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Rapid drug susceptibility testing of mycobacteria by culture on a highly porous ceramic support

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SUMMARY

BACKGROUND: Phenotypic, culture-based methods for drug susceptibility testing (DST) of *Mycobacterium tuberculosis* are relatively simple and may be particularly appropriate for resource-limited settings where tuberculosis (TB) is most prevalent. However, these methods can be slow and generate significant amounts of infectious waste. Low-cost digital imaging and a unique porous ceramic support for cell culture (Anopore) may offer opportunities to improve this situation.

OBJECTIVE: To test a rapid DST method based on fluorescence microscopy of mycobacteria grown for a few generations on Anopore.

DESIGN: Mycobacteria were cultured with and without drugs, and the resulting microcolonies were heat-killed and stained with the fluorogenic dye Syto16. Microscopy, image-capture with a charge-coupled device camera and digital processing were used to quantify the inhibition of growth by drugs. Rapid DST for rifampicin and isoniazid was performed for clinical isolates.

RESULTS: Mycobacteria could be cultured, killed, stained and imaged on Anopore. For DST, the Anopore method gave an accurate result in 3 days.

CONCLUSION: This is an unprecedented speed for culture-based DST for this group of organisms and results in minimal infectious waste (<20 000 colony forming units). Analysis of mycobacteria by fluorescence and electron microscopy on Anopore also opens up research possibilities.

KEY WORDS: tuberculosis; rapid DST; Anopore

THE DEVELOPMENT and spread of drug-resistant strains of *Mycobacterium tuberculosis* is a matter of global concern that necessitates drug susceptibility testing (DST) of clinical isolates.[1] Tuberculosis (TB) is prevalent in the developing world, where cost and lack of infrastructure are barriers.[2] Culture is rate limiting for many forms of DST. Methods that require the formation of visible *M. tuberculosis* colonies on agar take up to 3 weeks.[3] There are two approaches to reducing this time: detecting growth earlier, or using predictive molecular markers.[4–7] However, covering all possible markers at high confidence is not possible for all drugs. Molecular testing is also less practical in settings where expertise and infrastructure are limited.[6] Rapid phenotypic methods use the detection of viability, growth or metabolism to speed diagnosis. Their simplicity and low cost may make them suitable for the developing world. Many phenotypic DST methods use dyes or radioactive tracers[6,8,9] or turbidometry.[10] Imaging microcolonies on solid media is another route to faster culture-based DST. Optical imaging of microcolonies on agar plates for DST takes 5–13 days.[11] Glass slides can also be used to culture *M. tuberculosis*; the resulting microcolonies are stained with fluorogenic dyes and can be imaged within 7 days.[12] Microscopy is also usable in liquid culture; the microscopic-observation drug-susceptibility assay (MODS) takes 7 days to yield results.[13,14] DST by fluorescence-activated cell sorting (FACS) of microencapsulated *M. tuberculosis* takes 5–7 days.[15] Bacteriophage-based methods require 2 days, but are primarily confined to testing resistance to rifampicin (RMP).[16]

Rapid culture methods appear promising, but they are not implemented in most clinical laboratories. We intended to make microcolony-based DST faster and to develop a format with potential as a standard assay. To do this, a novel porous ceramic (Anopore or PAO, PamGene International, 's-Hertogenbosch, The Netherlands)[17] was used as a culture support. This material has previously been identified as an effective matrix for microbial culture. Culture on Anopore, with nutrients provided from beneath through the pores, allows microcolonies to be grown, readily manipulated in situ (for example, killed or stained) and imaged.[18] Antibiotic sensitivity testing of Gram-negative rods has been demonstrated on Anopore with only a few rounds of division in a few hours.[19] Slow-growing organisms should provide greater time savings compared to

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conventional culture-based methods if a similar number of divisions can be used. Anopore has not, however, been applied to the culture of mycobacteria, which presents challenges in terms of growth rate, staining and safe handling. We combined the culture, killing and staining of clinical isolates of *M. tuberculosis* on Anopore with quantitative imaging methods to perform culture-based DST with unprecedented speed.

MATERIALS AND METHODS

Ethics approval for this study was not required.

Culture of mycobacteria

Routine culture of mycobacteria was performed on Löwenstein-Jensen (LJ) medium supplemented with 0.75% glycerol or LJ broth, both at 37°C under aerobic conditions.[3,20]

Drug susceptibility testing

Culture and DST in BacT 3D Alert TB bottles was implemented and interpreted as recommended by the manufacturer (BioMerieux, Marcy l'Etoile, France). The Anopore method was performed by inoculating a culture of the appropriate strain onto heat-sterilised strips of Anopore (36 × 8 mm, 0.2 μm pores, 60 μm thick) placed on 7H10 agar plates with no drug, 0.1 μg/ml isoniazid (INH) or 1 μg/ml RMP, as appropriate. Samples taken from 2- to 5-week LJ slants were dispersed by bead grinding with 1 g of 1 mm diameter sterile glass beads to 1 ml culture (at least 10^4 colony forming units [cfu]/ml) using a vortex mixer at 250 revolutions per minute for 2 min. After 1–8 days' culture at 37°C, the mycobacteria on the strips were heat-killed by transfer of the Anopore to a hot block at 80°C for 20 min. Staining was performed by transfer to a slide covered with a film of low-melting-point agarose containing Syto16 at a concentration of 2.5 μg/ml (Invitrogen, Heidelberg, Germany). Imaging was by fluorescence microscopy, as previously described, with at least 100 microcolonies analysed for each strain and condition.[19]

Image processing

Tagged image file format (TIFF) digital files of fuorescently-stained microcolonies were processed using the publicly available digital imaging package, ImageJ.* Pictures were processed using a median filter (radius 7 pixels), then converted to a binary image and the microcolony areas calculated. Sub-cellular particles (<40% of the area of a typical cell) and objects partially on the image were excluded. At least 100 microcolonies were measured for each condition.

* Rasband W S. Image J. Bethesda, MD, USA: National Institutes of Health, 2004. http://rsb.info.nih.gov/ij/ Accessed March 2008.

Statistical methods and calculations

For each strain, the uncultured inoculum was used to calculate a threshold value and the microcolony area above which <5% of the population was distributed. After culture, the percentage of microcolonies above the threshold was calculated. Where culture was performed with and without drugs, these percentages were expressed as ratios: the treated condition divided by the control. A value significantly below 1 indicated growth inhibition.

Electron microscopy

Scanning electron microscopy (SEM) of *M. tuberculosis* strain 473 was performed essentially as described.[19] Anopore strips were transferred to 7H10 agar plates containing 3% (v/v) glutaraldehyde (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) to fix the cells through the pores of the Anopore with minimal disruption of the microcolonies. Fixation was for 3 h at room temperature to ensure complete killing and was followed by dehydration, critical point drying and imaging.

RESULTS

Culture and imaging on Anopore

Clinical isolates of *M. tuberculosis* could be cultured on sterile strips of Anopore placed on nutrient agar plates. This was apparent by the formation of visible colonies in a time period similar to that of culture directly on agar (2–3 weeks), and which were of a similar size and appearance. The results were similar for the strains of non-tuberculous mycobacteria (NTM) cultured (listed in the Table), where colonies on Anopore grew in a similar time frame (1–2 weeks) to those cultured directly on agar. Treatment with methanol (20 min), glutaraldehyde (3 h) or heat (80°C, 20 min) were all sufficient to kill all bacteria, as judged by sterile transfer of the strips to LJ broth and incubation for 3 weeks, which produced no turbidity. All three killing treatments left the Anopore support undamaged.

To visualise microcolonies, a 1-step staining protocol was developed. Staining without any destaining step was performed using the fluorogenic DNA stain Syto16 (Figure 1). Heat-killed microcolonies allowed for staining at higher contrast and with less disruption than those treated with methanol (data not shown). Generally, microcolonies large enough to indicate that growth had occurred could be detected in 2–3 days. The data-processing procedure used a median filter combined with a cut-off value in pixels to ensure colonies were treated as individual units (Figure 2).

For all strains tested (Table), significant growth was seen within 3 days ($P < 0.001$, comparing the microcolony area of the inoculum with the 3-day culture using Student's *t*-test). A simple way of expressing growth was to use a threshold value, set as the

Table Strains used and comparison of DST with BacT/ALERT 3D and Anopore methods. Results of the BacT method are shown with the time to detection for the proportionate control shown in days*

Species	Strain number	BacT control, days	INH BacT	RMP BacT	INH ratio Anopore	RMP ratio Anopore	INH Anopore	RMP Anopore
M. tuberculosis	268	8.2	R	R	1.26	1.28	R	R
M. tuberculosis	450	7.3	R	S	1.29	0.35	R	S
M. tuberculosis	473	9.3	R	S	0.96	0.24	R	S
M. tuberculosis	502	8.7	S	R	0.23	0.78	S	R
M. tuberculosis	533	9.3	I	S	0.46	0.26	I or S	S
M. tuberculosis	555	7.7	R	R	1.00	0.80	R	R
M. tuberculosis	609	10.3	S	S	0.23	0.20	S	S
M. tuberculosis	614	9.7	S	S	0.37	0.54	S	S
M. tuberculosis	660	8.8	S	S	0.18	0.23	S	S
M. tuberculosis	719	7.5	S	S	0.35	0.38	S	S
M. malmoense	717	5.0	R	R	0.94	1.07	R	R
M. kansassi	720	5.0	S	R	0.06	1.18	S	R
M. chelonae	730	6.0	R	R	1.16	1.19	R	R
M. bovis	742	6.5	S	S	0.27	0.34	S	S
M. avium	751	8.5	R	R	1.09	1.28	R	R

* Sensitivity ratios for the Anopore method were calculated as described in the Materials and Methods and cut-offs used to classify strains as susceptible, resistant and intermediate. Results for the agar dilution method using the same antibiotic concentrations as Anopore also matched the above results.
INH = isoniazid; RMP = rifampicin; R = resistant; S = susceptible; I = intermediate.

microcolony area below which 95% of the initial (uncultured) inoculum was distributed. The grinding reduced most cfu to single cells, with a relatively small number of larger masses. For *M. tuberculosis* strain 473, inoculated repeatedly on the same day from the same grinding medium, the variance in the threshold was 1.27 fold ($n = 6$). For the 10 *M. tuberculosis* strains listed in the Table with inoculation on different days, the overall variance in the threshold value was 1.71 fold.

After 3 days, there were at least three times more microcolonies above the threshold for all the strains listed in the Table. After 2–5 days' growth, a distinctive microcolony morphology emerged, with discrete areas within the microcolonies not stained with Syto16. Examination by SEM revealed these areas to be holes within the otherwise tightly packed and nearly fused cells (Figure 1). With <8 days' culture, the microcolonies were effectively a monolayer, with an average density of 1.3 cells/μm^2. The cell density within growing microcolonies did not vary at 2, 3, 5 and 7 days' culture of *M. tuberculosis* strain 473 (analysis of variance with Tukey's post hoc test, $P > 0.5$ for all pairwise comparisons, $n = 20$). Calculation of a doubling time was possible from the microcolony areas. After deducting the unstained areas, the microcolony area was considered proportionate to the number of cells. For strain 473, the doubling time was calculated to be 21.1 h.

Drug susceptibility testing

Ten clinical isolates of *M. tuberculosis* were assessed by growth on Anopore for resistance to RMP and INH by cultivation on plates containing the same drug concentrations as the agar dilution method or without a drug. The results after 3 days were compared with the agar dilution and the BacT methods (Table). Break points for the classification of drug susceptibility were calculated based on the comparison of the microcolonies above threshold, with and without an antimicrobial agent. For RMP, DST ratios of 0.06–0.54 classified strains as susceptible. This indicates that microcolonies above the threshold were 2–50 fold less

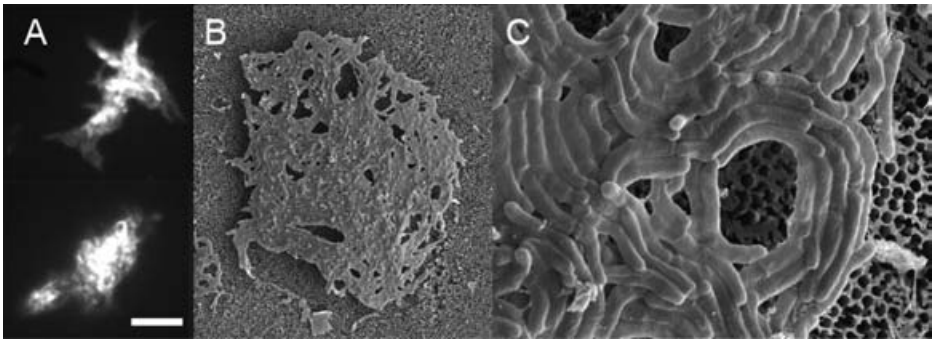

Figure 1 Images of *M. tuberculosis* cultured on Anopore. **A.** Microcolonies stained with Syto16 and imaged by fluorescence microcopy after 3 days. **B.** A typical microcolony after 5 days' growth imaged by SEM. **C.** Detail of panel B showing characteristic cell morphology and holes in colony through which the porous structure of the underlying Anopore is visible. Scale bar is 6 μm when applied to **A**, 8 μm in **B** and 1.5 μm for **C**. SEM = scanning electron microscopy.

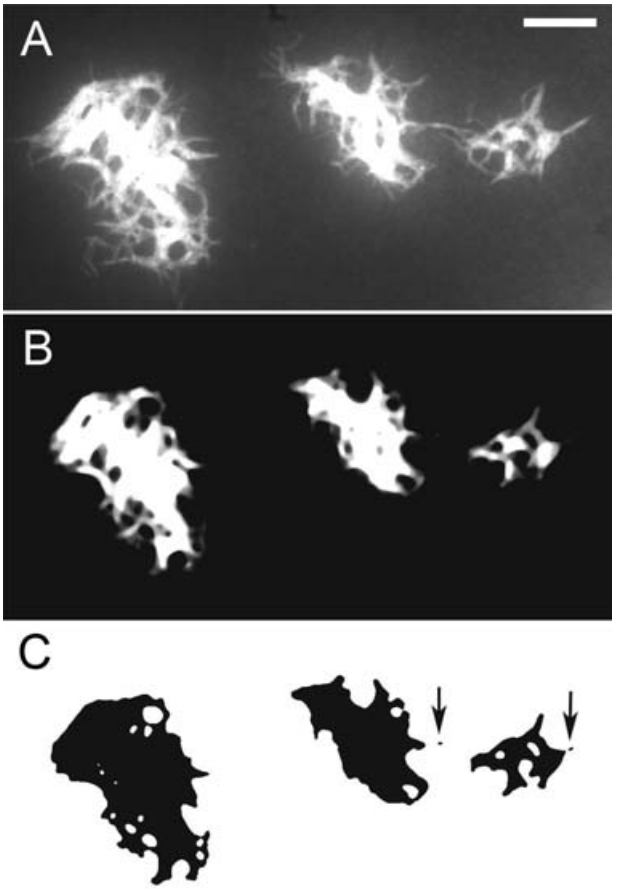

Figure 2 Illustration of digitisation processes. **A.** Image containing three microcolonies with characteristic growth pattern. **B.** The same image after a median filter (radius 7 pixels) was applied. **C.** The same picture after thresholding to a binary image immediately before calculation of microcolony area. Two additional areas of residual signal (shown with arrows) were excluded as being below the cut-off value with the area of the three larger colonies calculated. Scale bar is 10 μm.

abundant with the antibiotic. Resistant strains fell into the 0.78–1.30 range, i.e., the larger microcolonies were about as abundant with the drug as without. INH-susceptible strains had a growth ratio of 0.18–0.37 and resistant strains of 0.96–1.28, with the single intermediate strain falling between these ranges, at 0.46. The DST ratios of the *M. tuberculosis* strains were recalculated using the average threshold values for the 10 strains rather than the individual values. The assignation of susceptible vs. resistant was unchanged (data not shown). This suggests that the variance in threshold values was not a major cause of error and a fixed value may be used in future. All three techniques could be used to classify the strains identically, but the Anopore method was faster, generally by a few days, and resulted in less infectious waste. No variation in the final result was seen using inocula from 2- to 5-week LJ slants (data not shown). Also, four replicate tests for strain 473 on different days gave the same final result (susceptible vs. resistant) for both RMP and INH. In addition to *M. tuberculosis*, *M. bovis* and NTM were tested with similar results with 3 days' incubation. However, for the more rapidly growing strains (*M. malmoense, M. kansassi, M. chelonae* and *M. bovis*), a result could be achieved within 2 days.

CONCLUSIONS

Anopore has been shown to be an effective support for *M. tuberculosis* complex and related bacteria. The material is highly porous and, as has been shown for other organisms, nutrient access is sufficient not to limit growth rates.[19,21] A key additional advantage is that the organism can be manipulated in situ after culture. Such processing can include killing treatments that permit safe handling and imaging methods. A one-step microcolony staining procedure was developed using the fluorogenic nucleic acid stain Syto16. After the cells were heat-killed, the dye appeared to penetrate all cells (microcolony sizes determined by SEM and Syto16 were similar), which is consistent with the study of Pina-Vaz et al.[22] Syto16 was chosen because it was unnecessary to destain the samples, which can be heat-killed and stored desiccated for weeks before staining. This latter point makes Syto16 superior to fluorescein diacetate, which is a vital dye and known to be less effective in staining *M. tuberculosis* samples after transport.[23]

The Anopore DST method, based on the detection of microcolonies, is extremely simple. Although a larger number of clinical isolates must be analysed, this method is promising. The results were accurate for RMP and INH for a range of isolates, both members of the *M. tuberculosis* complex and NTM strains. RMP and INH were the drugs tested, but the method is flexible and can accommodate any drug for which an effect can be seen when incorporated into an agar plate. To date, an extremely wide range of microbial pathogens have been cultured on Anopore and chemically diverse antibiotics tested.[18,19,21] Outside the TB field, susceptibility to bacteriostatic and fungistatic agents has been successfully assessed on Anopore.[19] Given that the method measures the inhibition of growth, there is reason to believe that drugs that inhibit the growth of TB without killing can be assayed. This suggests rapid adaptability of the method to new TB drugs, for drugs without convenient molecular markers and to deal with emerging variants such as extremely drug-resistant TB. This is a general method, with a greater value for slow-growing organisms such as mycobacteria and fungi. It is also faster than other microcolony methods. We attribute this to two factors: first, microcolonies can be manipulated (moved, stained, killed) with minimal disruption due to the rigid support; second, the application of digital processing of images using a publicly available software package (ImageJ) allowed quantification of growth in only a few generations. ImageJ scripts that can be applied to this method are relatively simple. Such customised scripts are easy

to share between laboratories, facilitating standardisation. A significant additional advantage is in waste disposal and safety. The volume of infectious waste from a stack of Petri dishes, tubes or BacT bottles is both expensive to dispose of and represents an unnecessary enrichment of a dangerous organism; both methods require culture to visible levels ($\geqslant 10^8$ cells). We estimate that a valid Anopore assay can be performed with only 10^4 cells cultured on Anopore and at least 1 ml of 10^4 cfu/ml providing the inoculum. This represents a reduction of at least 10-fold in mass/volume of waste compared to culture bottles.

For direct application in the clinic, some modification to the basic Anopore strip is desirable. First, the Anopore needs to be reinforced to facilitate handling. Second, a large number of drugs should be localised on a single strip (or culture chip). This would reduce the cost per assay and the hands-on time per test. We calculate the current cost/assay (drugs, fluorogenic dye, Anopore, grinding beads nutrient agar) to be €1.2–€5 (assuming 1–4 tests per Anopore strip, 4 strips per plate are required). Further miniaturisation and economies of scale (e.g., in the bulk purchase of dyes and Anopore) can only improve this situation. A vortex mixer, hot plate and basic fluorescence microscope with charge-coupled device camera are also required (around €14 000). However, as simple light-emitting diode (LED) systems are capable of detecting fluorescently stained bacteria (including *M. tuberculosis*), a LED reader may replace the microscope.[24]

In addition, rapid assessment of culture and viability, coupled with imaging, has other applications. Direct quantification of *M. tuberculosis* from sputum samples, conceivably coupled with direct DST, has not been attempted, but appears possible. The Anopore method is adaptable to research applications: microcolony imaging is well suited to the analysis of phenotypic heterogeneity in bacteria.[21] Heterogeneity of subpopulations during the infection and treatment of individual patients is an important issue in TB, but has not been extensively studied.[25] Finally, Anopore can be used as a microarray platform for molecular assays, offering the prospect of coupling *M. tuberculosis* culture with molecular testing.[26]

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RÉSUMÉ

CONTEXTE : Des méthodes phénotypiques basées sur la culture pour la détermination de la sensibilité de *Mycobacterium tuberculosis* aux médicaments (DST) sont relativement simples et peuvent être particulièrement appropriées dans les contextes à ressources limitées où la tuberculose est la plus prévalente. Toutefois, ces méthodes peuvent être lentes et produire des quantités significatives de déchets infectieux. Des occasions d'améliorer cette situation sont offertes par l'imagerie digitale à faible coût et un support céramique poreux sans précédent pour les cultures de cellules (Anopore).

OBJECTIF : Tester une méthode rapide de DST se basant sur la microscopie par fluorescence des mycobactéries croissant sur Anopore pendant quelques générations.

SCHÉMA : On a cultivé les mycobactéries avec ou sans médicaments et on a tué les microcolonies qui en résultaient par la chaleur ; on les a colorées par le colorant fluorogénique Syto 16. L'examen microscopique et la capture d'images par une caméra couplée à la charge ainsi que leur traitement digital ont été utilisés pour quantifier l'inhibition du développement par les médicaments. On a pu pratiquer ainsi des DST rapide pour la rifampicine et l'isoniazide provenant d'isolats cliniques.

RÉSULTATS : Les mycobactéries ont pu être cultivées, tuées, colorées et transformées en image sur Anopore. La méthode Anopore a donné un résultat précis de DST en 3 jours.

CONCLUSION : Il s'agit ici d'une rapidité sans précédent grâce à la DST basée sur la culture pour ce groupe d'organismes. La technique entraîne des déchets infectieux minimaux (<20 000 cfu). De plus, l'analyse des mycobactéries par fluorescence et par microscopie électronique sur Anopore ouvre la voie à de nouvelles possibilités de recherche.

RESUMEN

MARCO DE REFERENCIA : Los métodos fenotípicos basados en el cultivo para las pruebas de sensibilidad (DST) de *Mycobacterium tuberculosis* a los medicamentos son relativamente sencillos y pueden ser particularmente apropiados en medios con recursos limitados, donde se observa la mayor prevalencia de tuberculosis. Sin embargo, estos métodos pueden ser lentos y generar cantidades considerables de desechos infecciosos. Una técnica de imágenes numéricas de bajo costo, con un soporte único de cerámica porosa para el cultivo celular (Anopore), podría ofrecer oportunidades de mejorar la situación actual.

OBJETIVO : Evaluar una DST rápida a los medicamentos basada en la microscopia de fluorescencia de micobacterias cultivadas durante pocas generaciones en el sistema Anopore.

MÉTODO : Se cultivaron las micobacterias con y sin medicamentos y las microcolonias obtenidas se inactivaron por calor y se colorearon con la tinción fluorogénica Syto16. Se utilizó un procedimiento de microscopia con captura de imágenes acoplada a un sistema numérico, a fin de cuantificar la inhibición del crecimiento por los medicamentos. Se aplicó esta DST rápida a la rifampicina y a la isoniazida en aislados clínicos.

RESULTADOS : Las micobacterias se cultivaron, se inactivaron por calor, se colorearon y se observaron en el Anopore. Este método aportó un resultado exacto de sensibilidad a los medicamentos en 3 días.