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Comparative evaluation of egg based solid media for primary isolation of Paratuberculosis bacilli

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Paratuberculosis caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a prevalent infection and is highly expensive to livestock industry. Paratuberculosis also has zoonotic concerns with Crohn's disease. Isolation of MAP from infected individuals/samples is the most vital for downstream studies. However, MAP is extremely fastidious organism and primary isolation is extremely difficult. Mycobacterial growth indicator tube (MGIT) medium is most recommended for primary isolation, but this medium is highly expensive and requires sophisticated instrument and is unaffordable for developing and underdeveloped world. Present study aimed to investigate the comparative performance of three different egg based solid media (MB7H10, LJ and HEYM) for the primary isolation of MAP from clinically suspected animals. Animals having signs of paratuberculosis were sampled either from farms in Jaipur District of Western India or slaughterhouse. Slaughtered animals were sampled having swollen & enlarged mesenteric lymph nodes (MLN) with thickened/ corrugated intestine near ileo-cecal junction (ICJ). Fecal samples were collected from suspected cattle, buffalo, goat and sheep. MB7H10 medium showed maximum recovery of MAP isolates from clinical samples. Most of the isolates grew on MB7H10 (41.9 %) followed by LJ (22.3%) and HEYM (6.25 %). In case single medium is to be used MB7H10 is the first choice and combination of MB7H10+LJ is better. In conclusion, combination of MB7H10+LJ is the best choice for solid media for the isolation of the MAP in laboratories.

Keywords: Buffalo, Cattle, Crohn's disease, Johne's Disease, Goat, Livestock, MAP, *Mycobacterium avium* subspecies *paratuberculosis*, Sheep

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease (JD)/ paratuberculosis. MAP is pathogenic mycobacteria affecting dairy cattle and other ruminants globally[1]. The disease is slowly progressive (chronic) and occurs in different stages. Initial stage (silent stage) with no signs of the disease progresses to sub-clinical stage, where occasional shedding of MAP in feces, milk, etc can be without signs of the disease. Sub-clinical stage often progresses to clinical stage wherein continuous weight loss with or without diarrhea are prominent signs[2,3]. Paratuberculosis reduces production as a result of decline in milk yield and quality, poor conversion of feed, higher susceptibility to other diseases, reduced reproductive competence, treatment expenses, premature culling, reduced slaughter value, etc[4]. Recent studies also advocate the zoonotic role of MAP in inflammatory bowel disease (IBD)/ Crohn's disease (CD)[5]. Therefore control and prevention of paratuberculosis has become priority for governments. Control and prevention of MAP is largely dependent on diagnosis (followed by culling or segregation) with implementation of epidemiological measures of disease restriction at farms[4].

Culture, PCR, ELISA, DTH etc based diagnostic tests are available for paratuberculosis and have variable performance depending upon the disease stage, however, isolation of MAP from infected animal is still considered as Gold Standard. Isolation of MAP has not only diagnostic importance, but is critical for epidemiological investigations and also for the production of antigens and developing vaccines, besides study of various other disease associated parameters.

Although MAP is a member of the *Mycobacterium avium* complex (MAC), compared to other members of MAC, isolation of MAP is notoriously difficult. MAP is extremely fastidious organism and primary isolation takes minimum of 12-16 weeks[6]. Further,

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in vitro growth of MAP is dependent on iron-chelating compound, mycobactin J[7]. Mycobactin J dependency is the vital characteristic of MAP identification in *in vitro* isolations and differentiation from other mycobacteria. Range of media is available for primary isolation and subsequent subculture of MAP and liquid media BACTEC (radiometric based) & MGIT (fluorescence based) have become popular for their improved sensitivity of MAP isolation. Being radioactivity based, BACTEC media has been discontinued and MGIT is available. However, MGIT is costly and may not be afforded by developing countries. Instrument for MGIT culture system costs about 35 lakh rupees with recurring cost of Rs 120 per tube[8]. Therefore we need to identify alternate economic media with good efficacy of isolation. Present study compared the performance of three egg based media (Herrold's Egg Yolk Medium- HEYM, Lowenstein-Jensen Medium- LJ and Middle Brook 7H10 Medium-MB7H10) for the primary isolation of MAP from disease suspected animals. These media were modified from their standard composition in the present study.

Materials and Methods

Sample collection

Animals having signs of paratuberculosis were sampled either from farms in Jaipur District of Western India (Table 1) and slaughterhouse (Chainpura, Jaipur). Farm animals were suspected for para-tuberculosis with clinical signs of rough, dry skin, ribs visible from distance, suffering from diarrhea which is non-treatable, loose feces, intermittent diarrhea, weakness, continued loss in body condition, body weight either static or loosing body weights with or without diarrhea, reduced reproductive rate or reduced fertility and cases of repeat breeding and lowered milk production. Slaughtered animals were sampled having swollen & enlarged mesenteric lymph nodes (MLN) with thickened/ corrugated intestine near ileo-cecal junction (ICJ).

Among the total 112 samples were collected, 62 fecal (Table 1), 25 mesenteric lymph nodes (MLN) and 25 intestines near ileo-cecal junction (ICJ). Fecal samples were collected from suspected cattle, buffalo, goat and sheep. Tissue samples were only collected from slaughtered buffaloes.

Culture Media

Three egg based solid media were tested in the present study; Herrold's egg yolk medium (HEYM), Middlebrook 7H10 medium (MB7H10) and Lowenstein-Jensen medium (LJ) (all media were supplemented with Mycobactin J).

HEYM was prepared as per Merkal and Curran, (1974)[9] with modifications as follows (per/ liter); peptone (9.0 g; Himedia), sodium chloride (4.5 g; Sigma), agar (17.0 g; Himedia), beef extract (2.7 g; Himedia), Glycerol (27.0 mL; Sigma), malachite green (100 mg; Sigma), freshly prepared egg yolk from fertilized eggs (120 mL), and mycobactin J (2.0 mg). The medium was autoclaved prior to the addition of egg yolk and mycobactin J. Antibiotics were added to this medium.

MB7H10 was prepared as described by Whittington *et al.*[10] with modifications and comprised (per/liter), Middlebrook 7H10 agar base (19.0 g; Himedia), casien digest (1.0 g;enzymatic digest of casein by Himedia), glycerol (5.0 mL), PANTA plus antibiotic mixture (Himedia) (Polymyxin B at 37,600 U, Amphotericin B at 3.8 mg, Nalidixic acid at 15.0 mg, Trimethoprim at 3.8 mg and Azlocillin at 3.8 mg) and 100 mL ADC supplement (Himedia) containing Bovine Albumin at 7.6 g, Dextrose at 3.0 g, and Catalase at 6.0 mg, freshly prepared egg yolk from fertilized eggs (200 mL) and mycobactin J (2.0 mg). The antibiotic mixture, ADC supplement, egg yolk, and mycobactin J were added after autoclaving.

LJ medium was prepared as suggested by Kalis *et al.*[11] with modification and contained (per/liter), LJ medium base (23.3 g, Difco), glycerol (7.5 mL, Sigma) homogenate of fertilized whole egg (625 mL), antibiotic mix (as for MB7H10), and mycobactin J (2.0 mg). The medium was sterilized using inspissations at 85°C for 45 min.

Processing of fecal samples

Fecal samples were processed as per Whipple *et al.*[12] with modifications. Briefly, 2.0 g of fecal sample was homogenized in sterile mortar-pestle using 20 mL of

Table 1 — Collection of fecal samples from suspected animals

Farm	Species	No. of animals sampled	Remarks
BorajGaushala, Boraj, Jaipur	Cattle	15	Community shelter for let off cattle, poor health condition in general
Yadav Dairy Farm, Sirsi, Jaipur	Buffalo	15	Good health condition in general
Farmer's Herd, Sirsi, Jaipur	Goat	15	Poor health condition in general
Farmer's Herd, Sirsi, Jaipur	Sheep	17	Poor health condition in general

sterile phosphate buffered saline (PBS). Homogenate was transferred to the tubes and allowed to stand for 30 minutes at room temperature (RT). Supernatant was transferred to sterile 50 mL test tube containing 20 mL of 0.9% hexa-decyl-pyridinium chloride (Sigma); mixture was allowed to stand at room temperature for overnight followed by centrifugation at 900 g for 30 min. Pellet was re-suspended in 0.5 mL of sterile PBS. 100 µL of this suspension was inoculated on media slopes. Each media was inoculated in triplicates. Tubes were incubated at 37°C and were examined every 15 days till appearance of growth, tubes were observed for one-year post inoculation.

Processing of tissue samples

Tissue samples were processed as per Whittington *et al.*[13]. About 5.0 g of tissue was, chopped into small pieces, followed by homogenization in 20 mL sterile PBS using Stomacher Machine for 5 min at a maximum speed. Contents of the stomacher bag were transferred to a sterile 50 mL tube containing 20 mL of 0.9% HPC. Tube was allowed to stand at room temperature for overnight. After decontamination supernatant was transferred into sterile tube and centrifuged at 3800 g for 30 min at 4°C. Pellet was re-suspended in 1.0 mL of sterile PBS and centrifuged at 6500 g for 15 min at 4°C. Pellet was re-suspended in 0.5 mL of sterile PBS and 100 µL of re-suspended material was used for inoculation of media. Tubes were incubated at 37°C and were examined every 15 days till appearance of growth, tubes were observed for one year post inoculation.

Identification of MAP colonies

Suspected colonies were subjected to acid fast staining using Himedia ZN Staining Kit as per manufacturer instructions. Acid fast colonies were subject to IS*900* PCR using Vary Primers, direct colony PCR was done as per Sohal *et al.*[14], colonies yielding typical 229 bp amplicon were identified as MAP. Briefly, a single visible colony was harvested with a sterile toothpick in 10 µL of PCR grade water and heated at 95°C for 10 min. The mixture was then transferred to 40 µL of a PCR mixture consisting of 5 µL of 10XPCR buffer, 200 µM deoxynucleoside triphosphates (dNTPs), 1.5 mM $MgCl_2$, 1.0 µM primers (IS*900* FP: 5'- CCG CTA ATT GAG AGA TGC GAT TGG -3'; IS*900* RP: 5'- AAT CAA CTC CAG CAG CGC GGC CTC G -3') and 1 U of *Taq* polymerase.

Amplification was performed under the following conditions: one cycle of 4 min at 94°C and 40 cycles of 10 s at 94°C, 10 s at 61°C, and 1 min at 72°C.

Results

Out of 112 fecal (n=62) and tissues (n=50) samples tested, MAP was isolated from 59 samples (52.6%) (Fecal- 37 and tissue- 22). Acid fast isolates (Fig. 1) having positive IS*900* PCR reaction (Figs. 2 & 3)

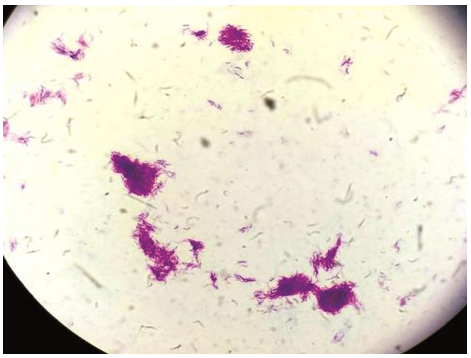

Fig. 1 — Acid fast staining of MAP bacilli

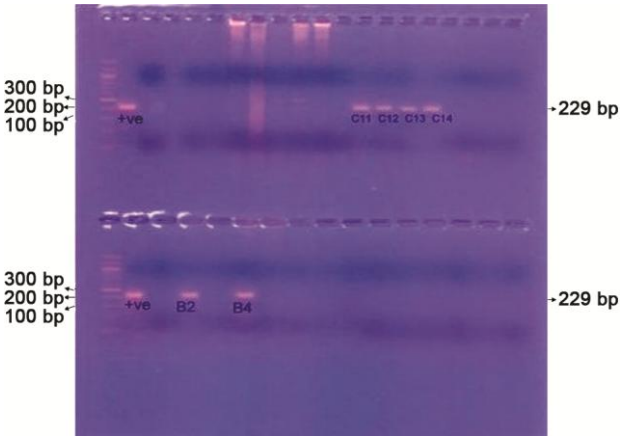

Fig. 2 — IS*900* PCR on suspected colonies from cattle and buffalo (229 bp). [Upper lanes: Lane 1, DNA ladder (100 bp); Lane 2, positive control; Lanes 13-16, cattle positive sample; Lower lanes: Lane 1, DNA ladder (100 bp); Lane 2, positive control; and Lanes 4 and 6, buffalo positive sample]

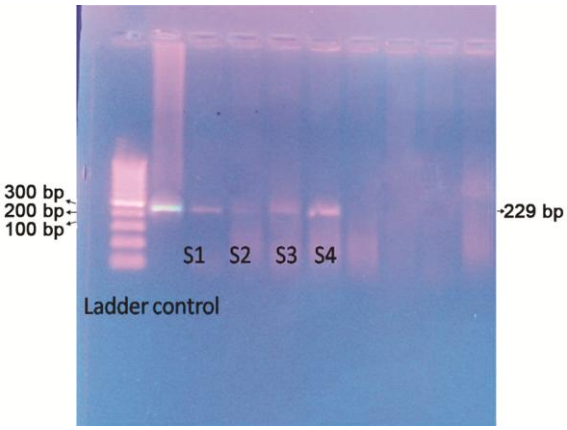

Fig. 3 — IS*900* PCR on suspected colonies from sheep (229 bp). [Lane 1, DNA ladder (100 bp); Lane 2, positive control; and Lanes 3-6, sheep positive sample]

were only considered as MAP. Of the 25 ICJ samples, 13 showed typical corrugations (Fig. 4). Maximum isolations were observed on MB7H10 medium, 47 (41.9%) samples, followed by LJ, 25 (22.3%) and HEYM, 07 (6.25%) samples (Tables 2 & 3). Out of the positive isolations, 28 (47.4%) samples were exclusively positive on MB7H10 followed by LJ, 08 (13.5%) and HEYM, 03 (5.08%) (Table 4). Out of 59 isolates, 39 (66.1%) grew only on a single medium (either MB7H10 or HEYM or LJ) and 20 (33.8%) sample grew on more than one medium (Table 4). None of sample was positive in all three media and combination of MB7H10+LJ recovered, 16 (27.1%) followed by HEYM+MB7H10, 03 (5.08%) and HEYM+LJ, 01 (1.69%) (Table 4). For all types of clinical samples, maximum isolations were observed on MB7H10 (47) followed by LJ (25) and HEYM (07) (Tables 3-5). Also, species-wise maximum isolations were observed on MB7H10 followed by LJ and HEYM, except for cattle, wherein there was no isolate appearing on LJ (Table 5). Growth pattern was also different for each medium (Table 6). The colonies on HEYM are rougher, larger, and medium colour also changed. Colonies on MB7H10 and LJ are small in size and smooth in appearance and uniformly distributed in whole culture tube (Fig. 5).

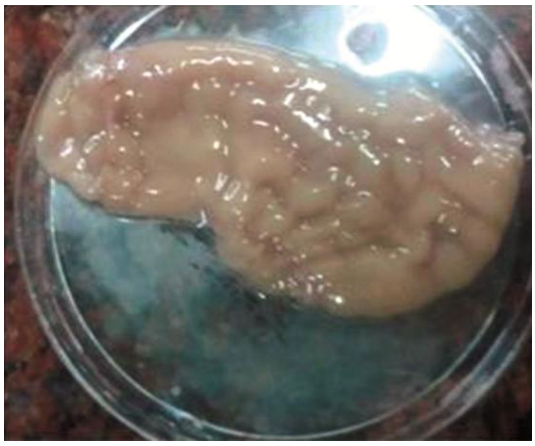

Fig. 4 — Corrugated ICJ of buffalo

Table 2 — Samples positive for isolation of MAP

Sample	Number Positive for Isolation of MAP (%)
Feces (62)	37 (59.6)
MLN (25)	8 (32.0)
ICJ (25)	14 (56.0)
Total (112)	59(52.6)

Table 3 — Growth of fecal and tissue MAP isolates in different solid medium

Sample	HEYM (%)	MB 7H10 (%)	L J (%)
Feces (62)	5 (8.06)	28 (45.1)	19 (30.6)
MLN (25)	1 (4.0)	7 (28.0)	2 (8.0)
ICJ (25)	1 (4.0)	12 (48.0)	4 (16)
Total (112)	7 (6.25)	47 (41.9)	25 (22.3)

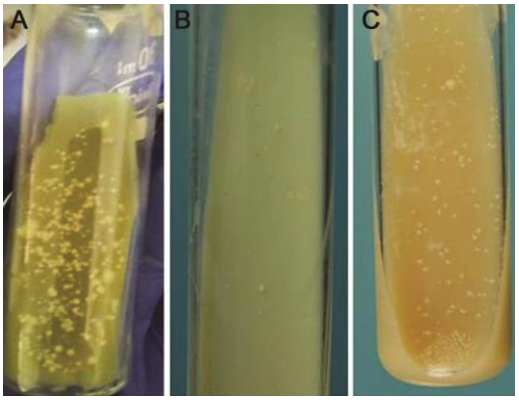

Fig. 5 — Appearance of MAP colonies on different media (A) HEYM; (B) MB 7H10; and (C) LJ

Table 4 — Exclusive growth of MAP isolates in different medium

Positive Samples	HEYM (%)	MB7H10 (%)	LJ (%)	HEYM + MB7H10 (%)	HEYM + LJ (%)	MB7H10 + LJ (%)	HEYM+MB7H10+ LJ (%)
Feces (37)	03 (8.1)	14 (37.8)	05 (13.5)	01 (2.7)	01 (2.7)	13 (35.1)	00
MLN (08)	00	05 (62.5)	01 (12.5)	01 (12.5)	00	1 (12.5)	00
ICJ (14)	00	09 (64.2)	02 (14.2)	01 (7.14)	00	02 (14.2)	00
Total (59)	03 (5.08)	28 (47.4)	08 (13.5)	03 (5.08)	01 (1.69)	16 (27.1)	00

Table 5 — Species wise performance of culture media

Species	No. of Samples	Total Positive (%)	HEYM (%)	MB7H10 (%)	LJ (%)	HEYM+ MB7H10 (%)	HEYM+LJ (%)	MB7H10+ LJ (%)	HEYM+ MB7H10+ LJ (%)
Cattle	15	03 (20.0)	02 (66.6)	02 (66.6)	00	01 (33.3)	00	00	00
Buffalo	65	26 (40.0)	02 (7.6)	22 (84.6)	07 (26.9)	02 (7.6)	00	03 (11.5)	00
Sheep	17	15 (88.2)	03 (17.6)	08 (47.05)	07 (41.1)	00	01 (5.8)	02 (11.7)	00
Goat	15	15 (100)	00	15 (100)	11 (73.3)	00	00	11 (73.3)	00
Total	112	59 (52.6)	07 (11.8)	47 (79.6)	25 (42.3)	03 (5.08)	01 (1.69)	16 (27.1)	00

Table 6 — Special observations on colony characteristics, incubation period and other parameters

Media	Colony morphology	Size of colonies	Incubation period (wk.)	Colour change in media	Drying of Media
HEYM	Rough, speared on whole slant	Large	9-36	Observed	Moderate
MB7H10	Smooth, distinguished	Small	13-36	Not observed	Frequent
LJ	Smooth, speared on whole slant	Small	20-34	Not observed	Not observed

Discussion

Widely, Western world in particular, MGIT liquid culture system based on the fluorescence by Becton-Dickinson has replaced the conventional BACTEC radiometric culture system for primary isolation of MAP, though it is still not popular in the developing and underdeveloped countries due to high cost and requirement of sophisticated instrument. Apart from affordabiligy, MGIT does not support the growth of sheep type MAP strains too[15]. Therefore, alternate media that are affordable and support the growth of all type of MAP strains becomes a necessity. Further, genetically different strains of MAP have different nutritional requirements[16]. Also, prevalence of particular MAP strain may vary depending upon the geographical region[17,18]. The present study, in this context, becomes relevant to both these observations because nutritional requirements of Indian MAP strains have not been evaluated so far. Solid media are more commonly used to culture MAP because they are cheaper, less instrumentation required, easy to handle, and observation of organism is also simpler. This study evaluated comparative performance of three solid media (HEYM, and MB 7H10 and LJ) for primary isolation of MAP from suspected animals.

The primary finding of present research advocates that MB7H10 is the most suitable media for the primary isolation of MAP followed by LJ, it seems that HEYM is not preferable media (Tables 2-4). There is evidence that genetically diverse MAP strains prefer different type of media[16], however, Whittington *et al.*[19] showed that MB7H10 supports growth of all type of MAP strains (14 genotypes were tested), but the size of colonies was small. In the present study also MB7H10 supported growth of majority of isolates and the size of the colonies was small compared to HEYM. A study conducted in Quebec, Canada also found MB7H10 as most suited media for primary isolation of MAP (Personal Communication). Finding of Donaghy *et al.*[20] also confirms the observations on MB7H10. LJ medium was the second preference of MAP isolates in this study; this finding is contrary to study of Carvalho *et al.*[21], wherein HEYM was preferred over LJ by MAP isolates. However, findings of Juste *et al.*[22] are in accordance with our findings wherein LJ has better isolation rate compared to HEYM. Stevenson *et al.*[23] also report that HEYM is not a preferred medium for many MAP strains. Further, considering the exclusive isolations of MAP on each medium used in the present study (Table 4), it cannot be denied that nutritional requirements of MAP strains are dependent upon the genotype; there are previous reports on the same[15,16]. Literature review suggests that there are no consensuses on one single medium but, it can be concluded that earlier studies used LJ and HEYM as preferred media and lately focus is on use of MB7H10 as preferred solid media[10,19].

In the present study, MB7H10 outperformed other media and supported growth of maximum number of MAP isolates (Table 4). This may be attributed to the nutritional composition of this medium. This medium contains ADC supplement (albumin, dextrose and catalase) in addition other general nutrient components. It has been shown that albumin protects the mycobacterial inoculum from toxic effects of esterified fatty acids[24]. Also, catalase protects the initial inoculum from toxic intermediates[25], mycobacteria possess gene for catalase. However, under stress there may be low expression, therefore providing the catalase in the medium will protect the inoculum. Dextrose is the ready carbon source that can be utilized directly in the glycolytic pathway as source of instant energy that may be required during early stages (lag phase of growth). Casein digest has also been added to medium, compared to peptone, casein digest is not only source of amino acids but it is also provides nucleotide precursors, vitamins and other metabolites that these cells would otherwise have to synthesize[26]. Further, it has been shown that mycobacteria are not able to digest complex protein/peptides, therefore availability of free amino acids in form of casein digest enhances mycobacterial growth[26]. Egg yolk is the major constituent of this medium, besides proving the nutrient source egg yolk is also source of lecithin[27], lecithin contains choline that binds with phospho groups in the cell membrane, enhances cell permeability thereby improving the nutrient uptake especially lipids[28].

Lipids are essentially required by mycobacteria because of high requirement in cell wall. Lecithin also protects the inoculum from the effect of residual disinfectants[29]. MB7H10 is providing ready-to-use glutamic acid and it has been shown that presence of glutamic acid in the medium enhances mycobacterial growth[30]. Further, glutamic acid is an essential component of mycobacterial cell wall[31], and hence its presence in the medium would promote the growth. This medium is also a source of ammonium ions that has regulatory role in amino acid assimilation[32]. This

medium also ensures the presence of iron in form of ferric ammonium citrate. Other media tested in the study may vary in the iron source batch to batch. MB7H10 is also a ready-to-use source of pyridoxine and biotin, and it has been shown that these vitamins are essential for mycobacterial survival[33] as cofactor for many mycobacterial enzymes[34]. Therefore, presence of pyridoxine and biotin would promote the growth of stressed cells in the inoculum and thereby enhance their recovery. These benefits may be attributed to the higher recovery rates of MAP on MB7H10 compared to other media used in the present study. In general, MAP requires 12-20 weeks for primary isolation on solid medium. However, in the present study, minimum incubation period was 9 weeks on HEYM and maximum was 36 week on HEYM and MB7H10. Therefore, it is suggestive to keep the slants for enhanced period. ICJ and MLN belonged to the same animal, but, ICJ had better recovery rate for isolation of MAP compared to MLN (Table 2).

Conclusion

Considering the genotypic diversity of MAP, it can be concluded that using multiple media may satisfy the nutritional requirements of diverse MAP isolates. Use of extended panel of media will ensure recovery of enhanced panel of MAP isolates useful for the epidemiological studies. In case of single medium, MB7H10 should be the first choice and combination of MB7H10+LJ proved better.

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Conflict of Interest

Authors declare no conflicts of interest.

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