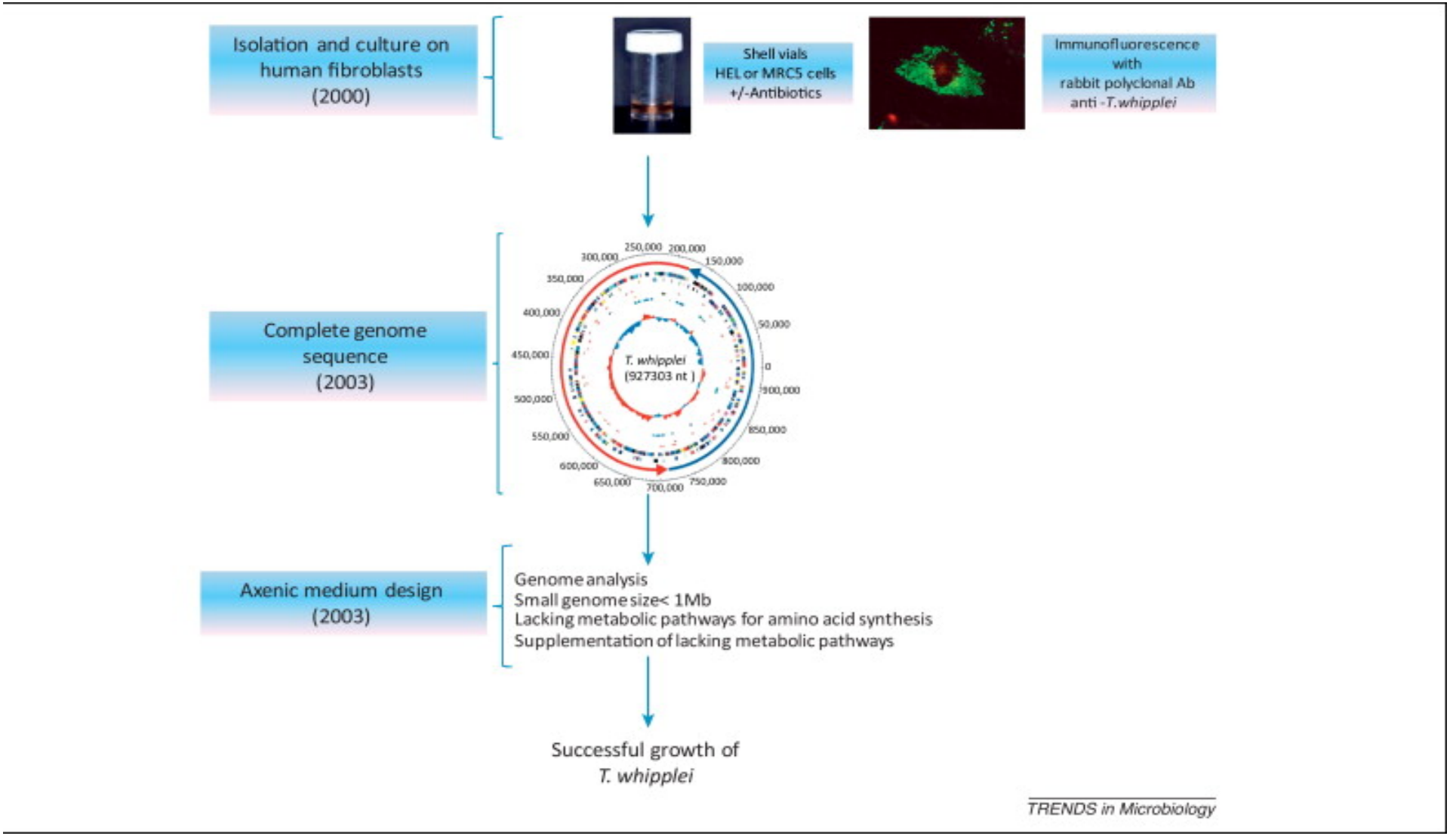
Isolating "Uncultivable" Microorganisms in Pure Culture in a Simulated Natural Environment

T. Kaeberlein *et al.*

Science **296**, 1127 (2002);
DOI: 10.1126/science.1070633

- Uncultivable microorganisms might grow in pure culture if provided with the chemical components of their natural environment
- Microorganisms separated by the sediment, serially diluted and mixed with warm agar prepared with sea water, the assembled diffusion chambers were incubated in mock natural environments
- Successful isolation of novel bacteria previously not described
- Novel bacteria could grow on artificial media only in the presence of other community members.





Strategy for the design of Tropheryma whipplei axenic medium. Circular representation of the T. whipplei twist genome, reproduced, with permission, from <ce:cross-ref refid="bib0145"> [29]</ce:cross-ref> . Abbreviation: Ab, antibody.

Sudhir Singh , Carole Eldin , Malgorzata Kowalczewska , Didier Raoult

Axenic culture of fastidious and intracellular bacteria

Trends in Microbiology Volume 21, Issue 2 2013 92 - 99
http://dx.doi.org/10.1016/j.tim.2012.10.007

Directed culturing of microorganisms using metatranscriptomics. Bomar L et al. mBio. 2011 April 29;2(2).

- *Hirudo verbana* (medicinal leech) simple symbiotic gut microbiome in its “crop"(*Aeromonas veronii* and a *Rikenella*-like bacterium)
- One component eluded culture despite repeated attempt
- Metatranscriptome of the leech at the time of estimated active proliferation of the elusive bacteria
- High expression levels of the mucin and glycan utilization genes – identification of source of energy
- Modification of Eggerth-Gagnon medium by replacing glucose with mucin from bovine submaxillary glands
- Successful isolation and confirmation of *Rikenella*-like bacterium

CMI
CLINICAL MICROBIOLOGY AND INFECTION

ORIGINAL ARTICLE

Microbial culturomics: paradigm shift in the human gut microbiome study

J.-C. Lagier[1,†], F. Armougom[1,†], M. Million[1], P. Hugon[1], I. Pagnier[1], C. Robert[1], F. Bittar[1], G. Fournous[1], G. Gimenez[1], M. Maraninchi[2], J.-F. Trape[3], E. V. Koonin[4], B. La Scola[1], D. Raoult[1]

Article first published online: 3 OCT 2012

DOI: 10.1111/1469-0691.12023

Issue

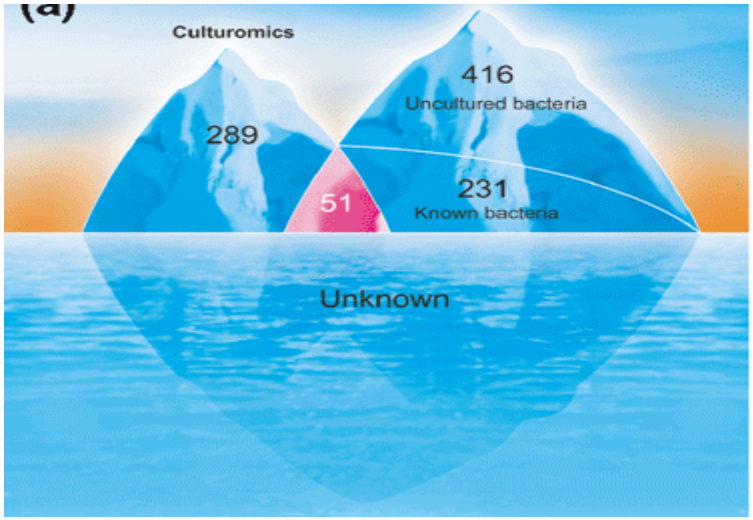
Clinical Microbiology and Infection

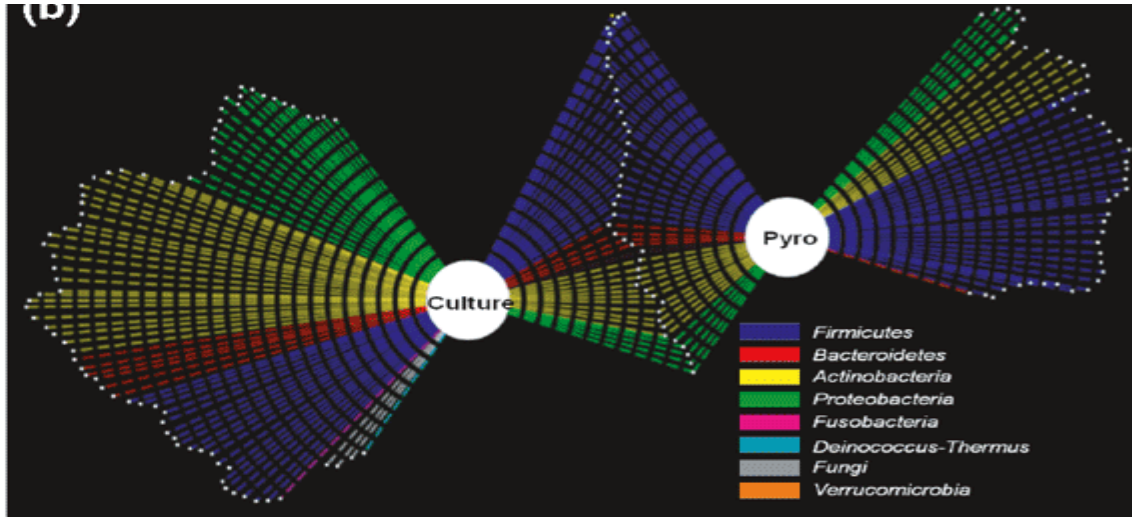
Volume 18, Issue 12, pages 1185–1193, December 2012

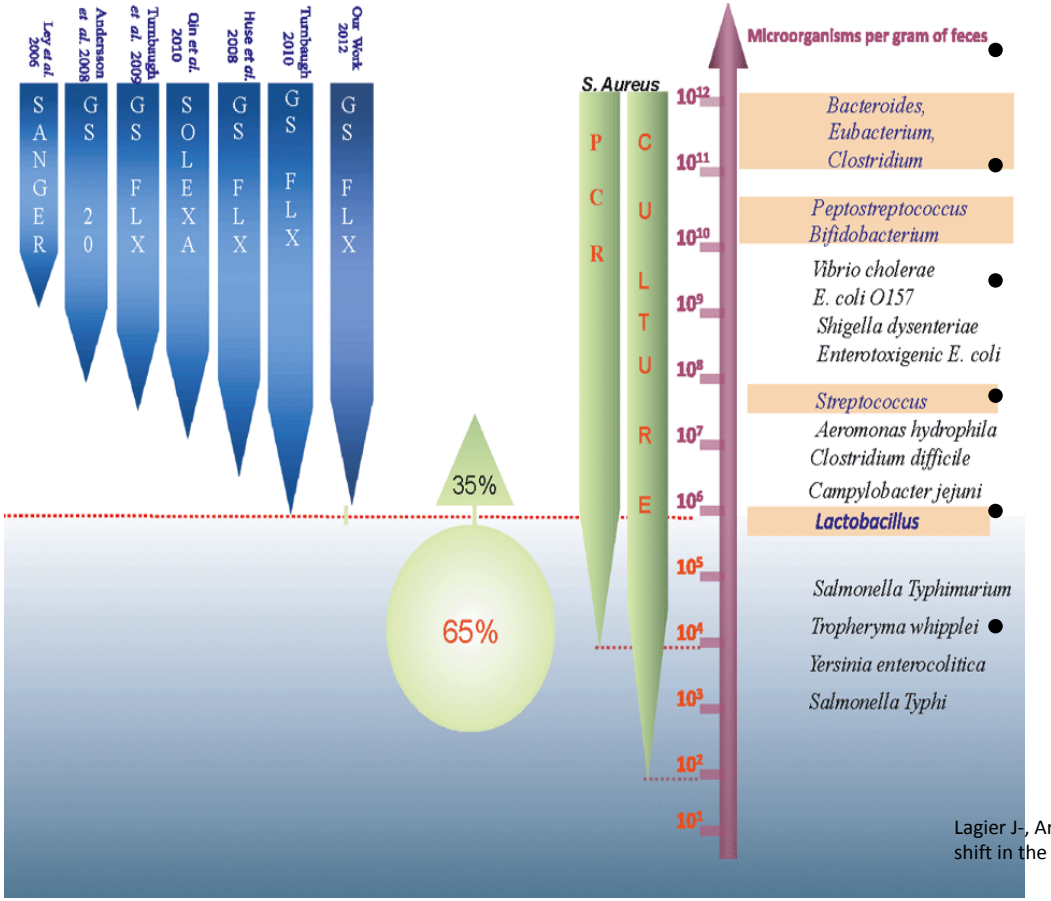
Two stool specimen from healthy young Senegalese males and stool specimen from a French obese individual
212 culture conditions used with variations in physicochemical conditions, pre-incubation in blood culture bottles, rumen fluid and sterile stool extract to mimic the natural environment , co culturing with Amoeba spp for fastidious bacterial
Strategies to select minority population: active and passive filtration, and bacteriophages.
Cultures observed on day 1, one week, two weeks and 1 month after incubation
Total no of colonies studied: 32500
Identification strategies: MALDI-TOF MS
For un identified colonies the 16S rRNA gene sequencing

Comparison: pyro sequencing of a 16S rRNA amplicon targeting the V6 region, the most variable region,
Novel viruses and bacteria: The new bacterial genera and species were sequenced

Individual	Isolates	16s rRNA sequencing phylotypes	
1 Healthy	99 bacterial spp, 42 have not been found in human gut before, 2 novel spp	126 previously known	416 previously unknown
2 Obese	191 bacterial spp, 2 new genera and 6 new spp	138 previously known	
3 Healthy	219 bacterial spp, 5 fungi, 3 new genera, 18 new spp	157 previously known	







- 340 bacterial spp identified in total by culturonomics
- Total 17 new taxa not cultivable by routine axenic methods
- Culturomic core microbiome had only 45 spp
- 100% of species grew in 70 conditions and 73% grew under 20 conditions
- Only 51 species were commonly represented in both methods
- Most metagenomic + culture – ones were anaerobes

Lagier J-, Armougom F, Million M, Hugon P, Pagnier I, Robert C, et al. Microbial culturomics: Paradigm shift in the human gut microbiome study. Clinical Microbiology and Infection. 2012;18(12):1185-93.

- 1: Pfleiderer A, Lagier JC, Armougom F, Robert C, Vialettes B, Raoult D. Culturomics identified 11 new bacterial species from a single anorexia nervosa stool sample. Eur J Clin Microbiol Infect Dis. 2013 Nov;32(11):1471-81. doi: 10.1007/s10096-013-1900-2. Epub 2013 Jun 2. PubMed PMID: 23728738.
- 2: Hugon P, Mishra AK, Lagier JC, Nguyen TT, Couderc C, Raoult D, Fournier PE. Non-contiguous finished genome sequence and description of Brevibacillus massiliensis sp. nov. Stand Genomic Sci. 2013 Apr 15;8(1):1-14. doi: 10.4056/sigs.3466975. eCollection 2013 Apr 15. PubMed PMID: 23961307; PubMed Central PMCID: PMC3739172.
- 3: Ramasamy D, Lagier JC, Gorlas A, Raoult D, Fournier PE. Non contiguous-finished genome sequence and description of Bacillus massiliosenegalensis sp. nov. Stand Genomic Sci. 2013 Jun 5;8(2):264-78. doi: 10.4056/sigs.3496989. PubMed PMID: 23991258; PubMed Central PMCID: PMC3746431.
- 4: Mishra AK, Hugon P, Lagier JC, Nguyen TT, Couderc C, Raoult D, Fournier PE. Non contiguous-finished genome sequence and description of Enorma massiliensis gen. nov., sp. nov., a new member of the Family Coriobacteriaceae. Stand Genomic Sci. 2013 Jun 6;8(2):290-305. doi: 10.4056/sigs.3426906. PubMed PMID: 23991260; PubMed Central PMCID: PMC3746427.
- 5: Mishra AK, Lagier JC, Robert C, Raoult D, Fournier PE. Genome sequence and description of Timonella senegalensis gen. nov., sp. nov., a new member of the suborder Micrococcinae. Stand Genomic Sci. 2013 Jun 13;8(2):318-35. doi: 10.4056/sigs.3476977. PubMed PMID: 23991262; PubMed Central PMCID: PMC3746429.
- 6: Ramasamy D, Lagier JC, Nguyen TT, Raoult D, Fournier PE. Non contiguous-finished genome sequence and description of Dielma fastidiosa gen. nov., sp. nov., a new member of the Family Erysipelotrichaceae. Stand Genomic Sci. 2013 Jun 13;8(2):336-51. doi: 10.4056/sigs.3567059. PubMed PMID: 23991263; PubMed Central PMCID: PMC3746426.
- 7: Hugon P, Ramasamy D, Lagier JC, Rivet R, Couderc C, Raoult D, Fournier PE. Non contiguous-finished genome sequence and description of Alistipes obesi sp. nov. Stand Genomic Sci. 2013 Feb 25;7(3):427-39. doi: 10.4056/sigs.3336746. PubMed PMID: 24019990; PubMed Central PMCID: PMC3764931.

- Same methodology applied on further atypical stool samples in order to identify hitherto unknown bacterial spp

European Journal of Clinical Microbiology & Infectious Diseases
May 2013, Volume 32, Issue 5, pp 637-645

The gut microbiota of a patient with resistant tuberculosis is more comprehensively studied by culturomics than by metagenomics

G. Dubourg, J. C. Lagier, F. Armougom, C. Robert, I. Hamad, P. Brouqui, D. Raoult

European Journal of Clinical Microbiology & Infectious Diseases
November 2013, Volume 32, Issue 11, pp 1471-1481

Culturomics identified 11 new bacterial species from a single anorexia nervosa stool sample

A. Pfleiderer, J.-C. Lagier, F. Armougom, C. Robert, B. Vialettes, D. Raoult

Patient with MDR TB

- Patient: 63 year old female with MDR TB treated with an extensively large group to antibiotics
- 9 g of stool collected and stored in aliquots at – 80 ^{o}C.
- Serially diluted aliquots cultured at 70 pre selected culture conditions selected to isolate a diverse bacterial range
- Novel culture media to suite the patients gut physiology created by adding different antibiotics

Patient with anorexia nervosa

- Patient: 21 year old Caucasian female with severe restrictive anorexia for 9 years
- 97 g of stool, aliquoted and stored at – 80 ^{o}C
- Serially diluted aliquots cultured at 88 pre selected culture conditions selected to isolate a diverse bacterial range
- In addition to methods mentioned in the primary study, empirical new media based on the products used in the patients dietary habits were included

Patient with MDR TB

- 4000 isolates tested using MALDI TOF spectrometry
- Maximum 100 peaks used for identification, compared to a database updated from previous studies
- None identified colonies verified by 16s rRNA and spectra added to the database
- Novel species were whole genome sequenced and assembled de novo
- Fungal identification by direct sequencing of 18s RNA and ITS
- Electron microscopy, gram staining and bacterial counting performed on selected aliquots
- Pyrosequencing performed on DNA extracted directly from stool samples by sequencing v6 region

Patient with anorexia nervosa

- 12700 isolates tested using MALDI TOF spectrometry
- Maximum 100 peaks used for identification, compared to a database updated from previous studies
- None identified colonies verified by 16s rRNA and spectra added to the database
- Novel species were whole genome sequenced and assembled de novo
- Fungal identification by direct sequencing of ITS1 and ITS4R
- Pyrosequencing performed on DNA extracted directly from stool samples by sequencing v6 region

Patient with MDR TB

- 39 bacterial species isolated
- 3 have not been previously described from the human gut
- One novel species identified
- The culture condition producing the best yield was pre incubation in an anaerobic blood culture bottle with sheep rumen and sheep blood
- Gram staining and electron microscopy did not reveal any bacterial spp
- Pyrosequencing resulted in 89469 reads from 4 phyla. (19 OTUs)
- Only 23 isolates were commonly identified in both groups

Patient with anorexia nervosa

- 133 bacterial species including 11 new ones described
- 36 bacterial species in addition to the ones previously described from the human gut by culturomics
- The novel bacterial species included 9 new species from three phyla and two new genera
- The culture condition producing the best yield was pre incubation in an anaerobic blood culture bottle with thioglycate
- Pyrosequencing resulted in 83950 reads from 7 phyla (1268 OTUs)
- Only 2 isolates were commonly identified in both groups

TABLE I. Comparison between metagenomics and culturomics

	Metagenomics	Culturomics
Definition	Method allowing the description of the microbial composition by high-throughput sequencing	Method allowing the description of the microbial composition by high-throughput culture
Methodology	Pyrosequencing of 16S rRNA amplicons and/or direct metagenomics without amplification step	Use of various selective and/or enrichment culture conditions coupled to MALDI-TOF MS identification
Limitations	Does not provide a strain for further studies Misses minority population (depth bias)[a] Only detects eubacteria[b] Does not provide information on enzymatic abilities[b]	Misses so-called 'non-cultivable' microorganisms Does not directly provide information on enzymatic abilities Major workload
Advantages	Detects 'non-cultivable' microorganisms	Detect minority populations Open approach Detects only viable bacteria[c]
Rate of success	Approximately 200 bacterial species/sample[d]	Approximately 100 bacterial species/sample[d]
Possible future developments	Increased depth of sequencing because of new technology Coupling pyrosequencing with direct metagenomics	Automated detection of microbial growth[e] Automated identification[f] Miniaturization Other innovative culture conditions

MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.
[a]Limitation of direct metagenomics (no amplification step).
[b]Limitation of pyrosequencing of 16S rRNA amplicons.
[c]Relevance of dead microorganisms lower than that of viable microorganisms (no metabolic activity).
[d]Mean numbers of bacterial species recovered from one stool sample by Lagier *et al.* [7] when comparing both approaches.
[e]Smart incubators, pH indicators in broth, microcalorimetry, etc.
[f]Automated colony picking coupled to MALDI-TOF MS and/or full laboratory automation.

Greub G. Culturomics: a new approach to study the human microbiome. Clin Microbiol Infect. 2012 Dec;18(12):1157-9.

Future and impact on clinical microbiology

- Identification of pre determined culture conditions from combined WGS (metabolic pathways), metagenomic (diversity and depth) and culturomic approach (identification of the exact media needed and the spectrum of organisms)
- Development of culture panels needed for syndromic diagnostics accordingly

References

1. - Gan M, - Su J, - Wang J, - Wu H, - Chen L. - A scalable microfluidic chip for bacterial suspension culture. - Lab Chip. (- 23):- 4087.

2. Bertelli C, Greub G. Rapid bacterial genome sequencing: Methods and applications in clinical microbiology. Clinical Microbiology and Infection. 2013;19(9):803-13.

3. Bomar L, Maltz M, Colston S, Graf J. Directed culturing of microorganisms using metatranscriptomics. mBio. 2011 April 29;2(2).

4. Dubourg G, Lagier JC, Armougom F, Robert C, Hamad I, Brouqui P, et al. The proof of concept that culturomics can be superior to metagenomics to study atypical stool samples. Eur J Clin Microbiol Infect Dis. 2013 Aug;32(8):1099.

5. Dubourg G, Lagier JC, Armougom F, Robert C, Hamad I, Brouqui P, et al. The gut microbiota of a patient with resistant tuberculosis is more comprehensively studied by culturomics than by metagenomics. Eur J Clin Microbiol Infect Dis. 2013 May;32(5):637-45.

6. Fournier PE, Drancourt M, Colson P, Rolain JM, La Scola B, Raoult D. Modern clinical microbiology: New challenges and solutions. Nat Rev Microbiol. 2013 Aug;11(8):574-85.

7. Goodman AL, Kallstrom G, Faith JJ, Reyes A, Moore A, Dantas G, et al. Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. Proceedings of the National Academy of Sciences. 2011 March 21.

8. Greub G. Culturomics: A new approach to study the human microbiome. Clinical Microbiology and Infection. 2012;18(12):1157-9.

9. Ingham CJ, Sprenkels A, Bomer J, Molenaar D, van den Berg A, van Hylckama Vlieg JET, et al. The micro-petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms. Proceedings of the National Academy of Sciences. 2007 November 13;104(46):18217-22.

10. Kaeberlein T, Lewis K, Epstein SS. Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science. 2002 Ma 10;296(5570):1127-9.

11. Lagier J-, Armougom F, Million M, Hugon P, Pagnier I, Robert C, et al. Microbial culturomics: Paradigm shift in the human gut microbiome study. Clinical Microbiology and Infection. 2012;18(12):1185-93.

12. Long SW, Williams D, Valson C, Cantu CC, Cernoch P, Musser JM, et al. A genomic day in the life of a clinical microbiology laboratory. Journal of Clinical Microbiology. 2013 April 01;51(4):1272-7.

13. Miller RR, Montoya V, Gardy JL, Patrick DM, Tang P. Metagenomics for pathogen detection in public health. Genome Med. 2013 Sep 20;5(9):81.

14. Pfleiderer A, Lagier JC, Armougom F, Robert C, Vialettes B, Raoult D. Culturomics identified 11 new bacterial species from a single anorexia nervosa stool sample. Eur J Clin Microbiol Infect Dis. 2013 Nov;32(11):1471-81.

15 Renesto P, Crapoulet N, Ogata H, La Scola B, Vestris G, Claverie JM, et al. Genome-based design of a cell-free culture medium for tropheryma whipplei. Lancet. 2003 Aug 9;362(9382):447-9.

16. Seng P, Abat C, Rolain JM, Colson P, Lagier JC, Gouriet F, et al. Identification of rare pathogenic bacteria in a clinical microbiology laboratory: Impact of matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol. 2013 Jul;51(7):2182-94.

17. Singh S, Eldin C, Kowalczewska M, Raoult D. Axenic culture of fastidious and intracellular bacteria. Trends Microbiol. 2013 2;21(2):92-9.

Thank you!