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Article

Comparison of selective agars recommended by method ISO 11290-1 and chromogenic agars for the isolation of *Listeria* sp. in refrigerated sausages

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The aim of this study was to determine the prevalence of *Listeria* sp. in refrigerated sausages, and to compare the performance of the selective plating media employed in the ISO 11290-1 method (PALCAM and Oxford agars) with chromogenic agars (Chromogenic *Listeria* agars CM 1080 (OCLA) and CM 1084). The prevalence of *Listeria* sp. detected was 52.9%, comprising 13.7% *L. monocytogenes* strains. The efficacy of the four agars for the isolation of *L. monocytogenes* proved to be satisfactory. Despite differences in composition of the chromogenic media assessed, these disparities did not affect concordance among results. However, PALCAM agar was shown to suppress other microorganisms more effectively, being more applicable for detecting *Listeria* strains present in lower quantities. Based on these results, the use of PALCAM agar, in combination with a chromogenic media, is recommended for enhanced isolation of atypical *Listeria* sp. strains in meat products.

Uniterms: *Listeria* sp./detection/meat products. Meat products/qualitative analysis. Refrigerated sausages/qualitative analysis. PALCAM Agar. Oxford Agar. Chromogenic Agar.

Este estudo teve como objetivo a análise da prevalência de *Listeria* sp. em linguiças resfriadas e a comparação dos meios seletivos utilizados no plaqueamento do método ISO 11290-1 (Ágar PALCAM e Ágar Oxford), e ágares cromogênicos (Ágares *Listeria* Cromogênico CM 1080 (OCLA) e CM 1084 (ISO)). A frequência de *Listeria* sp. foi de 52,9%, sendo que destas, 13,7% corresponderam à *L. monocytogenes*. A eficácia dos quatro ágares para o isolamento de *L. monocytogenes* demonstrou-se satisfatória. Apesar de haver algumas diferenças nas composições dos meios cromogênicos analisados, estas não pareceram influenciar nas concordâncias entre os resultados expressos. Contudo, o ágar PALCAM mostrou-se mais eficaz na supressão de outros micro-organismos, aumentando, assim, a possibilidade de detecção de espécies de *Listeria* presentes em número reduzido. Através deste trabalho sugere-se a utilização do ágar PALCAM associado a um meio cromogênico para aumentar a chance de isolamento de cepas atípicas de *Listeria* sp. em produtos cárneos.

Unitermos: *Listeria* sp./detecção/produtos cárneos. Produtos cárneos/análise qualitativa. Lingüiças resfriadas/análise qualitativa. Ágar PALCAM. Ágar Oxford. Ágar cromogênico.

INTRODUCTION

The scientific community was first alerted to the dangers of listeriosis during the 1980s following a series of outbreaks in North America and Europe. In 1988, the World Health Organization (WHO) established that the consumption of contaminated foodstuffs was the primary route of transmission of *L. monocytogenes* in humans (Faber, Peterkin, 1991; Oliveira, 1993).

Listeria sp. is found widely in nature, explaining the frequency of its emergence in foods, from production to end-consumption (Beuchat, 1996).

Research into *L. monocytogenes* in ready-to-eat foods has become increasingly important, particularly

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given that the food products involved in the outbreaks and sporadic cases of listeriosis were predominantly industrially processed refrigerated foods with a long shelf life.

As *Listeria* has specific nutritional requirements, the cultivation stage involving enrichment broths plays a key role in the recovery and multiplication of viable cells from the microorganisms present in the sample. However, in the stages following the enrichment, selective media can hamper microorganism recovery due to sensitivity to the antibiotics in the media, resulting in their suppression by these inhibitory agents (Trabulsi, Althertum, 2008).

Two different selective agars, PALCAM and Oxford, recommended by the International Organization for Standardization (ISO) (1996) have been adopted in protocols for the detection/enumeration of *L. monocytogenes*. These methods incorporate added esculin which forms greyish-green to black colonies, sometimes evidenced by blackening of the media, aiding visualization and identification of colonies. However, this traditional method is unable to differentiate *L. monocytogenes* colonies form other *Listeria* sp. species. Consequently, the detection of *L. monocytogenes* requires additional identification procedures entailing pain-staking tests, which are both time-consuming and costly.

However, researchers have sought to improve selectivity by proposing modifications in the formulas used. Hemolytic ceftazidime lithium chloride agar – HCLA, "enhanced haemolysis agar" – EHA and modified forms, *Listeria monocytogenes* blood agar - LMBA, Rapid'L Mono developed by L' Mono de Foret, Dorey (1997), and Chromogenic *Listeria* agar Base (Oxoid *chromogenic Listeria* agar) (OCLA) CM 1080 and (ISO) CM 1084, both supplied by Oxoid (Ottaviani *et al.,*1997), are examples of media able to differentiate *Listeria monocytogenes* from *Listeria* sp. (Vlaemynck *et al.,* 2000).

The aim of the present study was to analyse the prevalence of *Listeria* sp. in refrigerated sausages produced in Paraná State, Brazil, and to compare the performance of selective plating media employed in the ISO 11290-1 method (PALCAM and Oxford agars) with chromogenic agars (Chromogenic *Listeria* agars CM 1080 (OCLA) and CM 1084) in terms of efficacy for the detection of *Listeria* sp. in naturally contaminated samples.

MATERIAL AND METHODS

Selection of samples

A total of 51 samples of pre-packed refrigerated sausages, purchased from different commercial outlets in the State of Paraná and produced in the South of Brazil, were collected. The minimum amount of 250 g was acquired for each sample to ensure representativity as recommended by the RDC (Board of Commerce Resolution) 12 of 02/01/2001 published by ANVISA (National Health Surveillance Agency) (Brazil, 2001).

Methods

The conventional culture method was performed as per ISO 11290-1 recommendations. However, in the present study, for the third step of the ISO 11290-1 for the identification of *Listeria* sp. involving the process of isolating the bacterium, an assessment using four commercially available agars was performed, namely: PALCAM agar supplemented with SR 0150E (polymyxin B 5 mg, acriflavine HCl 2.5 mg) (Oxoid, England); Oxford agar selective for *Listeria* supplemented with *Listeria* Selective Supplement (Oxford Formulation) SR0140E (cycloheximide 200 mg, colistin sulphate 10 mg, acriflavine 2.5 mg, cefotetan 1mg, phosphomycin 5 mg) (Oxoid, England); Chromogenic *Listeria* agar CM 1080 (OCLA) (Oxoid, England) supplemented with Chromogenic *Listeria* Selective Supplement SR 0227E (nalidixic Acid 13 mg, polymyxin B 38,350 IU, amphotericin 5 mg, ceftazidime 3 mg) (Oxoid, England) and Chromogenic *Listeria* Differential Supplement SR 0228E (lecithin solution 20 mL); Chromogenic *Listeria* Agar CM 1084 (Oxoid, England) supplemented with Chromogenic *Listeria* Differential Supplement SR 0228E and Chromogenic *Listeria* Selective Supplement (ISO) SR 0226E (nalidixic Acid 10.0 mg, polymyxin B 38,350 IU, amphotericin 5 mg, ceftazidime 10 mg) (Oxoid, England). Agar plates were incubated at 37 °C for 48 hours.

From 3 to 5 colonies per plate containing typical colonies were selected for subsequent confirmation.

Typical colonies were streaked onto tubes containing Tryptic Soy agar supplemented with 0.6% yeast extract (TSA-YE) (Laborclin, Brazil) and incubated at 30 °C for 24 and 48 hours.

Typical colonies were confirmed by motility tests performed through inoculation of enriched broth in tubes of sulfide-indole motility with the addition of 0.05% triphenyltetrazolium chloride (modified SIM) (Laborclin, Brazil) and incubation at 25 °C for up to 7 days under daily observation. In parallel, strains were identified using the API® *Listeria* system (Biomerieux, France).

Results for the four agars analyzed were grouped by characteristics. Comparative analysis was carried out based on two groups: Chromogenic agars (CM 1080 and CM 1084) and Classic agars (PALCAM and Oxford). Analysis of concordance between positive and negative

results was carried out by comparing the ISO method for classic versus chromogenic agars for the detection of *Listeria* sp. in refrigerated sausages according to the model proposed by Jekel *et al.* (1999).

RESULTS AND DISCUSSION

Based on results of the 51 samples analyzed using the selective media employed in the present study, the prevalence of *Listeria* sp. detected in refrigerated sausages was 52.9%, comprising 13.7% *L. monocytogenes*. *L. grayi* was found in 19.7% of the samples studied whereas *L. innocua* and *L. welshimeri* were identified in 13.7% and 5.9% of cases, respectively. The above results agreed with those of samples confirmed by the API® *Listeria* system.

Separate assessment of the selective media revealed 52.9% positivity for PALCAM agar (n=27), 47.0% for Oxford agar (n=24), 43.1% for Chromogenic *Listeria* agar CM 1080 (n=22), and 45.1% for Chromogenic *Listeria* agar CM 1084 (n=23).

Congruent with the final result, PALCAM agar showed no false-negatives, whereas Oxford agar, Chromogenic *Listeria* CM 1084 and CM 1080 agars had 3, 4 and 5 false-negatives, respectively.

These results confirm that election of a single selective media other than PALCAM agar would lead to approval of false-negative samples (contaminated but undetected in analysis) for consumption, posing a potential health risk since infected food batches may be cleared due to non-detection of the pathogen.

Comparison of the results for chromogenic media (Table I) show positive concordance of 32 samples (62.7%) and negative concordance in 18 samples (35.3%), giving an overall agreement of 98.0% (Table II).

TABLE I - Positive and negative results comparing chromogenic agars for detection of *Listeria* sp. in refrigerated sausages

	CM1080 Vs. CM1084
Positive agreement	32
Positive disagreement	1
Negative disagreement	0
Negative agreement	18
Total	51

Positive disagreement was disclosed in only one sample (2.0%), while no negative disagreement was found.

The *kappa* test was used to compare chromogenic agars, yielding 96.0% agreement, representing excellent concordance among results obtained for the agars analysed, i.e. there was strong agreement among correlations assessed.

TABLE II - Concordance between positive and negative results comparing chromogenic agars for detection of *Listeria* sp. in refrigerated sausages

Degree of concordance	CM1080 Vs. CM1084
Overall percentage agreement	98.0%
Kappa	96.0%

Despite some differences in composition of the chromogenic media analyzed, these appeared to have no influence on concordance among the results.

Comparison of the results for classic media (Table III) revealed positive agreement for 34 samples (66.5%) and negative agreement in 18 samples (23.5%), giving an overall agreement of 90.0% (Table IV). These findings indicate a greater capacity of PALCAM agar for identifying lower concentrations of *Listeria* sp. compared with Oxford agar.

TABLE III - Positive and negative results comparing classic agars for detection of *Listeria* sp. in refrigerated sausages

	PALCAM Vs. OXFORD
Positive agreement	34
Positive disagreement	4
Negative disagreement	1
Negative agreement	12
Total	51

TABLE IV - Concordance between positive and negative results comparing classic agars for detection of *Listeria* sp. in refrigerated sausages

Degree of concordance	PALCAM Vs. OXFORD
Overall percentage agreement	90.0%
Kappa	76.0%

Positive disagreement was disclosed in 4 samples (7.8%), while negative disagreement was found in only one sample (2.0%).

The *kappa* test was used to compare classic agars, yielding 76.0% agreement, representing excellent concordance among results for the Agars analyzed.

The efficacy of the four Agars for the isolation of *L. monocytogenes* in the naturally contaminated samples proved satisfactory. However, PALCAM agar was shown to be more effective in isolating all the strains identified

in the present study, proving capable of detecting *Listeria* strains present in low concentrations.

Other studies have reported similar efficacy of PALCAM agar in suppressing accompanying microorganisms coupled with higher rates of isolation of *Listeria* sp. For instance, the study by Gunasinghe *et al. (*1994), comparing the performance of selective Oxford (Oxoid) and PALCAM (Merck) agars in varieties of Frankfurter sausages, salami and pate, found PALCAM agar to be superior and also noted more effective isolation of *Listeria.* Nayak *et al*. (2010), in studies of buffalo meat, noticed that PALCAM agar identified 100% of species analyzed and was in concordance with results on polymerase chain reaction (PCR). By contrast, Oxford agar showed a recovery rate of 60%. Capita (2001), examining cuts of raw poultry meat, found a significantly higher percentage of tested samples positive for *Listeria* sp. using PALCAM plating media compared with Oxford agar (95.0% versus 87.0%), respectively. Similarly, a higher percentage of samples was found to be contaminated with *L. monocytogenes* using PALCAM agar (31.0%) than with Oxford agar (27.0%). In a comparative assessment of different selective isolation media for the detection of *Listeria* sp., Art and Andre (1991) found that the PALCAM media offered excellent performance, superior to that of Oxford media, and urged for replacement of the latter by another media capable of differentiating *L. monocytogenes* from other *Listerias* strains.

However, some authors failed to confirm more favorable results using PALCAM agar. Pinto *et al.* (2001) for example, in a comparative assessment of food and environmental samples, found higher sensitivity for the recovery of *L. monocytogenes* using *Listeria monocytogenes* Blood agar (LMBA) compared with both PALCAM and Oxford agars. These authors were unable to distinguish any difference in results using the selective media recommended by the ISO. Becker *et al.* (2006) performed a comparison of the two selective chromogenic media, ALOA and RAPID´L Mono, with the official plating method recommended by ISO 11290-1, using different food samples. The group noted no significant performance difference among the media studied. Leclercq (2004), examining the performance of the selective PALCAM, Oxford, Rapid'L Mono and ALOA solid media found low recovery and enumeration capabilities, although confirmed ALOA agar as the best isolation media. Rodrigues *et al*. (2003) obtained superior results using hemolytic ceftazidime lithium chloride agar compared with both PALCAM and Lithium chloride phenylethanol moxalactam media.

PALCAM agar is known for its high selectivity and sensitivity, rendering it useful for the detection of *Listeria* sp. in products with high bacterial loads such as fresh sausages (Warburton *et al*., 1992). The selectivity of this media is achieved through the presence of lithium chloride, polymyxin B sulfate, acriflavine HCl and ceftazidime, which suppress growth of most non-*Listeria* species present in food (Gunasinghe *et al*., 1994).

Chromogenic media, such as those described in this study, together with their modified forms, are gradually gaining acceptance by the regulatory authorities (Reissbrodt, 2004; Gasanov *et al*., 2005). However, despite offering numerous advantages over other tests, such as low cost, ease of interpretation, and shorter execution times, according to the results attained, the performance of the chromogenic media, although satisfactory, remained analytically inferior to the gold standard. Nevertheless, the use of more than one isolation media, employing different selective agents and systems of identifying colonies, is important to increase the chance of isolating the target organism. This procedure is recommended for detecting bacteria whose presence in foods, even at low amounts, can expose consumers to serious risk, as is the case for *L. monocytogenes* (Rodrigues *et al*., 2003). Based on the findings of the present study, the use of a combination of different media, such as special alternative media (chromogenic agars) and media containing esculin (PALCAM agar) is recommended. These media are particularly applicable for the detection or enumeration of atypical strains of *L. monocytogenes*.

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