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Comparison of Media for the Isolation of *Salmonella* (XLD and Rambach) and *Listeria* species (ALOA and Palcam) in Naturally Contaminated Duck Samples

Frederick Adzitey[1, 2*], Nurul Huda[1], Rusul Gulam[1]

[1]*Food Technology Programme, School of Industrial Technology, Universiti Sains Malaysia, Minden, 11800, Pulau Pinang, Malaysia*
[2]*University for Development Studies, Animal Science Department, Box TL 1882, Tamale, Ghana*

Abstract

In this study, two media each, xylose lysine deoxycholate (XLD) agar and Rambach agar, for the isolation of Salmonella species, and listeria selective agar (ALOA) and Palcam agar, for the isolation of Listeria species were assessed on their efficacy to isolate these food-borne pathogens from naturally contaminated duck samples. A total of 144 and 128 duck samples were examined for the presence of Salmonella and Listeria species, respectively. Out of which 58 Salmonella's were observed on XLD agar and 51 on Rambach agar. Fifty one (51) Listeria's were observed on Palcam agar and 44 on ALOA. The study revealed that, the isolation of Salmonella and Listeria species on XLD and Palcam agars from naturally contaminated duck samples were relatively higher than their opponents. Improved efficacy of isolating these pathogens is essential for clinical and reporting purposes due to the public health implications associated with these pathogens.

Key words: *Salmonella* species, *Listeria* species, xylose lysine deoxycholate agar, Rambach agar, Palcam agar, listeria selective agar

Introduction

The history of isolating food-borne pathogens from animals, plants and environmental sources is well known and documented. There have been a number of studies on the improvement and comparison of existing media for the isolation and detection of *Salmonella* and *Listeria* species including those of Taylor (1965), Van Netten et al. (1989), Rambach (1990), Tate et al. (1990), El-Sherif and Elmossalami (1998) and Perez et al. (2003).

Current trends have involved the use of antibiotics and nutrients, based on the principles of the existing ones and modifications to increase the recovery of food-borne pathogens. Antibiotics such as ceftazidime, polymyxin B, and acriflavin; and nutrients such as yeast extract, glucose, xylose, sucrose, lactose, peptone, bacteriological agars, sodium thiosulphate, ferric ammonium sulphate, and sodium desoxycholate, have been used in media for isolating *Salmonella's* and *Listeria's* (Health Protection Agency (HPA) 2004a, Health Protection Agency (HPA) 2004b). Additionally, almost all media have indicators (e.g. phenol red in xylose lysine deoxycholate agar, aescullin in listeria selective agar, and phenol red, escullin, ferrous iron, and/or mannitol in Palcam agar) responsible for the characteristic colour change in media or colony that helps in the visual and presumptive identification of bacteria colonies. Xylose lysine deoxycholate and Rambach agars

* Corresponding author. Mailing address: School of Industrial Technology, University science of Malaysia, Minden, 11800, Pulau Pinang, Malaysia.Tel: +604-6532112, Fax: +604-6573678, E-mail: adzitey@yahoo.co.uk

do not require the addition of supplements while Palcam and listeria selective agars require the addition of supplements. The world has also seen major changes and additions in the isolation, detection, identification and/or confirmation of food-borne pathogens by various modern and sophisticated molecular methods.

Isolation of *Salmonella* serovars and *Listeria* species in ducks raised in Malaysia, the third world producer of duck meats, have already been reported (FAO 2009, Adzitey et al. 2010a, Adzitey et al. 2010b). The presence of *Salmonella* and *Listeria monocytogens* in ducks is of public health concern and thus efficient media, techniques and/or methods for isolating these pathogens are required for more accurate reporting and control measures. This study was therefore conducted to compare the ability of two media each of *Salmonella* (xylose lysine deoxycholate agar and Ranbach agar), and *Listeria* species (listeria selective agar and Palcam agar) to recover these pathogens in naturally contaminated duck samples.

Material and Methods

Location, duration and data collection. This study took place between September, 2009 and January, 2010. A total of 144 (for *Salmonella* species) and 128 (for *Listeria* species) duck samples collected from wet markets and duck farms in Penang were examined for presence of *Salmonella* and *Listeria* species. Duck samples examined were duck intestines and wash water samples (water used for washing ducks carcasses) from the wet market and duck faecal, soil and feed samples from duck farms. These samples were stored under 4 $^{\circ}$C during transportation and analyzed immediately upon arrival at the Microbiology and Food Safety Laboratory of the School of Industry Technology, University Science of Malaysia, Penang, Malaysia.

Isolation, confirmation and identification of *Salmonella* and *Listeria* species

***Salmonella* species.** Duck samples were pre-enriched in buffered peptone water (Merck, Germany) and incubated at 37 ^{o}C for 24 h under aerobic conditions. One gram (1 g) each of duck intestinal content, faecal and soil sample were pre-enriched in 9 ml buffered peptone water while and 10 ml of wash water and 10 g feed samples each were pre-enriched in 90 ml buffered peptone water. After pre-enrichment, 0.1 ml and 1 ml of pre-enriched aliquots were transferred into 10 ml rappaport and vassiliadis broth (Merck, Germany) and 10 ml selenite cystine broth (Merck, Germany), respectively for enrichment. Enrichment samples in rappaport and vassiliadis broth were incubated at 42 ^{o}C for 24 h while that of the selenite cystine broth were incubated at 37 ^{o}C for 24 h. Enriched aliquots (ca. 10 µl) were then streaked onto xylose lysine deoxycholate (Merck, Germany) and Rambach agar (Merck, Germany) and incubated at 37 ^{o}C for 24–48 h. Presumptive *Salmonella* species were purified on MacConkey and nutrient agar all purchased from Merck, Germany and confirmed using Gram staining, biochemical (triple sugar iron, lysine iron agar, urease, and iodole production) and serological (Salmonella H Antiserum Poly A-Z and Salmonella O Poly A-I & Vi Antiserum, Difco) methods.

***Listeria* species.** Similarly, duck samples (1 g intestinal content, faecal and soil samples for 9 ml enrichment broths; and 10 ml wash water and 10 g feed samples for 90 ml enrichment broths) collected were enriched in listeria enrichment broth (Merck, Germany), and Fraser broth (Merck, Germany, supplemented with ammonium iron (iii) citrate and Fraser selective supplement, Merck, Germany). In listeria enrichment broths, the samples were incubated at 30 ^{o}C for 24-48 hours while in the Fraser broth two steps were followed; first in half Fraser broth, incubated for 24 h and further in Fraser broth for additional 24 h. After enrichments, about 10 µl aliquots were streaked onto listeria selective agar (Merck, Germany, supplemented with listeria selective supplement, Merck, Germany) and Palcam (Merck, Germany, supplemented with Palcam selective supplement Merck, Germany). All incubation was done at 30 ^{o}C for 24 to 48 h under aerobic condition. Presumptive *Listeria* species were purified on trypticase soy agar (Merck, Germany) with 0.6 % yeast extract. They were confirmed and identified using Gram staining, oxidase, catalase, motility, haemolysis test, and dextrose, esculin, and maltose utilization.

Results

The results obtained for the comparison of xylose lysine deoxycholate (XLD) and Rambach agars for the isolation of *Salmonella* species is presented in Table 1. From Table 1, out of the 144 duck samples tested for *Salmonella* species 58 positives were observed on xylose lysine deoxycholate agar plates while 51 were found on the Rambach agar plates. A combination of the individual broths and agars revealed that enrichment and plating on rappaport and vassiliadis broth (RV) and xylose lysine deoxycholate agar plates was the highest (30 positives), followed by selenite cystine broth (SN) and xylose lysine deoxycholate agar plates (28 positives), rappaport and vassiliadis broth and Rambach agar plates (27 positives), and selenite cystine broth and Rambach agar plates (24 positives).

With regards to *Listeria* species as shown in Table 2, 128 duck samples were analyzed, out of which 51 positives were observed on Palcam agar plates and 44 positives on listeria selective agar plates (ALOA). Isolation of *Listeria* species from Fraser broth (FB) and Palcam agar plates (27) was the highest, followed by listeria enrichment broth (LEB) and Palcam agar plates (24), Fraser broth and listeria selective agar plates (23), and listeria enrichment broth and listeria selective agar plates (21).

Table 1: Comparison of the efficiency of isolating *Salmonella* species from naturally contaminated duck samples on XLD and Rambach agars

Type of isolating media	No. of duck samples tested	No. positive *Salmonella* spp.
RV + XLD	144	30
SN + XLD	144	28
RV + Rambach agar	144	27
SN + Rambach agar	144	24

XLD= Xylose lysine deoxycholate agar; RV= Rappaport and vassiliadis broth, SN= Selenite cystine broth

Table 2: Comparison of the efficiency of isolating *Listeria* species from naturally contaminated duck samples on Palcam agar and ALOA

Type of isolating media	No. of duck samples tested	No. positive for *Listeria* spp.
FB + Palcam agar	128	27
LEB + Palcam agar	128	24
FB + ALOA	128	23
LEB+ ALOA	128	21

ALOA= Listeria selective agar; FB= Fraser broth; LB= Listeria enrichment broth

Discussion

Salmonella and *Listeria* species are very important pathogens implicated in most food-borne diseases. Mead et al. (1999) estimated that *Salmonella* infection is the second largest cause of food-borne illnesses while *Listeria* infection has the highest mortality rate compared to all food-borne illnesses. Symptoms of *Salmonella* infection include abdominal pain, diarrhoea, fever, vomiting and occasionally septicaemia (Coburn et al. 2007). Pathogenic *Listeria* species causes gastroenteritis, meningitis, abortion, and sometimes death in systemic cases (Khelef et al. 2006; Adzitey and Nurul 2011). Therefore the use of media and methods that ensure the highest possible recovery of these pathogens is essential.

Xylose lysine deoxycholate (XLD) agar contains xylose that differentiates *Salmonella* species and other enteric pathogens from *Shigella* species because *Shigellas* are unable to ferment xylose to produce acid (Anonymous 2011a). The lysine added is decarboxylated by *Salmonellas* which keeps the pH neutral or slightly alkaline. At such pH, H_2S is produced from the reduction of sodium thiosulfate making ferric ammonium citrate to produce black or black centred colonies differentiating *Salmonellas* from other *Coliforms* (Health Protection Agency (HPA) 2004a, Anonymous 2011a). Sodium deoxycholate is also used in XLD to inhibit the growth of Gram positive bacteria and many stains of *Coliforms* (Anonymous 2011a). *Salmonella* on XLD agar appears as red or pink colonies with or without black centre, *Shigella* as red transparent colonies, *E. coli* as yellow colonies (due to fermentation of lactose to produce acid), *Citrobacter* as yellow sometimes with black centre, *Proteus* as red with black centre and fishy odour, and *Providencia* as red colonies (HPA 2004a, Wallace and Hammack 2007, Anonymous 2011a).

The appearance of some colonies such as *Proteus* on XLD agar can be difficult or misleading to identify visually and thus the need to further confirm suspected or presumptive *Salmonella* colonies by biochemical and serological tests. Furthermore, Rambach agar was

developed by Rambach (1990) to facilitate and improve upon the recovery and isolation of *Salmonellae*. Rambach agar contains nutritive substrates that enable *Enterobacteriaceae* to multiply readily, sodium desoxycholate to inhibit the growth of accompanying Gram positive bacteria, chromogene to differentiate *Salmonellae* from *Coliforms* due to galactosidase- splitting of *Coliforms* (Anonymous 2011b). The underlying principle in Rambach agar is the use of propylene glycol, in combination with or presence of a pH indicator, *Salmonella*e form acid with propylene glycol and produces a characteristic red colony (Rambach 1990, Gruenewald et al. 1991, Anonymous 2011b). Thus *Salmonella*e on Rambach agar appears as red colonies; *Coliforms* as blue-green or blue-violet colonies, because they produce beta-galactosidase; other *Enterobacteriaeceae* and Gram negative bacteria (*Proteus, Pseudomonas, Shigella, S. Typhi*, and *S. Parathyphi*) as colourless or yellow colonies (Rambach 1990, Anonymous 2011b).

Nonetheless, our results indicated that, isolation of *Salmonella*e on XLD agar (58) was higher than on Rambach (51) using naturally contaminated duck samples; although we observed that presumptive *Salmonella* colonies on Rambach agar were without much competitive flora compared to colonies on XLD agar. Conversely, El-Sherif Amal and Elmossalami (1998) found that the use of Rambach agar to detect *Salmonella*e excelled over the use of XLD agar in meat and meat products. They reported 33.3 % and 83.3 % false negative cases for raw meat products and ready to eat meat products respectively on XLD agar plates, although they recommended that Rambach agar should be used alongside with XLD agar for the isolation of enteric bacteria from either raw or heat-treated meat products. Freydiere and Gille (1991) in their work involving the use of Rambach agar and C8 esterase spot test, to detect 170 non-typhi *Salmonella* strains, reported that 92 and 97 % gave bright red colonies on Rambach agar after 24 and 48 h of incubation, respectively, while 100 % of 112 other members of the family *Enterobacteriaceaec* were of different colours (beige, blue, green, colourless). They also observed that five of five *Acinetobacter* isolates and one of three *Psuedomonas* isolate were red colonies on Rambach agar. Therefore the isolation of *Salmonellae* from food or other samples should rely on the use of one or more *Salmonellae* isolation method and/or media.

The use of Palcam agar for isolating *Listeria* species is based on two indicator systems that is esculin/ferrous iron and mannitol/phenol red. All *Listeria* species hydrolyses esculin to ecsuletin which reacts with ferrous irons resulting in the blackening of the medium and the formation of a black halo especially around *L. monocytogenes* colonies (HPA 2004b, Anonymous 2011d). Lithium chloride is included in the medium, together with ceftazidime, polymyxin B and acryflavine from the supplement, to inhibit the growth of non-*Listeria* species, which can also hydrolysed esculin (Anonymous 2011c). Mannitol is added to the medium to differentiate *Listeria monocytogenes* and other non mannitol *Listeria* species from contaminants such as *Enterococci* and *Staphylococci* (Anonymous 2011d). Columbia agar base provide rich nutrient base source for growth of *Listeria* species, yeast extract serves as a source of vitamins and ferric ammonium citrate improves upon the growth of *L. monocytogenes* in particular (Anonymous, 2011d). *Listeria* species on Palcam agar appears as green-gray colonies with black centre and black halo or dimpled brown/black coloured colonies with blach halo (Anonymous 2011c, Anonymous 2011d).

Listeria selective agar has similar composition and principle as Palcam agar. It contains Columbia agar base (a nutritive source for which selective inhibitors are added), Lithium chloride (to inhibit enterococci), acriflavine (which inhibits some Gram-negative and positive organisms), and other selective agents such as colistin, fosfomycin, cefotetan and cyclohexamide (HPA 2004b). It also contain esculin as a differential indicator of which *Listeria* species hydrolyses to form esculutin and reacts with iron salt to give black precipitate around the colonies (HPA 2004b). HPA (2004b) also recommended the use of Palcam agar for food samples and listeria selective agar for either clinical specimens or food samples. Colonies of *L. monocytogenes* on Listeria selective agar appears black surrounded by black/brown zone (HPA 2004b, Anonymous 2011c).

Ringle et al. (1991) preferred the use of Palcam agar to Oxoid agar for the isolation of *Listeria* species in cheese by direct plating because they found it more selective. Vazquez-Boland et al. (1992) reported that Palcam agar, a more selective agar was suitable for direct plating of silage sample but with Oxoid agar, there was the presence of large competitive flora.

Conclusion

Salmonella and *Listeria* species are very important food-borne pathogens that need to be isolated by effective and more accurate methods. Isolation of *Salmonella* species from naturally contaminated duck samples on xylose lysine deoxycholate agar plates (58) was found to be higher than on Rambach agar plates (51), while isolation of *Listeria* species on Palcam agar plates (51) was higher than on listeria selective agar plates (44). Presumptive *Salmonella* colonies on Rambach agar were with less accompanying flora and produced well separated single colonies compared to XLD. Therefore we recommend that the isolation of *Salmonella* and *Listeria* species from food, clinical, environmental or other samples will depend on the material to be tested and the test should rely on the use of one or more techniques, method and/or media. The study also confirms that healthy ducks are potential sources of *Salmonella* and *Listeria* species which are threats to the

food industry in terms of food safety, quality and their health related diseases.

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