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Development of a Selective Culture Medium for Primary Isolation of the Main *Brucella* Species[▽]

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Bacteriological diagnosis of brucellosis is performed by culturing animal samples directly on both Farrell medium (FM) and modified Thayer-Martin medium (mTM). However, despite inhibiting most contaminating microorganisms, FM also inhibits the growth of *Brucella ovis* and some *B. melitensis* and *B. abortus* strains. In contrast, mTM is adequate for growth of all *Brucella* species but only partially inhibitory for contaminants. Moreover, the performance of both culture media for isolating *B. suis* has never been established properly. We first determined the performance of both media for *B. suis* isolation, proving that FM significantly inhibits *B. suis* growth. We also determined the susceptibility of *B. suis* to the antibiotics contained in both selective media, proving that nalidixic acid and bacitracin are highly inhibitory, thus explaining the reduced performance of FM for *B. suis* isolation. Based on these results, a new selective medium (CITA) containing vancomycin, colistin, nystatin, nitrofurantoin, and amphotericin B was tested for isolation of the main *Brucella* species, including *B. suis*. CITA's performance was evaluated using reference contaminant strains but also field samples taken from brucella-infected animals or animals suspected of infection. CITA inhibited most contaminant microorganisms but allowed the growth of all *Brucella* species, to levels similar to those for both the control medium without antibiotics and mTM. Moreover, CITA medium was more sensitive than both mTM and FM for isolating all *Brucella* species from field samples. Altogether, these results demonstrate the adequate performance of CITA medium for the primary isolation of the main *Brucella* species, including *B. suis*.

Brucellosis is a worldwide zoonosis affecting mainly low-income countries (23). Besides two new species (*Brucella ceti* and *B. pinnipedialis*) isolated recently from marine mammals, the genus *Brucella* includes six classical species named according to their host preference (18). Among them, *B. melitensis*, *B. abortus*, *B. suis*, and *B. ovis* (which preferentially infect sheep and goats, cattle, pigs, and sheep, respectively) are the most important from a socioeconomic standpoint, since in addition to decreasing productivity in animals, the first three species are the main ones responsible for brucellosis in human beings (19). In infected animals, brucellae can be isolated from vaginal discharges, placental and fetal tissues, milk, and semen of live animals and, after necropsy, from the lymph nodes, spleen, liver, mammary glands, epididymides, and male sexual glands. Due to its specificity, the isolation and identification of *Brucella* in these animal fluids and tissues are the only incontestable demonstration of brucellosis in a given animal or flock. Since primary *Brucella* isolation requires 4 to 7 days of incubation, the presence in the above field samples of overgrowing fungi as well as commensal and environmental bacteria explains the frequent contamination of culture plates and the reduced sensitivity of bacteriological diagnosis. Thus, the use of adequate selective culture media is of paramount importance for a proper bacteriological diagnosis of brucellosis.

The simultaneous use of Farrell's medium (FM) (7) and modified Thayer-Martin medium (mTM) (15) is currently considered the strategy of choice for primary *Brucella* isolation from field veterinary samples (21). FM is probably the most widely used selective medium for the bacteriological diagnosis of brucellosis. This excellent medium, initially developed for the isolation of *B. abortus* bv. 2 from contaminated sources (6, 7), contains antibiotics that are highly inhibitory for most overgrowing contaminants present in field veterinary samples (14). Moreover, because it is translucent, it facilitates the presumptive identification of *Brucella* by assessing the colony morphology (1). However, due mainly to the nalidixic acid and bacitracin contained in its formulation, FM is inhibitory for *B. ovis* and also for some *B. melitensis* and *B. abortus* strains (15). Considering that mTM significantly improves the sensitivity of FM for the isolation of *B. ovis* and *B. melitensis* (3, 14), the simultaneous use of both culture media has been recommended for the routine bacteriological diagnosis of disease (21). However, mTM is significantly less inhibitory than FM for contaminant microorganisms. Moreover, mTM is not translucent due to the presence of hemoglobin as a basal component and thus is unsuitable for direct observation of colony morphology, probably the most practical procedure for the presumptive identification of *Brucella* (1). Finally, the performance of both FM and mTM for isolating *B. suis* has been the object of debate but has never been investigated thoroughly. One of the objectives of this work was to evaluate this subject and to investigate the susceptibility of *B. suis* to the different antibiotics contained in both selective media. Using the results obtained, we formulated a new selective culture medium (CITA medium) which, in addition to being translucent, allows the growth of all *Brucella* species and simultaneously inhibits

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TABLE 1. *Brucella* reference and field strains used in this work

Species	Biovar[a]	Strain	No. of strains or isolates
Reference strains			
B. abortus	1	2308	
	1	S19	
	3	Tulya	
B. melitensis	1	16 M	
	1	Rev1	
	2	63/9	
	3	Ether	
B. suis	1	1330	
	2	Thomsen	
	3	680	
	4	40	
	5	513	
B. ovis	NA	Bow	
	NA	Reo 198	
B. canis	NA	6/66	
Strains isolated from field samples			
B. abortus	1	S19	2
	1	RB51	26
	3		7
B. melitensis	1		61
	3		148
	1	Rev1	28
B. suis	1		5
	2		25
	3		7
	4		3
B. ovis	NA		32

[a] NA, not applicable.

TABLE 2. Compositions of the selective culture media compared in this study

Component	Concn or description[a]		
	FM	mTM	CITA
Basal medium	BMB-CS	GC-H	BAB-CS
Nalidixic acid (mg/liter)	5		
Bacitracin (IU/liter)	25,000		
Polymyxin B (IU/liter)	5,000		
Cycloheximide (mg/liter)	100		
Natamycin (mg/liter)	50		
Vancomycin (mg/liter)	20	3	20
Nystatin (IU/liter)	100,000	100,000	100,000
Colistin (mg/liter)		7.5	7.5
Nitrofurantoin (mg/liter)		10	10
Amphotericin B (mg/liter)			4

[a] FM, Farrell's medium (7); BMB-CS, brucella medium base (Oxoid, United Kingdom) supplemented with 5% CS (PanBiotech, Germany); mTM, modified Thayer-Martin medium (15); GC-H, GC agar base supplemented with 2 g Bacto agar and 1% hemoglobin (all from Difco); CITA, new selective culture medium; BAB-CS, blood agar base number 2 (Biolife, Italy) supplemented with 5% CS. FM was prepared using commercial antibiotic supplements containing either natamycin or cycloheximide (Oxoid). All antimicrobial agents not included in the commercial FM supplements were from Sigma.

most contaminant microorganisms present in veterinary samples.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and medium compositions. The *Brucella* reference and field strains used are summarized in Table 1. All *Brucella* strains were typed by standard bacteriological (1) and molecular (8) procedures and were kept lyophilized at 4°C in the culture collection of the CITA de Aragón (Zaragoza, Spain) until use. After rehydration, the strains were cultured on blood agar base plates (BAB no. 2; Biolife, Italy) supplemented with 5% sterile newborn calf serum (CS; PanBiotech, Germany) (BAB-CS) as described previously (9). *Brucella* suspensions were prepared in sterile buffered saline solution (BSS; pH 6.85) and adjusted to the desired concentration by spectrophotometry (suspensions with an optical density at 600 nm [OD_{600}] of 0.170 usually contain 1×10^9 CFU/ml), using BSS or Mueller-Hinton broth (MHB; Difco) supplemented with 0.5% yeast extract (YE; Difco) and 5% CS (MHB-YE-CS). The exact number of colonies (CFU/ml) contained in each bacterial suspension was determined retrospectively, as described elsewhere (9).

The microorganisms used as typical contaminants of veterinary samples were *Escherichia coli* (ATCC 285922), *Staphylococcus aureus* (ATCC 6538), *Aspergillus niger* (ATCC 16404), *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), and *Proteus* spp. (MicroBioLogics). All of these strains were provided by the Spanish Type Culture Collection (ECCT; University of Valencia, Spain) and cultured as recommended by the supplier. The growth or inhibition of these contaminant microorganisms on the corresponding selective culture media was assessed after 1 week of incubation at 37°C in both air and a 10% CO_2 atmosphere.

Both FM and mTM were prepared as described previously (15), and the compositions of these media are shown in Table 2.

Susceptibility of *B. suis* to both FM and mTM. Bacterial suspensions of the 5 *B. suis* reference strains and 40 field strains tested (Table 1) were prepared in BSS, and dilutions containing around 1×10^3 CFU/ml were cultured (0.1 ml/plate) in triplicate on plates of FM, mTM, and BAB-CS (control). The mean and standard deviation (SD) for the number of CFU/plate were determined for each strain in each culture medium after incubation of plates at 37°C for 3 days in a 10% CO_2 atmosphere. In addition, the 40 *B. suis* field strains were also studied for individual susceptibilities to each of the 9 antimicrobial agents contained in both FM and mTM, as well as to amphotericin B (Sigma Aldrich) (Table 2). This experiment was carried out in both liquid and solid media, as follows.

(i) Liquid medium assay. Bacterial suspensions of the above-mentioned *B. suis* strains in MHB-YE-CS were adjusted to 5×10^5 CFU/ml as described above, and 0.1 ml of each suspension was mixed in a 96-well sterile plate (Nunc microplate; ThermoFisher Scientific S.L.U., Spain) with 0.1 ml of MHB-YE-CS supplemented with adequate antibiotic concentrations. For this purpose, concentrated suspensions of each antibiotic were prepared in MHB-YE-CS (40 mg/liter nalidixic acid, 200,000 IU/liter bacitracin, 40,000 IU/liter polymyxin B, 800 mg/liter cycloheximide, 400 mg/liter natamycin, 800,000 IU/liter nystatin, 160 mg/liter vancomycin, 60 mg/liter colistin, 80 mg/ml nitrofurantoin, and 32 mg/liter amphotericin B) (all from Sigma Aldrich) and then 2-fold serially diluted in MHB-YE-CS. Wells containing only bacteria, antibiotics, or MHB-YE-CS were used as controls in each plate. Plates were incubated at 37°C in 10% CO_2, and bacterial growth was determined spectrophotometrically (OD_{595} readings) 4 and 7 days after incubation and confirmed by streaking out 25 μl/well in triplicate onto BAB-CS plates, which were incubated at 37°C in 10% CO_2 for 5 to 7 days. Isolation of at least one CFU was considered a positive result.

(ii) Solid medium assay. Bacterial suspensions of the above-mentioned *B. suis* strains were adjusted in BSS to 1×10^3 CFU/ml, and 0.1 ml of each suspension was smeared in triplicate on MH agar plates (MHA; Difco) supplemented with YE-CS as described above (MHA-YE-CS) and supplemented or not with the corresponding concentration of each antibiotic (Table 2). Nalidixic acid and bacitracin (both from Sigma Aldrich) were also tested at concentrations 2-fold above those contained in FM. The mean number of CFU/plate for each culture medium was determined after incubation at 37°C for 5 to 7 days in a 10% CO_2 atmosphere. The mean ($n = 3$) individual number of CFU/plate was assessed for each *Brucella* strain in each culture medium. Once equivalent numbers of CFU were obtained in the control BAB-CS medium for the 45 *B. suis* strains tested, the mean and SD for the number of CFU/plate were determined for each antimicrobial agent. Statistical comparisons of means were performed by a two-way (strain-medium interaction) analysis of variance (ANOVA) followed by *post hoc* Fisher's protected least significant difference (PLSD) test.

Development of new selective culture medium (CITA). Considering the importance and practical interest of using translucent media, the performances of the following basal culture media were assessed for isolating the main *Brucella* species: brucella medium base (BMB; Oxoid, United Kingdom), GC medium base (GC; Difco), Trypticase soy broth supplemented with 0.5% yeast extract (both products from Biolife, Italy) and 1.5% agar (Difco) (TSA-YE), and BAB. All of these basal components were supplemented with 5% sterile CS, and the resulting culture media (BMB-CS, GC-CS, TSA-YE-CS, and BAB-CS) were

compared for *Brucella* growth. Adjusted suspensions (see above) of representative *B. abortus*, *B. melitensis*, *B. suis*, and *B. ovis* strains (Table 1) were plated in the corresponding basal media and incubated for 5 to 7 days at 37°C in 10% CO_2, and the resulting mean CFU/plate was determined as described elsewhere (9). With the exception of GC-CS, which was somewhat inhibitory, the remaining media performed similarly for isolation of all *Brucella* species (results not shown). With its adequate performance and availability, BAB-CS was considered the basal component of choice for ensuing experiments.

In the second experimental phase, suspensions of representative *Brucella* strains (Table 1) and contaminants were inoculated on BAB-CS plates supplemented with all of the antibiotics contained in mTM (Table 2), but increasing the vancomycin (Sigma Aldrich) concentration to 20 mg/liter (as in FM) and adding several concentrations (2.5, 4, and 5 mg/liter) of amphotericin B (Sigma Aldrich) as a complementary antifungal agent. The same mTM formulation without amphotericin B (Sigma Aldrich) and BAB-CS alone were used as controls in this experiment. The resulting CFU of the corresponding contaminants and brucellae were determined in triplicate experiments after incubation at 37°C in 10% CO_2 for 3 and 5 days, respectively.

According to the results obtained in these previous experiments, 4 mg/liter of amphotericin B (Sigma Aldrich) and 20 mg/liter of vancomycin (Sigma Aldrich) were considered adequate enough to inhibit the contaminant microorganisms while allowing the growth of all *Brucella* species and strains tested. Thus, the CITA medium was formulated with BAB-CS as a basal component and with the following antibiotic supplements: vancomycin (20 mg/liter), colistin methanesulfonate (7.5 mg/liter), nitrofurantoin (10 mg/liter), nystatin (100,000 IU/liter), and amphotericin B (4 mg/liter) (all from Sigma Aldrich).

Diagnostic performance of CITA medium. The performance of the new selective culture medium was compared with those of both FM and mTM, using representative reference and field strains (i.e., 38 *B. abortus*, 35 *B. melitensis*, 45 *B. suis*, and 34 *B. ovis* strains). Adequate suspensions of these strains were cultured in triplicate, and the numbers of CFU per plate in each selective medium and in BAB-CS as a control were determined and statistically compared as described previously (9). Also, the growth or inhibition of contaminant microorganisms was determined for each culture medium after 72 h of incubation at 37°C in a 10% CO_2 atmosphere.

In the final step, the performance of CITA medium for direct primary isolation was compared to those of FM and mTM, with the last being supplemented with 4 mg/liter of amphotericin B (Sigma Aldrich) (mTMA). For this purpose, a total of 4,483 fresh field samples were obtained either *in vivo* (milk, vaginal swabs, and semen) or after necropsy (uterus/epididymides, spleen, and lymph nodes) from (i) rams experimentally infected with *B. ovis* PA (n = 528 samples), (ii) sheep suspected of *B. melitensis* infection (n = 3,247 samples), (iii) swine suspected of *B. suis* infection (n = 602 samples), and (iv) aborted cattle in an area where *B. abortus* is endemic and cattle are vaccinated with *B. abortus* RB51 (n = 106 samples) (see Table 4). All of these samples were taken and processed as described previously (2), and the same sample amounts (massively in the case of vaginal swabs and 0.5 ml of tissue homogenate/plate for the remaining samples) were cultured in duplicate plates of CITA and either mTMA or FM (see Table 4). One culture was considered positive when at least one CFU of *Brucella* was isolated. The suspected *Brucella* colonies isolated were identified and typed using both standard microbiological (1) and molecular (8) methods. The number of contaminated plates of each selective medium was also recorded.

RESULTS

***B. suis* studies.** The mean ± SD (n = 45) CFU/plate obtained with representative *B. suis* strains in FM (11.3 ± 5.7) was significantly lower ($P < 0.0001$) than those obtained with both mTM (69.4 ± 21) and BAB-CS (69.3 ± 20). The liquid medium assay evidenced that all *B. suis* strains were highly susceptible to nalidixic acid and bacitracin at concentrations close to and above those contained in FM (data not shown). These results correlated with those obtained in the solid medium assays (Table 3), in which the number of *B. suis* CFU was significantly inhibited ($P < 0.001$) by both antibiotics at the concentrations used in FM and at 2-fold higher concentrations (Table 3). In contrast, the remaining antibiotics tested did not show significant inhibitory effects on *B. suis*.

TABLE 3. Susceptibility of *B. suis* to the different antibiotics contained in FM and mTM

Antibiotic	Concn	Growth of *B. suis* (no. of CFU/plate [mean ± SD])[a]
Nalidixic acid	5 mg/liter	71.7 ± 8.1*
	10 mg/liter	2.9 ± 0.6**
Bacitracin	25,000 IU/liter	70.9 ± 6.9*
	50,000 IU/liter	13.8 ± 3.7**
Polymyxin B	5,000 IU/liter	81.4 ± 7.9
Cycloheximide	100 mg/liter	80.8 ± 7.5
Natamycin	50 mg/liter	78.8 ± 7.3
Vancomycin	20 mg/liter	74.3 ± 9.8
Nystatin	100,000 IU/liter	80.1 ± 7.7
Colistin	7.5 mg/liter	79.6 ± 7.9
Nitrofurantoin	10 mg/liter	80.7 ± 8.1
Amphotericin B	4 mg/liter	80.7 ± 6.6
None (control)		80.5 ± 7.8

[a] Growth of 40 representative *B. suis* strains (Table 1) in MHA-YE-CS supplemented with the indicated antibiotics at the concentrations used in FM or mTM. Nalidixic acid and bacitracin were also tested at 2-fold higher concentrations than those in FM or mTM. *, $P < 0.001$; **, $P < 0.0001$ with respect to MHA-YE-CS agar control plates without the corresponding antibiotic.

Development and performance of CITA selective medium. *B. suis* bv. 2 strains were significantly ($P < 0.0001$) inhibited when they were cultured on GC-CS basal medium (not shown). However, when hemoglobin was added to this basal medium, all *B. suis* strains grew as in BAB-CS (not shown). Although the difference was not significant, the BMB-CS basal medium negatively affected the growth of *B. ovis* (not shown). Thus, both media were discarded as basal components for formulating the new CITA medium. In contrast, both BAB-CS and TSA-YE-CS media showed similar performances (in terms of CFU numbers and colonial morphology and size) in supporting the growth of all *Brucella* species (not shown). For practical reasons, availability, and ease of preparation, BAB-CS was selected as the basal medium for CITA formulation.

The antibiotics contained in mTM (Table 2) allowed the growth of *A. niger* and *E. faecalis* while inhibiting the remaining contaminant microorganisms tested (not shown). However, *A. niger* was inhibited when 4 or 5 mg/liter (but not 2.5 mg/liter) of amphotericin B was added to either mTM or BAB-CS (not shown). Moreover, *E. faecalis* growth was fully inhibited when 20 mg/liter (but not lower concentrations) of vancomycin was added to either mTM or BAB-CS. Therefore, in addition to the antibiotics contained in mTM (Table 2), amphotericin B (4 mg/liter) and vancomycin (20 mg/liter) were selected as the antibiotic combination of choice for the definitive formulation of the new CITA selective medium.

The comparative efficacies of mTM, FM, and CITA medium to support the growth of the different *Brucella* species and biovars are shown as a box-plot graph in Fig. 1. Boxes represent the two central quartiles, the data between whiskers are 90% of the data obtained for each *Brucella* species (Table 1) in each culture medium, and horizontal lines inside boxes indicate the median values (CFU/plate) obtained. As expected, FM fully inhibited the growth of *B. ovis* and significantly reduced the growth of *B. suis* and *B. melitensis* ($P < 0.0001$), as well as that of some strains of *B. abortus* (not evident in Fig. 1), with respect to that on BAB-CS control medium. Moreover,

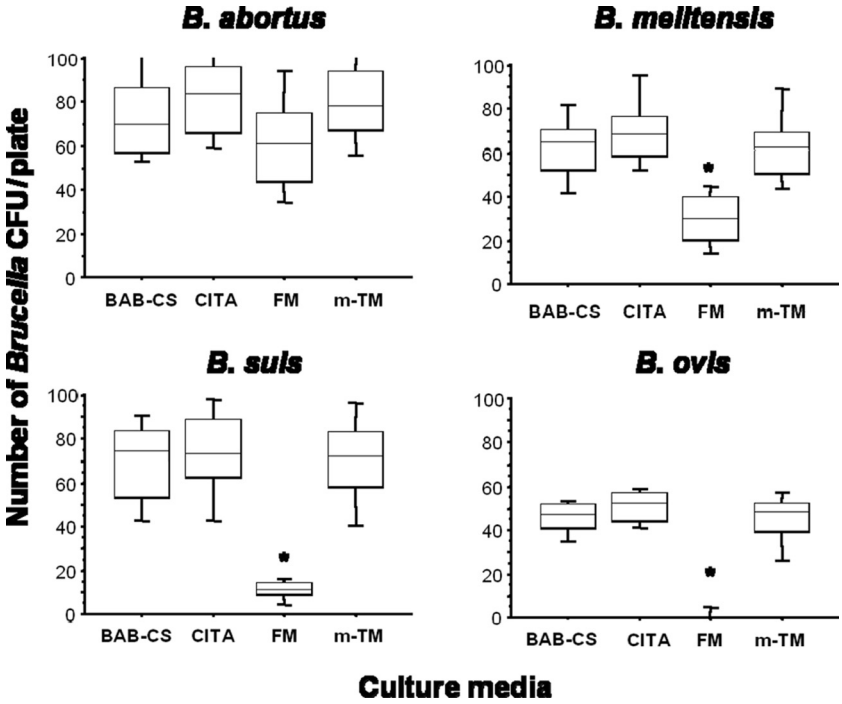

FIG. 1. Growth (number of CFU/plate) of representative *Brucella* species (see Table 1 for descriptions) on BAB-CS and three selective culture media: mTM (15), FM (15), and CITA (the new selective medium developed in this work). Statistical comparisons of means (numbers of CFU/plate) obtained for a given *Brucella* sp. for the different culture media were performed by Fisher's PLSD test. *, $P < 0.0001$ between FM and any other culture medium.

both CITA medium and mTM were as effective as BAB-CS medium without added antibiotics at supporting the growth of all *Brucella* species and biovars tested (Fig. 1). In testing the ability of these culture media to inhibit the growth of contaminants, both FM and CITA medium fully inhibited the standard contaminant microorganisms tested, while mTM allowed the growth of *A. niger* and *E. faecalis* (results not shown).

The comparative diagnostic performances of the three selective culture media for isolating *Brucella* from field samples are summarized in Table 4. The number of culture plates discarded due to the presence of overgrowing contaminants was lower for CITA medium than for mTM for samples from *B. ovis*-exposed rams but was similar to that for FM for the remaining samples. Regardless of the *Brucella* species considered, the new CITA medium showed a higher overall diagnostic sensitivity than the other selective media compared (Table 4).

DISCUSSION

The use of selective culture media is needed to increase the probability of success of bacterial culture, and it is compulsory for the adequate bacteriological diagnosis of brucellosis. The different studies conducted with the several selective culture media described until now (1, 3, 5, 7, 10, 11, 12, 13, 15, 16, 22, 24, 25, 26, 27) have concluded that the simultaneous use of both FM and mTM results in maximal sensitivity to isolate the most relevant *Brucella* species and biovars from field veterinary samples (21). FM is probably the most widely used selective medium for isolating *Brucella*, as it inhibits most contaminants

TABLE 4. Numbers of animal samples cultured in CITA and either FM or mTMA and the corresponding *Brucella* sp. strains isolated[a]

Brucella organism(s) detected (no. of isolates)	No. of samples cultured	No. of *Brucella* strains isolated (no. of contaminated plates)				
		CITA alone	FM alone	mTMA alone	Both FM and mTM	Total
B. ovis	528[b]	93 (0)	ND	50 (22)	151 (10)	294 (32)
B. melitensis bv. 1 strain Rev1 (28), field strains (331), and bv. 3 (139)	3,247[c]	157 (37)	39 (45)	ND	302 (19)	498 (101)
B. suis bv. 2	602[d]	46 (22)	16 (14)	ND	49 (11)	111 (47)
B. abortus bv. 1 strains S19 (1) and RB51 (24) and bv. 3 (7)	106[e]	11 (21)	0 (13)	ND	21 (10)	32 (44)

[a] See Table 2 for the exact composition of each selective culture medium. mTMA, mTM supplemented with 4 mg/liter of amphotericin B. ND, not determined.
[b] Semen and necropsy samples from rams experimentally infected with *B. ovis* PA.
[c] Vaginal swabs and milk and necropsy samples from sheep suspected of *B. melitensis* infection.
[d] Vaginal swabs and milk and necropsy samples from swine suspected of *B. suis* infection.
[e] Vaginal swabs and milk samples from cattle from areas where *B. abortus* is endemic and who were vaccinated with RB51.

present in field veterinary samples. However, some of the antibiotics contained in this medium are also highly inhibitory for *B. ovis* and some *B. melitensis* and *B. abortus* strains, including the RB51 vaccine strain (11, 15). In the cases of *B. abortus* and *B. melitensis*, this inhibitory effect is due mainly to the presence in FM of nalidixic acid and bacitracin at concentrations close to or higher than (Table 3) those allowing the growth of all strains of these *Brucella* species (15). The results obtained here confirm the above findings showing that FM fully inhibits the growth of *B. ovis* and significantly reduces the growth of *B. melitensis*, and moreover, they demonstrate that it is also highly inhibitory for *B. suis* (Fig. 1). Despite a somewhat reduced growth of *B. abortus* in FM (Fig. 1), the differences obtained were not statistically significant in this study.

As indicated above, FM is the preferred culture medium for the primary isolation of *B. abortus* from contaminated sources. Due to the presence of calf serum (7), this medium is very effective for isolating serum-dependent strains of *B. abortus* bv. 2 (6, 7). The presence of cycloheximide in the antibiotic supplement makes this medium highly effective for inhibiting the growth of fungi, which are among the main contaminants found in field veterinary samples. This agent inhibits the translation of mRNA by ribosomes, preventing protein synthesis of fungi. However, this drug has potentially toxic effects on mammalian cells, including teratogenesis, DNA damage, birth defects, sperm toxicity, and several others (20). The existence of these risks has resulted in the increasingly reduced availability and use of cycloheximide in recent years. Natamycin has been proposed as an alternative to cycloheximide for inclusion in commercially available antibiotic supplements for *Brucella* selective media (26). In fact, an antibiotic supplement containing natamycin is currently available (Oxoid), although it has been reported that this drug is somewhat less inhibitory than cycloheximide for fungal growth (26). Amphotericin B has been proposed as a safer and cheaper antifungal agent and has been recommended as a component of culture media for the primary isolation of *Mycobacterium* spp. (4) and *Campylobacter* spp. (17). This drug interacts with ergosterol, a steroid present in fungal membranes, and results in a loss of both membrane selective permeability and cytoplasmic components. However, amphotericin B does not affect bacteria, with the exception of *Mycoplasma* species. As demonstrated here, amphotericin B at 4 mg/liter was inhibitory for the reference fungal contaminants tested but did not affect the growth of *B. suis* (Table 3) or the remaining *Brucella* species tested when it was incorporated into the new CITA formulation (Fig. 1).

Altogether, the results of the different experiments allowed us to select the most adequate components to formulate a new selective medium (CITA) suitable for the isolation of all *Brucella* species. In addition to a suitable antibiotic combination (Table 2), this new medium contains BAB-CS as the basal component, resulting in a translucent medium, which is important for facilitating the presumptive identification of brucellae by assessing colony morphology under stereoscopic magnification (1). The addition of serum is also relevant, since this component allows the growth of the most fastidious *Brucella* strains, including *B. ovis*. These components of the CITA medium were proven highly effective in the final experiment assessing its diagnostic performance on a large collection of animal field samples (Table 4).

Both FM and mTM have been reported as highly inhibitory for the *B. abortus* RB51 vaccine strain (10, 11). Our results agree with these reports, since FM inhibited over half of the *B. abortus* strains isolated, most of which were RB51 (Table 4). In conclusion, the new CITA selective culture medium should be considered a useful tool for successful isolation of all *Brucella* species from field veterinary samples. However, the simultaneous use of CITA plus FM or mTMA results in the best diagnostic performance for isolating *B. melitensis* and *B. suis* or *B. ovis*.

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