

(1) Title

Direct Tuberculosis Culture in Selective Broth Without Decontamination or Centrifugation

(2) Running Title.

Direct Tuberculosis Culture

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ABSTRACT

30 Tuberculosis culture usually requires sputum decontamination and centrifugation to prevent cultures from being overgrown by contaminating bacteria and fungi. However, decontamination destroys many tuberculous bacilli, and centrifugation is not often possible in resource-poor settings. We therefore assessed the performance of *Mycobacterium tuberculosis* culture in unprocessed samples plated directly using tuberculosis-selective media compared to conventional culture using centrifuge-decontamination. Quadruplicate aliquots of H37RV strain were cultured in 7H9 broth with and without selective antimicrobials and after centrifuge-decontamination. Subsequently the comparison was made with 715 sputum samples. Split paired sputum samples were cultured conventionally with centrifuge-decontamination and by direct culture in tuberculosis-selective media containing antibiotics. Centrifuge-decontamination reduced tuberculosis H37RV colonies by 78% ($P<0.001$) whereas direct-culture in tuberculosis-selective media had no inhibitory effect. Similarly, in sputum cultures that were not overgrown by 40 contaminants, conventional culture yielded fewer tuberculosis colonies than direct-culture ($P<0.001$). However the sensitivity of conventional culture was greater than direct-culture because samples were less affected by contamination. Thus, of the 340 sputum samples that were tuberculosis culture-positive, conventional culture detected 97%, whereas direct-culture detected 81% ($P<0.001$). Conventional and direct-cultures both took a median of 8.0 days to diagnose tuberculosis ($P=0.8$). In those direct cultures that detected drug resistance or susceptibility, there was a 97% agreement with conventional culture (Kappa 0.84, $P<0.001$). Direct-culture is a simple, low-tech and rapid technique for diagnosing tuberculosis and determining drug susceptibility. Compared with conventional culture, direct-culture has reduced sensitivity because of bacterial overgrowth but in basic laboratories this may be outweighed by ease of use.

INTRODUCTION

Worldwide, the majority of tuberculosis patients are diagnosed by direct smear examination of sputum(20). However culture diagnosis is more sensitive and allows for simultaneous drug susceptibility to be determined(12). In resource poor settings, the success of treatment is threatened by multi-drug resistant (MDR) and extensively drug resistant (XDR) tuberculosis(7) that highlight the need for a rapid, simple and cost effective method of culture and susceptibility testing that can be carried out as close as possible to the point of care(15).

Microscopic-Observation Drug-Susceptibility (MODS) testing allows both rapid and low-cost tuberculosis diagnosis in liquid culture with simultaneous determination of drug susceptibilities(3, 12, 13). However, MODS requires sodium hydroxide decontamination and centrifuge concentration prior to culture in order to prevent contamination by bacteria and fungi. Centrifugation is costly and must only be performed in facilities with adequate biosafety(4, 21), while decontamination with sodium hydroxide is time consuming, uses expensive consumables and kills many viable tuberculous bacilli(14, 16).

Direct tuberculosis culture with media made selective by the addition of antibiotics has been used to determine the early bactericidal activity of drugs(5, 6, 17-19) to detect tuberculosis in sputum(16) and extra-pulmonary specimens(9-11). In this study, the ability of direct culture to detect tuberculosis and simultaneously determine drug susceptibility was compared to that of conventional decontaminated culture using the MODS technique, first with H37RV strain samples and subsequently in sputum.

MATERIALS AND METHODS

Clinical Samples. In 2006, 715 sputum samples were collected from 485 patients presenting with tuberculosis symptoms to health posts in a Peruvian shantytown. Participants included symptomatic contacts of tuberculosis patients and new tuberculosis patients who had been diagnosed clinically or by positive sputum smear. New tuberculosis patients had just begun or were about to begin treatment. HIV-testing was not done, but nationally, 3% of tuberculosis patients and 0.2% of adults are HIV-positive(8).

Preliminary Studies. In initial experiments (Fig. 1), the effect of adding antimicrobials to the culture broth and the effect of centrifuge-decontamination on growth in culture broth were determined using the H37RV laboratory strain of *M. tuberculosis*.

Logarithmic-phase cultures were diluted to MacFarlane 1 turbidity ($\sim 3 \times 10^8$ colony forming units (cfu)/ml). Quadruplet 50 μ l aliquots were cultured in 450 μ l culture media under four different conditions: (1) control media (Middlebrook 7H9 and 12.5% OADC (oleic acid, albumin, dextrose, and catalase, Becton Dickinson, USA)); (2) with control media and PANTA (a selective media designed for the inoculation of decontaminated samples, Becton Dickinson, USA, polymixin 40 U/ml, amphotericin B 4 μ g/ml, nalidixic acid 16 μ g/ml, trimethoprim 4 μ g/ml and azlocilin 4 μ g/ml); (3) with control media and Selectatab antimicrobials (polimixin 200 U/ml, ticarcillin 0.1 mg/ml, amphotericin B 10 μ g/ml and trimethoprim 10 μ g/ml from Mast Diagnostics, Bootle, UK); and (4) after centrifuge-decontamination in control media with Selectatab anti-microbials. The samples were cultured undiluted and with three serial 1:20 dilutions. Colonies were counted on day 15.

Sample Preparation. Samples were transported from the health post to the laboratory at ambient temperature. On arrival at the laboratory they were stored at 4°C until processed. Samples less than 2 ml in volume were made up with Phosphate Buffered Saline at pH 6.8 (PBS) to 2 ml and sputum in excess of 2 ml was discarded. All samples were liquefied with 500 µl of 0.5% N-acetyl cysteine and 2.9% sodium citrate, and mixed for approximately 1 minute. Two aliquots of 50 µl were removed for direct culture and the remaining 2.4 ml underwent centrifuge decontamination.

Direct Culture Technique. One 50 µl aliquot was inoculated into a detection well of a 24 well tissue culture plate (Becton Dickinson) containing 450 µl of control media (see above) supplemented with Selectatab antimicrobials. The other 50 µl aliquot was inoculated into an MDRTB testing well which was identical to the detection well other than containing 0.2 µg/ml rifampicin and 1.0 µg/ml isoniazid in addition. The samples were cultured undiluted and with three serial 1:20 dilutions.

Conventional Centrifuge-Decontamination Technique. The remainder of the sample was decontaminated by mixing with an equal volume (2.4 ml) of 2% sodium hydroxide. After 20 minutes, 10 ml PBS was added. This was followed by centrifugation at 2500xg for 15 minutes at 17°C. The pellet was re-suspended in PBS to the original volume (2.4ml) and two aliquots of 50 µl were cultured (as described below).

Conventional Culture Technique. The two 50 µl aliquots of decontaminated centrifuged sputum were inoculated into detection wells and MDRTB wells as for direct culture, except that the broth was supplemented with PANTA instead of Selectatab antimicrobials. The samples were cultured undiluted and with three serial 1 in 20 dilutions.

Dilutions. Three serial dilutions were made from both the detection and MDRTB wells in both direct and conventional culture. After mixing the inoculated well, 25 μ l was removed, and then inoculated into another well containing 475 μ l of the same culture media. Two further serial dilutions of 1 in 20 were made in the same manner. To minimize the risk of cross-contamination and occupational exposure, immediately after inoculation lids were applied to the culture plates that were then sealed inside plastic zip lock bags. The bags were sealed for the duration of culture and were transparent, allowing microscopy to be performed.

Culture Reading Methods. Cultures were incubated at 37°C and read with an inverted light microscope with x40 magnification as described(3). Positive *M. tuberculosis* cultures were identified by characteristic cord formation and non-tuberculous mycobacteria by their lack of cording or, for *M. chelonae* by overgrowth before day 5. A positive sample was defined as one in which *M. tuberculosis* was visualized in any of the sample dilutions. Cultures were examined for *M. tuberculosis* growth 3 times per week from day 5-35 and colony-forming units (cfu) counted on days 15 and day 35. Bacterial contamination was easily distinguished from the filamentous hyphae of fungal contamination on the basis of morphology. Contamination by bacteria and fungi could prevent visualization of tuberculosis in the well. Partial contamination was defined as contamination of any kind that did not obscure visualization of the well. A negative culture well was defined as one in which neither *M. tuberculosis* grew nor contamination was seen.

Sputum Drug Susceptibility Testing. The MDR-testing well was read as soon as *M. tuberculosis* growth was identified in the detection culture-well and MDRTB was defined by the presence of any *M. tuberculosis* growth in the isoniazid and rifampicin containing culture. On day 15, all MDR-testing wells were read regardless of growth in the detection wells. A non-MDR sample was defined as one in which growth was observed in a detection culture with no

growth in the isoniazid and rifampicin containing well. Confirmatory testing of drug susceptibility with the Tetrazolium Microplate Assay (TEMA)(2) was performed on drug resistant cultures. Any sample detected as resistant in MDRTB testing that had not recovered tuberculosis in any of the detection wells was considered a new positive sample in this phase of the analysis.

Analysis of Combined Cultures. When the combined result of all four dilutions was used to compare direct culture against conventional culture. A negative culture was defined as one in which there was no tuberculosis growth and no contamination in the most concentrated dilution, with no tuberculosis growth in more dilute wells. In order to not underestimate the effect of contamination (or over estimate the number of true negative samples), a negative-contaminated culture was defined as one in which the most concentrated dilution was contaminated with no tuberculosis growth in the dilutions below. Consequently the negative-contaminated samples had at least one negative well although the most concentrated dilution was contaminated. A positive culture was defined as one in which there was tuberculosis growth in any culture well.

Statistical Analysis. Results were analyzed using STATA 9 software. The data generated with pure laboratory tuberculosis strains was analyzed using an unpaired Student's t-test. The median times to culture positivity was analyzed using the Wilcoxon signed rank test. The paired Students t-test was used to compare colony counts in the clinical study. The Z-test of proportions was used to compare the differences in sensitivity. All P values were two-sided and $P < 0.05$ was considered statistically significant.

RESULTS

160 The 715 sputum samples were provided from 485 patients with a median age of 23 years and a male to female ratio of 1.2:1. Patients were treated for a median of 1 day prior to sample collection and 276 samples were Ziehl-Neelson positive (Table 1).

The Effect of Centrifuge Decontamination on H37RV strain. Conventional centrifuge-decontamination of pure laboratory tuberculosis cultures caused a 78% reduction in the number of *M. tuberculosis* colonies recovered, as assessed by serial dilutions of H37RV strain (1,320 cfu/μl in control cultures vs. 320 cfu/μl after decontamination, 0.56 log decrease, $P<0.001$). Colony counts did not differ significantly between cultures in control broth with no antimicrobial additives compared with cultures in broth containing PANTA or Selectatab antimicrobial mixtures (Control 1,060 cfu/μl, PANTA 2,520 cfu/μl $P=0.1$, Selectatabs 1,320 cfu/ml $P=0.2$).
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Mycobacterial Growth in Direct and Decontaminated Culture. Considering only sputum cultures that were free from bacterial or fungal growth in both direct-culture and decontaminated-culture, direct-cultures yielded higher *M. tuberculosis* colony-counts than decontaminated-cultures in all dilutions ($P<0.001$ combined dilutions, Fig. 2) and direct-culture yielded more positive tuberculosis cultures than conventional culture when diluted to 1:8000 ($P<0.001$). Both techniques diagnosed tuberculosis in a median of 8.0 days (Fig. 3, $P=0.8$).

Sensitivity of Direct-Culture vs Decontaminated-Culture. The greater sensitivity of direct than conventional culture for samples that yielded interpretable results with both techniques was outweighed by more frequent direct-culture failure because of bacterial and
180 fungal overgrowth (Fig. 4a). Eighteen percent (126/715) of samples were contaminated (either partially or totally) in all direct-culture dilutions. Thirty two percent (232/715) of samples were

negative-contaminated. The corresponding contamination rates for decontaminated cultures were 3% (20/715) and 8% (57/715), both significantly less than direct culture ($P < 0.001$). Consequently, the diagnostic sensitivity of direct-culture was less than decontaminated-culture (81% vs. 97% sensitivity comparing combined results of all dilutions $P < 0.001$, Fig 4b and Table 2). Comparing the best performing single culture-wells, direct-culture was most sensitive when sputum was diluted 1 in 400 which detected 70% (239/340) of positives vs. 93% (316/340) in the best performing, undiluted decontaminated-culture ($P < 0.001$).

Drug Susceptibility Testing with Direct and Decontaminated-Culture. All 715 samples were tested for MDRTB (Table A1). The additional MDRTB culture wells yielded two positive (resistant) samples that were not detected in any of the detection wells. Four positive samples were detected in direct culture alone and 3 positive samples in conventional culture alone. Hence, the total number of samples that recovered *M. tuberculosis* (resistant or susceptible) by both tests was 342. Direct culture detected 280 (82%) of these samples and conventional culture detected 334 (98%). For the 272 samples with interpretable MDRTB results (resistant or susceptible) from both direct and decontaminated culture, there was a 97% agreement in MDRTB detection between the two methods (Kappa 0.84, $P < 0.001$, Table A1). Of the 86 samples that were determined to be MDRTB in decontaminated culture, 83 strains underwent confirmatory testing with TEMA and 76 (92%) yielded concordant results, 2 samples had TEMA results indicating mono-resistance and 5 pan-susceptibility. Of the 73 samples that were determined to be MDR by direct culture, 68 samples underwent confirmatory indirect drug susceptibility testing with TEMA, and 65 (96%) yielded concordant results, 2 samples had TEMA results indicating mono-resistance and 1 sample pan-susceptibility.

Contamination. Partial contamination had a significant affect on diagnostic sensitivity (Table 2, Table A2). Excluding all contaminated samples, the sensitivity of direct culture matched that of conventional culture (96 vs 98% P=0.4), however when partial contamination alone was included the sensitivity dropped (84% vs 97% P<0.001). Direct-culture was more often contaminated with bacteria than fungi (84% vs 21% of all contaminated wells, P<0.001; 5% contaminated with both). The proportion of bacterial to fungal contamination was similar for
210 undiluted culture and sputum cultured at a 1:8000 dilution (75% vs 82%, P=0.2). Diluting sputum samples increased the sensitivity of direct-culture, by reducing contamination, and decreased the sensitivity of decontaminated-culture.

Sample Storage and Transport. The median time between sample collection and sample storage at 4°C was 2 days and increased delay was not associated with higher contamination rates (49% vs 53% for 0-3 days and 4-7 days transport respectively using definition B, P=0.4). The median time between sample collection and sample processing was 7 days and increased delay to processing was not associated with higher contamination rates (53% vs 49% for 0-5 days and 6-10 days respectively, using definition B, P=0.4).

DISCUSSION

220 This is the first report comparing direct-culture with conventional MODS culture and our
adaptation to the rapid and sensitive MODS technique is also novel. In both H37Rv strain and
paired sputum cultures not affected by contamination, direct culture grew more colonies than
conventional culture. In the most diluted cultures, direct-culture was also more likely to detect
tuberculosis than decontaminated-culture. Serial dilution of sputum samples improved the
sensitivity of direct culture and only required minimal equipment. However, contamination was
too frequent to be operationally acceptable in most settings. Increased bacterial resistance to
antibiotics as a result of widespread medical, veterinary and farming use may explain the higher
rate of contamination in our study compared with earlier reports(10, 11, 16). Furthermore,
previous studies used agar, which has lower sensitivity for tuberculosis culture than broth, but is
230 less affected by bacterial and fungal contamination.

Studies to determine the character of the contaminants and to evaluate the combination of
selective media with disinfectants are underway. If bacterial and fungal overgrowth could be
efficiently prevented, direct culture would be expected to have a greater diagnostic sensitivity
than culture after centrifuge-decontamination. The significant effect of partial contamination on
tuberculosis growth in liquid culture is important. Partial contamination impairs tuberculosis
growth and may cause false reporting of negative samples. We recommend that negative samples
with partial contamination should be re-cultured with more thorough decontamination.

A limitation of the current research was the lack of comparison with the Petroff technique,
in which sputum is cultured after decontamination but without centrifugation(22). In this long-
240 established technique that is used in parts of the developing world, sodium hydroxide is added to

sputum and after an interval, excess buffer is added and the resultant diluted sample is applied directly to acidified egg-based media. The Petroff method however has previously been shown to yield fewer colony forming units than direct culture in selective media(1).

Processing of sputum directly in selective culture media without centrifugation or decontamination is designed to allow reliable drug susceptibility testing in understaffed or poorly equipped laboratories. This study demonstrated that direct culture can rapidly and reliably diagnose tuberculosis, identify MDRTB, and has the potential for implementation in basic laboratories, at the cost of reduced sensitivity compared with conventional centrifuge-decontaminated culture. Ongoing work aims to reduce the contamination of directly cultured sputum by improving the selective media in order to increase the diagnostic sensitivity.

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ETHICAL APPROVAL

Ethical approval for the study was obtained from Imperial College London and Universidad Peruana Cayetano Heredia, Lima.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest in relation to this work.

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Figure 1. Overview of methodology

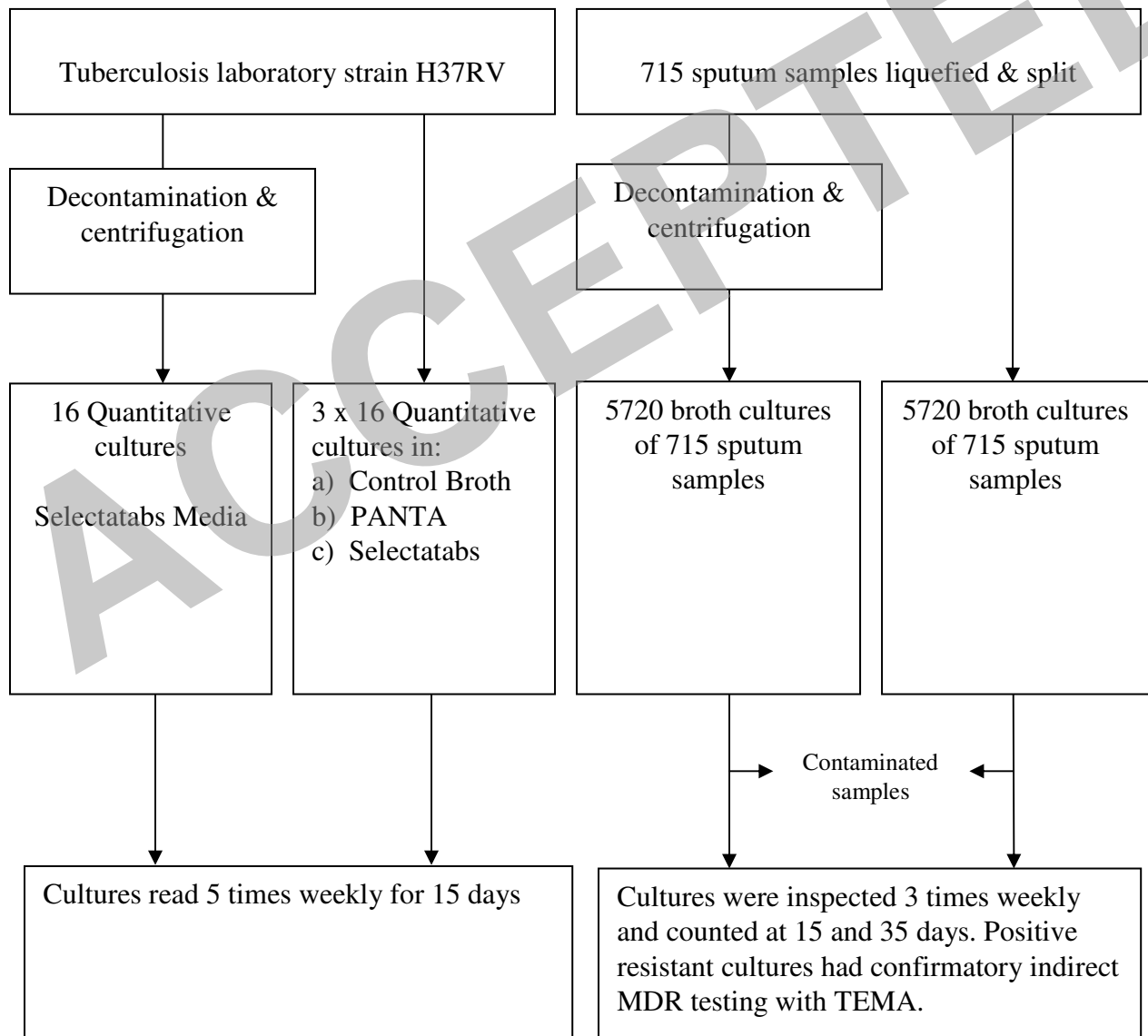


Figure 2. Growth of Mycobacterium Tuberculosis in Direct and Decontaminated Culture. The comparison was made for those cultures not affected by contamination. Overall difference between the two groups ($P < 0.001$). Individual P values for dilutions $P = 0.14$ (1:1), $P < 0.001$ (1:20), $P < 0.001$ (1:400) and $P < 0.001$ (1:8000). Error bars indicate 95% confidence intervals. Colony forming units in both culture techniques appear to increase with increasing dilution but this is simply artefact caused by false negative contaminated cultures occurring more frequently at higher sample concentrations.

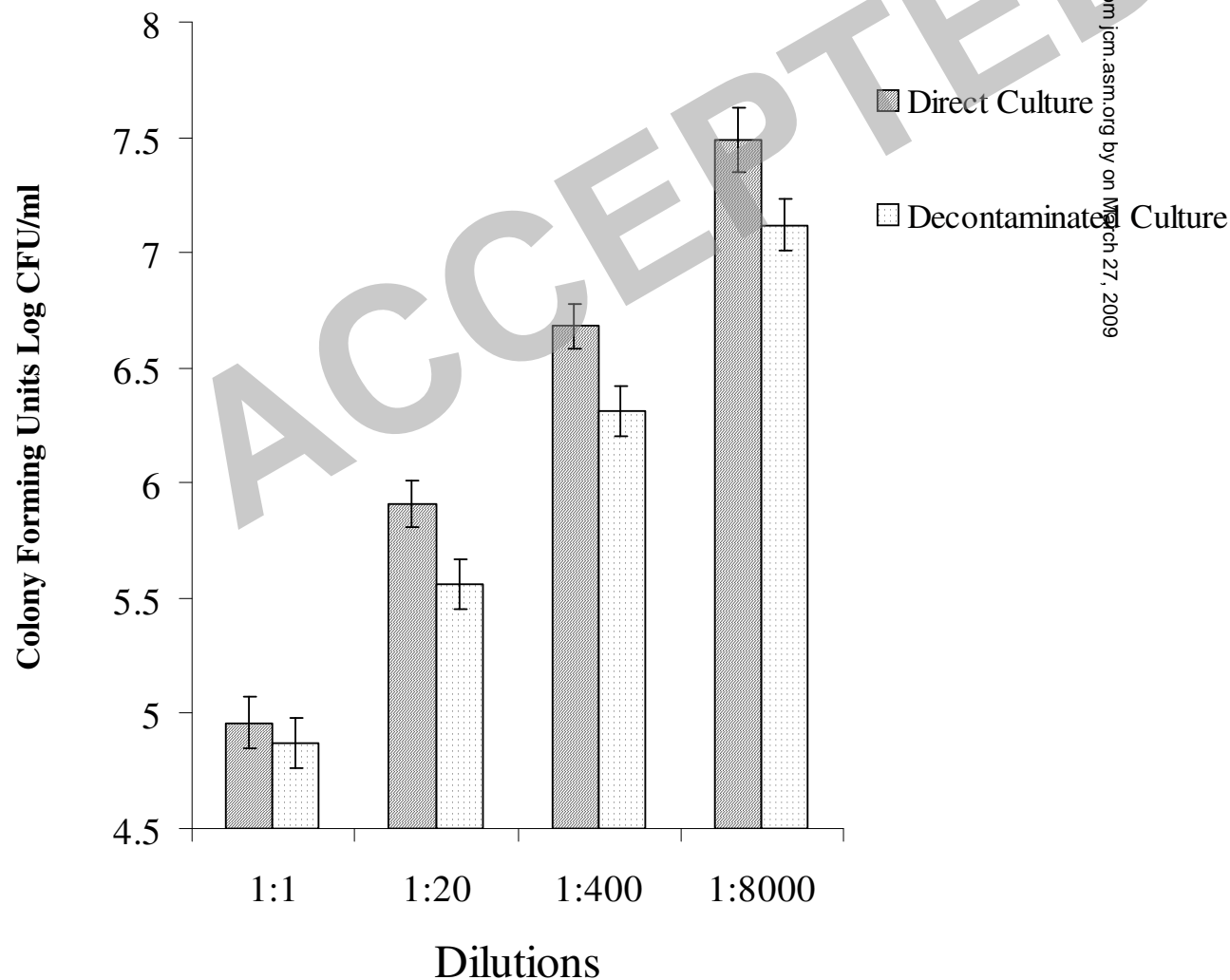
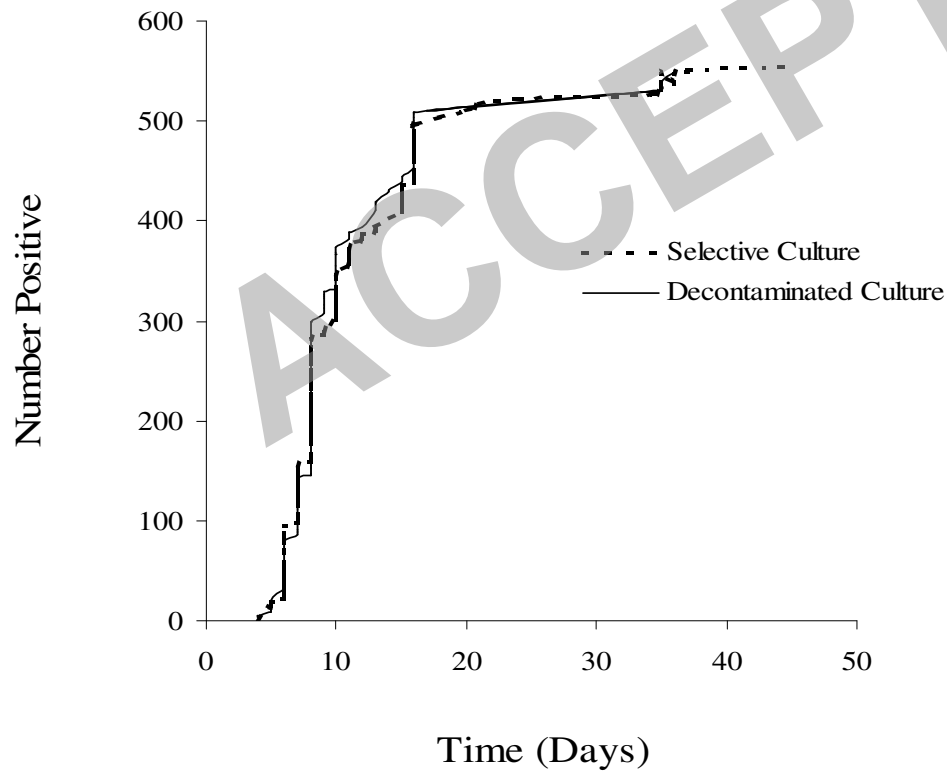


FIGURE 3. The number of days from sample processing until the detection of tuberculosis growth is shown for the samples that were positive in both direct culture and decontaminated culture. Five from 715 samples were read after the usual 35 days.



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FIGURE 4a. The results for each of the 8 culture wells are shown for the 715 sputum samples.

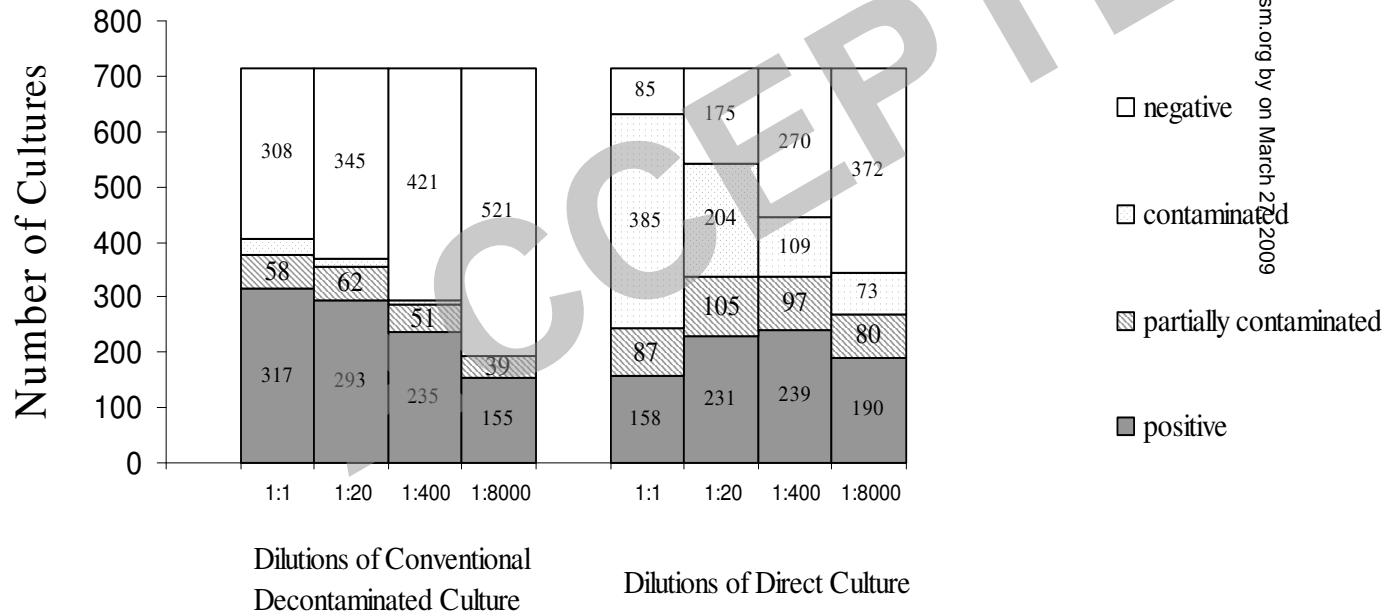


Figure 4b. Results of all culture dilutions combined

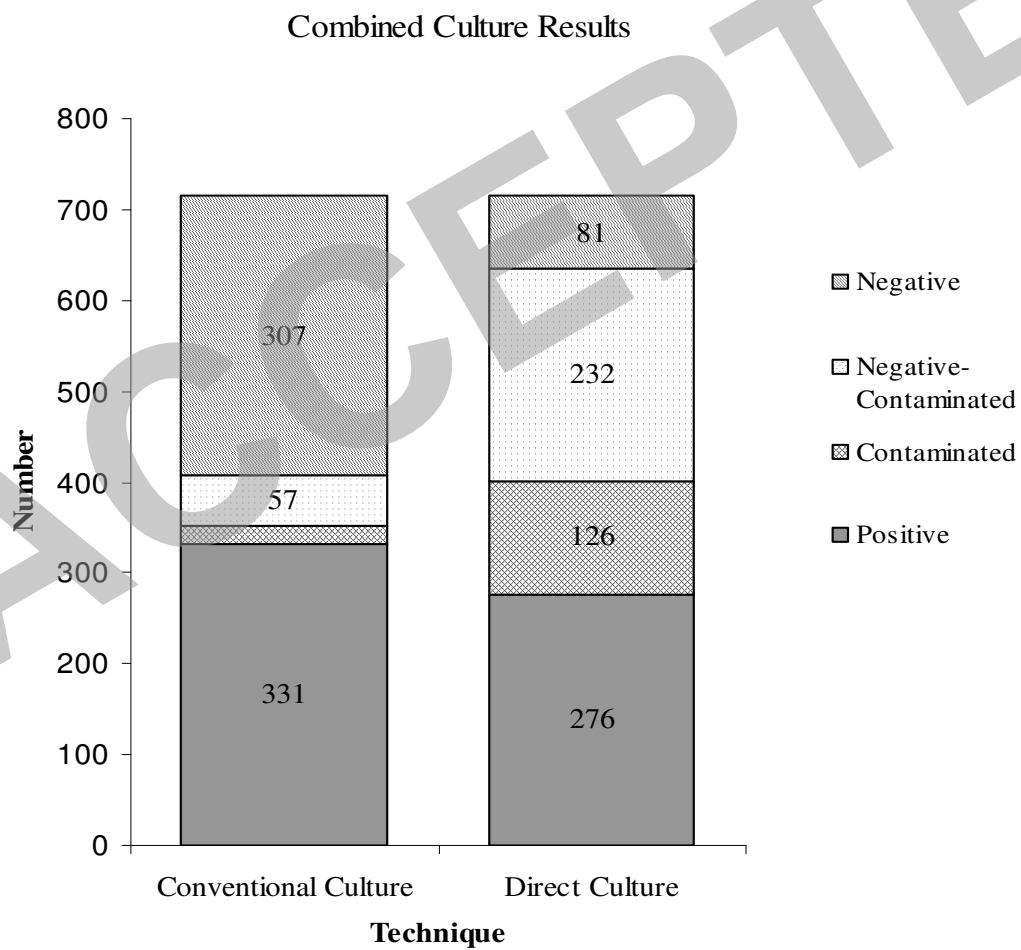


Table 1. Patient Demographics

	Samples from new tuberculosis patients: (n=542)	Samples from tuberculosis suspects: (n=173)	Total samples: (n=715)
Age: median (IQR)	24 (12-36)	20.5 (11.5-30)	23 (12-34)
Age range (months / years)	1m – 85 y	1y – 77y	1m – 85y
Gender: n (% female) ^a	235 (43)	91 (53)	326 (46)
Number of individuals: n	328	157	485
Number of sputum samples per Patient (Mean)	1.7	1.1	1.5
Tuberculosis treatment days prior to sample: median (IQR)	31 (1-206)	0 (0-0)	1 (0-125)
Ziehl-Neelsen sputum smear positivity: number (%) ^b	268 (49)	8 (5)	276 (39)

^aPatient gender data was not available for 7 samples

^bZiehl-Neelsen sputum smear data was unknown for 88 samples

Table 2. Performance of direct culture vs. decontaminated culture

	Conventional Decontaminated culture (n=715)	Direct Culture (n=715)	P value
Days to positive: median (IQR)	8.0	8.0	P=0.8
Agreement MDRTB testing	97%, Kappa=0.84		P<0.001
Sensitivity All cultures combined dilutions	97% (331/340)	81% (276/340)	P<0.001
Most sensitive single dilution (sensitivity)	Undiluted (93%)	1:400 (70%)	P<0.001
Sensitivity of the most concentrated culture well:			
If all contamination excluded	98% (158/162)	96% (155/162)	P=0.4
If partial contamination included	97% (180/185)	84% (156/185)	P<0.001