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´´EVALUACIÓN DE TRES MEDIOS DE CULTIVO ALTERNATIVOS PARA LA DETECCIÓN DE *Mycobacterium tuberculosis* Y DETERMINACIÓN DE LA SUSCEPTIBILIDAD DE FÁRMACOS MEDIANTE LA TÉCNICA MODS´´

TESIS PARA OPTAR EL GRADO DE MAESTRA EN MICROBIOLOGÍA

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DEDICATORIA

La presente tesis, está dedicada Dios por acompañarme en este proceso, a mis familiares y amigos por ser el motor para lograrlo, sin ellos esta investigación no hubiese sido posible. Finalmente dedico esta tesis a mi propia persona, por haber mantenido la perseverancia y determinación en alcanzar esta meta. Espero que este triunfo sea el primer paso hacia un futuro lleno de logros y satisfacciones.

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RESUMEN

La tuberculosis resistente a fármacos es un importante problema de salud a nivel global, El ensayo MODS (Microscopic Observation Drug Susceptibility Assay) en un método que usa un cultivo líquido que detecta a *M. tuberculosis* (MTB) y evalúa la susceptibilidad a isoniazida (INH) y rifampicina (RIF) a partir de muestras de esputo. Una adaptación del ensayo MODS combinado con la prueba Wayne, MODS-Wayne es una prueba fenotípica que evalúa indirectamente la susceptibilidad a pirazinamida (PZA) mediante la detección de ácido pirazinoico, producto enzimático de la pirazinamidasa. La detección de la susceptibilidad a PZA se realiza con MGIT 960 y el ensayo de Wayne, el problema de estas pruebas son su baja confiablidad, dificultad de utilización y el elevado costo. En este estudio evaluamos tres medios de cultivo deshidratados alternativos con el objetivo de obtener medios de fácil preparación reconstituyendo el medio con agua. Los medios deshidratados fueron: (1) medio estéril-liofilizado, (2) medio en polvo mezclado no esterilizada y (3) medio en polvo irradiado. Se evaluó el crecimiento de MTB y la susceptibilidad a los fármacos para INH, RIF y PZA. 282 muestras de esputo con frotis ácido-resistente positivo fueron evaluados. La positividad de la tuberculosis mostró una correlación de 0,925, 0,889 y 0,866 entre cada uno de los tres medios alternativos y el convencional. 47 muestras fueron multirresistentes (MDR) y 24 muestras fueron resistentes a la PZA. Las pruebas de susceptibilidad para MDR y PZA mostraron una excelente concordancia de 1 para MDR y PZA, entre los medios de cultivo alternativos y el estándar de consenso de referencia. El medio mezclado presentó mayor contaminación con respecto al medio convencional. Este análisis

demuestra que los medios de cultivo alternativos son apropiados y ventajosos para ser utilizados en el MODS en entornos de bajos recursos.

PALABRAS CLAVE

PIRAZINAMIDA, MODS-WAYNE, TUBERCULOSIS.

ABSTRACT

Drug-resistant tuberculosis is a major global health problem. The MODS (Microscopic Observation Drug Susceptibility Assay) trial is a method that uses a liquid culture that detects M. tuberculosis (MTB) and assesses susceptibility to isoniazid (INH) and rifampicin (RIF) from sputum samples. An adaptation of the MODS assay combined with the Wayne test, the MODS-Wayne test is a phenotypic test that indirectly assesses susceptibility to pyrazinamide (PZA) by detecting pyrazinoic acid, the enzyme product of pyrazinamidase. Detection of susceptibility to PZA is performed with MGIT 960 and Wayne's assay. The problem with these tests is their low reliability, difficulty of use, and high cost. In this study we evaluated three alternative dehydrated culture media with the objective of obtaining media that are easy to prepare by reconstituting the medium with water. The dehydrated media were: (1) sterile-lyophilized medium, (2) non-sterile mixed powdered medium, and (3) irradiated powdered medium. MTB growth and drug susceptibility to INH, RIF, and PZA were assessed. 282 sputum samples with positive acid-fast smears were evaluated. The positivity of tuberculosis showed a connection of 0.925, 0.889 and 0.866 between each of the three alternative means and the conventional one. 47 samples were multidrug resistant (MDR) and 24 samples were resistant to PZA. Susceptibility tests for MDR and PZA showed an excellent agreement of 1 for MDR and PZA, between the alternative culture media and the reference consensus standard. The mixed medium presented higher contamination with respect to the conventional medium. This analysis demonstrates that alternative culture media are appropriate and advantageous for use in MODS in low-resource settings.

KEY WORDS

PYRAZINAMIDE, MODS-WAYNE, TUBERCULOSIS.

I. INTRODUCCIÓN

La tuberculosis (TB) es una enfermedad infecciosa causada por el bacilo Mycobacterium tuberculosis, que afecta principalmente los pulmones y se transmite a través de la inhalación de microgotas expulsadas por pacientes con TB (1). El tratamiento estándar para la TB se basa en el uso de medicamentos como rifampicina, isoniazida, etambutol y pirazinamida (2). Sin embargo, el uso inadecuado de estos medicamentos puede llevar al desarrollo de cepas resistentes, conocidas como cepas multidrogo resistentes (MDR-TB), que son resistentes a isoniazida y rifampicina (3). La resistencia a los fármacos utilizados en el tratamiento de la TB es un problema de salud pública a nivel mundial. En 2020, se informaron 9,9 millones de casos de tuberculosis y 1,5 millones de muertes en todo el mundo (4). Además, se notificaron 206.000 nuevos casos de tuberculosis MDR-TB, donde Perú representa el 13,4% de todos los casos de MDR-TB notificados en la región de las Américas (5).

La prueba MODS (Microscopic Observation Drug Susceptibility Assay) es una técnica que se utiliza para detectar la presencia de MTB y evaluar su susceptibilidad a isoniazida y rifampicina a partir de muestras de esputo (6). Esta prueba se basa en la visualización de cordones de MTB en una placa de 24 pozos donde se encuentra la muestra de esputo descontaminada y resuspendida en caldo Middlebrook 7H9 suplementado con OADC (ácido oleico, albúmina, dextrosa y catalasa) y un cocktail de antibióticos (PANTA) (7). Aunque la prueba MODS es altamente sensible y específica para detectar la TB y la MDR-TB, en Perú su uso esta centralizado a solo 4 laboratorios a nivel nacional debido a la dificultad en la preparación del OADC ya que su preparación requiere de al menos 53 horas (8).

Las pruebas para detectar la susceptibilidad a PZA son BACTEC MGIT 960, secuenciamiento del gen pncA y el ensayo de Wayne, el problema de estas pruebas es el elevado costo, la dificultad de utilización y que son realizadas a partir de cepas de MTB, por lo que el tiempo de obtención de resultados es más prolongado (9). Una adaptación del ensayo MODS combinado con la prueba Wayne, MODS-Wayne es una prueba fenotípica que evalúa indirectamente la resistencia a PZA mediante la detección de POA, el producto enzimático de la pirazinamidasa a partir de muestras de esputo (10).

Con el objetivo facilitar el diagnóstico de la tuberculosis, Sheen et al., 2022, evaluaron preparaciones alternativas del medio de cultivo MODS, proponiendo variantes de medios de cultivo que podrían optimizar la preparación, el transporte y la manipulación de los reactivos necesarios para el ensayo MODS (11). Su descubrimiento en cuanto a la detección del crecimiento de TB ha permitido obtener "medios modificados" denominados: medio liofilizado (LM), mezclado no esterilizado (MM) y mezclado irradiado con radiación gamma (IM), estas presentaciones deshidratadas están listas para ser usados para comparar el crecimiento. En este contexto, el presente estudio plantea la evaluación de 3 presentaciones deshidratadas, listas para ser usadas, del medio 7H9-OADC-PANTA para la determinación de la susceptibilidad a INH, RIF y PZA en muestras de esputo usando el método MODS, esto contribuiría a descentralizar la prueba y facilitar su implementación en más laboratorios a nivel nacional, mejorando así el diagnóstico y el tratamiento adecuado de la TB en el Perú.

Objetivos:

Objetivo General:

Evaluar 3 presentaciones deshidratadas del medio 7H9-OADC-PANTA para la detección de crecimiento y evaluación de susceptibilidad a INH, RIF y PZA de M. tuberculosis mediante el método de cultivo MODS a partir de muestras de esputo.

Objetivos Específicos:

- Evaluar el crecimiento de *M. tuberculosis* a partir de muestras de esputo en los medios de cultivo liofilizado, mezclado no esterilizado y mezclado irradiado con radiación gamma con respecto al medio Middlebrook 7H9-OADC convencional.
- Evaluar la susceptibilidad a INH y RIF de *M. tuberculosis* mediante la técnica MODS en los medios de cultivo liofilizado, mezclado no esterilizado y mezclado irradiado con radiación gamma.
- Evaluar la susceptibilidad a PZA de *M. tuberculosis* mediante la técnica MODS-Wayne en los medios de cultivo liofilizado, mezclado no esterilizado y mezclado irradiado con radiación gamma.

II. METODOLOGÍA

1.1. Preparación de los medios de cultivos.

El medio convencional se preparó siguiendo el protocolo de MODS, mientras que los medios deshidratados se realizaron siguiendo las indicaciones de Sheen *et al*., 2022. Brevemente, para el medio convencional se pesaron y mezclaron los componentes y fue esterilizado mediante autoclave, una vez enfriado se enriqueció con el OADC. El medio liofilizado fue preparado de la misma manera similar, el medio estéril fue alicuotado en viales de 25 mL para una posterior congelación y liofilización. Los medios mezclados fueron preparados agregando todos los componentes en un vial de 25 mL (sin esterilizar), los medios mezclados irradiados fueron enviados al IPEN para ser esterilizado por radiación gamma. Se muestran los componentes que contiene cada medio de cultivo (tabla 1). El ácido oleico no fue agregado al medio debido a que es un componente líquido y su adición no afecta al crecimiento de las micobacterias (12); en el caso del medio mezclado, no se agregó catalasa debido a que su adición, a pesar de que ejerce un efecto positivo en el crecimiento de *M. tuberculosis*, también incrementa la contaminación del medio (11). Al momento del uso, los viales fueron resuspendidos con 25 mL de agua destilada estéril.

Tabla1. Componentes del medio convencional y los medios alternativos.

Azul: componentes del OADC

Convencional	**Liofilizado**	**Mezclado sin esterilizar**	**Mezclado Irradiado**
7H9 Middlebrook	7H9 Middlebrook	7H9 Middlebrook	7H9 Middlebrook
Casitona	Casitona	Casitona	Casitona
Glicerol	Glicerol	Glicerol	Glicerol
PANTA	PANTA	PANTA	PANTA

Albúmina	Albúmina	Albúmina	Albúmina
NaCl	NaCl	NaCl	NaCl
D-Glucosa	D-Glucosa	D-Glucosa	D-Glucosa
ácido oleico	ácido oleico	-	-
catalasa	catalasa	-	catalasa

1.2. MODS y MODS Wayne de las muestras de esputo

Las muestras de esputo fueron digeridas y descontaminadas de otros microorganismos mediante el método Petroff modificado usando una solución NaOH-Citrato de Sodio + NALC (12). El descontaminado fue transferido a 4 tubos que contenían medios MODS convencional, liofilizado, mezclado sin esterilizar y mezclado irradiado con radiación gamma. Cada suspensión fue alicuotado (900 µl) en 6 pozos de una placa de 24 pozos. En cada pozo fue adicionado 100 µl de medio y antibióticos de acuerdo a la Figura 1. La placa fue incubada a 37ºC.

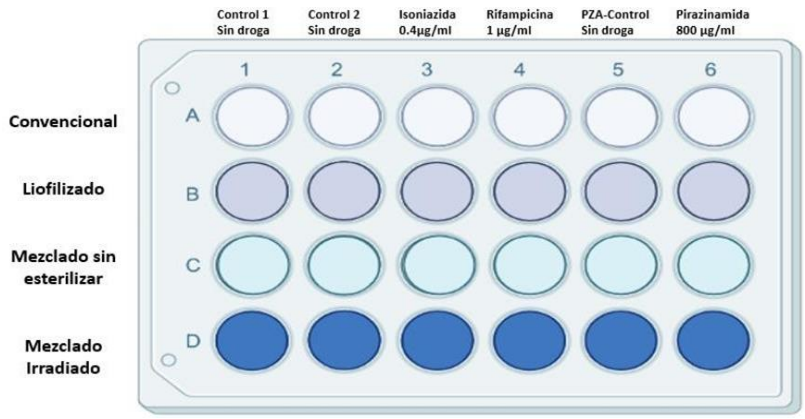

Figura 1. Representación del esquema empleado en l evaluación de cada variante de medio de cultivo en una placa de 24 pozos.

La contaminación de los medios fue revisado a las 24 h y el crecimiento de los cordones de *M. tuberculosis* a partir del día 5. La fecha de crecimiento y la cantidad

de crecimiento fue registrado el primer día de observación de cordones (positividad). El crecimiento bacteriano fue registrado también los días 7 y 14. El crecimiento bacteriano se describió según las categorías: 1, crecimiento que cubre el 25% del pozo; 2, crecimiento que cubre 25–50% del pozo; 3, crecimiento que cubre 50–75% del pozo; y 4, crecimiento cubriendo el 100% del pozo (Figura 2).

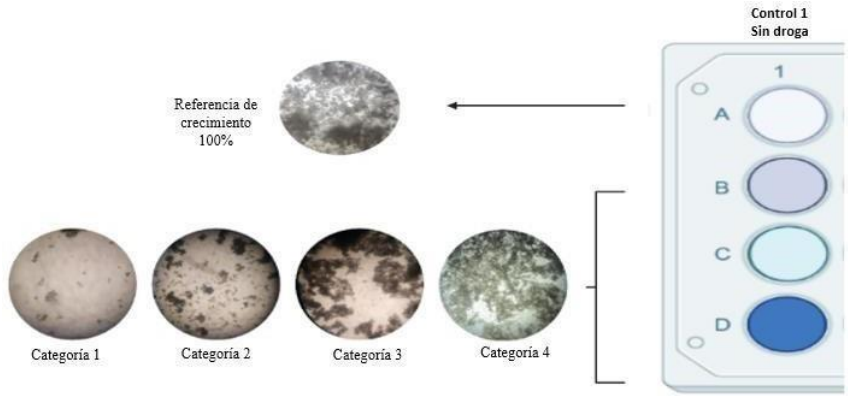

Figura 2. Observación microscópica de cordones de *M. tuberculosis*. El crecimiento bacteriano se informó usando una escala cualitativa que estimaba el área del pozo cubierta por el crecimiento bacteriano.

En ensayo del MODS-Wayne se realizó agregando PZA al pozo 6 luego de observar crecimiento en los pozos 5 y 6. Con el objetivo que la PZA sea convertida en POA, la placa fue incubada 3 días más; luego se adicionó 100 µl de 10% sulfato de amonio ferroso (SAF) al pozo control y al pozo PZA. La presencia de color rojizo, producto de la reacción del SAF con el POA, indicó la producción de POA (sensible a PZA) y la ausencia de color indicó la ausencia de POA (resistente a PZA). La intensidad del color fue registrada desde baja a muy alta (Figura 3).

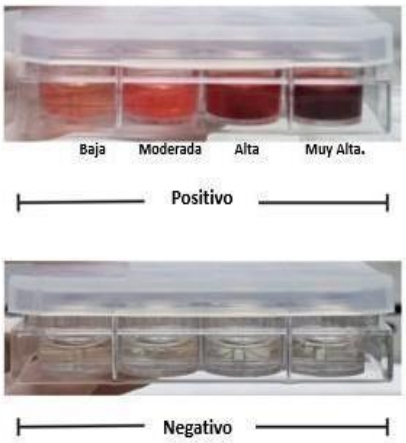

Figura 3. Lectura MODS-Wayne y Wayne Convencional: Observación del complejo rojizo producto de la reacción del SAF y POA en muestras sensibles a PZA (positivo) y ausencia del complejo rojizo en muestras resistentes (negativo).

1.3. Determinación de la susceptibilidad a PZA a partir de cepas: secuenciamiento *pncA*, Wayne convencional y MGIT

La susceptibilidad a PZA fue determinado en las cepas MDR y monoresistentes (INH o RIF) mediante s**ecuenciamiento del gen *pncA*** a través de la extracción de ADN mediante fenol cloroformo y amplificación del gen *pncA* con los primers P1 y P6 (13, 14). El fragmento amplificado fue secuenciado por Psomagen y analizado con herramientas bioinformáticas para encontrar mutaciones a lo largo de la región del gen *pncA*; **Wayne convencional** a través del cultivo de las cepas en medio Dubos a 37°C durante 7 días. La producción de POA fue revelado con la adición de sulfato de amonio ferroso al 1%. El color fue registrado: Color rojizo: Positivo (PZA susceptible); sin color: Negativo (PZA resistente) (15) y **MGIT960PZA (MGIT)** a través del cultivo de las cepas (3 x 108 ufc/ml) en medio 7H9 como control (sin PZA) y en medio 7H9 con PZA. Los tubos fueron colocados en el equipo BACTEC e incubados entre 8 y 15 días (16). La fluorescencia en el equipo BD BACTEC desde 0 (no se observa crecimiento) hasta 400 (crecimiento típico de

las micobacterias: en la base en forma de grumos). Los resultados fueron interpretados cuando el tubo control presentó crecimiento; la cepa fue determinada como susceptible cuando la fluorescencia en el tubo con PZA fue de cero y resistente cuando la fluorescencia del tubo con PZA fue 400.

1.4. Análisis estadísticos

El crecimiento de MTB, tasa de positividad y de contaminación entre todos los medios de cultivo se compararon utilizando las pruebas de rango con signo de Wilcoxon para muestras pareadas. El día de positividad fue analizado con el test de anova de una vía y la correlación con el coeficiente se correlación de Spearman. La frecuencia de sensibilidad antibiótica a INH y RIF se comparó entre todos los medios de cultivo utilizando la prueba de proporciones. Para probar la hipótesis de que no hay diferencias en la semana de crecimiento entre los medios de cultivo, se realizó una prueba de Friedman para diseños de bloques completamente aleatorizados, considerando como factores: la semana en la que se registró el crecimiento bacteriano (inicial, semana-1 y semana-2) y el tipo de medio de cultivo. Para todos los análisis, cada medio de cultivo se comparó con el medio de cultivo MODS convencional (CM). Finalmente, se estimó la concordancia entre los resultados dicotómicos del MODS-Wayne en cada variante mediante el índice Kappa.

III. RESULTADOS

1.5. Rendimiento de los medios de cultivos alternativos y en las variantes deshidratadas.

Un total de 282 muestras de esputo se cultivaron en los medios CM, LM y MM, y 253 de las 282 muestras se evaluaron en el medio IM. La tasa de contaminación global se situó entre el 8,2% y el 13,5%. La tasa de contaminación más alta se registró en el medio MM. No se observó ninguna diferencia significativa en la tasa de contaminación de IM y LM en comparación con el medio convencional (CM) (Tabla 2). Las tasas de positividad de los medios no mostraron una diferencia significativa (P > 0,1) en comparación con el medio CM (Tabla 2). No se observaron diferencias significativas en el tiempo medio de crecimiento entre LM y MM en comparación con el medio CM, sin embargo, el tiempo de crecimiento en IM fue significativamente menor. (Tabla 2). El crecimiento bacteriano en MM, LM e IM fue muy similar al de CM en todas las categorías, sin embargo, se observó una diferencia significativa en MM con respecto a CM (p=0.0413). El medio liofilizado fue el que mostró un patrón de cobertura más similar al medio convencional (p=0.4049), mientras que IM, aunque mostró un porcentaje mayor de scores altos, la desviación no fue significativa (p=0.0599) (Tabla 3). Más del 90% de las muestras mostraron un crecimiento bacteriano que iba de bajo a alto (es decir, un crecimiento que cubría hasta el 75% del área del pozo) (Figura 4, Tabla 3).

Tabla 2. Rendimiento de los medios de cultivo alternativos en comparación con el medio estándar MODS. Se muestran las tasas de contaminación y positividad.

Medio de cultivo	Contaminación		Positividad		Tiempo de positividad		Correlación
	% (n/N)	p-value	% (n/N)	p-value	Mediana	p-value	
Convencional (CM)[$]	8.2 (23/282)	-	86.8 (225/259)	-	9.0	-	-
Liofilizado (LM)	8.9 (25/282)	**0.617**	89.5 (230/257)	0.818	8.5	**0.131**	**0.925**
Mezclado no esterilizado (MM)	13.5 (38/282)	**0.005**	86.47 (211/244)	0.637	9.5	**0.621**	**0.889**
Mezclado irradiado (IM)	8.3 (21/253)	**0.563**	83.6 (194/232)	0.106	8.0	**0.007**	**0.866**

$ El medio convencional MODS fue considerado como el medio de cultivo de referencia para la comparación.

Tabla 3. Nivel de crecimiento bacteriano en cuatro medios de cultivo en el ensayo de cultivo MODS el día de la detección de MTB.

Medio decultivo	# Muestras	Categorías de crecimiento bacteriano*			
		Categoría 1	Categoría 2	Categoría 3	Categoría 4
		1 – 25% well area	25 – 50% well area	50 – 75% well area	75 – 100% well area
Convencional (CM)[$]	225	30.1 (78)	25.8 (67)	25.4 (66)	5.4 (14)
Liofilizado(LM)[a]	230	31.1 (80)	26.8 (69)	25.6 (66)	5.8 (15)
Mezclado sin esterilizar (MM)[b]	211	30.3 (74)	24.5 (60)	27.0 (66)	4.5 (11)
Mezclado irradiado(IM)[c]	194	28.9 (67)	25.9 (60)	21.5 (50)	7.3 (17)

*Porcentaje y número de muestras reportadas; $ El medio convencional MODS fue considerado el medio de cultivo de referencia para la comparación.

a: Z=-0.833, p=0.4049. *b:* Z=2.040, p=0.0413. *c:* Z=1.88, p=0.0599.

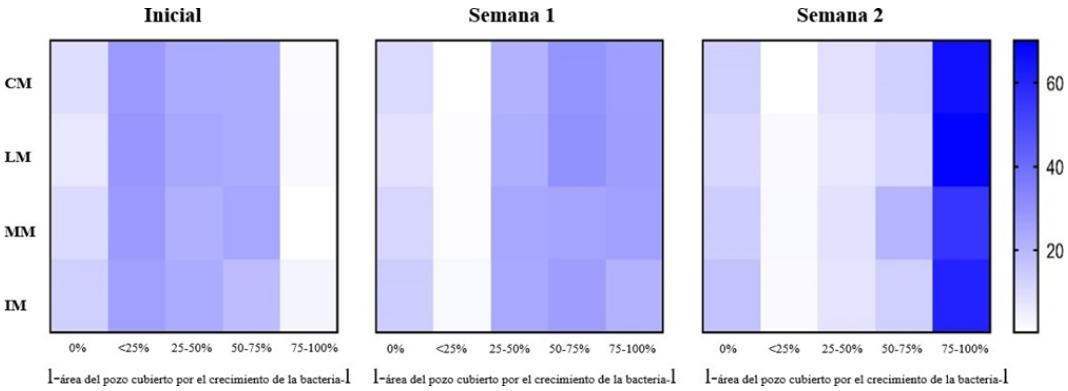

Figura 4. Niveles de crecimiento bacteriano registrados en los medios de cultivo evaluados en tres tiempos diferentes de lectura de la placa.

1.6. Determinación de susceptibilidad a isoniacida y rifampicina

En general, para los medios de cultivo alternativos, la sensibilidad y la especificidad fueron del 100% para la detección de MDR-TB en comparación con el medio MODS convencional, y no hubo diferencias significativas entre LM (P = 1000), MM (P = 1000) y IM. (P = 1000) en comparación con el medio MODS convencional (MM) (Tabla 4).

Tabla 4. Rendimiento del medio de cultivo para la determinación del perfil de resistencia a la isoniazida y a la rifampicina en el cultivo de MODS.

Medio de cultivo	**Isoniacida**		**Rifampicina**		**MDR**		**Concordancia**
	Sen* % (n/N)	Spe# % (n/N)	Sen % (n/N)	Spe % (n/N)	Sen % (n/N)	Spe % (n/N)	Kappa index
Liofilizado (LM)	100 (32/32)	100 (137/137)	100 (3/3)	100 (137/137)	100 (47/47)	100 (137/137)	1

Mezclado sin esterilizar (MM)	100 (31/31)	100 (124/124)	100 (3/3)	100 (124/124)	100 (43/43)	100 (124/124)	1
Mezclado irradiado (IM)	100 (29/29)	100 (110/110)	100 (3/3)	100 (110/110)	100 (42/42)	100 (110/110)	1

*Sen, sensibilidad; Spe, especificidad. El medio convencional MODS fue considerado como el medio de cultivo de referencia para la comparación

1.7. Susceptibilidad a pirazinamida

Se realizó el estándar de referencia para pirazinamida (CRT) a las 83 muestras que mostraron resistencia a la RIF, a la INH y a ambas (MDR). De las 83 muestras, 24 se clasificaron como resistentes a PZA por CRT, 38 (45,78%) eran resistentes a PZA según el MGIT, la prueba clásica de Wayne indicó que 19 (22,89%) eran resistentes a PZA, mientras que en 25 muestras (30,12%) se identificó al menos una mutación en el promotor o en la secuencia del gen pncA (Tabla 5).

Según el protocolo MODS-Wayne, el número de aislados resistentes a PZA osciló entre el 13 y el 16% en los medios de cultivo alternativos y en el medio CM estándar (tabla 6). No se encontraron diferencias significativas entre el MODS-Wayne de CM (P =0.763), LM (P = 0.366), MM (P = 0.527) y IM. (P = 0.527) en comparación con el CRT (Tabla 7). Los resultados en el presente trabajo arrojaron una perfecta concordancia entre los medios alternativos y el CM en el MODS-Wayne con valores de kappa = 1 (Tabla 7).

Tabla 5. Resultados de la determinación de la resistencia a la PZA.

Número de muestras	Perfil de Resistencia a PZA				Secuenciación *pncA* *
	MODS Wayne	CRT	MGIT BACTEC 960	Prueba clásica Wayne	
39	Sensible	Sensible	Sensible	Sensible	Sin mutación
12	Sensible	Sensible	Resistente	Sensible	
1	Resistente	Sensible	Resistente	Sensible	
5	Resistente	Sensible	Sensible	Sensible	
1	Resistente	Resistente	Resistente	Resistente	
8	Resistente	Resistente	Resistente	Resistente	A152G – H51R
2	Resistente	Resistente	Resistente	Sensible	
1	Sensible	Sensible	Sensible	Sensible	
4	Resistente	Resistente	Resistente	Resistente	A29G – Q10R
2	Resistente	Resistente	Resistente	Sensible	
2	Sensible	Resistente	Resistente	Resistente	
1	Sensible	Resistente	Resistente	Sensible	G371C – G124A
1	Sensible	Resistente	Resistente	Resistente	T11 – L4S
1	Resistente	Resistente	Resistente	Resistente	A424G – T142A
1	Resistente	Resistente	Resistente	Resistente	A170T – H57L
1	Resistente	Sensible	Sensible	Sensible	T26G – V9G
1	Resistente	Resistente	Resistente	Resistente	A478C – T159P

* Las mutaciones H51R, Q10R, L4S, T142A y V9G están relacionadas con la resistencia fenotípica a la PZA.

Tabla 6. Perfil de resistencia a la PZA según el ensayo MODS-Wayne.

Medio de cultivo	# muestras*	Frecuencia de muestras % (N)		
		PZA resistente	PZA sensible	Contaminación
Convencional (CM)	225	15.6 (35)	79.1 (178)	5.3 (12)
Liofilizado (LM)	230	13.7 (29)	78.7 (166)	7.6 (16)
Mezclado sin esterilizar (MM)	211	14.8 (34)	77.8 (178)	7.4 (17)
Mezclado irradiado (IM)	194	16.1 (31)	74.6 (144)	9.3 (18)

*Diferencia en el número de muestras se debe a la contaminación.

Tabla 7. Rendimiento de MODS-Wayne evaluado en tres medios de cultivo alternativos y comparado con una prueba de referencia de consenso.

Medio de cultivo	# muestras*	Sensibilidad % (n/N)	Especificidad % (n/N)	p-value	Kappa index
Convencional (CM)	78	82 (18/22)	88 (49/56)	0.763	-
Liofilizado (LM)	74	81 (17/21)	87 (46/53)	0.366	1
Mezclado sin esterilizar (MM)	71	79 (15/19)	88 (46/52)	0.527	1
Mezclado irradiado (IM)	71	82 (18/22)	88 (43/49)	0.527	1

*Diferencia en el número de muestras se debe a la contaminación

IV. DISCUSIÓN

En este análisis comparativo, se demuestra que tres preparaciones alternativas de medios de cultivo deshidratados permiten la detección del crecimiento de MTB utilizando el protocolo de prueba de MODS (6). Los medios alternativos mostraron un porcentaje similar de crecimiento de MTB y un tiempo de respuesta de prueba comparable al medio convencional MODS y no se observaron diferencias significativas en el patrón de crecimiento y formación morfológica de MTB en los medios alternativos (6,17). La esterilidad de los medios de cultivo es crucial, y se mencionan diferentes métodos de esterilización, incluyendo la radiación gamma (18,19). El uso de la mezcla de antibióticos PANTA se informa como una forma efectiva de reducir la contaminación en los cultivos de MTB (6). Los medios alternativos (LM, MM e IM) no presentan diferencias significativas en la tasa de contaminación en comparación con el medio MODS convencional, los estándares aceptables de contaminación y se sugiere que una mejora en el proceso de descontaminación puede contribuir al rendimiento de los medios de cultivo alternativos, principalmente en el MM que presentó un porcentaje de contaminación por arriba del rango aceptable (20). Debido a la complejidad de la preparación del medio MODS convencional se sugiere que una metodología más sencilla podría facilitar su implementación a mayor escala. Los medios deshidratados se presentan como opciones viables, especialmente el MM por su costo y preparación sencilla (11).

La tuberculosis farmacorresistente es un problema global, especialmente en países de bajos recursos (4). Las pruebas automatizadas y moleculares son limitadas en su aplicación en estos países, por lo que se utilizan pruebas basadas en medios de

cultivo sólidos (21). La prueba MODS es una opción que permite detectar la resistencia a múltiples fármacos (7,22). En este estudio, se compararon medios de cultivo alternativos y se encontraron resultados comparables en términos de sensibilidad y especificidad para detectar resistencia a fármacos. Sin embargo, la determinación de la resistencia a la PZA sigue siendo un desafío y se ha propuesto una variante de la prueba MODS, la prueba MODS-Wayne, que muestra resultados prometedores (10). En resumen, el uso de medios alternativos en la prueba MODS puede fortalecer el diagnóstico y la detección de resistencia a fármacos, especialmente en entornos con recursos limitados.

V. CONCLUSIONES

- La positividad fue similar en todos los medios de cultivo evaluados con un rango de 83 al 89%.
- La contaminación fue mayor en el medio mezclado no esterilizado (13.5%) con respecto al medio convencional (8%), mientras que en los medios liofilizado y mezclado irradiado la contaminación fue similar al medio convencional.
- El porcentaje de monoresistentes a RIF e INH y MDR en los medios evaluados fue similar al medio convencional.
- El porcentaje de susceptibles y resistentes a PZA fue similar en los medios evaluados con respecto al medio convencional.

VI. RECOMENDACIONES

- Se recomienda realizar un estudio más extenso que incluya muestras tanto positivas como negativas para poder determinar la sensibilidad y especificidad de diagnóstico de cada medio alternativo.

- Se recomienda realizar un estudio de la estabilidad en el tiempo de los componentes de cada medio de cultivo, y la temperatura optima de almacenamiento.

- Se recomienda mejorar la técnica de descontaminación de esputo y evaluar el medio mezclado sin esterilizar ya que es el candidato más sencillo de producir, de menor costo y muestra buenos resultados en crecimiento y detección de la susceptibilidad.

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VIII. ANEXOS

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Evaluation of three alternatives cost-effective culture media for *Mycobacterium tuberculosis* detection and drug susceptibility determination using the microscopic observation drug susceptibility (MODS) assay

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ABSTRACT

Tuberculosis phenotypic detection assays are commonly used in low-resource countries. Therefore, reliable detection methods are crucial for early diagnosis and treatment. The microscopic observation drug susceptibility (MODS) assay is a culture-based test to detect Mycobacterium tuberculosis and characterize drug resistance in 7–10 days directly from sputum. The use of MODS is limited by the availability of supplies necessary for preparing the enriched culture. In this study, we evaluated three dry culture media that are easier to produce and cheaper than the standard one used in MODS [1]: an unsterilized powder-based mixed (Boldú et al., 2007) [2], a sterile-lyophilized medium, and (Sengstake et al., 2017) [3] an irradiated powder-based mixed. Mycobacterial growth and drug susceptibility were evaluated for rifampin, isoniazid, and pyrazinamide (PZA). The alternative cultures were evaluated using 282 sputum samples with positive acid-fast smears. No significant differences were observed in the positivity test rates. The positivity time showed high correlations (Rho) of 0.925, 0.889, and 0.866 between each of the three alternative media and the standard. Susceptibility testing for MDR and PZA showed an excellent concordance of 1 compared to the reference test. These results demonstrate that dry culture media are appropriate and advantageous for use in MODS in low-resource settings.

1. Introduction

Tuberculosis is an endemic infectious disease in low- and middle-income countries (LMIC), and drug-resistant tuberculosis is a signifi- cant global health concern. In 2020, the WHO reported 9.9 million tuberculosis cases and 1.5 million deaths. Additionally, 206,000 new multidrug-resistant tuberculosis (MDR-TB) cases have been reported [1]. MDR-TB is caused by Mycobacterium tuberculosis (MTB) strains that are resistant to the first-line antibiotics rifampin (RIF) and isoniazid (INH). Pyrazinamide (PZA) is another antibiotic used as first-line MTB therapy; however, PZA resistance has been reported in at least 50% of MDR-TB cases [2,3]. MDR-TB is especially problematic in low- and middle-income countries (LMICs), with Peru representing 13.4% of all reported MDR-TB cases in the Americas [4]. Mismanagement of anti-biotics used to treat MTB increases the incidence of MDR-TB in TB-endemic countries [5]. Rapid and affordable TB diagnostic and drug susceptibility tests with high sensitivity and specificity are necessary for the early detection and personalized TB treatment.

Phenotypic and molecular approaches have been used for drug susceptibility testing (DST). Molecular approaches are based on the detection of mutations that confer drug resistance [6,7]. Molecular DST offers high sensitivity and specificity and is recommended by WHO. However, despite multiple options for DST, such as line-probe assay,

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Abbreviations

MM	Standard MODS culture medium
PM	Powder-component mixed culture medium
LM	Lyophilized liquid culture medium
IM	Irradiated powder-component mixed culture medium
RIF	Rifampin
INH	Isoniazid
PZA	Pyrazinamide.
PANTA	Antibiotic mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin
CRT	Consensus reference test
MGIT	Mycobacteria growth indicator tube

high-resolution melting, and Gene-Xpert, molecular DST remains costly and resource-prohibitive in LMICs, where phenotypic DST methods are still commonly used [8–10]. Phenotypic DST methods include classic assays such as the agar proportion method, absolute concentration method, resistant ratio method, and automated mycobacterial growth indicator tube (MGIT) [7,11]. All phenotypic DST methods rely on the use of a critical drug concentration that directly affects the DST performance [10,12]. In addition, these methods require prior isolation of the patient's MTB strain, which significantly increases the overall turnaround time of the test. Currently, the MGIT assay is considered the standard test for first- and second-line anti-TB drug susceptibility testing [13,14]. Nevertheless, because of its high cost, many LMICs do not routinely use MGIT [11].

For PZA, a discrepancy between in vitro and in vivo sensitivities has been previously reported [15]. Therefore, there is no reference DST for determining PZA susceptibility [16]. The WHO recommends DNA sequencing of *pnc*A and MGIT-BACTEC-960 as molecular and phenotypic DST, respectively [17–19]. However, technical limitations of both tests have been reported. In DNA sequencing, not all mutations reported in the *pnc*A gene are related to PZA resistance, and the prevalence of mutations is highly variable depending on geographical distribution [20,21]. However, high technical expertise is required to prepare MTB isolates for MGIT-960. The bacterial load of the inoculum and the PZA concentration used are critical factors in the performance of MGIT-960 to determine PZA susceptibility [18,19]. A consensus reference test (CRT) has been proposed as an alternative for evaluating PZA susceptibility. CRT uses more than one DST to determine the PZA resistance profile based on consensus results [16]. The microscopic observationdrug susceptibility (MODS) assay is a validated alternative low-cost phenotypic DST for susceptibility characterization of both first- and second-line MTB antibiotics [22,23]. The MODS test is based on observing specific cords during MTB growth in liquid culture medium using inverted microscopy [22]. The MODS-Wayne test is an adaptationof the MODS test, based on the classic Wayne assay [17], which indi- rectly determines PZA resistance by detecting the lack of pyr- azinamidase enzymatic activity of MTB through the detection of the pyrazinoic acid metabolite. The MODS assay has shown a shorter turn- around time compared to traditional phenotypic DST, simultaneous evaluation of drug susceptibility profile, low cost per test, high sensi- tivity and specificity, and being patent-free [22,23]. However, this diagnostic assay is not routinely used. Two technical factors limit the use of the MODS assay:1) the high cost and limited availability of the supplies necessary for the enriched culture media and 2) the limited availability of equipment and trained personnel for preparing enriched culture media [24].

The MODS culture medium is Middlebrook 7H9 medium supplemented with glycerol, casitone, and oleic acid, albumin, dextrose, and catalase (OADC) to promote MTB growth [24,25]. A PANTA antibiotic mixture (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) is added to minimize contamination from sputum sam- ples [26]. Reagents, such as OADC and PANTA, are commercially available. However, home-based reagents, such as OADC, can be usedfor enriched culture medium to reduce the assay implementation cost [24]. Nonetheless, the test turnaround time could increase by at least 72 h, which represents a limitation in laboratories with high diagnostic demand. A commercial culture kit (Hardy Diagnostic, USA) containing liquid medium was used for the MODS assay [27]. The culture perfor- mance of the kit was compared to that of the conventional MODS culture medium, and no significant differences were observed between the kit and the conventional MODS assay [28]. However, the kit requires continuous cold chain (4 °C) transportation for all components, which increases the cost and limits its use in LMIC settings.

In a previous study, we evaluated cost-effective alternatives to the standard MODS media, which included a powder-component mixed medium (PM) that does not require sterilization to prepare, a sterile lyophilized liquid medium (LM), and an irradiated medium (IM) [29]. These media contain all the MODS media components as a dry presentation and only require water and PANTA to be used. The results showed that all of them had similar performance to the standard MODS culture medium when evaluating the growth of MTB isolates and sputum samples [29], although PM was the simplest to prepare.

Here, we evaluated three alternative culture media against the standard 7H9 in a MODS assay to determine drug susceptibility to RIF, INH, and PZA from clinical sputum samples. Drug susceptibility profiles were compared for RIF and INH against the standard MODS assay, andfor PZA against a consensus reference test that included pncA sequencing, MGIT-PZA, and the classic Wayne assay.

1. Material and methods

1.1. Clinical samples

A total of 282 remnants of clinical sputum samples were obtained from recently diagnosed and treated patients. Samples were collected from the Hospital Nacional Cayetano Heredia, Lima, Peru; Hospital Hipólito Unanue, Lima, Peru; and the Regional Tuberculosis Reference Laboratory, Callao, Lima, Peru. Ethical approval was obtained from the Universidad Peruana Cayetano Heredia (345-15-19).

Samples were decontaminated according to a standardized protocol [30]. Briefly, the sample was mixed with a fresh 2% N-acetyl-L-cystei- nesodium hydroxide (NALC-NaOH) solution at a ratio of 1:1 to a final volume of 4 mL and vortexed. The sample was then incubated at room temperature for 15 min and centrifuged at 3,000 rpm at 17 °C for 15 min. Finally, the pellet was resuspended in 2.5 mL of PBS.

1.2. Evaluated culture media

1.2.1. Standard MODS culture medium (MM)

Middlebrook 7H9 broth was prepared as described previously [31]. The culture broth base (500 mL) was enriched with 625 mg BactoTM Casitone (BD, USA) and 1.55 mL glycerol (J.T. Baker, USA). The medium was sterilized and stored at 4 °C. Before use, commercial OADC (2.5 mL) and PANTA (500 μL) were added to a 22.5 mL aliquot.

1.2.2. Powder-component mixed media (PM)

The media were prepared according to the MODS protocol [27] with the following modifications: Aliquots were prepared with 0.148 g of Middlebrook 7H9 broth base (BD, USA), 0.031 g BactoTM Casitone (BD, USA), 0.125 g bovine serum albumin (Sigma-Aldrich, USA), 0.021 g NaCl (Merck, USA), and 0.05 g D-Glucose (Sigma-Aldrich, USA). The PM culture medium was not sterilized. Before use, aliquots were resuspended with 23.45 mL of distilled water, then 1.55 mL glycerol and 500 μL PANTA were added.

1.1.1. Lyophilized liquid media (LM)

Lyophilized culture medium was prepared according to the standard MODS protocol [27]. After sterilization by autoclaving, the LM medium was enriched with a homemade OADC and aliquoted into 25 mL aliquots. Aliquots were frozen for 24 h at —80 °C. Lyophilization was performed at —46 °C with a pressure between 50 × 10−3 and 70 × 10-3 MBAR in LYPHLOCK 12 (Labconco, USA). Before use, aliquots were resuspended in 25 mL of distilled water, and 500 µL PANTA was added.

1.1.2. Irradiated powder-component mixed media (IM)

The irradiated culture medium was prepared using the same protocol as for PM. IM aliquots were irradiated with gamma radiation at 5 kGy

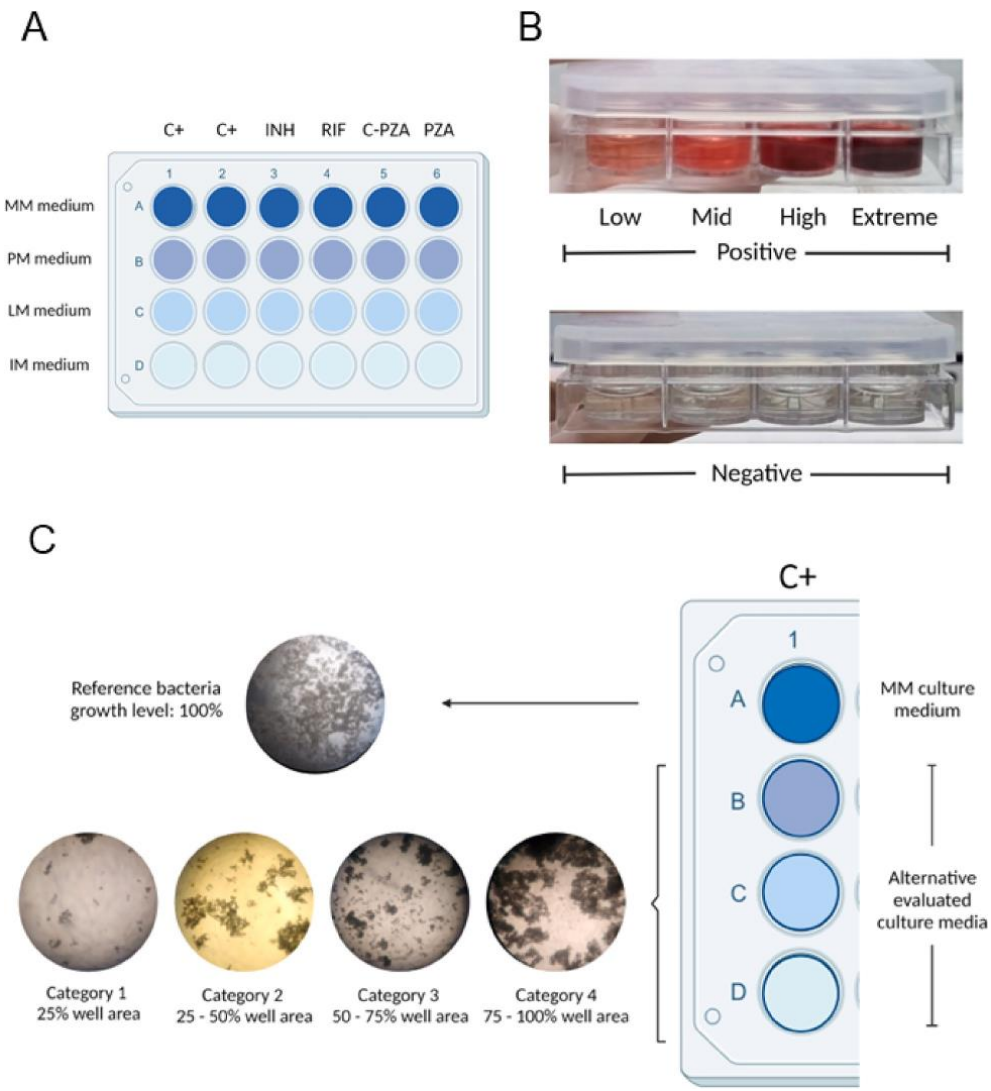

Fig. 1. Culture 24-well plate design and drug susceptibility evaluation for the MODS assay. By microscopical evaluation, drug susceptibility to isoniazid (INH) was evaluated using a critical concentration of 0.4 µg/mL, and rifampin (RIF) at 1 µg/mL. By visual color detection, drug susceptibility to pyrazinamide (PZA) was evaluated using a critical concentration of 800 µg/mL. (A) One decontaminated sputum sample was cultivated per plate, and each culture medium was evaluated per plate row; (B) Drug susceptibility to PZA was indirectly determined by detecting pyrazinoic acid (POA) using 10% ferrous ammonium sulfate (FAS). The reaction between POA and FAS produces a reddish-colored compound whose intensity ranges from low to extreme. Sensitive isolates produce POA that can be detected in the supernatant liquid culture medium; (C) Bacterial growth was reported using a qualitative scale estimating the well area covered by the bacterial growth. The growth level was determined using the results observed in the control growth wells. C+: Control growth well; C-PZA: Control test well for PZA resistance determination; MM: Standard MODS culture medium; PM: Powder-component mixed culture medium: LM: Lyophilized liquid culture medium; IM: Irradiated powder-component mixed culture medium. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

using Co-60 gamma radiation at 19 °C and 968 hPa, with the Gamma cell 220 irradiator type 1 at the Instituto Peruano de Energía Nuclear. Before use, aliquots were resuspended with 23.45 mL of distilled water, then 1.55 mL of glycerol and 500 µL of PANTA were added.

1.1. Mycobacterial culture assay

The culture assay was performed in 24-well plates (Fig. 1). From the decontaminated samples, 500 µL was added to independent tubes containing 7 mL of each culture medium (MM, PM, LM, and IM). Next, 900 µL of the diluted sample was transferred to each well. Then, 100 µL of culture media, 4 µg/mL INH, or 1 µg/mL RIF was added according to the plate design (Fig. 1A). PZA was added only after bacterial growth was observed in control wells. One sputum sample per culture plate was evaluated. The plate was covered with a silicone cap and incubated at 37 °C for up to 21days. MTB strains, such as H37Rv and DM97, were used as drug-sensitive and drug-resistant controls, seeded at a concentration of 2 x 10^5 CFU/mL.

Bacterial growth was recorded as positive from day five of incubation onward at MTB day detection (initial reading), using an inverted microscope (Nikon TS100) at 40× magnification. Bacterial growth was described according to the following scale (Fig. 1C): Category 1, growth covering 25% of the well; Category 2, growth covering 25–50% of the well; Category 3, growth covering 50–75% of the well; and Category 4, growth covering 100% of the well. Additionally, bacterial growth was recorded seven (week-1 reading) and fourteen (week-2 reading) days after the positivity day. Drug susceptibility to INH and RIF was reported when cordon-pattern growth was observed in control wells (i.e., without antibiotics) [32]. Drug susceptibility to PZA was evaluated according to the MODS-Wayne protocol [17]. Briefly, 6 days after the first bacterial growth in the control wells, 100 µL of 8 mg/mL PZA was added, and the plate was incubated for another three days. On day 9 post-bacterial growth, 100 µL of 10% ferrous ammonium sulfate (FAS) was added. A PZA-sensitive result was indicated by the reddish color observed after FAS addition; color intensity was classified as low (+), mid (++), high(+++), and extreme (++++) (Fig. 1B). Resistance to PZA was indicated by the absence of color.

Contamination was determined the day five when the wells were observed for the first time. Contamination was determined when fungus or bacteria were observed by rapid overgrowth and clouding in wells (Fig. 2) [22].

1.2. Consensus reference test for PZA susceptibility determination (CRT)

In this study, only MDR and mono-resistant isolates were assessed using the CRT test to determine PZA susceptibility. Non-resistant isolates were not assessed because of the low prevalence of PZA resistance. Three assays were considered for CRT: BACTEC MGIT 960, DNA sequencing of the promoter and *pncA* genes, and the classic Wayne assay. A PZA-sensitive result was defined as samples that showed sensitivity in at least two of the CRT assays. A PZA-resistant result was defined as a sample that demonstrated resistance in at least two of the CRT assays [16,33].

To perform the assays for CRT, a 100 µL aliquot from the control wells was inoculated onto agar 7H10 enriched with OADC and incu- bated at 37 °C for three weeks. MTB isolates were pre-treated as follows for each assay.

1.2.1. BACTEC MGIT 960

A bacterial suspension was prepared by suspending one full loop of MTB after 14 days of culture in 500 µL physiological saline. The suspension was vortexed and allowed to rest for 20 min. 3 mL of suspension were adjusted to a turbidity of 0.5 McFarland using additional physiological saline. Two dilutions were prepared at ratios of 1:5 and 1:50 from the MTB suspension. 0.5 mL of each dilution was transferred to a PZA-MGIT test tube and control tube [34]. PZA was added to the test tube at a final concentration of 100 µg/ml. Tubes were placed in the BACTEC machine and were incubated for 8 and 15 days at which times the fluorescent signal of the sample tubes was compared to the control tube.

1.2.2. DNA sequencing

DNA extraction was performed using a previously described modified proteinase K-chloroform protocol [35], and amplification of the *pncA* gene was performed using previously described primers P1 and P6 and protocol on a T100 thermocycler (Biorad, USA) [36]. The 720-bp amplification product was sequenced using the same primers used for PCR (Psomagen, USA). A pairwise sequence alignment was performed against the nucleotide sequence of the MTB H37Rv reference strain (NCBI RefSeq accession no. NC_000962.3) to detect the presence of mutations in the promoter and pncA genes.

1.2.3. Classic wayne assay

Agar Dubos was prepared according to Ref. [37] with 100 µg/mL PZA in 10 mL glass tubes. After 21 d of culture, two heavy loops of MTB were inoculated onto the agar surface. The tubes were incubated at 37 °C for seven days before reading. Sensitive (H37Rv) and resistant(DM97) controls were used. For the assay, 1 mL of fresh 1% FAS solution was added to each tube and incubated in the dark at 4 °C for up to 3 h. Following incubation, a reddish color indicated that the MTB was sensitive to PZA, whereas the absence of color indicated that the MTB sample was resistant to PZA.

1.3. Statistical analysis

MTB growth, day post-inoculation of a positive test, and contamination rates among all culture media were compared using Wilcoxon signed-rank tests for paired samples. Only paired samples free of contamination and with culture test availability were included. The frequency of antibiotic sensitivity to INH and RIF was compared among all culture media using a test of proportions. To test the hypothesis that there were no differences in the week of growth between the culture media, a Friedman test was performed for completely randomized block designs, considering the following factors: the week in which bacterial growth was recorded (initial, week 1, and week 2) and the type of

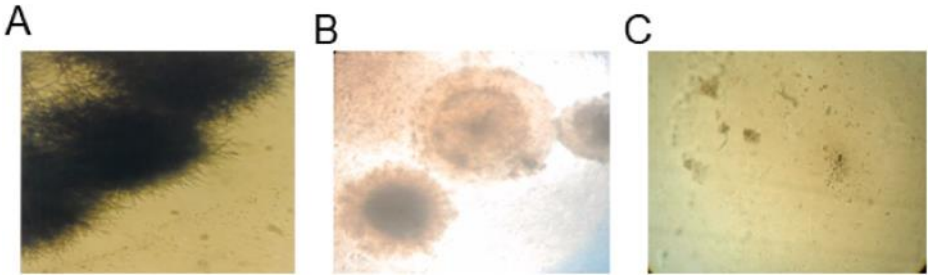

Fig. 2. Examples of contaminated well during MODS assay. (A–B) Contamination by fungus. (C) Contamination by bacteria (clouding). All images were takenusing a magnification of 40×.

culture. For all analyses, each culture medium was compared to the standard MM culture medium. Finally, the agreement between the MODS-Wayne dichotomous results and the CRT was estimated using the Kappa index.

1. Results

1.1. Performance of alternative media

A total of 282 sputum samples were cultured in LM, PM, and MM, 253 of the 282 samples were evaluated using IM. The overall contami-nation rate ranged from 8.2% to 13.5%. The highest contamination rate was reported for PM. No significant difference was observed in the contamination rates of IM and LM compared with the standard MM media (Table 1). The test positivity rates of PM (86.5%), LM (89.5%), and IM (83.6%) were not significantly different (P > 0.1) from those of the MM medium (Table 1). No significant difference was observed in the median time to growth between LM (8.5 days; P 0.131) and PM (9.5 days; P 0.621) media compared to MM media (9 days) (Table 1). However, the positivity time in the IM group was significantly shorter than that in the MM group (8 vs. 9 days; P 0.007). Bacterial growth inPM, LM, and IM was very similar to that in MM in all categories. PM and IM showed statistical significance (PM (P 0.041) and IM (P 0.059)); however, this difference was evident only in one category, without showing a clear trend (Fig. 3). More than 90% of the samples showed bacterial growth ranging from low to high (i.e., growth covering up to 75% of the well area) (Fig. 3). Bacterial growth was evaluated on two additional days of incubation after seven (week 1) and 14 days (week 2), to determine the effect of longer incubation periods on bacterial growth. The evaluated culture media showed similar bacterial growth levels independent of the reading plate time (Fig. 3).

1.2. Drug susceptibility determination for INH and RIF

Using the MODS standard medium, 85 (37.7%) samples showed antibiotic resistance. Of these, three (3.53%) were resistant to RIF only, 33 (38.82%) were resistant to INH only, and 49 (57.64%) were resistant to both RIF and INH (MDR). Overall, for all alternative culture media, the sensitivity and specificity were 100% for MDR-TB detection compared to standard MODS media, and there were no significant dif- ferences (P 1000) compared to standard MODS medium (MM) (Table 2).

1.3. PZA susceptibility

The CRT was conducted on 83 samples that showed resistance to RIF, INH, and both (MDR). Of the 83 samples, 24 (28.92%) were classified as PZA-resistant. According to each test used for CRT, 38 (45.78%) samples were resistant to PZA, as indicated by the MGIT, 19 (22.89%) were resistant to PZA, as indicated by the Wayne test, while 25 samples (30.12%) had at least one mutation in the promoter or pncA gene sequence (Table 3). On the other hand, considering the resistance profile to RIF and INF, of the 47 MDR samples, 21 (44%) were classified as PZA- resistant according to the CRT. Of the 33 samples with resistance only to INH, three (9.1%) were also PZA-resistant by CRT. PZA resistance was not observed in RIF-resistant samples.

According to the MODS-Wayne protocol, the number of PZA- resistant isolates ranged from 13% to 16% in the alternative culturemedia and standard MM medium (Table 4). The MODS-Wayne perfor- mance was evaluated based on 83 samples that reported resistance toRIF and INH. Sensitivity and specificity were calculated by comparison with the PZA resistance profile determined using CRT. No significant differences were observed between the alternative culture media and standard MM medium (P > 0.5). A 100% agreement was observed for each of the evaluated culture media compared with the MM medium (Table 5).

2. Discussion

The MODS test was specially designed for application in an LMIC setting [38]. In Peru, the MODS test is still only used in some healthreference laboratories (Callao, Lima Sur, Arequipa, and Ica), despite being the country where the test was developed and validated [39]. One factor limiting the widespread use of the MODS test is the relatively elaborate and time-consuming preparation of culture media, which is a limitation for diagnostic laboratories that require high-throughput MTB testing. This study demonstrates that three alternative preparations of dry culture media allow the detection of MTB growth using the MODS testing protocol, with a similar percentage of MTB growth and without significant differences in the test turnaround time when compared with the standard MODS culture medium (MM). MTB exhibited the same growth pattern in all evaluated culture media (Fig. 3), and ~8 days of culture, over 90% of the samples showed low to moderate growth. After an additional 14 days of incubation, more than 90% of the samples showed similar growth in both the alternative culture media (PM, LM,and IM) and MM medium. At least subjectively, no differences were observed in the morphological pattern formation characteristic of MTB growth in MODS cultures [26], which is consistent with other studies evaluating MODS [40].

Additionally, sterility of the culture medium is a critical factor for diagnostic purposes. Different methods for sterilizing culture media include steam heat, dry heat, pasteurization, sterile filtration, and high- energy radiation [41,42]. In this study, we evaluated the use of antibi- otics and gamma irradiation to control contamination. The use of anti- biotics is a low-cost and efficient method for controlling contaminationin microbiological cultures. Therefore, they are routinely used [24,29]. The commercial antibiotic mixture PANTA has been reported to reduce the viability of the contaminating flora of clinical samples when eval- uated in culture for the diagnosis of tuberculosis [26]. The use of PANTA in liquid culture media has been reported to reduce contamination (2.2%) compared to solid media such as Lowenstein-Jensen (6%) [28]. Gamma radiation is used commercially to sterilize agricultural products,

Table 1

Culture performance of alternative culture media compared to the standard MODS medium. Contamination and positivity rates are shown. Contamination was determined at 24 h of incubation. Positivity values were recorded on the first day of cording observation in each culture medium. Percentage values for positivity werecalculated against the number of samples without contamination in culture. MM, Standard MODS medium; PM, Powder-component mixed media; LM, Lyophilized liquid media; IM, Irradiated PM.

Culture media	Contamination rate		Positivity rate		Incubation days for positivity		Correlation[b]
	% (n/N)	p-value	% (n/N)	p-value	Median	p-value	
MM[a]	8.2 (23/282)	-	86.8 (225/259)	-	9.0	-	-
PM	13.5 (38/282)	0.005	86.5 (211/244)	0.637	9.5	0.621	0.925
LM	8.9 (25/282)	0.617	89.5 (230/257)	0.818	8.5	0.131	0.889
IM	8.3 (21/253)	0.563	83.6 (194/232)	0.106	8.0	0.007	0.866

[a] The standard MODS medium was considered the reference culture media for the comparison.

[b] Spearman correlation values were calculated against the standard MODS medium.

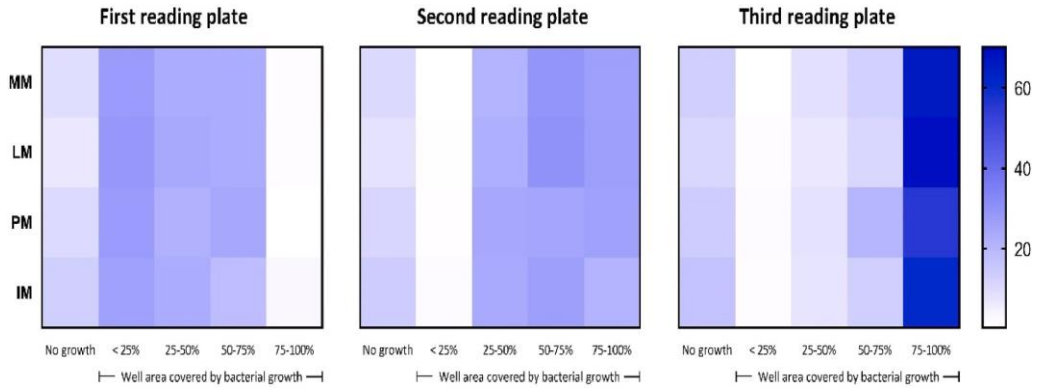

Fig. 3. Bacterial growth levels registered in the evaluated culture media at three different reading plate times. Bacterial growth was reported qualitatively using a percentage scale (see Fig. 1C). The level of growth incremented along with the incubation days in the same way for the four evaluated culture media. Columns in the heat map indicate the bacterial growth observed in the control wells. The percentage of samples per culture medium and bacterial growth is displayed with continuous color shading. MM, Standard MODS culture medium; LM, Lyophilized liquid media; PM, Powder-component mixed media; and IM, Irradiated powder- component mixed media. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Culture medium performance for the determination of isoniazid and rifampin resistance profile in MODS culture.

Culture media	Isoniazid			Rifampin			MDR			Kappa index
	Sen[a] % (n/N)	Spe[b] % (n/N)	p-value	Sen % (n/N)	Spe % (n/N)	p-value	Sen % (n/N)	Spe % (n/N)	p-value	
PM	100 (31/31)	100 (124/124)	1.000	100 (3/3)	100 (124/124)	1.000	100 (43/43)	100 (124/124)	1.000	1
LM	100 (32/32)	100 (137/137)	1.000	100 (3/3)	100 (137/137)	1.000	100 (47/47)	100 (137/137)	1.000	1
IM	100 (29/29)	100 (110/110)	1.000	100 (3/3)	100 (110/110)	1.000	100 (42/42)	100 (110/110)	1.000	1

[a] Sen, sensitivity.
[b] Spe, specificity; PM, Powder-component mixed media; LM, Lyophilized liquid media; IM, Irradiated PM.

Table 3
Pyrazinamide resistance determination results.

Number of isolates	PZA resistance profile[#]				*pncA* sequencing*
	MODS	CRT	MGIT	Classic	
	Wayne		BACTEC 960	Wayne test	
39	S	S	S	S	Wild type
12	S	S	R	S	
1	R	S	R	S	
5	R	S	S	S	
1	R	R	R	R	
8	R	R	R	R	A152G –
2	R	R	R	S	H51R
1	S	S	S	S	
4	R	R	R	R	A29G – Q10R
2	R	R	R	S	
2	S	R	R	R	
1	S	R	R	S	G371C – G124A
1	S	R	R	R	T11 – L4S
1	R	R	R	R	A424G – T142A
1	R	S	S	S	A170T – H57L
1	R	R	R	R	T26G – V9G A478C – T159P

Table 4
Pyrazinamide resistance profile according to the MODS-Wayne assay.

Culture medium	# samples[a]	Number of samples % (N)		
		PZA resistant	PZA sensitive	Contaminated
MM	225	15.6 [35]	79.1 (178)	5.3 [12]
PM	211	14.8 [34]	77.8 (178)	7.4 [17]
LM	230	13.7 [29]	78.7 (166)	7.6 [16]
IM	194	16.1 [31]	74.6 (144)	9.3 [18]

MM, Standard MODS medium; PM, Powder-component mixed media; LM,Lyophilized liquid media; IM, Irradiated PM.
[a] Difference in the number of samples is due to contamination (see Table 2).

Table 5
MODS-Wayne performance evaluated in three alternative culture media and compared to a Consensus Reference Test.

Culture medium	[b] samples[a]	Sensitivity % (n/N)	Specificity % (n/N)	p-value	Kappa index[b]
MM	78	82 (18/22)	88 (49/56)	0.763	–
PM	71	79 (15/19)	88 (46/52)	0.527	1
LM	74	81 (17/21)	87 (46/53)	0.366	1
IM	71	82 (18/22)	88 (43/49)	0.527	1

contamination rates for MODS assay vary between 2 and 8.1% [22,28, 38,39]. Contamination values for culture media that do not use anti- biotic supplementation as PANTA (i.e., Lowenstein Jensen) report values ranging from 12.4 to 27.2% [22,45–47]. Our study reported a contamination rate between 8.2% and 8.9%, with the exception of PM. The PM culture medium was not subjected to any sterilization method during its preparation, and although PANTA was added to the medium once hydrated, a contamination rate of 13.5% was observed. Similarly,it has been reported that thin layer agar (TLA) has a contamination rateof 26% when evaluating sputum culture samples; however, this assay is still recommended because it is rapid and does not require an inverted microscope [44]. In contrast, a very low contamination rate (less than 3%) suggests an overly stringent decontamination process, which can also affect the growth of MTB and adversely affect diagnostic sensitivity [44]. An improvement in the decontamination process can contribute to the diagnostic culture performance of alternative culture media, espe- cially PM. Therefore, further research is required in this regard. In aprevious study, it was demonstrated that PM, LM, and IM did not show any growth contamination after culturing the media only [29]. This confirmed that no source of microbiological contamination was present in these media. Therefore, the slight increase in the contamination rate observed in PM compared with MM (13.5% vs. 8.2%) may not be attributed to an external source of contamination present in the PM. It is possible that the components present in PM may have a particular property, based on the conditions of its preparation, which favors the growth of contaminants present in the processed sputum sample. Further studies are required to confirm this hypothesis.

Implementing a more straightforward methodology in terms of handling, preparing, and sterility of the culture media for the MODS test could facilitate the implementation of this assay on a larger scale. A commercial kit for the MODS test was developed and marketed in 2014 [27]; however, its production was discontinued. According to our re- sults, the three dry media evaluated here are viable options for use in the MODS test, while PM is also cheaper and easier to produce. We believe that despite its higher level of contamination, PM is a good option for laboratories with limited resources and infrastructure. LM and IM media may also be suitable alternatives but require more initial technical ca- pacity for irradiation and lyophilization, which may be cost- andinfrastructure-prohibitive. Further studies are required to evaluate the stability and requirements of the cold chain during transportation, which could affect the cost.

Regarding antibiotic resistance determination, in our study, the evaluated culture media did not show significant differences in diag- nostic performance values. Our test showed sensitivity and specificity values of 100% for the determination of INH resistance. When deter- mining resistance to RIF, a sensitivity and specificity of 100% were also found; however, only three samples with mono-resistance to RIF were obtained. Sensitivity and specificity values of 100% have been reported for the detection of MDR TB isolates. These values are comparable to the reported sensitivity (96.95%) and specificity (96.8%) for other micro- biological and molecular susceptibility assays, such as the MTBDRplus assay [48–50].

Finally, no DST is currently considered the gold standard for deter- mining the PZA resistance [50]. Tests such as BACTEC MGIT or DNA sequencing of promoter and *pnc*A genes are recommended by the WHO to determine susceptibility to PZA [17–19]. However, both tests have technical limitations, including false-negative results due to the amountof inoculum used, PZA concentration, and pH of the culture medium for the MGIT assay [18,19]. Additionally, the low frequency and diversity of mutations, different geographical prevalence of mutations, and the fact that not all possible mutations in the pncA gene can be associated with a resistant phenotype restrict the use of molecular assays on a large scale [20,21,51,52]. Our research group reported a variant of the MODS testto assess susceptibility to PZA: the MODS-Wayne assay [17]. This test is based on the detection of POA produced by MTB in the presence of PZA. This molecule is generated after PZA deamination by pyrazinamidase (PZasa). Mycobacterial strains sensitive to PZA have a functional PZAse that transforms PZA to POA, which is released into the extracellular environment and can be detected by adding FAS [34]. Unlike the BACTEC MGIT assay, the MODS-Wayne test does not require acidic pH.It can be performed directly from cultures of clinical samples, elimi- nating the need for prior isolation [17]. It is worth noting that the results in the MODS-Wayne in all the culture media, including the standard MODS media, were 100% correlated, and the minimal differences in sensitivity and specificity among them are likely due to distinct samples evaluated in each medium due to the differential contamination rate.

1. Conclusion

Our results demonstrate that it is possible to use alternate, less expensive, faster, and simpler culture media for the MODS assay without compromising the quality parameters of the test. The different forms of preparation and manipulation of the alternative culture media did not affect the diagnostic performance for determining susceptibility to three first-line antibiotics (INH, RIF, and PZA) used to treat MTB. However, a study with a larger sample size should be conducted to further validate these results. Based on the results of LM and IM culture media, we consider that these alternative culture media are candidates appropriate for use as a base medium for the design of a new MODS test diagnostickit. However, we believe that the LM media has the great advantage of being available more extensively, as gamma irradiation facilities are not likely to be available in low resources settings. This will allow decen- tralization of the MODS test to more regional laboratories in LMIC set- tings, which would lead to earlier and more appropriate TB treatments.

Author contribution

Patricia Sheen: Conceptualization, Methodology, Formal Analysis,Review and Editing, Supervision, Project Administration, Funding Acquisition. **Jhojalith Rodriguez:** Methodology, Investigation, Formal Analysis, Writing – Original Draft. **Roberto Alcántara**: Formal Analysis, Writing Original – Draft, Visualization. **Joseline Rodriguez:** Method- ology, Investigation. **Johnny Vargas**: Methodology. **Elisa Roncal:** Methdology, Investigation. **Ricardo Antiparra:** Methodology, Investi- gation. **Robert H. Gilman**: Conceptualization. **Louis Grandjean**: Re- view & Editing. **David Moore**: Conceptualization. **Mirko Zimic**: Formal Analysis, Supervision, Funding Acquisition.

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