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Brief Communication

Multicenter evaluation of AYC.2.2 agar for the isolation of mycobacteria from clinical samples☆,☆☆,☆☆☆

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ABSTRACT

Purpose: The aim of this multicenter study is to evaluate AYC.2.2 agar for the isolation of mycobacteria from clinical samples.

Methods: Totally 5559 media were tested in 7 centers. AYC.2.2 agar media for the study were prepared by C1 and sent to other centers under appropriate conditions. Other media except AYC.2.2 agar were purchased commercially.

The media were subjected to routine laboratory operations in the center where they were sent. After the samples received for routine processing (in all centers, samples were processed with the same method (NALC-NaOH)), they were cultivated on routine media and AYC.2.2 agar afterward.

Results: C1: Average growth time was determined as 12.74±3.74 days with MGIT 960 system; 24.42±4.75 days with LJ and 24.37±4.96 days with AYC.2.2 agar. C2: Average growth time was determined as 18.25±9.32 days with TK-Medium, 28.73±7.44 days with LJ, and 31.72±6.35 days with AYC.2.2 agar. C3: Average growth time was determined as 20.48±7.24 days with Ogawa medium, 20.74±7.12 days with LJ, and 20.26±7.43 days with AYC.2.2 agar. C4: Average growth time was determined as 15.27±6.37 days with MGIT 960 system, 22.14±9.1 days with LJ, and 22±8.45 days with AYC.2.2 agar. C5: Average growth time was determined as 13±4.24 days with MGIT 960 system, 32.16±6.23 days with LJ, and 33±5.73 days with AYC.2.2 agar. C6: Average growth time was determined as 9±3.11 days with MGIT 960 system, 18.68±5.32 days with LJ, and 18.34±4.63 days AYC.2.2 agar. C7: Average growth time was determined as 14.74±7.65 with MGIT 960 system, 26.01±8.21 days with LJ, and 26.24±7.88 days with AYC.2.2 agar.

Conclusions: In conclusion, similar results were obtained with LJ and Ogawa media and AYC.2.2 agar. Furthermore, more studies should be conducted for isolation of *M. tuberculosis* and performing antibiotic susceptibility tests using AYC.2.2 agar before it can be used as a routine media in the laboratories.

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1. Introduction

Tuberculosis is still one of the oldest unsolved health problem worldwide and is the 10th deadliest disease in the Worldwide. According to the 2018 data of the World Health Organization (WHO), 23% of the world population, approximately 1.66 billion people, are infected with tuberculosis bacilli. The incidence of tuberculosis is 10 million according to 2017 data, and the mortality due to tuberculosis is 1.6 million (0.3 million are HIV positive) and 6.4 million of these patients are registered and 3.6 million are unregistered [1]. In our country, there is a decrease in the number of cases by half from 2005 (20.535 cases) to 2017 (12.046) [2]. Emerging increase in drug-resistant (multidrug-resistant, common drug-resistant) isolates over the years shows that the disease still maintains its importance. There are several media available for the isolation of *M. tuberculosis* isolates from clinical samples. The best known solid media are Lowenstein-Jensen (LJ) medium, Middlebrook 7H10, and 11 agar. LJ medium is difficult to prepare and bacteria require 3–6 weeks to grow and obtain visible colonies. Middlebrook 7H10 and 11 agars require an expensive enhancer (OADC). However, there are also automated systems developed to provide rapid detection of tuberculosis bacilli from clinical samples. The most widely used one of these systems is Bactec MGIT 960 (Becton Dickinson Diagnostic Systems, Sparks, MD) but BacT/ALERT (the bioMerieux, France), TK-Medium (Innovative Biotechnology Organization Ltd. Istanbul, Turkey) and Versa-Trek (TREK Diagnostics) are also used less frequently. Although automated systems provide much faster results; however, since the automated systems are expensive, using these systems is limited especially in developing countries and rural areas [3,4].

This study aims to evaluate the performance of the newly developed AYC.2.2 agar for the growth of mycobacteria in routine mycobacteriology laboratories.

2. Materials and methods

2.1. Centers

Center 1; Ondokuz Mayıs University Medical Faculty Department of Medical Microbiology, Samsun, Center 2; Samsun Dr. Kamil Furtun Chest Disease and Chest Surgery Hospital, Tuberculosis Laboratory Samsun, Center 3; Ankara Atatürk Chest Disease and Chest Surgery Education and Training Hospital, Ankara, Center 4; Celal Bayar University Medical Faculty Department of Medical Microbiology, Manisa, Center 5; Mersin University Medical Faculty Department of Medical Microbiology, Mersin, Center 6; Istanbul University Istanbul Medical School, Department of Medical Microbiology, Istanbul, and Center 7; Dr. Suat Seren Chest Disease and Chest Surgery Education and Training Hospital, İzmir.

In the study, no additional samples were taken from the patients for this study. It was used for the remaining material inoculation during routine work. Therefore, ethical permission was not required. However, in order to carry out the study, institutional permissions were obtained by 2 centers.

Table 2
Average growth time of isolates inoculated to different media.

Centers	Media	Average growth time (day)
Center 1	MGIT 960 system	12.74±3.74
	LJ medium	24.42±4.75
	AYC.2.2 agar	24.37±4.96
Center 2	TK-medium	18.25±9.32
	LJ medium	28.73±7.44
	AYC.2.2 agar	31.72±6.35
Center 3	Ogawa medium	20.48±7.24
	LJ medium	20.74±7.12
	AYC.2.2 agar	20.26±7.43
Center 4	MGIT 960 system	15.27±6.37
	LJ medium	22.14±9.1
	AYC.2.2 agar	22±8.45
Center 5	MGIT 960 system	13±4.24
	LJ medium	32.16±6.23
	AYC.2.2 agar	33±5.73
Center 6	MGIT 960 system	9±3.11
	LJ medium	18.68±5.32
	AYC.2.2 agar	18.34±4.63
Center 7	MGIT 960 system	14.74±7.65
	LJ medium	26.01±8.21
	AYC.2.2 agar	26.24±7.88

2.2. Media preparation

The patent filed AYC.2.2 agar prepared by the developer and sterilized by autoclave at 121 °C and 1 atm. The medium removed from the autoclave and cooled in a water bath to 45–50 °C and 5% inactivated and filter sterilized sheep serum (Sigma, Germany) was added. To prevent contamination, polymixin (3 μg/ml), azlocillin (3 μg/ml), nalidixic acid (15 μg/ml), trimethoprim (3 μg/ml) and amphotericin (3 μg/ml) were added. Then it was dispensed into tubes (16 × 160 mm) to be as 5 ml. Tubes were placed in a slanted position and solidified. The prepared solid AYC.2.2 agar media were then sent to the test centers, providing cold chain conditions and stored at + 4 °C until used. AYC.2.2 agar media for the study were prepared by C1 and sent to other centers under appropriate conditions. Other media except AYC.2.2 agar were purchased commercially.

2.3. Testing media

In the study, media were tested in October-November-December 2016 and January-February-March 2017 in Center 1–6 and in May-June-July-August 2017 in Center 7.

The media were subjected to routine laboratory operations in the center where they were sent. After the samples received for routine processing (in all centers, samples were processed with the same method (NALC-NaOH)), they were cultivated on routine media and AYC.2.2 agar afterward. AYC.2.2 agar was evaluated in terms of Mycobacterium spp. growth and contamination once every three days during the first week and then observed once a week. After 45 days of observation since cultivation, negative media were separated and reported. When acid-fast

Table 1
Distribution of growth and contamination rates by centers.

Centers	Number of samples	MGIT		LJ		AYC.2.2 agar		Ogawa medium		TK-medium	
		Pos	Con (%)	Pos	Con (%)	Pos	Con (%)	Pos	Con (%)	Pos	Con (%)
C1	249	11	6,04	9	15,32	7	10,08	–	–	–	–
C2	288	–	–	34	17,01	18	24,65	–	–	21	16,66
C3	1878	–	–	142	4,2	118	8,8	150	2,8	–	–
C4	530	11	4,1	7	6,03	8	9,8	–	–	–	–
C5	256	14	1,9	12	5,4	13	5,8	–	–	–	–
C6	1400	51	1,28	40	2,71	35	1,92	–	–	–	–
C7	958	112	2,1	94	4	75	4,7	–	–	–	–

Pos: positive number; con: contamination rate.

Table 3
Comparison of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of AYC.2.2 agar vs MGIT 960 system, AYC.2.2 agar vs LJ medium and MGIT 960 system vs LJ medium.

		Center 1	Center 2	Center 3	Center 5	Center 6	Center 7
AYC.2.2 agar * MGIT 960 system	Sensitivity	70	53.33	95.24	92.86	100	100
	Specificity	99.52	94.97	100	100	100	100
	PPV	87.50	47.06	100	100	70	100
	NPV	98.57	96.05	28.57	99.61	98.87	100
AYC.2.2 agar * LJ medium	Sensitivity	80	62.50	95.41	91.67	85	100
	Specificity	100	98.25	100	99.22	99.92	100
	PPV	100	83.33	100	84.62	97.14	100
	NPV	98.97	94.92	28.57	99.61	99.54	100
MGIT 960 system * LJ medium	Sensitivity	90	100	99.23	85.71	74	100
	Specificity	99.49	93.23	100	100	100	100
	PPV	90	55.17	100	100	100	100
	NPV	99.49	100	66.67	99.22	99.2	100

growth was confirmed, *M. tuberculosis*/non-tuberculosis mycobacteria (NTM) were reported by making distinctions. Growth was evaluated in terms of time, type of the automated system and solid media used in each center.

3. Results

In this study; totally 5559 media were tested (249 in Center 1, 288 in Center 2, 1878 in Center 3, 530 in Center 4, 256 in Center 5, 1400 in Center 6, and 958 in Center 7). When routine samples were processed, periodic evaluations were made in terms of growth by simultaneously planting on AYC.2.2 agar. The growth and contamination rates by center are summarized in Table 1. It was observed that some centers had high contamination rates. It is thought that the reason for the high contamination rates is that the clinical samples coming to these centers were collected from different centers and transferred.

1st Center: A total of 249 samples were tested. *M. tuberculosis* growth was detected in 11 samples, 9 samples and 7 samples using the MGIT 960 system, LJ medium and AYC.2.2 agar, respectively. Comtamination rates of the MGIT 960 system, LJ medium and AYC.2.2 agar were 6.4%, 15.32% and 10.08%, respectively (Table 1). The mean growth time was 12.74 ± 3.74 days with MGIT 960 system, 24.42 ± 4.75 days with LJ medium, and 24.37 ± 4.96 days with AYC.2.2 agar (Table 2).

2nd Center: TK-medium was used as an automated system, while the LJ medium was used as a solid medium. A total of 288 pulmonary samples were tested. *M. tuberculosis* growth was detected in 21 samples, 34 samples and 18 samples using TK-medium, LJ medium and AYC.2.2 agar, respectively. Comtamination rates of TK-medium, LJ medium and AYC.2.2 agar were 16.66%, 17.01% and 24.64%, respectively (Table 1). The mean growth time; was 18.25 ± 9.32 days for TK-medium, 28.73 ± 7.44 days for LJ medium, and 31.72 ± 6.35 days for AYC.2.2 agar (Table 2).

3rd Center: A total of 1878 samples were tested in this center and they reported only positive values. This center used Ogawa medium, LJ medium, and AYC.2.2 agar for the cultivation and there was no automated system. *M. tuberculosis* growth was detected in 150 samples, 142 samples and 118 samples using Ogawa medium, LJ medium and AYC.2.2 agar, respectively. Comtamination rates of Ogawa medium, LJ medium and AYC.2.2 agar were 2.8%, 4.2% and 8.8%, respectively (Table 1). The average growth time in AYC.2.2 agar was determined as 20.26±7.43 days (Table 2).

4th Center: A total of 530 clinical samples were tested. Mycobacteria growth was detected in 11 of these samples. *M. tuberculosis* growth was detected in 11 samples, 7 samples and 8 samples using the MGIT 960 system, LJ medium and AYC.2.2 agar, respectively. Comtamination rates of the MGIT 960 system, LJ medium and AYC.2.2 agar were 4.1%, 6.03% and 9.8%, respectively (Table 1). The mean growth time was 15.27±6.37 days for MGIT 960 system, 22.14±9.1 days for LJ medium, and 22±8.45 days for AYC.2.2 agar (Table 2).

5th Center: A total of 256 clinical samples were tested. *M. tuberculosis* growth was detected in 14 samples, 12 samples and 13 samples using the MGIT 960 system, LJ medium and AYC.2.2 agar, respectively. Comtamination rates of the MGIT 960 system, LJ medium and AYC.2.2 agar were 1.9%, 5.4% and 5.8%, respectively (Table 1). The mean growth time was 13±4.24 days for MGIT 960 system, 32.16±6.23 days for LJ medium, and 33±5.73 days for AYC.2.2 agar (Table 2).

6th Center: A total of 1400 clinical samples were tested and 51 mycobacterial growth was detected. *M. tuberculosis* growth was detected in 51 samples, 40 samples and 35 samples using the MGIT 960 system, LJ medium and AYC.2.2 agar, respectively. Comtamination rates of the MGIT 960 system, LJ medium and AYC.2.2 agar were 1.28%, 2.71% and 1.92%, respectively (Table 1). Mean growth time was 9±3.11 days for MGIT 960 system, 18.68±5.32 days for LJ medium, and 18.34±4.63 days for AYC.2.2 agar (Table 2).

7th Center: A total of 958 samples were tested in Center 7. *M. tuberculosis* growth was detected in 112 samples, 94 samples and 75 samples using the MGIT 960 system, LJ medium and AYC.2.2 agar, respectively. Comtamination rates of the MGIT 960 system, LJ medium and AYC.2.2 agar were 2.1%, 4% and 4.7%, respectively (Table 1). The mean growth time for the MGIT 960 system was 14.74±7.65 days, 26.01±8.21 days for LJ medium, and 26.24±7.88 days for AYC.2.2 agar (Table 2).

The sensitivity, specitivity, positive predictive value (PPV), negative predictive value (NPV) of AYC2.2 agar/MGIT 960 system, AYC2.2 agar/LJ medium and MGIT 960 system/LJ medium were given in Table 3. Average growth time of isolates inoculated to the media were comparatively summarized in Table 2. Colony morphology and growth characteristic of *M.tuberculosis* in AYC.2.2 agar was presented in Fig. 1. Because Center 4 sent only the data related to AYC 2.2 agar, the other data from this center not included in Table 3.

According to the results of this stage, it was detected that the liquid automatized system (MGIT 960 system) was more efficient than the other media in terms of growth and time, and also similar results were obtained with AYC.2.2 agar and LJ medium in terms of growth.

4. Discussion

Blood agar (BA) is the most widely used and easily accessible medium in microbiology laboratories. In recent studies, it has been shown that BA can be used in the growth of *M. tuberculosis* at least as effectively as LJ. Also, BA was validated to test the susceptibility of the primary antituberculosis agents INH, RIF, EMB, and STM, and critical concentrations for each antibiotic were determined [5–11]. BA medium containing 5% whole sheep blood is not promising in terms of colony appearance. Although the growth of tuberculosis bacilli on BA is quite good, the clarity of the appearance of colonies is not as good as transparent media. Colonies can be observed more easily on Middlebrook 7H10 and 11 agar because of the transparent ground color. Studies have been conducted to

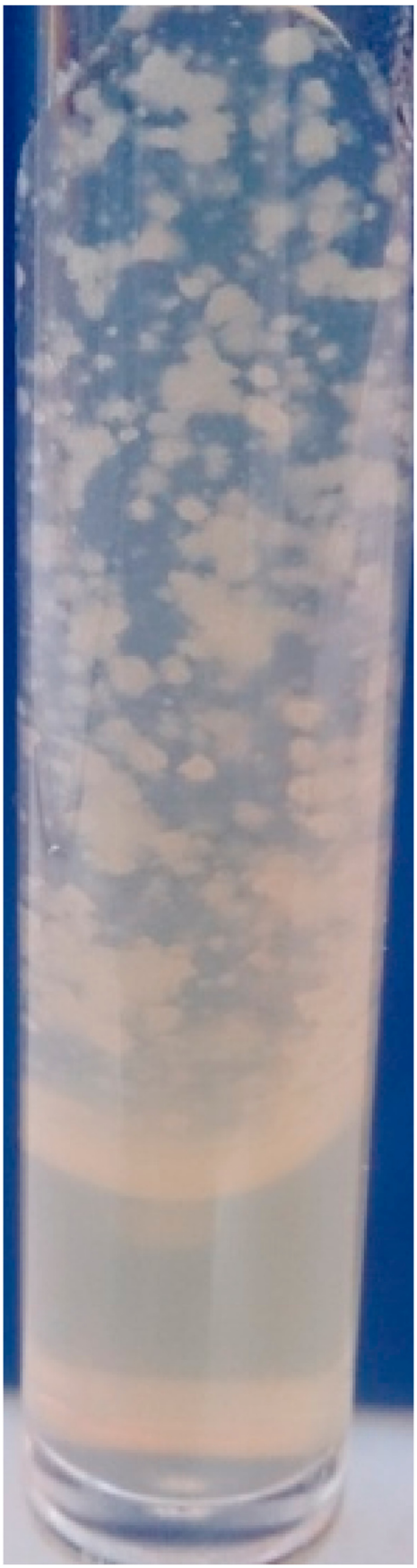

Fig. 1. Colony morphology and growth characteristic of *M.tuberculosis* in AYC.2.2 agar.

deal with this issue follow-up experiment performed and shed the light over the problem that when sheep serum was added instead of sheep blood when preparing blood agar medium, it was observed that the medium became transparent. This procedure allowed colonies to be determined in a shorter time [12].

The developed medium can be used both for the isolation of *M. tuberculosis* isolates from clinical samples and for detecting antibiotic susceptibility. Middlebrook 7H10 and 11 agar used in the growth of mycobacteria, especially *M. tuberculosis* isolates, are the media where the proportion method is applied, which is the reference method recommended for susceptibility testing. When applying this method, 10% OADC-enrichment should be added to Middlebrook 7H10 and 11 agar. OADC is a solution that is commercially available in liquid form and has a relatively short shelf life of 365 day [13]. However, sheep serum can be stored at −20 –C for up to 5 years without losing its performance [14]. Also, considering that OADC is used at a rate of 10% and sheep serum at a rate of 5%, it is observed that the use of sheep serum is much cheaper than the OADC.

In the study, although the cost of the automated system is high, it seems advantageous in terms of both growth and time. It was shown that there was no difference in growth time between AYC.2.2 agar and LJ medium and considering the preparation of the LJ medium, AYC.2.2 agar can be used as a solid medium in addition to liquid medium in mycobacteriology laboratories. It was also observed that some of the mycobacteria growing on LJ medium did not grow on AYC.2.2 agar. According to the feedback from some of the centers participating to the study, it was stated that some samples were first inoculated on routine media, and the remaining amount on AYC.2.2 agar. After some samples were inoculated into routine medium, there were not enough samples left for AYC.2.2 agar. We think that one of the most important reason for the low growth rates on AYC.2.2 agar is the small amount of inoculated samples.

5. Conclusion

In conclusion, when the data obtained were reviewed, it was thought that advanced multi-center studies in which all standards were metare a need for determining more precise results. Furthermore, more studies should be conducted for isolation of *M.tuberculosis* and performing antibiotic susceptibility tests using AYC.2.2 agar before it can be used as a routine media in the laboratories.

Authors contribution

AYC conceived and designed research. AYC conducted experiments. AYC contributed analytical tools. AYC analyzed data. AYC wrote the manuscript. IC, MU, GEG, CB, NO, SS, OY, GA, NK, YTC performed the laboratory tests and all authors read and approved the final manuscript.

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CRediT authorship contribution statement

Ahmet Yilmaz Coban: Formal analysis, Writing – original draft, conceived and designed research, conducted experiments, contributed analytical tools, analyzed data, wrote the manuscript. **Ismail Ceyhan:** performed the laboratory tests and all authors read and approved the final manuscript. **Meltem Uzun:** performed the laboratory tests and all authors read and approved the final manuscript. **Gonca Erkose Genc:** performed the laboratory tests and all authors read and approved the final manuscript. **Can Bicmen:** performed the laboratory tests and all authors read and approved the final manuscript. **Nuri Ozkutuk:** performed the laboratory tests and all authors read and approved the final manuscript. **Suheyla Surucuoglu:** performed the laboratory tests and all

authors read and approved the final manuscript. **Ozlem Yanar:** performed the laboratory tests and all authors read and approved the final manuscript. **Gonul Aslan:** performed the laboratory tests and all authors read and approved the final manuscript. **Nurbanu Kurnaz:** performed the laboratory tests and all authors read and approved the final manuscript. **Yeliz Tanriverdi Cayci:** performed the laboratory tests and all authors read and approved the final manuscript.

Declaration of competing interest

The author declares that he has no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijmmb.2021.12.002.

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