JOURNAL OF FOODBORNE AND ZOONOTIC DISEASES
Journal homepage: www.jakraya.com/journal/jfzd

ORIGINAL ARTICLE

Comparison of different cultural techniques in isolation of *Listeria monocytogenes* from various samples

[1]Ajay Kumar VJ*, [2]Latha C, [3]Sunil B and [4]Dhanya VR

[1]Assistant Professor, Department of VPE, RIVER, Puducherry, India.
[2]Associate Professor and Head, Department of Veterinary Public Health, COVAS, Thrissur, Kerala, India.
[3]Associate Professor and Head, Department of Veterinary Public Health,, COVAS, Wayanad, Kerala, India.
[4]Veterinary Surgeon, Veterinary Clinical Lab, Palakkad, Kerala, India.

*Corresponding author

Ajay Kumar VJ

E-mail: ajaykins@gmail.com

Received: 01/03/2014
Revised: 29/03/2014
Accepted: 29/03/2014

Abstract

Two different enrichment procedures and plating methods for isolation of *Listeria monocytogenes* were compared while screening 1878 samples from animals (cattle- 475 Buffalo-70 and goats - 385), human beings (240) and from environment (708). The methods of enrichment included a single step enrichment procedure using Buffered Listerial broth (BLEB) and another one with two step enrichment procedure using University of Vermount (UVM) broth. Polymixin Acriflavin Lithium Chloride Ceftazidime Mannitol agar (PALCAM) and Oxford agars were the solid media used. The results of the study showed that the two step enrichment procedure using UVM and PALCAM media combination yielded maximum isolates (4.04 per cent) followed by two step enrichment using UVM and plating to Oxford media (3.35 per cent), single step enrichment using BLEB and plating to PALCAM (2.02 per cent) and finally, single step enrichment using BLEB and plating to Oxford agar (1.91 per cent). The results indicated the superiority of two step enrichment especially when sample contained more background microflora.

Keywords: PALCAM, Oxford, *Listeria*, UVM, BLEB.

Introduction

Listeria monocytogenes is a multisystemic invasive pathogen, capable of colonising multiple host tissues, causing a range of clinical conditions in animals and human beings. This causes considerable mortality and morbidity in humans and livestock and is considered as one of the important food borne pathogen. The case fatality rate for this foodborne pathogen, which ranges from 15.0 to 30.0 per cent with the highest hospitalization rates (90.5 per cent) amongst known food-borne pathogens (CDC, 2000). It is a significant food borne pathogen due to its ability to survive in a wide range of environmental conditions including at refrigeration temperatures.

All the members under the genus *Listeria* share almost the same growth requirements and can grow in the media used for *L. monocytogenes*. *Listeria*, being a non fastidious organism, can be sub cultured on most common bacteriological media such as tryptose agar, nutrient agar and blood agar. However, attempted isolation of *Listeria* from inoculated or naturally contaminated food and clinical specimens by use of nonselective media is often challenging. Difficulties encountered in isolating *L. monocytogenes* dates back to initial detection of this pathogen in 1926 when Murray and his co-workers stated, "The isolation of the infecting organism is not easy and we found this to remain true even after we had established the cause of the disease."

The selective medium developed by McBride and Girard (1960) was among the first solid media suitable for recovering *L. monocytogenes* from mixed cultures. The selective agent used by them was potassium tellurite. The media was selective/differential for *Listeria* that reduces tellurite to tellurium, producing black colonies. In 1986, Lee and McClain described lithium chloride–phenylethanol–moxalactam (LPM) agar, a modification of McBride *Listeria* agar. In 1988, Netten *et al.,* developed a solid media that included acriflavine, phenylethanol, esculin, mannitol and egg yolk emulsion which was suitable for enuemerating *L. monocytogenes.* This media was later developed as Polymixin, Acriflavin, Lithium Chloride, Ceftazidime and Mannitol agar (PALCAM) by adding polymixin

B, lithium chloride and ceftadizime and is still a widely recommended media for detection of *Listeria*. In 1989, Curtis *et al.,* developed an agar, Oxford agar (OXA), and it was prepared from Columbia agar base to which several selective agents, including colistin sulfate (20 mg/L), fosfomycin (10 mg/L), cefotetan (2 mg/L), cycloheximide (400 mg/L), lithium chloride (15 g/L), and acriflavine (5 mg/L), were added. Esculin and ferric ammonium citrate also were added as differential agents to produce black *Listeria* colonies from esculin hydrolysis. This medium was slightly modified by McClain and Lee by incorporating moxalactam; this new medium was designated modified Oxford agar (MOX) (Carnevale and Johnson, 1989). Curtis and Lee (1995), reviewed different types of enrichments procedure and plating media for *L. monocytogenes* and commented that the preference of media and methods are governed by the type of sample, number and nature of competing flora and cost.

The efficacy of differential enrichment system in USDA-FSIS system was assessed to detect its' sensitivity and specificity (Capita *et al.,* 2000). They commented that the sensitivity of 98.95 per cent and specificity of 40 per cent obtained in the experiment was not satisfactory especially when working with samples containing other esculin positive organisms. Capita *et al.* (2001), compared the USDA, PALCAM and Modified Oxford agar for their ability to detect *Listeria* from chicken, they found that there was no considerable difference between these methods in their ability to detect the organism. Parihar *et al.* (2008) followed a two step enrichment procedure using University of Vermount Broth (UVM I) and UVM II broths followed by plating in PALCAM and Dominguez-Rodriguez Isolation Agar DRIA. They found the enrichment procedure was useful in the isolation of the organism. The overgrowth of *L. monocytogenes* by other *Listeria* in samples undergoing enrichment was studied by Besse *et al.* (2010) and they observed some isolates grew faster than others.

Materials and Methods

The occurrence of *Listeria* spp. in a total of 1878 samples from animal, human and environmental samples collected from different parts of Kerala, India, was determined by different culture techniques. The samples collected from animals included 475 from cattle (260 faecal samples, 145 samples of aborted materials and 70 milk samples), 70 from buffaloes (50 milk and 20 faecal samples) and 385 were from goats (200 faecal samples, 170 samples from aborted materials and 15 milk samples). Two hundred and forty human faecal samples were also collected. The environmental samples included 270 soil samples, 328 water samples and 110 samples of vegetables (40 cabbage samples and 70 leafy vegetable samples).

All the samples were screened for the presence of *Listeria* spp. using two different methods for enrichment and plating.

First method of enrichment: In the first method, selective enrichment using buffered listerial enrichment broth (BLEB) was done. Twenty five grams (millilitres in case of liquid samples) of each sample was added to 225 ml of BELB. The mixture was homogenized using a laboratory blender for two minutes and incubated at 30^0C for 4 h. Then the broth was added with selective ingredients such as cycloheximide, nalidixic acid and acriflavin and it was incubated for another 44 hours at 30^0C before inoculating to solid media.

Second method of enrichment: In this method, a two step enrichment procedure using UVM I and II broth was followed. In primary enrichment, UVM I was used. Acriflavin (6 mg) and Nalidixic acid (10 mg) are the selective agents used in the UVM I media. Twenty five grams (millilitres in case of liquid samples) of each sample was added to 225 ml of UVM I. The mixture was homogenized using a laboratory blender for two min and incubated at 30^0C for 24 h.

The secondary selective enrichment was carried out in UVM II broth, which contained the selective agents at higher concentration (Acriflavin 12.5 mg and Nalidixic acid 10 mg). From the primary enriched UVM I broth culture, 0.1 ml was transferred to 10 ml of UVM II broth and incubated at 30 ^{0}C for 24 h.

Selective plating: After the enrichment procedure using BLEB and UVM broth the selective plating was done using two different media. The samples enriched by both methods were plated into two different selective agars.

One media used was Oxford agar. After the enrichment in the BLEB and UVM II broth, a loopful of the inoculum was streaked onto Oxford agar plates. The media contains acriflavin, colistin sulphate and lithium chloride which will prevent the growth of other organisms. The plates were incubated at 37^0C for 48 h. On oxford agar, *Listeria monocytogenes* produced brown-green coloured colonies with a black halo due to hydrolyses of esculin to esculetin which forms a black complex with iron ions.

The second media used was PALCAM agar. After the period of incubation in UVM II broth, a loopful of the inoculum was streaked to PALCAM agar

plates. The plates were incubated at 37^0C for 48 h. On PALCAM Agar, colonies of *Listeria* appear gray-green with a black sunken centre and a halo. Identification of *Listeria* spp. on PALCAM agar plates was based on aesculin hydrolysis and mannitol fermentation.

For confirmation, five or more suspected colonies from Oxford and PALCAM agar plates were transferred on to Brain Heart Infusion Agar (BHIA) plates and incubated at 30^0C for 48 h. The selection of five colonies ensured that multiple species of *Listeria*, if at all present, will be identified. The suspected individual colonies were then transferred to Brain Heart Infusion Broth (BHIB) (HiMedia, India) and incubated at 37^0C for 24 h. The isolates were then subjected to a series of biochemical tests for identification.

All the chemicals and dehydrated media used in the study were procured from HiMedia, India.

Results

Out of 1878 samples were processed using two different cultural techniques, maximum number of *Listeria* isolates (76) was obtained by two step enrichment in UVM broths. After this enrichment, plating onto PALCAM and Oxford agars yielded isolates from 4.04 per cent and 3.35 per cent of the samples, respectively. The combination of double step enrichment using UVM broths and plating to PALCAM agar was successful in obtaining maximum *Listeria* organisms from the samples when compared to other methods (Table 1.)

Of the 76 isolates, 54 were *L. innocua*, nine were *L. grayi*, seven were *L. ivanovii* and two were *L. welshimerii*. Four *L. monocytogenes* isolates were also obtained. One was from cattle fecal sample, another from a case of goat abortion and one each from soil and cabbage samples. It was observed that the UVM enrichment was capable of detecting all four *L. monocytogenes* isolates. But enrichment using BLEB was unable to detect the presence of *L. monocytogenes* the faeces.

On statistical analysis by proportion test using the *chi*-square, it was found that the third method, that is, two step enrichment in UVM broth followed by - plating in PALCAM was significantly better than other methods. However, when two step enrichment in UVM broth, followed by plating in PALCAM was compared with the fourth method, that is, two step enrichment in UVM broth and plating to Oxford agar by using '*Z*' test, no significant difference ($p>0.05$) was found between them.

Discussion

Different cultural techniques were compared by using the methods in isolating the organisms from 1878 samples. The results showed that two step enrichment in UVM broth followed by plating onto PALCAM agar was superior to other methods. Same method of enrichment followed by plating into Oxford agar was also found effective. The enrichment using BLEB was found inferior when compared with other methods.

The need for different types of enrichment and plating while screening for *Listeria* from different samples was suggested by Capita *et al.* (2001). The study also observed that by using parallel enrichment and plating methods, recovery of organisms can be improved. They also observed that plating onto PALCAM yielded more isolates as observed in the present study.

Evolution of Listeria populations in food samples undergoing enrichment was studied in detail by Besse *et al*. (2005). They observed that *L. innocua* was able to overgrow the *L. monocytogenes* during enrichment and based on the study of growth curve and analysis of enrichment media they suggested that *L. innocua* is capable of producing certain bacteriocins so that *L. monocytogenes* will not be able to grow. However they could not suggest any solution to overcome this problem.

It was also observed that even among *L. monocytogenes*, different serotypes behave differently during enrichment. In a study using FDA enrichment protocol and plating onto MOX agar, Gorski *et al.* (2006) noticed that serotype 1/2a when co-inoculated to BLEB with other serotypes of *L. monocytogenes* had shown highest fitness.

This was proved by the recovery of only 1/2a serotype after enrichment. Similar observation was made by Bruhn *et al.* (2005) while studying the UVM 1 medium.

The results of comparison of culture techniques in present study clearly show the advantage of two step enrichment procedure. None of the samples negative by this enrichment procedure was positive by single step enrichment. Most of the isolates were from soil, fecal samples and vegetables. These samples were rich in background microflora and thus only a highly selective enrichment procedure would have allowed the isolation of the organism. Since the selectivity is very high there is a chance that organisms may fail to grow especially when they are stressed or in very small numbers. The results would have been different if the isolation was attempted from processed foods or clinical samples with less number of background microflora (blood and CSF). For example the BLEB contains sodium pyruvate, a content that can repair stressed *Listeria* cells and promote the growth.

Table 1: Comparison of culture techniques used in the study

Sl No.	Method of selective enrichment	Solid media plating	Samples screened	Samples Positive for *Listeria* spp.	
				No.	%
1.	BLEB- single step enrichment	PALCAM	1878	38	2.02
2.	BLEB- single step enrichment	Oxford	1878	36	1.91
3	UVM broth- two step enrichment	PALCAM	1878	76	4.04
4	UVM broth- two step enrichment	Oxford	1878	63	3.35

This will help the stressed bacteria to grow, even though they are present in small numbers. But as it focuses more on recovery, selectivity is compromised to some extent. Same argument can be applied to PALCAM and Oxford agars as PALCAM is more selective.

Conclusion

From the results it can be concluded that two step enrichment procedures along with plating to PALCAM is best method when there is background microflora in the samples to be screened. However, to confirm the identity of an isolate, a combination of biochemical, immunological and genetic tests should be used as all the media used can promote growth of different species of *Listeria* and the organism frequently demonstrates enormous variations in biological, biochemical and immunological characteristics.

Acknowledgement

The authors express immense gratitude to Indian Council of Agriculture and Research for providing financial assistance to the work. The encouragement from Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala is also acknowledged.

References

Besse NG, Audinet N, Kerouanton A, Colin P and Kalmokoff S (2005). Evolution of *Listeria* populations in food samples undergoing enrichment culturing. *International Journal of Food Microbiology,* 104: 123-134.

Besse NG, Barre L, Buhariwalla C, Vignuad ML, Khamissi E, Decourseulles E, Nirsimloo M, Chelly M and Kalmokoff M (2010). The overgrowth of *Listeria monocytogenes* by other *Listeria* species in food samples undergoing enrichment cultivation has a nutritional basis. *International Journal of Food Microbiology,* 136: 345-351.

Bruhn JB, Vogel BF and Gram L (2005). Bias in the *Listeria monocytogenes* Enrichment Procedure: Lineage 2 Strains Outcompete Lineage 1 Strains in University of Vermont Selective Enrichments. *Applied and Environmental Microbiology*, 71(2): 961-967.

Capita R, Callega CA, Arias MTG, Moreno B and Ferrnandes MCG (2000). Centers for Disease Control and Prevention. Annual Report-2000. CDC/USDA/FDA Foodborne Disease Active Surveillance Network. CDC's Emerging Infections Program. Revised 27 June 2003. URL:http://www.cdcgov/foodnet/annual/1998/html/1998p.

Capita R, Callega CA, Moreno B and Ferrnandes MCG (2001). Occurrence of *Listeia* species in retail poultry meat and comparison of a cultural/ immnunoassay for their detection. *International Journal of Food Microbiology,* 65: 75-82.

Carnevale RA and Johnston RW (1989). Method for the isolation and identification of *Listeria monocytogenes* from meat and poultry products.USDA-FSIS, Laboratory Communication No. 57, Revised May 24. USDA, Washington D.C. 68p.

Centres for Disease Control and Prevention (2000). URL–http://cdc.gov/nczed/divisions/dfbmd/diseases/listeriosis.

Curtis GDW and Lee WH (1995). Culture media and methods for the isolation of *Listeria monocytogenes*. *International Journal of Food Microbiology,* 26: 1-13.

Curtis GDW, Mitchell RGW, King F and Griffen EJ (1989). A selective differential medium for the isolation of *Listeria monocytogenes*. *Letter's in Applied Microbiology,* 8: 95-98.

Gorski L, Flaherty D and Mandrell RE (2006). Competitive fitness of *Listeria monocytogenes* serotypes 1/2a and 4b in mixed cultures with and without food in US-FDA protocol. *Applied and Environmental Microbiology*, 72: 776-780.

Lee WH and McClain D (1986). Improved *Listeria monocytogenes* selective agar. *Applied and Environmental Microbiology*, 52: 1215-1218.

McBride ME and Girard KF (1960). A selective medium for the isolation of *Listeria monocytogenes* from mixed bacterial populations. *Journal of Laboratory and Clinical Medicine,* 55: 153-157.

Murray EGD, Webb RA and Swann MBR (1926). A disease of rabbits characterized by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus, *Bacterium monocytogenes*. *Journal of Pathology and Bacteriology,* 29: 407-439.

Netten PV, Van de VA, Perales I and Mossel DAA (1988). A selective and diagnostic medium for use in the enumeration of *Listeria* spp. in foods. *International Journal of Food Microbiology,* 6: 187-198.

Parihar VS, Barbuddhe SB, Chakurkar EB, Danielsson T and Tham W (2008). Isolation of *Listeria* species from farm bulk milk at the receiving dairy plant and cervicovaginal swabs. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases,* 27(1-2): 53-55.