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Cultural and Immunological Detection Methods for *Salmonella* spp. in Animal Feeds – A Review

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ABSTRACT

Food-borne salmonellosis continues to be a major public health concern, and contamination with *Salmonella* spp. in pre-harvest animal production is considered a primary contributor to this problem. Animal feeds can easily become contaminated during primary production, feed mixing and processing as well as during feeding. Consequently, monitoring and surveillance of feeds and feed ingredients for *Salmonella* spp. contamination may be useful or necessary in the prevention and control of this organism. Cultural and immunological detection methods for salmonellae have been used or suggested as possible approaches for use in animal feeds. Cultural methods remain advantageous owing to their ability to detect viable bacterial cells, while immunological methods have the capability of detecting nonculturable bacterial cells. Advancements and improvements in both methodologies offer opportunities for eventual routine use of these detection technologies in animal feed assays.

Keywords: foodborne *Salmonella*, animal feeds, detection, culture methods, immunological methods

Abbreviations: cfu, colony-forming unit(s); ELISA, enzyme-linked immunosorbent assay; FDA, US Food and Drug Administration; FSIS, USDA Food Safety and Inspection Service; BG, Brilliant green; HE, Hektoen enteric; IgM, immunoglobulin M; IMS, immunomagnetic separation; MSRV, modified semisolid RV; *S.*, *Salmonella*; SC, selenite cystine; RV, Rappaport–Vassiliadis; TSA, tryptic soy agar; TT, tetrathionate; USDA, US Department of Agriculture; XLD, xylose lysine desoxycholate

INTRODUCTION

Estimates of economic loss due to food-borne salmonellosis vary depending on which social factors (medical costs, work productivity, potential earnings and other factors) are included in the calculation of 'cost' (Roberts, 1988; Angulo and Swerdlow, 1998). Mead and colleagues (1999) estimated that in the United States 1.2% of the over 1.4 million patients suffering from nontyphoidal salmonellosis are hospitalized annually, with nearly 600 deaths. Salmonellosis may cause severe illness in infants, the elderly and immunocompromised patients (Cross *et al.*, 1989; Tauxe, 1991; Smith, 1994; Ziprin, 1994; Bäumler *et al.*, 2000).

The phenotypic and virulence characteristics of *Salmonella* spp. have been reviewed (Bäumler *et al.*, 2000; Grimont *et al.*, 2000). *Salmonella* spp. are Gram-negative, facultative anaerobic, straight rods of the family *Enterobacteriaceae* that can ferment glucose. Most strains are motile with peritrichous flagella and are able to reduce nitrate to nitrite (Grimont *et al.*, 2000). Reeves and colleagues (1989) noted that a bacterial 'species' should be grouped by a genetic relatedness of at least 55%. On the basis of this criterion, Reeves and colleagues (1989) suggested that *Salmonella* serovars may be classified into two species, *S. enterica* and *S. choleraesuis*. Grimont and colleagues (2000) suggested six subspecies for *S. enterica*, namely *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae*, and *S. enterica* subsp. *indica*. However, an official ruling on *Salmonella* nomenclature has not been completed (Ezaki *et al.*, 2000). Therefore, in this review, specific *Salmonella* serovars will be named according to the nomenclature of Grimont (Grimont *et al.*, 2000; e.g. *Salmonella* ser. Typhimurium).

Food-borne *Salmonella* spp. can be encountered at any phase in pre-harvest food animal production. Given its ability to exist in multiple environments, *Salmonella* can easily infect individual animals and, depending upon host animal status and housing conditions, can easily infect large numbers of animals or flocks of chickens. One of the more likely transmission routes that can lead to ingestion by animals and their infection by *Salmonella* spp. is the feed consumed by the animal. The ecology and potential control measures have been extensively reviewed previously (Williams, 1981a, c). Briefly, animal feeds can become contaminated by *Salmonella* spp. at several stages during primary production, mixing and processing at the feed mill or during storage of the mixed feed or feed ingredients prior to feeding. The potential for contamination does not end here, however, as feed can easily become contaminated with *Salmonella* spp. during the course of animal feeding if the feed comes into contact with insects, wild animals and birds carrying the bacterium. Consequently, environmental surveillance and monitoring of food animal production for contamination should probably include feed analysis for *Salmonella* spp. A number of methods have been used for and/or have potential for detection of *Salmonella* spp. in feeds and these include growth culture/selective media and immunological and molecular approaches (Williams, 1981b; Ricke *et al.*, 1998). Although molecular methods have potential, there remain issues regarding specificity and reliability before they can be incorporated into routine analysis (Ricke *et al.*, 1998). These issues will not be further discussed here. Both cultural and immunological approaches remain viable options for detection of *Salmonella* spp. in animal feeds. Cultural methods have advantages due to their ability to detect viable bacterial cells, while immunological methods offer the ability to detect viable nonculturable cells (Table I). This review focuses on improvements and the potential applicability of cultural and immunological detection methods for routine *Salmonella* spp. analysis in feeds.

TABLE I
Relative advantages and disadvantages of cultural and immunological methods for the isolation and detection of *Salmonella* spp. in animal feeds

Methods	Principles	Advantages	Disadvantages
Cultural	Selective enrichment of *Salmonella* spp. from animal feeds using media that exploit the bacterium's unique biochemical and physiological properities	Proven technology Sensitive due to multiple enrichment steps Kits available for running multiple biochemical tests simultaneously	Requires 5–7 days for isolation Labour-intensive Possible false positives owing to competing bacteria with similar biochemical and physiological properties
Immunological	Use of mono- or polyclonal antibodies for somatic or flagellar antigens to detect *Salmonella* spp. in animal feeds	More rapid than cultural methods and specific owing to adsorption between antibody and antigen Can be used to either detect bacterial antigens in animal feeds or antibodies in exposed animals In combination with magnetic beads beads can enhance the isolation of *Salmonella* spp. in large samples Can be automated to reduce time and labour	Still requires pre-enrichment steps Cross-reactivity with antigens in closely related bacteria may cause false positives Possible false negatives owing to variations in sampling techniques and processing Automated and immunomagnetic procedures are expensive May not detect damaged or stressed bacterial cells

CULTURAL DETECTION OF *SALMONELLA* spp.

Cultural methods for isolating *Salmonella* spp. in animal feeds and feed ingredients have been well reviewed by Williams (1981b) and further discussed by Guthrie (1992), Cox (1988) and Ricke and colleagues (1998). Currently, the method that is recommended by both the US Food and Drug Administration (FDA) and the Food Safety and Inspection Service (FSIS) of the US Department of Agriculture (USDA), for the detection of salmonellae still centres around cultural selection, both in selective liquid media and on agar plates (Andrews *et al.*, 1995; Rose, 1998). For feed, Andrews and colleagues (1995) suggests pre-enrichment for 24 h in lactose broth, followed by selective enrichment in Rappaport–Vassiliadis (RV) and tetrathionate (TT) broths. Hammack and colleagues (1999) confirmed the selectivity of these media in a comparison of RV, TT and selenite cystine (SC) broths, and suggested optimal incubation temperatures of 35°C and 42°C for TT and RV media, respectively. After 24 h of selective enrichment, Andrews and colleagues (1995) recommended that positive cultures should be streaked onto bismuth sulphite, xylose lysine desoxycholate (XLD) and Hektoen enteric (HE) agars for isolation. Characteristic colonies must then be stab-inoculated into triple sugar iron and lysine iron agars and biochemically characterized using the urease and indole tests, as well as negative growth on malonate and potassium cyanide media, followed by serological typing with both somatic (O) and flagellar (H) antisera.

Advances in conventional media for *Salmonella* spp. centre around increasing the ease, specificity and reliability of detection, and reducing cost and labour (Weenk, 1992). For example, individual biochemical tests have been replaced by standard kits such as the API 20E (BioMérieux, Hazelwood, MO, USA) and the Crystal Enteric/Nonfermenter (E/NF: Becton Dickinson, Franklin Lakes, NJ, USA), which run numerous tests simultaneously (Micklewright and Sartory, 1995; Ricke *et al*, 1998). Recovery of injured microorganisms, especially those that may still be infectious, is of particular interest, as injured pathogens may not be detectable but may still recover and multiply in the gastrointestinal tracts of animals. Strategies for the recovery of injured bacteria centre around two overlay methods. An enriched isolate may either be grown on tryptic soy agar (TSA) for 2 h and then overlaid with selective media (McCleery and Rowe, 1995) or be grown on selective media overlaid with nonselective TSA (Kang and Fung, 1999). Kang and Fung (2000) noted that a similar strategy of overlaying XLD with TSA was more effective in cultivating heat-damaged *Salmonella* spp. than the use of XLD alone.

With other pathogenic organisms such as *L. monocytogenes*, recovery has also been improved through the use of anaerobic Hungate roll tubes, Fung's double-tube method, or oxygen scavengers such as thioglycollate or Oxyrase (Oxyrase, Inc., Knoxville, TN, USA; Hoffmans *et al.*, 1997). Oxyrase, when incorporated into a commercial medium (SPRINT enrichment broth for *Salmonella* spp., Oxoid, Basingstoke, UK), has been shown to increase the recovery of injured salmonellae from ice cream and milk powder after enrichment (Baylis *et al.*, 2000).

Amendments to media and novel media for isolation and detection of *Salmonella* spp. have also been investigated. Novobiocin has long been added to HE, XLD, TSA,

and Brilliant green (BG) agars to enhance the isolation of *Salmonella* spp. from food and faecal samples (Restaino *et al.*, 1977; Devenish *et al.*, 1986). Cycloheximide has been noted to decrease fungal overgrowth when isolating *Salmonella* spp. from environments with a large mycoflora (Ha *et al.*, 1995a,b; Ricke *et al.*, 1998), and nitrofurantoin has been used to isolate *S.* ser. Enteritidis (de Boer, 1998). A biochemical test involving L-pyroglutamic acid and *p*-dimethylaminocinnamaldehyde has been used to distinguish *Citrobacter* spp. (which possess pyrrolidonyl peptidase and thus elicit a colour change) from salmonellae (Bennett *et al.*, 1999). New media have been investigated, based on α-galactosidase activity in the absence of β-galactosidase activity, catabolism of glucuronate, glycerol and propylene glycol, and H_2S production (de Boer, 1998; Shelef and Tan, 1998; Peng and Shelef, 1999; Perry *et al.*, 1999; Mallinson *et al.*, 2000; Miller and Mallinson, 2000). Commercial chromogenic agar (CHROMagar, CHROMagar Microbiology, Paris, France) has been shown to be superior to Hektoen enteric agar for the isolation of *Salmonella* spp. from faecal material, especially after the addition of cefsulodin (Gaillot *et al.*, 1999). Other chromogenic and fluorogenic media, which exploit specific metabolic pathways in *Salmonella* spp. to facilitate detection and isolation, include SM-ID agar (BioMérieux, Marcy l'Etoile, France), Rambach agar (Merck, Darmstadt, Germany), MUCAP-test (Biolife, Milan, Italy), Rainbow *Salmonella* agar (Biolog, Hayward, CA, USA), and Chromogenic *Salmonella* esterase agar (PPR Diagnostic Ltd, London, UK) (Manafi, 2000). Enrichment media may even affect alternative detection assays. Huang and colleagues (1999) noted that, even though *Salmonella* spp. numbers were greater in RV media than in TT–Brilliant green media, enrichment in RV media either inhibited the ELISA reaction or resulted in lower titres after enrichment in brain–heart infusion broth.

The accepted isolation method for *Salmonella* spp. (Andrews *et al.*, 1995) pre-enrichment for 24 h in lactose broth, followed by selective enrichment in RV and TT broths, and plating on bismuth sulphite, XLD and HE agars – does possess drawbacks, chief among them being the time required for a confirmed result. Cultural confirmation requires 5–7 days. For a feed mill producing 40 metric tonnes per hour of feed or practising 'just in time' feed processing (Jones and Ricke, 1994), storing a batch for 5–7 days would present difficulties, in both logistics and expense. The use of XLD as a selective medium is also in question. Both Braun and colleagues (1998) and Gomez and colleagues (1998) reported that a selective motility enrichment on modified semisolid RV (MSRV) medium resulted in a greater isolation of *Salmonella* spp. when compared to XLD and *Salmonella-Shigella* agar, respectively. Heyndrickx and colleagues (2002) reported that when RV, MSRV and DIASALM, another selective semi-solid agar, were used to detect *Salmonella* spp. in 3150 broiler faecal and environmental samples, DIASALM and MSRV exhibited greater sensitivity than the RV (93.9%, 79.2% and 61.3%, respectively). While rapid results can be obtained by direct plating, the selective enrichment steps cannot be shortened without the risk of increased false positive results, as *Enterobacter cloacae* and *Citrobacter freundii* may be mistaken for salmonellae using MSRV media (Gomez *et al.*, 1998) and XLD media (Coleman *et al.*, 1995b), respectively. In addition, neither MSRV nor DIASALM are recommended for the isolation of non-motile *Salmonella* spp. However, as non-motile

salmonellae represent $<1\%$ of the isolates from clinical samples and animal feeds and are far less virulent than their motile counterparts, this is not considered a major problem (Poppe *et al.*, 2004)

IMMUNOLOGICAL METHODS AND APPLICATIONS

Immunological methods for the detection of *Salmonella* spp. centre around the enzyme-linked immunosorbent assay (ELISA), though other dot blot enzyme assays have been explored (Blais *et al.*, 1998). In the ELISA assay an antigen that is specific to the target of interest is captured onto a solid matrix and bound to an enzyme-labelled antibody (Cox, 1988). The members of the genus *Salmonella* are serotyped by their antigenic formula of somatic (O) and flagellar (H) antigens. The presence and/or concentration of the antigen can subsequently be measured by a colorimetric or fluorescent product produced by the enzymatic cleavage of a substrate.

Alternately, researchers and producers may use ELISA to detect the presence of antibodies in the blood of animal herds or flocks to monitor exposure to *S.* ser. Choleraesuis, *S.* ser. Enteritidis, *S.* ser. Infantis, or *S.* ser. Typhimurium (Gast and Holt, 1998; Wiuff *et al.*, 2000). This can be especially useful in detecting subclinical cases of salmonellosis or carrier animals. However, *Salmonella* ser. Enteritidis colonizes the oviduct and often cannot be directly detected from blood samples (Gast *et al.*, 1997; Withanage *et al.*, 1999; Zamora *et al*, 1999a,b). ELISA assays have also been instrumental in the creation and evaluation of vaccines against *S.* ser. Enteritidis and *Helicobacter pylori*, using recombinant *S.* ser. Typhimurium (Gómez-Duarte *et al.*, 1998; Meenakshi *et al.*, 1999).

The combination of antibody binding and magnetic particles is gaining popularity as a method of recovering and concentrating *Salmonella* spp. from large samples. Lim and colleagues (1998) also detected antibodies against *S.* ser. Typhi in a competitive assay by the use of two types of latex particles, one coated with anti-O9 IgM and a second type that is magnetic and coated with lipolysaccharide from *S.* ser. Typhi. Ten Bosch and colleagues (1992) reported that the use of immunomagnetic separation (IMS) resulted in a 2000-fold improvement in the detection level of *Salmonella* spp. in milk powder and the elimination of an enrichment step. Commercial products such as Dynabeads anti-*Salmonella* (Dynal, Oslo, Norway) require pre-enrichment in nonselective media, but may decrease required enrichment times and increase recovery from selective media (Cudjoe and Krona, 1997). Streptavidin-coated magnetic beads (Dynal) have been used in conjunction with a sensor (Threshold, Molecular Devices, Sunnyvale, CA, USA) to measure at least 1 cfu *Escherichia coli* O157:H7 per gram of ground beef after a minimum of 5 h of enrichment. Immunomagnetic beads have been used to increase specificity and decrease the detection time of *Salmonella* spp. in animal feed, cheese, eggs, ground beef, ice cream, raw chicken carcasses, sausages and skimmed milk powder (Coleman *et al.*, 1995a,b; Shaw *et al.*, 1998; Ripabelli *et al.*, 1999; Baylis *et al.*, 2000). Marriott and colleagues (1999) even used antibody-coated magnetic beads to increase recovery of *Salmonella* spp. from cell culture as a measure of invasion.

An advantage of using ELISA is that the assay be automated to produce a testing system that is rapid, less labour-intensive, and with the ability to handle large numbers of samples. Several automated ELISA systems are commercially available, such as the EIAFoss (Foss Electronics, Hillerød, Denmark) and VIDAS (BioMérieux, Hazelwood, MO, USA), and are currently being used in the meat and poultry processing industries to test for bacterial contamination. Several studies have compared the specificity and sensitivity of automated ELISA systems to conventional culturing methodology in detecting *Salmonella* spp. in infected milk and meat samples (Keith, 1997; Massó and Oliva, 1997; Uyttendaele *et al*., 2003). In general, these studies suggested that the detection rates of the two methods were comparable. When DIASALM and VIDAS were used to test for the presence of *Salmonella* spp. in naturally infected pork, beef and poultry samples, 95% agreement between the two methods was obtained (Uyttendaele *et al*., 2003). When MSRV and EIAFoss tests were used to assay 216 raw meat samples, no significant difference in the detection rates of salmonellae were found: 31.9% and 29.2%, respectively (Massó and Oliva, 1997). EIAFoss had a calculated sensitivity of 95.3% and a specificity of 100%. Keith (1997) noted that the sensitivity of VIDAS assay was not greatly affected by the presence of competing microflora, such as *Citrobacter freundii*; the sensitivity of the assay dropped from 96% to 95% with the presence of *C. freundii*. However, conventional culture methods were better able to detect severely stressed bacteria (Uyttendaele *et al*., 2003). When *S* ser. Enteritidis was held at –18°C for 7 days, DIASALM detected all eight of the artificially contaminated samples, whereas VIDAS detected only five of the eight.

The ELISA assay does have some potential disadvantages for the detection of bacteria such as *Salmonella* spp. in animal feeds, however, including limits in sensitivity and antigen variability (Ricke and Pillai, 1999). The minimum sensitivity of the assay (approximately 10^5/ml; Cox, 1988; Ricke *et al*., 1998) requires enrichment in a standard medium for production of cell surface antigens and detection. An assay investigated by Bolton and colleagues (2000), for example, required 36 h to detect 1 cfu *Salmonella* spp. per 25 g of food matrix. Peplow and colleagues (1999) noted that the sensitivities of a commercial ELISA (Reveal, Neogen Corp., Lansing, MI, USA) varied widely between sampling times and sample processing methods, and led to false-negative results. Dill and colleagues (1999) could detect as few as 10^2 *S.* ser. Typhimurium/ml of chicken wash using a combination of monoclonal and polyclonal antibodies in a commercial filtering system (Threshold, Molecular Devices) but noted that complex matrices may clog a filtering step and produce false-positive signals. Cross reactivity is also an issue. Westerman and colleagues (1997) noted that the $O30_1$ lipopolysaccharide of *Salmonella* is identical to the O157 antigen of *E. coli*. Antigens may also be altered by acetylation, changing recognition by assay antibodies (Kim and Slauch, 1999).

The IMS technique also exhibits some difficulties. Ripabelli and colleagues (1999) found recovery from ground beef to be superior using SC broth. Lucore and colleagues (2000) noted that IMS was organism-specific and expensive, and required small sample sizes, preferring to use metal hydroxides such as zirconium hydroxide instead. Coleman and colleagues (1995a) noted that organisms were lost from beads during

separation from samples with high fat content, and reported difficulty with non-specific binding of *Citrobacter freundii* and coliforms, especially coliforms with mucoid layers. However, immunological techniques, especially IMS, possess the potential to increase recovery of heat-injured cells when used in conjunction with pre-enrichment, cultural detection, or detection by molecular assays (Rijpens *et al.*, 1999).

CONCLUSIONS

Animal feeds potentially represent a source of *Salmonella* spp. for infection of food animals. Monitoring and surveillance require detection technologies that are reliable and predictable given the complexities of the multitude of feed matrices. Cultural and immunological detection assays continue to be improved and are becoming more amendable for routine use in feed analysis. However, issues remain regarding time restraints between test results and feed production time, maintaining the assays' sensitivity and specificity, and devising a representative sampling strategy. As improvements continue to be made for cultural and immunological assays, routine detection of feed *Salmonella* spp. should become even more feasible.

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