Evaluation of *Mycobacterium avium* subsp. *paratuberculosis* faecal culture protocols and media[1]

Paula Ristow[2*], Marlei Gomes Silva[2], Leila de Souza Fonseca[2] and
Walter Lilenbaum[3]

ABSTRACT.- Ristow P., Silva M.G., Fonseca L.S. & Lilenbaum W. 2006. [**Evaluation of *Mycobacterium avium* subsp. *paratuberculosis* faecal culture protocols and media**.] *Pesquisa Veterinária Brasileira 26(1):1-4.* Mycobacteria Laboratory, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Cidade Universitária, Rio de Janeiro, RJ 21941-590, Brazil. E-mail: paularistow@bigfoot.com

Paratuberculosis is an important enteritis of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*). The disease is officially considered exotic in Brazil, but recent serological surveys and the isolation of the agent suggest it may occur in our herds. The aim of this study was to evaluate three different formulations of Herrold's egg yolk agar with mycobactin J (HEYM) and four faecal culture protocols considering their ability for *Map* growth as well as cost and ease of application. Three formulations of HEYM were inoculated with two suspensions of *Map*. Spiked faeces and naturally contaminated faecal samples were treated by the four faecal culture protocols. Centrifugation protocol and HEYM recommended by OIE showed the best results on the recovery of *Map*.

INDEX TERMS: Paratuberculosis, bovine, diagnosis, mycobacterium.

RESUMO.- [**Avaliação de protocolos de cultivo fecal e meios para a cultura de *Mycobacterium avium* subsp. *paratuberculosis*.**] A paratuberculose é uma importante enterite de ruminantes causada por *Mycobacterium avium* subsp. *paratuberculosis* (*Map*). A enfermidade é oficialmente considerada exótica no Brasil, mas inquéritos sorológicos recentes e o isolamento do agente etiológico sugerem que ela pode estar presente em nossos rebanhos. O objetivo do estudo foi avaliar três diferentes fórmulas do Ágar gema de ovo de Herrold suplementado com micobactina J (HEYM) e quatro protocolos de cultura fecal quanto ao crescimento de *Map*, bem como custo e facilidade de implementação. Três fórmulas de HEYM foram inoculadas com duas suspensões de *Map*. Fezes contaminadas artificialmente e naturalmente com *Map* foram tratadas pelos quatro protocolos de cultura fecal. O protocolo da centrifugação e a fórmula de HEYM recomendada pela OIE demonstraram os melhores resultados quanto à recuperação de *Map*.

TERMOS DE INDEXAÇÃO: Paratuberculose, bovinos, diagnóstico, mycobacterium.

INTRODUCTION

Paratuberculosis is a fatal chronic granulomatous enteritis caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) that affects ruminants. It occurs throughout the world and causes serious economic losses (Chiodini et al. 1984). The disease is considered rare and exotic in Brazil and was reported only sporadically, in cases evolving imported animals (Dacorso Filho et al. 1960, Driemeier et al. 1999). Nevertheless, recent serological surveys (Fonseca et al. 2000, Ferreira et al. 2001) and the isolation of the agent (Dias et al. 2002, Ristow et al. 2004) suggest the disease may be present on Brazilian herds.

The isolation of the agent from faeces or tissues is the gold-standard diagnostic method (Stabel 1997), but due to a lack of qualification of veterinary bacteriology laboratories on mycobacteriology and to difficulties inherent to *Map*'s cultivation, it is infrequently performed in Brazil. *Map*'s culture is very slow, takes 8-16 weeks of incubation and is mycobactin dependent (Whipple et al. 1991). Cetylpyridinium chloride (HPC) is the most commonly employed decontaminant for *Map* culture

and many protocols using it were proposed (Stabel et al. 2002), including some with two decontamination steps (Whitlock & Rosenberger 1990). Besides the decontamination steps, culture protocols include the concentration of specimens by sedimentation (Whipple et al. 1991) and/or centrifugation (Whitlock & Rosenberger 1990, Stabel 1997). Although centrifugation increases contamination (Stabel 1997), it increases culture sensitivity nearly three-fold (Whitlock & Rosenberger 1990). Several culture media can be used for *Map*'s cultivation like the modified Dubos´s, Middlebrook 7H9, 7H10, 7H11, 7H12 Bactec and Löwenstein-Jensen, all supplemented with mycobactin (World Organization for Animal Health 2004), but the most widely used is the Herrold's egg yolk agar supplemented with mycobactin J (HEYM). The incorporation of antibiotics to media is opportune to control the growth of contaminants (Stabel et al. 2002).

Beyond the inherent difficulty on the cultivation of *Map*, there is no consensus about HEYM's in house formulations and faecal culture protocols in the literature (Whipple et al. 1991). Although HEYM is commercially available, its cost and import difficulties may limit its use in Brazilian laboratories. The purpose of this work was to evaluate three different formulations of HEYM and four faecal culture protocols, considering their ability for *Map* growth as well as cost and ease of application. Paratuberculosis should be considered a sanitary threat in Brazil and due to our important beef and dairy industries, it is utterly necessary to prevent and control it. This study will probably contribute to the bacteriological diagnosis of paratuberculosis in Brazil.

MATERIALS AND METHODS

HEYM's evaluation

Three Herrold's egg yolk agar formulations supplemented with mycobactin J (Allied Monitor, USA) were evaluated. Formulations were prepared as described below. HEYM named HEYM-OIE is recommended by the World Organization for Animal Health (2004) and was processed following OIE standards, adjusting the pH of the liquid medium to 7.0 and autoclaving it at 121°C for 25 minutes. HEYM named HEYM-W is recommended by Whipple et al. (1991) and was processed as HEYM-OIE, adjusting the pH of the suspension prior to autoclaving to 8.1-8.4. Addition of amphotericin B, as recommended by the authors, was not employed. HEYM named HEYM-P was processed as HEYM-OIE, adding 6.25 g of sodium pyruvate. The pH of the liquid medium was adjusted to 7.5 and it was autoclaved at 121°C for 20min. Fresh eggs from a flock that does not receive antibiotics were used in all media formulations. Following quality control procedures for HEYM (Whipple et al. 1991), all slopes were incubated at 37ºC for 48h and 10% of those for seven days before use to check sterility.

To access the ability for *Map* growth of each of HEYM formulations, a field strain (Malele 734 - RFLP standard A) isolated from a clinical case of bovine paratuberculosis in Argentina (Moreira et al. 1999) grown in HEYM - OIE formulation was used. A Mc Farland number one suspension, containing approximately 10^7 *Map* cfu mL^{-1}, was prepared in sterile distilled water by vortexing colonies on a tube with sterile glass beads. Successive dilutions were prepared in sterile distilled water. Following the recommendations of Whipple et al. (1991), 0.1mL of two light bacterial suspensions containing approximately 10^3 and 10^2 *Map* cfu mL^{-1} were inoculated in duplicate on HEYM's slopes of each formulation. Tubes were incubated at 37ºC and observed in two weeks intervals until 16 weeks for bacterial growth.

Culture protocols evaluation

Evaluation of faecal culture protocols was done with artificially spiked faeces, artificially spiked water and naturally infected faeces. Faeces were collected for the spiking with *Map* was from a healthy, serologically and culture negative bull, from a paratuberculosis-free beef herd. Naturally infected faecal samples were collected from four cows with positive faecal culture (identified as 103, 120, Jacuba and Humorista) obtained from a paratuberculosis-positive dairy herd in Rio de Janeiro, Brazil (Ristow et al. 2004). Two of them (Jacuba and Humorista) were from high shedder cows with characteristic clinical symptoms and the other two (numbers 103 and 120), from clinically asymptomatic medium shedder cows.

Spiking of faeces. A Mc Farland number one suspension of *Map* was prepared as described above. Successive dilutions were prepared in sterile distilled water. Suspensions containing approximately 10^5, 10^4 and 10^3 *Map* cfu ml^{-1} were prepared, attempting to reproduce high, medium and low shedding conditions, as usually observed in infected herds. Spiking was prepared adding one ml of each suspension to one gram of faeces, so that there were approximately $5.0x10^4$; $5.0x10^3$ and $5.0x10^2$ *Map* cfu g faeces^{-1}. Samples were homogenized in 50ml plastic tubes with glass beads on vortex for one min and processed by the four culture protocols. As a recovery control, distilled water was spiked by the same procedure.

Culture protocols. a) Sedimentation was performed as described by the World Organization for Animal Health (2004). Briefly, one gram of faeces was added to 20 ml of sterile distilled water, tubes were shaken for 30 min and then allowed to stand undisturbed for 30 min. Five ml of the supernatant were added to 0.75% HPC (Sigma, USA). Tubes were inverted several times and allowed to stand undisturbed for 18 hours at room temperature for decontamination. HEYM slopes were inoculated with 0.1 ml of the undisturbed sediment.

b) Centrifugation was performed as described by Stabel (1997), with brief modifications. Until the decontamination stage it was performed consistently like the sedimentation protocol, employing 25 ml of 0.9% HPC overnight. Tubes were centrifuged at 1700×g for 20 min, the supernatant was decanted and the pellet was resuspended in 1ml of 50μg/ml amphotericin B (Bristol Meyers Squibb, Brazil). HEYM slopes were inoculated with 0.2 ml of the suspension.

c) Double incubation was performed as described by Whitlock & Rosenberger (1990), with brief modifications. Two grams of faeces were added to 35ml of sterile distilled water with sterile glass beads, tubes were shaken for 30min and then allowed to stand undisturbed for 30 min. Five ml of the supernatant were added to a decontaminant mixture containing 25ml of 0.9% HPC and 30ml of BHI and the samples were allowed to decontaminate overnight at 37ºC. Samples were centrifuged at 900×g for 30 min and the supernatant discarded. Pellets were resuspended in 1 ml of antibiotic brew containing 50% BHI broth with 100 μg/ml vancomicin (Elly Lily, Brazil); 100 μg/ml nalidixic acid (Merck, Germany) and 50 μg/ml amphotericin B. Tubes were vortexed and incubated overnight at 37ºC. HEYM slopes were inoculated with 0.25 ml of the suspension.

d) Double centrifugation/decontamination or NADC (National Animal Disease Center) was performed as described by Stabel (1997). Until the settling of particles for 30 min it was performed consistently like the double incubation protocol. Then, the entire supernatant fraction was centrifuged at 1700×g for 20 min, the supernatant discarded and the pellets resuspended in 30mL 0.9% HPC/BHI solution. After overnight decontamination at 37ºC, samples were centrifuged at 1700×g for 20min and the supernatant discarded. Pellets were resuspended in 1ml of antibiotic solution containing 100μg/ml vancomicin, 100μg/ml nalidixic acid and 50μg/mL amphotericin B.

Tubes were incubated overnight at 37ºC and HEYM slopes were inoculated with 0.2 ml of the suspension.

Each sample was cultivated in duplicate on HEYM slopes (OIE formulation) with and without antibiotics (50μg/ml nalidixic acid, 50μ/ml amphotericin B and 50μg/ml vancomicin), totalling four HEYM slopes per sample. Antibiotic solutions were prepared according to the fabricants instructions and were filtered on Millipore membranes (0.22μm). Tubes were incubated at 37ºC and observed in a two weeks-interval for 16 weeks and the colonies enumerated.

Statistical analysis
Student's *T* test and Group Analysis of Variance (ANOVA) were employed to analyze the colony counts.

RESULTS AND DISCUSSION

The growth time of *Map* in the three different HEYM formulations was 60 days and no contamination was observed on the tubes. When suspension 10^3 was used, a mean of 435.0 ($\pm$ 15.0) colonies was observed in HEYM-OIE; 250.0 ($\pm$ 50.0) in HEYM-W and 137.0 ($\pm$ 7.0) in HEYM-P. Statistics showed significant difference between HEYM-OIE and HEYM-W (p = 0.003), but not between HEYM-OIE and HEYM-P (p = 0.071) or HEYM-P and HEYM-W (p = 0.155). With suspension 10^2, a mean of 50.0 ($\pm$3.0) colonies in HEYM-OIE, 5.0 ($\pm$ 2.0) in HEYM-W and 19.5 colonies ($\pm$ 1.5) in HEYM-P was observed. HEYM-OIE was ten-fold more sensitive on the colony detection than HEYM-W (p = 0.06) and 2.5-fold more sensitive than HEYM-P (p = 0.012). HEYM-OIE was considered the most appropriate for *Map* culture and from then on has been adopted in our laboratory as the routine medium. Beyond being recommended by the World Organization for Animal Health (2004), this formula is also used by many authors (Whitlock & Rosenberger 1990; Stabel 1997).

The highest recovery of *Map* on spiked samples was on the centrifugation protocol, with a mean of 44.5 CFU/slope (Table 1). The double incubation protocol recovered 30.6 CFU/slope, followed by NADC (26.2 CFU/slope) and sedimentation (14.3 CFU/slope). There was no contamination on spiked samples processed by sedimentation, double incubation or NADC protocols, but when centrifugation protocol was used, one HEYM slope without antibiotics presented contamination on sample faeces/10^3 (4.2%). The growth of *Map* was faster (30

days) in the centrifugation and NADC protocols, indicating a better sensitivity of those protocols.

On naturally infected faeces, the highest recovery of the bacterium was also obtained with the centrifugation protocol, with a mean of 42.7 CFU/slope and 25% of contamination. NADC protocol recovered 20.3 CFU/slope with a contamination rate of 31.2%; followed by double incubation protocol, with 13.3 CFU/slope and a contamination rate of 25% and sedimentation, with 3.9 CFU/slope and a contamination rate of 6.2%. Despite all protocols were able to recover *Map* from the two high shedders' samples (Jacuba and Humorista), none of them recovered bacteria from the two medium shedders' samples (numbers 103 and 120). There was high contamination (40.6%) on slopes of samples 103 and 120, what could explain the absence of growth. The low sensitivity of *Map* culture could also be responsible for not recovering *Map* from samples 103 and 120 (Stabel 1997).

As to the decontamination ability of each protocol, on both naturally infected and spiked samples, centrifugation and NADC protocols showed the greatest number of contaminations (12.5%), followed by double incubation protocol (10%) and sedimentation (1.6%). A high contamination level using the centrifugation protocol was previously described by Stabel (1997), yet minimal contamination was observed when the NADC method was used. Eamens et al. (2000) observed no contamination using the sedimentation protocol, what agrees with the results found in this study; nevertheless, for the double incubation protocol the contamination rate observed was 0.6%. It is important to observe that contamination was only observed on HEYM slopes with no antibiotics. This is a very useful finding and endorses the necessity of inclusion of antibiotics in media when processing faecal samples, which agrees with the recommendation of Stabel et al. (2002).

Comparison between the culture protocols is hindered due to the different characteristics of the protocols. The amount of faeces used on the double incubation and NADC protocols (2 g) is bigger than the amount used on the sedimentation and centrifugation protocols (1g) and furthermore, different amounts of inoculum were seeded onto HEYM slopes for the different protocols: 0.1ml for sedimentation, 0.2ml for centrifugation and NADC and 0.25ml for double incubation.

This study evaluated four culture protocols and three different HEYM formulations. It permitted us to choose the centrifugation protocol with inoculation on HEYM- OIE formula with and without antibiotics as a routine procedure for the cultivation of faeces for *Mycobacteruium avium* subsp. *paratuberculosis*. The centrifugation protocol presented the most satisfactory recovery of *Map* and the contamination of slopes was avoided by the addition of antibiotics to media. It is also less labour-intensive, faster and less expensive when compared to the double incubation and NADC protocols, which are major advantages when processing larger number of faecal samples.

Table 1 . Mean recovery of *Map* (CFU per HEYM tube) on the four culture protocols per sample on spiked samples

Sample/ suspension	CFU per HEYM tube on protocols			
	Sedimen-tation	Centrifu-gation	Double incubation	NADC
Water/10^5	10.5	87.5	58.5	0.5
Water/10^4	1.75	8.75	7.25	0
Water/10^3	0	1.5	0.25	0
Faeces/10^5	66.25	100	90	100
Faeces/10^4	5.75	61.25	26.5	55
Faeces /10^3	1.5	8	1	1.5
Mean total recovery[a]	343/24	1068/24	734/24	629/24
	14.3	44.5	30.6	26.2

[a] per protocol, obtained by the mean on the 24 four inoculated tubes.

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