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Efficient elimination of airborne pathogens: a study on aerosolized *Mycobacterium tuberculosis* and SARS-CoV-2 using ZeBox technology

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SUMMARY

Background: Despite multifactorial evidence, the safe and effective elimination of free-floating micro-organisms remains a significant scientific challenge. ZeBox Technology exploits microbial Zeta Potential, to extract and eliminate them from free-flowing air, using a non-ionizing electric field, in combination with a microbicidal surface.
Aim: Evaluation of ZeBox Technology against aerosolized SARS-CoV-2 and *Mycobacterium tuberculosis* under controlled conditions.
Methods: SARS-CoV-2 and *M. tuberculosis* H37Ra were used in this study. Individual micro-organisms were aerosolized using a Collison nebulizer inside an air-sealed test chamber. Air samples were collected from the chamber on to a Mixed Cellulose Ester membrane, at various time points, and used for enumeration. SARS-CoV-2 was enumerated using qRT-PCR, while *M. tuberculosis* H37Ra was quantified using standard microbiological procedures.
Findings: We established a viable aerosolized microbial load of ~10E9 and ~10E6 for SARS-CoV-2 and *M. tuberculosis* H37Ra, respectively, inside the test chamber. Under ideal conditions, the floating microbial load was at a steady-state level of 10E9 for SARS-CoV-2 and 10E6 for *M. tuberculosis*. When the ZeBox-Technology-enabled device was operated, the microbial load reduced significantly. A reduction of ~10E4.7 was observed for *M. tuberculosis*, while a reduction of ~10E7 for SARS-CoV-2 was observed within a short duration. The reduction in airborne SARS-CoV-2 load was qualitatively and quantitatively measured using fluorescence analysis and qRT-PCR methods, respectively.
Conclusion: This validation demonstrates the efficacy of the developed technology against two of the deadliest micro-organisms that claim millions of lives worldwide. In conjunction with the existing reports, the present validation proved the true broad-spectrum elimination capability of ZeBox technology.

Introduction

Infection-causing bioaerosols that comprise micro-organisms (viruses, bacteria, fungi), often transmitted interpersonally as airborne particles, have been known to the

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medical fraternity for over a century. This phenomenon is now in the public limelight due to the COVID-19 pandemic [1,2]. Bioaerosols are often generated by an infected human while sneezing (a process defined by a sudden involuntary expulsion of air from the nose and mouth, where millions of droplets of water and mucus are expelled at about 100 m/s). These droplets initially are about 10—100 μm in diameter but dry rapidly depending on the relative humidity of the ambient air to form droplet nuclei (1—4 μm) containing micro-organisms [3]. This phenomenon is the primary means of transmitting viral pathogens causing diseases such as COVID-19, SARS, MERS, chickenpox, flu, measles, and bacterial pathogens causative of whooping cough and meningitis diphtheria, pneumonia and tuberculosis [4,5]. It would be of global medical significance if we could eliminate SARS-CoV-2 and *Mycobacterium tuberculosis* from the air with a non-intrusive technology that is safe to be operated in the presence of humans.

Filtration, electrostatic precipitation, chemical fumigation, and ultraviolet germicidal irradiation (UVGI; using 254-nm light) are the frequently used clean-air technologies to address the rising concern of indoor air quality [6]. Filtration and electrostatic precipitation are examples of non-germicidal technologies. Microbes trapped inside filters can proliferate in situ and pose severe hazards during disposal [7]. Electrostatic precipitation uses an electric field to attract and trap pre-charged aerosols generated by corona discharge; however, it frequently produces gases such as ozone that can cause serious health issues [8]. Although they cannot be deployed in the presence of humans, chemical fumigation and UVGI can disinfect an entire room. UVGI is often used to disinfect upper room air and air circulating through ventilation ducts. However, the microbicidal action of UVGI depends on environmental parameters such as humidity and requires a minimum duration of exposure to microbes [9].

In a recent publication [10], we described a novel technology called 'ZeBox' that exploits microbial Zeta Potential to trap and eliminate free-flowing micro-organisms from the air. The technology utilizes the fact that all micro-organisms are naturally charged and characterized by their Zeta Potential (Table I); hence, their movement can be manipulated by applying an electric field. The technology uses a non-ionizing electric field to trap free-flowing micro-organisms in the air on to a three-dimensional surface coated with a long chain of quaternary ammonium salt (QAS). Once trapped, the microbes are eliminated by a synergistic effect of electric field and QAS.

Table I
Reported Zeta Potential of various micro-organisms, including SARS-CoV-2

Micro-organism	Zeta potential (mv)	References
Escherichia coli	−47	[12]
Staphylococcus aureus	−38	[12]
Pseudomonas aeruginosa	−40	[12]
Mycobacterium tuberculosis	−28.8	[13]
Aspergillus niger spores	−15	[14]
MS2 Coliphage	−10	[15]
SARS-CoV-2	−25.675	[16]
PhiX174 Coliphage	−31.78	[14]

It is important to note that regardless of the origin or type, Zeta Potential is always negative for microbes.

The technology and its device variants are proven to eliminate a wide range of micro-organisms under laboratory conditions and in clinical settings [11]. We extended the validation exercise and evaluated the efficacy of the technology in eliminating two of the most pernicious airborne pathogens known to man, SARS-CoV-2 and *M. tuberculosis*.

Materials and methods

Cells and virus

VeroE6 (CRL-1586, ATCC®) was used for infection experiments. Cells were cultured in complete media prepared using Dulbecco's modified Eagle's medium (DMEM; 12100—038, Gibco) supplemented with 10% HI-FBS (16140—071, Gibco), 100 U/mL Penicillin-Streptomycin (15140122, Gibco) and GlutaMAX™ (35050—061, Gibco). SARS-CoV-2 (Isolate Hong Kong/VM20001061/2020, NR-52282, BEI Resources, NIAID, NIH) was propagated and titrated by plaque assay in Vero E6 cells as described in the literature [17].

Quantification of viral load by quantitative reverse transcription polymerase chain reaction

Total RNA from infected cells was isolated using TRIzol (15596018, Thermo Fisher). A 10-μL reaction mixture with 100 ng of RNA per sample in a 384-well block was used to quantify viral RNA using the AgPath-ID™ One-Step RT-PCR kit (AM1005, Applied Biosystems). The following primers and probes targeting the SARS CoV-2 N-1 gene were used. Forward primer: 5′-GACCCCAAAATCAGCGAAAT-3′ and Reverse primer: 5′-TCTGGTTACTGCCAGTTGAATCTG-3′, Probe: (6-FAM/BHQ-1) ACCCCGCATTACGTTTGGTGGACC. The Ct values were used to determine viral copy numbers by generating a standard curve using SARS CoV-2 genomic RNA standard.

Qualitative analysis using fluorescence microscopy

Cells were washed once with warm phosphate-buffered saline (PBS) and fixed with 4% formalin for 10 min. After two washes with sterile 1 × PBS, pH 7.2, cells were permeabilized using 0.1% Triton X-100 for 5 min, washed and incubated with blocking buffer (0.01% Triton X-100, 2% BSA in PBS) for 2 h. Cells were then incubated for 4 h with SARS-CoV/SARS-CoV-2 spike antibody (GTX632604, GeneTex), washed, and then incubated for 2 h with anti-mouse Alexa 488 secondary antibody (A110011, Invitrogen). Both antibodies were diluted (1:1000) in the blocking buffer. Cells were incubated for 10 min in 0.1 μg/mL DAPI (D9542-10 MG, Merck) in PBS to label nuclei. Images were taken using an EVOS M5000 fluorescence microscope at 20 × magnification.

Test set-up for SARS-CoV-2

The entire experiment was performed in the Viral BSL3 facility at the Centre for Infectious Disease Research, Indian Institute of Science, Bangalore, India. All safety protocols were strictly followed while performing the experiment. A test chamber with dimensions 2 ft × 2 ft × 1 ft was built and placed inside a biosafety level 2 laminar flow hood while performing the experiments. The air-decontamination unit was placed

inside the test chamber. Proper care was taken to seal off all open ends around the test chamber before any nebulization experiment. During each control experiment, 3 mL of SARS CoV-2 was nebulized into the test chamber for 5 min; using a six-jet Collison Nebulizer. Dry air from a compressed air cylinder at a pressure of 10 psi was used to operate the nebulizer. Immediately after this, the nebulized virus was collected on mixed cellulose esters (MCE) membrane of 0.22-μm pore size (Millipore, GSWP04700) assembled within a filter unit (Tarsons 521090) connected to a vacuum unit. The collection was performed for a total duration of 15 min at 0.04 MPa pressure. The same procedure was repeated where the air-decontamination unit was operated during the nebulization process to decipher the efficiency of the technology.

Upon completion of each experiment, disinfection was carried by nebulizing 70% ethanol into the chamber for 30 min. The chamber and associated apparatus were then exposed to ultraviolet light for 1 h.

Quantitative analysis of SARS-CoV-2 collected from air samples

Viral particles collected in the MCE membrane filters were placed in a six-well cell culture dish containing 2 mL OptiMEM reduced serum medium (31985062, Gibco) per well. The plate was incubated at room temperature for 1 h, followed by incubation at 4 °C for 12 h. After 12 h of incubation, the reduced serum medium was aspirated and passed through a 0.45-μm syringe filter before using this as a sample for infection. Vero E6 cells were seeded to reach 80% confluency in 24-well cell culture dishes at the time of infection. The medium in wells was aspirated, and 100 μL of the sample was added per well in triplicate. After 1 h of virus adsorption with an intermittent rocking of the plates to ensure even spread of the inoculum, the medium was topped up with 400 μL per well DMEM containing 2% foetal bovine serum. Forty-eight hours post-infection, cells were harvested for immunofluorescence assay and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis.

Cultivation of M. tuberculosis H37Ra

M. tuberculosis H37Ra (MTCC 300) was procured from MTCC India. The microbes were revived in Lowenstein–Jensen Selective Medium (Himedia Labs, M162) at 37 °C for 21 days as instructed by the supplier. Post revival, *M. tuberculosis* H37Ra were cultivated using Middlebrook 7H9 Broth Base (Himedia Labs, M198) supplemented with Middlebrook ABC supplement (Himedia labs, FD019) as media and incubated at 37 °C for two weeks. Cells were pelleted down at 3500 × *g* for 15 min, resuspended with sterile 1 × PBS pH 7.2, and aerosolized using a six-jet Collison Nebulizer.

Test set-up for M. tuberculosis

An air-sealed test chamber of dimensions 8 ft × 6 ft × 7 ft was built with multiple sampling and nebulization ports. The environmental parameters such as relative humidity and temperature could be monitored using a probe inside the chamber.

A six-jet Collison Nebulizer was used to aerosolize the test microbes for a definite period in the test chambers. The nebulizer produces bioaerosols of 2–5 μm diameter that allow them to be suspended in the air in the test chamber for a definite period. Dry air from a compressed air cylinder at a pressure of 10 psi was used to operate the nebulizer.

The nebulized microbes were collected after different time intervals (with or without ZeBox operation) on an MCE membrane of 0.22 μm pore size (Millipore, GSWP04700) assembled within a filter-unit (Tarsons 521090) connected to a vacuum unit. The collection was performed for a total duration of 15 min at 0.04 MPa.

Enumeration of M. tuberculosis H37Ra

The MCE membrane of 0.22 μm pore size collecting the microbes was resuspended in 1 × PBS pH 7.2, and samples were then plated on blood agar [18] and incubated at 37 °C for six days. After six days of incubation, individual colonies were enumerated.

Results

The experimental strategy evaluated the efficiency of the ZeBox Technology-enabled unit in decontaminating a confined space of micro-organisms. M. tuberculosis H37Ra, a surrogate for the virulent strain, and SARS-CoV-2 were used in this study.

Reduction in SARS-CoV-2 load during challenge test

Infection in Vero E6 cells was used for testing the efficacy of ZeBox in abrogating infectious virus particles in aerosols generated by nebulization within the test chamber. Under ideal conditions, i.e, when the device was not operated, the floating viral load was at a steady-state level between ~10E9 and ~10E8. In contrast, there was a viral load reduction of ~10E7 to 10E6 when the device was operated. Fluorescence microscopic analysis carried out 48 h post-infection showed SARS-CoV-2 spike positive infected cells in virus control. Upon virus nebulization within the chamber, this was partially reduced in control experiments (colocalization of DAPI and SARS-CoV-2 spike antibody signals) and almost completely abolished during test experiments when the ZeBox technology-enabled unit was operated as indicated by the reduction in green SARS-CoV-2 spike antibody signal (Figure 1a). Upon quantification by qRT-PCR, this translated to a 7 $\log_{10}$ decrease in viral RNA copy number from cells infected with the virus collected during the test experiment (with ZeBox operation) (Figure 1b).

Reduction in M. tuberculosis H37Ra load during challenge test

At first, control experiments (without ZeBox operation) were performed to determine the timeframe within which the device could be operated for testing. Under ideal conditions, i.e, when the device was not employed, the floating bacterial load was at a steady-state level, settling at ~10E6 over 20 min. In contrast, when the device was operated, there was a reduction of ~10E4.5 within 20 min (Figure 2).

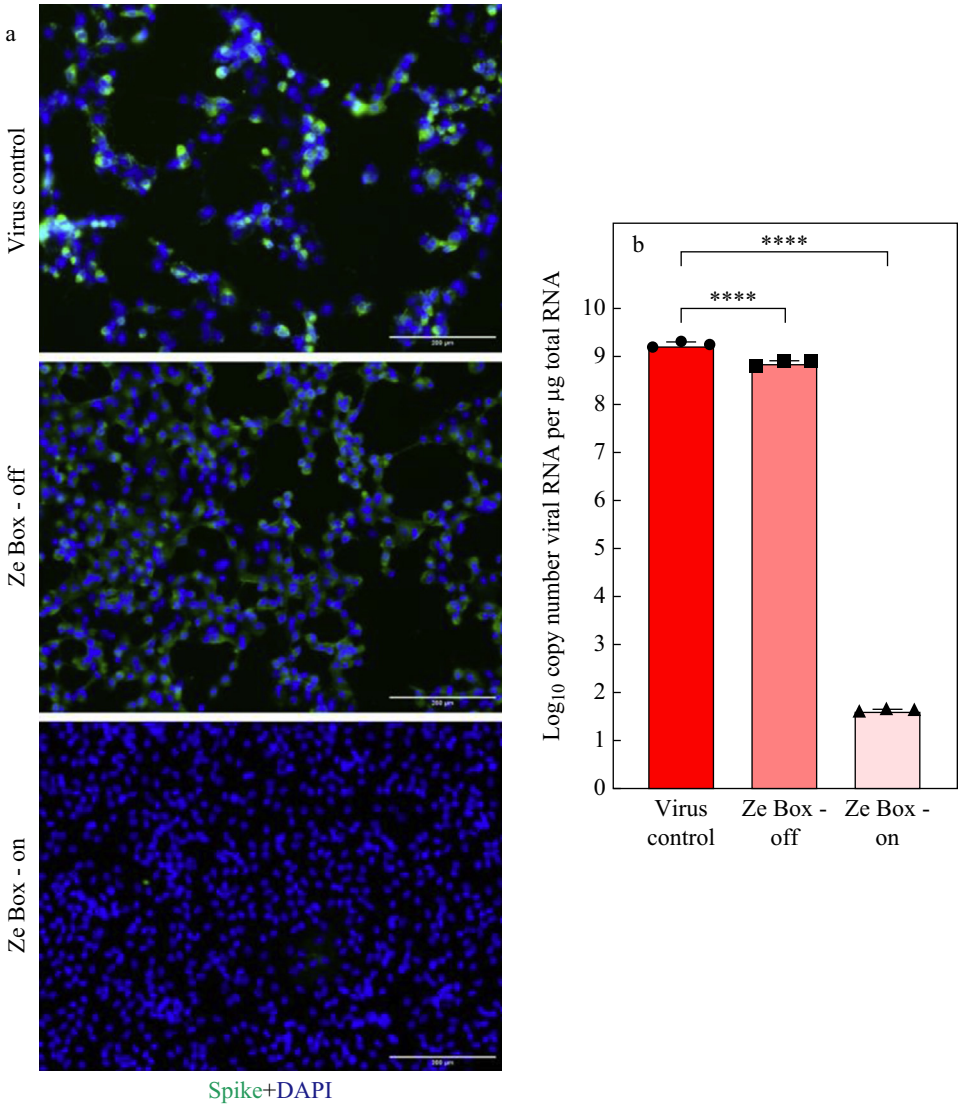

Figure 1. ZeBox abolishes aerosolized SARS-CoV-2 within the test chamber. SARS-CoV-2 was aerosolized within the air-sealed test chamber, and ZeBox operation was initiated. The virus collected from the air samples of the test chamber was used to infect Vero E6 cells and incubated for 48 h. SARS-CoV-2 virus stock was used a virus control. (a) Immunofluorescence assay images showing SARS-CoV-2 spike positive cells (green) and nuclei (blue) for samples from different conditions. (b) quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of viral RNA copy number from different conditions as indicated.

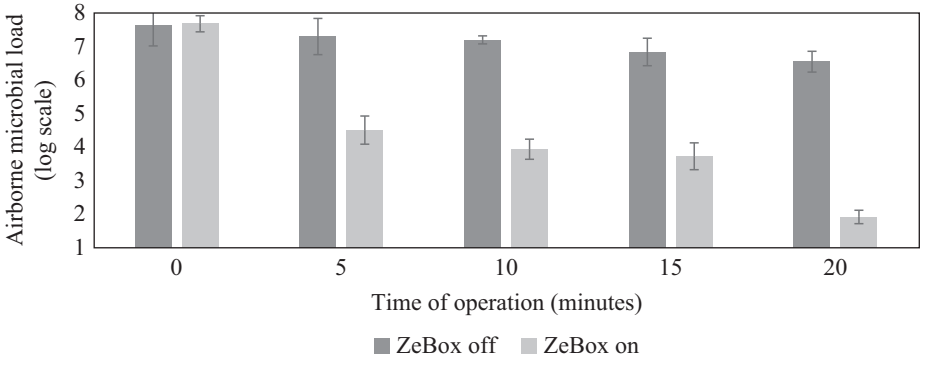

Figure 2. Reduction in Mycobacterium tuberculosis H37Ra load inside the test chamber. As observed, the microbial load inside the chamber settled down by ~1 log when the chamber was undisturbed; when ZeBox was operated, the microbial reduction was ~4.5 $\log_{10}$, indicating a 99.99% reduction in airborne M. tuberculosis H37Ra under a controlled environment.

Discussion

This validation demonstrates the efficacy of the ZeBox technology against two of the deadliest micro-organisms, claiming more than 5 million lives worldwide. Studies have shown that more than 10 million tuberculosis cases are reported yearly [16]. Globally, it is the 13th leading cause of death and the second leading infectious killer after COVID-19. It is prevalent in all countries and age groups. Therefore, diseases such as tuberculosis, which pose a global threat due to the emergence of MDR-TB and XDR-TB, will continue to claim more and more lives in the coming years, especially in an era where therapeutic options are limited. Respiratory pathogens such as SARS-CoV-2 and tuberculosis are transmitted via aerosol, and deploying proven air-decontamination technologies has become imperative. In conjunction with the existing reports [7,8], the present validation demonstrated the true broad-spectrum elimination capability of ZeBox technology. The fact that the killing efficiency of the device is independent of the microbial strain or species further gives it an edge in today's world, where the prevalence of hospital-acquired infections and the emergence of different antimicrobial-resistant strains are major issues.

Several air decontamination technologies are available, but most have certain drawbacks. Technologies such as HEPA filters can only trap microbes but are not bactericidal. Electrostatic-precipitation-involving technologies produce noxious gases such as ozone, and methods such as UVGI and chemical fumigation cannot be carried out in the presence of humans. As compared with these existing decontamination technologies, ZeBox has several advantages. It can trap and kill free-floating microbes without chances of further growth. It can be operated in the presence of humans, is effective against a wide range of robust microbes, and does not produce harmful chemical emissions or ozone. It also has low energy utilization as the airflow is parallel to the antimicrobial surface with almost no resistance. It can be used with existing technologies such as HEPA filters or independently in different settings such as hospitals, homes and office spaces. Therefore, this technology can be a validated starting point for our future war to prevent pandemics caused by airborne pathogens.

Author contributions

A.G., S.T. and S.D. designed the experiments; R.N. and D.K. performed the experiments. All authors contributed to writing the manuscript. A.G. and S.T. managed funding.

Conflict of interest statement

R.N. and S.T. declare no conflicts. D.K., A.G. are employed by Biomoneta Research Private Limited, India. S.D. is employed by Bugworks Research Inc, USA.

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References

[1] Baloch S, Baloch MA, Zheng T, Pei X. The coronavirus disease 2019 (COVID-19) pandemic. Tohoku J Exp Med 2020;250:271—8.
[2] Gonzalez-Martin C. Airborne infectious microorganisms. Encyclopedia of Microbiology 2019:52—60.
[3] Nicas M, Nazaroff WW, Hubbard A. Toward understanding the risk of secondary airborne infection: emission of respirable pathogens. J Occup Environ Hyg 2005;2:143—54.
[4] Leung NHL. Transmissibility and transmission of respiratory viruses. Nat Rev Microbiol 2021;19:528—45.
[5] Leonardo Martinez, Renu Verma, Julio Croda, C. Robert Horsburgh Jr, Katharine S. Walter, Nicholas Degner, et al. Detection, survival and infectious potential of Mycobacterium tuberculosis in the environment: a review of the evidence and epidemiological implications. Eur Respir J 2019; 53: 1802302.
[6] Liu G, Xiao M, Zhang X, Gal C, Chen X, Liu, et al. A review of air filtration technologies for sustainable and healthy building ventilation. Sustain. Cities Soci 2017;32:375—96.
[7] Forthomme A, Joubert A, Andrès Y, Simon X, Duquenne P, Bemer D, et al. Microbial aerosol filtration: growth and release of a bacteria—fungi consortium collected by fibrous filters in different operating conditions. J Aerosol Sci 2014;72:32—46.
[8] Boelter KJ, Davidson JH. Ozone generation by indoor, electrostatic air cleaners. Aerosol Sci. Technol 1997;27(6):689—708.
[9] Memarzadeh F, Olmsted RN, Bartley JM. Applications of ultraviolet germicidal irradiation disinfection in health care facilities: effective adjunct, but not stand-alone technology. Am J Infect Control 2010;38(5):S13—24.
[10] Phadke KS, Madival DG, Venkataraman J, Kundu D, Ramanujan KS, Holla N, et al. Novel non-intrusive continuous use ZeBox technology to trap and kill airborne microbes. Sci Rep 2021;11:22779.
[11] Nagaraj S, Chandrasingh S, Jose S, Sofia B, Sampath S, Krishna B, et al. Effectiveness of a novel, non-intrusive, continuous-use air decontamination technology to reduce microbial contamination in clinical settings: a multicentric study. J Hosp Infect 2022;123:15—22.
[12] Ferreyra Maillard APV, Espeche JC, Maturana P, Cutro AC, Hollmann A. Zeta potential beyond materials science: Applications to bacterial systems and to the development of novel antimicrobials. Biochim Biophys Acta Biomembr 2021;1863:183597.
[13] Ayala-Torres C, Hernández N, Galeano A. Novoa-Aponte L., Soto C.-Y. Zeta potential as a measure of the surface charge of mycobacterial cells. Ann Microbiol 2014;64:1189—95.
[14] Wargenau A, Fleissner A, Bolten CJ, Rohde M, Kampen I, Kwade A. On the origin of the electrostatic surface potential of Aspergillus niger spores in acidic environments. Res Microbiol 2011;162:1011—7.
[15] Chrysikopoulos CV, Syngouna VI. Attachment of bacteriophages MS2 and ΦX174 onto kaolinite and montmorillonite: extended-DLVO interactions. Colloids Surf B Biointerfaces 2012 Apr 1;92:74—83.
[16] Ghatak S, Khona DK, Sen A, Huang K, Jagdale G, Singh K, et al. Electroceutical fabric lowers zeta potential and eradicates coronavirus infectivity upon contact. Sci Rep 2021;11:21723.
[17] Case JB, Bailey AL, Kim AS, Chen RE, Diamond MS. Growth, detection, quantification, and inactivation of SARS-CoV-2. Virology 2020;548:39—48.
[18] Drancourt M, Carrieri P, Gévaudan MJ, Raoult D. Blood agar and Mycobacterium tuberculosis: the end of a dogma. J Clin Microbiol 2003 Apr;41(4):1710—1.