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Evaluation of a selective medium for *Brucella* isolation using natamycin

J.A. Stack, M. Harrison[1] and L.L. Perrett

Veterinary Laboratories Agency, Addlestone, and [1]Oxoid Ltd, Basingstoke, UK

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Aims: To select an anti-fungal agent to replace cycloheximide in the media used for isolation of *Brucella*.

Methods and Results: One potential agent, natamycin, was evaluated using 28 *Brucella* isolates, 18 yeasts and 14 fungi. The material for the evaluation included 37 bovine milk samples, six bovine vaginal swabs and 45 milk samples artificially infected with *Brucella*. The recovery of *Brucella* only from the artificially-inoculated milk samples increased with the use of the medium containing natamycin instead of cycloheximide, at the same time significantly inhibiting the growth of yeasts, fungi and other bacteria. The inclusion of either anti-fungal agent allowed growth of the 28 *Brucella* isolates and totally prevented the growth of all 18 yeasts and 13 of the 14 fungi.

Conclusions: Based on the results it was concluded that natamycin would be a suitable alternative to cycloheximide.

Significance and Impact of the Study: Cycloheximide has become unavailable worldwide and is currently an anti-fungal constituent of the medium often used for isolation of *Brucella* organisms. The use of natamycin as a replacement in the formulation did not inhibit growth of *Brucella* and was effective at eliminating most contaminants.

INTRODUCTION

Brucellosis is a serious disease affecting all mammalian species of farm livestock, causing severe economic loss. Infection may be transmitted to man to cause a very severe disease, the clinical manifestations of which include chills, fever, malaise and headaches, requiring prolonged treatment.

The genus *Brucella* currently comprises six nomen species, all biovars of which were used in this trial. The slow growth of *Brucella*, coupled with the high level of contamination with other organisms often found in the diagnostic material (abortion material, placental tissue, milk, semen) means that a variety of antibiotics is commonly added to the agar base to enable successful isolation and to inhibit contaminants.

Kuzdas and Morse (1953) first introduced the concept of selective media for the isolation of *Brucella* by including cycloheximide, circulin, bacitracin and polymixin B to Albimi Agar. Numerous selective media have been derived since, including those of Mair (1955), Morris (1956), Jones and Morgan (1958) and Morgan (1960). Ryan (1967) and Ewalt *et al*. (1983) included a variety of agents which were successful in eliminating contaminating organisms in milk. According to Corbel and MacMillan (1998), some antibiotic mixtures may be inhibitory for strains of *Brucella abortus* biovar 2, 3 and 4, and some strains of *Brucella melitensis* and *Brucella ovis* (Marin *et al*. 1996).

The selective medium commonly used for primary isolation from contaminated sources is that developed by Farrell (1974). Recent studies have identified some constituents of Farrell's medium (nalidixic acid and bacitracin) that are responsible for the inhibition of some strains of *Br. ovis, Br. melitensis* and *Br. abortus* when comparing the constituent antibiotics (Marin *et al*. 1996).

The objective of this trial was to compare plates containing Farrell's formulation of medium containing cycloheximide with others containing the replacement fungicide, natamycin, with regard to their ability to support the growth of *Brucella* while inhibiting contaminants. Natamycin was chosen as an alternative to cycloheximide because it had previously been evaluated by Edelstein and

Correspondence to: J.A. Stack, Veterinary Laboratories Agency, Addlestone, Surrey, UK (e-mail: j.a.stack@vla.defra.gsi.gov.uk).

Edelstein (1996) for the growth of *Legionella* spp., because it has similar anti-fungal properties and is non-hazardous to manufacture.

MATERIALS AND METHODS

Medium

Serum dextrose agar (SDA) is the base from which Farrell's medium is prepared. It consists of Oxoid Blood Agar Base no. 2 with 5 or 10% equine serum and 1% glucose added aseptically after autoclaving. Oxoid Brucella Selective Supplement (BSS) is added to this base following the formulation developed by Farrell (1974).

The supplements under test incorporated 50 μg ml^{-1} natamycin instead of 100 μg ml^{-1} cycloheximide and were incorporated into the SDA medium. SDA medium was used as a control for the *Brucella* isolations.

Brucella strains

The study examined all 18 reference strains of *Brucella* and five recent isolates of *Br. abortus* from Northern Ireland, as shown in Table 1. Also included in the trial were five strains (three *Br. abortus* and two *Br. melitensis*) isolated in Spain which were reported to grow poorly on Farrell's medium by Marin *et al*. (1996). The inclusion of *Br. abortus* biovar 2 was considered important because its *in vitro* environmental demands are the strictest among the three classical species (*Br. abortus*, *Br. suis* and *Br. melitensis*) and, except for *Br. ovis*, all derivative organisms are less demanding (Meyer 1990).

A suspension of 10^{10} organisms ml^{-1} for each organism under test, measured by nephelometric analysis, was inoculated onto each of the media using a modification of the ecometric technique of Mossel *et al*. (1983). The ecometric technique is based on streaking an inoculum of bacteria to extinction. It gives a numerical measurement of media performance that can form the basis of records suitable for analysis. Briefly, on duplicate plates, one 10 μl loop of the suspension is streaked 20 times onto the medium, without replenishing the loop, and the plates are incubated at 37°C for 4 days in an aerobic atmosphere containing 10% carbon dioxide (CO_2). The absolute growth index (AGI) is calculated as the mean percentage growth of the two plates and is determined by counting the numbers of continuous lines of growth for each medium type under test. Batches of media providing a mean value of greater than 50% AGI are considered acceptable for use (with a minimum of 25% on an individual plate).

Clinical samples

In total, 88 samples were examined using two plates of each medium for every sample. These samples comprised 37 milk samples and six vaginal swabs taken from cattle after abortion, for screening for the absence of *Brucella*. In addition, to mimic infected field samples, 45 bovine milk samples were artificially infected with *Br. melitensis* biovar 3, reference B524 (chosen as a representative strain of *Brucella*). Swabs were plated directly onto plates set up in duplicate, and the cream from the milk was spread onto duplicate plates using a swab. Plates were incubated at 37°C for 4 days in an aerobic atmosphere containing 10% CO_2.

Yeasts

Eighteen yeasts were obtained from either the American Type Culture Collection (ATCC) or Oxoid Culture

Table 1 *Brucella* strains used for ecometric test comparison

Brucella	Biovar	Strain	*Brucella*	Biovar	Strain
abortus	1	544	*suis*	1	1330
abortus	2	86/8/59	*suis*	2	Thomsen
abortus	3	Tulya	*suis*	3	870
abortus	4	292	*suis*	4	63/75
abortus	5	B3196	*suis*	5	C68
abortus	6	870	*melitensis*	1	16M
abortus	9	C68	*melitensis*	2	63/9
neotomae	–	5K33	*melitensis*	3	Ether
ovis	–	63/290	*canis*	–	RM6/66
abortus	3	B3†	*abortus*	1	Y38 – Y42*
abortus	3	B8†	*melitensis*	3	B524†
abortus	3	B9†	*melitensis*	1	B548†

Strains were from the culture collection held at the VLA.

*Strains received from Northern Ireland.

†Strains received from Unidad de Sanidad Animal, Diputacion General de Aragon, Spain.

Table 2 Fungi and yeasts used to compare cycloheximide with natamycin

Fungi		Yeasts	
Aspergillus niger	ATCC 9642	*Pichia fermentans*	ATCC 10651
Aspergillus niger	ATCC 16404	*Yarrowia lipolytica*	ATCC 8661
Aspergillus parasiticus	ATCC 28285	*Candida albicans*	ATCC 2091
Aspergillus terreus	ATCC 10690	*Candida albicans*	ATCC 10231
Aspergillus tamarii	ATCC 10836	*Candida pseudotropicalis*	ATCC 2512
Aspergillus flavus	ATCC 22543	*Candida krusei*	ATCC 6258
Penicillium notatum	ATCC 9178	*Candida kefyr*	ATCC 8555
Penicillium notatum	ATCC 9179	*Rhodotorula rubra*	ATCC 9449
Mucor racemosus	ATCC 42647	*Saccharomyces cerevisiae*	ATCC 9763
Microsporum gypseum	ATCC 24102	*Saccharomyces carlsbergensis*	ATCC 2700
Fusarium oxysporum	OCC123	*Schizosaccharomyces carlsbergensis*	OCC 215
Fusarium culmorum	OCC 121	*Schizosaccharomyces pombe*	OCC216
Fusarium crockwellense	OCC120	*Candida vini*	OCC 244
Aspergillus spp.	OCC 109	*Candida valida*	OCC 243
		Rhodotorula rubra	OCC 213
		Saccharomyces spp.	OCC 218
		Saccharomyces cerevisiae	OCC 201
		Candida tropicalis	ATCC 750

Collection (OCC) and are shown in Table 2. An overnight broth culture from each yeast suspension was plated onto each of the media and incubated at 35°C for 84 h; at the same time, they were maintained on Sabauraud agar slopes for viability checks.

Fungi

A range of 14 fungi was obtained from the ATCC or OCC (Table 2). A stab inoculation from a fungal suspension was made into the centre of each plate; at the same time, they were maintained on Sabauraud agar slopes for viability checks and incubated as for yeasts. The colony diameter (mm) of fungal growth was recorded.

RESULTS

Brucella strains

A comparison of the results obtained by ecometric testing showed agreement between the ability of the SDA plates containing cycloheximide or natamycin to support the growth of *Brucella* (Table 3). All plates passed within the accepted criterion of > 50% of the AGI. Variations of between 70 and 100% are often accepted during routine work and are considered as repeatable results.

Table 3 Results of ecometric tests: number of strains of *Brucella* at different API

API result (%)	SDA + BSS + cycloheximide	SDA + BSS + natamycin	Control SDA
100	24	21	26
> 70–95	4	7	2
< 70	0	0	0

Clinical samples

Milk. A total of 37 milk samples was examined on 74 plates (Table 4). When grouped into plates with or without growth of contaminants (bacterial and fungal), there was no significant difference between natamycin or cycloheximide using the χ^2 test ($P = 0{\cdot}4877$). When plates were grouped into those with or without fungal growth, there was a highly significant reduction in growth when using natamycin (V^2 analysis, $P = 0{\cdot}0015$). A total of 62 plates containing cycloheximide and 73 containing natamycin prevented fungal growth totally; 12 plates with cycloheximide and only one with natamycin permitted growth of fungi. There was, however, no significant difference (χ^2 test, $P = 0{\cdot}4109$) between plates with natamycin or cycloheximide using the same grouping for growth of bacteria only. A total of 52

Table 4 Number of plates inoculated with milk samples showing growth of contaminating organisms

Colony growth	SDA + BSS + cycloheximide	SDA + BSS + natamycin
Nil growth	44	48
Bacterial growth	18	25
Fungal growth	8	0
Bacterial and fungal growth	4	1

plates containing cycloheximide and 49 containing natamycin totally prevented bacterial growth; 22 plates with cycloheximide and 26 plates with natamycin permitted growth of bacteria. It must be noted that four plates containing media with cycloheximide permitted heavy growth of both bacteria and fungi which would obscure the potential presence of *Brucella* colonies.

Swab. Results from the small number of available swabs were divided into growth or no growth of bacterial contamination (there was no fungal contamination on any of the plates). Bacteria grew on four of the plates containing cycloheximide and eight plates were totally inhibited, whereas 10 plates with natamycin showed growth and only two had no growth. It was therefore demonstrated (using the V^2 analysis, $P = 0{\cdot}0150$) that plates containing cycloheximide were significantly better at inhibiting bacterial contaminants than those containing natamycin. *Brucella* was not isolated from any of these milks or swabs (which was to be expected because the samples were collected from Officially Brucellosis Free animals).

Artificially-infected milk samples. Medium containing natamycin improved the inhibition of contaminating bacterial, yeast and fungal growth; only 15 plates showed a light growth of these contaminants and 75 plates permitted the pure growth of *Brucella* only. Growth of *Brucella* was supported by the medium containing cycloheximide on 88 of the 90 plates used and in addition, 30 plates allowed the growth of contaminating organisms. Under normal circumstances, these contaminants, coupled with small numbers of *Brucella* organisms present in the milk sample, could possibly mask the recognition of the small colonies of *Brucella*. Using the χ^2 test, natamycin was better at preventing contamination and did not inhibit *Brucella* growth ($P = 0{\cdot}0039$). There was no significant difference between the two media for the propagation of *Brucella*.

Fungi

Only one species of fungus grew on the medium containing natamycin.

Yeasts

None of the yeasts in Table 2 grew on any of the media containing the antibiotic supplements.

DISCUSSION

Brucella are fastidious and relatively slow-growing organisms cultured from clinical material that is often grossly contaminated. It is therefore necessary to use selective media for attempting isolation, which must inhibit the growth of contaminants. There are many selective media available for the isolation of *Brucella* from contaminated material. Farrell's medium is currently widely recognized and used as an agreed international formulation recommended by the Office International des Epizooties (MacMillan and Stack 2000).

All media in the trial passed the ecometric assessment, indicating that the new formulation will provide an acceptable medium for isolation of *Brucella* even if the sample contains small numbers of viable organisms. Both media inhibited all selected fungi and most of the yeasts chosen in the trial, suggesting that the supplements are comparable.

The results indicate that cycloheximide can be replaced in Oxoid Brucella Selective Supplement with natamycin at 50 μg ml^{-1}, but it must be noted that only one strain was involved when artificially infecting the milk samples. This strain was chosen because it was suspected to grow poorly on Farrell's medium (Marin *et al.* 1996). This indicates that other strains of *Brucella* might grow successfully on media containing the new formulation. Cycloheximide was significantly better at reducing contaminants in vaginal swabs. This is possibly because the types of bacteria contaminating swabs may be different from those commonly found in milk.

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