RECENT PROGRESS IN THE DIAGNOSIS AND CONTROL
OF SALMONELLA INFECTIONS IN POULTRY

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Summary: Salmonella remains a threat to both animal and public health. Its economic significance remains high and changes in public perception mean that some measures for control may be necessary even in the absence of financial loss. Diagnosis is an important part of control. A great deal of new information has become available over the last few years as a result of research into various aspects of both diagnosis and control. This article seeks to summarise the progress in both these areas in the context of other recent reviews. New solid culture media, particularly Rambach agar, have become available which are selective for Salmonella. It is more reliable than either XLD or brilliant green agar but some Citrobacter species cause confusion. Enrichment-serological methods can shorten presumptive identification times. ELISAs for detection of Salmonella organisms are now available commercially but are limited by their cost and relatively low sensitivity. Their specificity is directly related to the type and quality of the detecting antibodies. Polymerase chain reaction technology has been assessed experimentally. Although it is not yet suitable for extensive use, its specificity and sensitivity suggests that it will have many advantages over other systems for rapid detection of Salmonella organisms. ELISAs are also being increasingly used for serological detection of invasive Salmonella infections in poultry flocks. They are used as an integral part of the control measures against Salmonella enteritidis and S. typhimurium in some countries. They could now also be used routinely for S. gallinarum and S. pullorum.

Salmonella control measures are dependent on the size of the poultry industry and the public perception of the problem in each country. They must include attempts to improve the microbiological quality of the stock, feed and housing. Stock improvement must start with elite, grandparent and then parent flocks. Additional measures such as combining competitive exclusion approaches together with antibiotics are being used. The risk of development of resistance to antibiotics under these conditions is real. Live, attenuated vaccines are also now in use but these have not yet been tested thoroughly. Vaccination soon after hatching can induce early protection by an exclusion effect that occurs between Salmonella strains. Other potential measures still in the experimental stage should be explored to the full.
1. INTRODUCTION

Despite the fact that avian salmonellosis can be controlled under many circumstances using well-tried methods, a combination of a number of factors has required that new approaches to the problem should be explored. These factors include changes in the epidemiology influenced by changes in husbandry, host and bacterium, and alterations in the attitude of the industry and general public to the problem. Salmonellosis used to be an animal health problem of considerable economic significance. In many countries it is now a major public health problem, again of considerable economic significance. The public health significance is likely to become greater in most countries in the next few years.

From the point of view of disease and pathogenesis Salmonella can be divided into two groups. This division affects the methods used for diagnosis and control. A small group of serotypes including Salmonella typhi, S. dublin and S. cholerae-suis produce typhoid-like diseases in a limited number of host species. The only serotype from this group seriously affecting the poultry industry is S. gallinarum-pullorum. In many countries which have had an intensive, integrated poultry industry for several decades, fowl typhoid and pullorum disease no longer pose serious economic problems. However, in countries which have begun to intensify their industries they can still cause problems and in some countries are once again increasing in importance. From the point of view of control the epidemiology is relatively simple since no non-avian animals routinely carry the organism and extensive long-term faecal excretion does not occur.

The second group generally does not produce epidemic disease in immunologically mature poultry. They are less virulent except in very young birds or in those subjected to stress, and their ability to colonise the alimentary tract of animals leads to their entry into the food chain producing increasing numbers of cases of human food-poisoning. The most recent major epidemiological change has been the establishment of a number of unrelated phage types of S. enteritidis in the breeder, meat and egg sectors of the industry in several different countries (58). Some systemic spread occurs, resulting in vertical transmission, and extensive faecal excretion results in environmental contamination which may become difficult to eliminate.

The following 33 Member Countries acknowledged the request for reports sent out by the OIE in August 1994:

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The countries can be grouped according to whether their poultry industries are intensive or have recently begun to intensify. In the former S. enteritidis and S. typhimurium are the main problems; whereas in the latter S. gallinarum and S. pullorum remain important problems. The latter two organisms are largely controlled by monitoring followed by slaughter of infected or carrier stock. Antibiotics and vaccination are occasionally used. Approximately equal numbers of countries approach the control of S. enteritidis and S. typhimurium by the application of competitive exclusion or antibiotics; by an extensive programme of monitoring followed by vaccination, chemotherapy or slaughter; or by no more than simply monitoring the situation. A very small number of countries have been successful in controlling the problem and one or two island countries have virtually no Salmonella problem.
2. ESTABLISHED METHODS FOR DIAGNOSIS

The established methods recognised internationally for diagnosis and control have been well documented. *S. gallinarum* and *S. pullorum* infections may be diagnosed serologically using whole blood and stained antigen in a rapid slide agglutination test (57). Other agglutination based tests are also available (61). Infections identified in this way should be confirmed bacteriologically using standard methods (32, 57). These can involve a pre-enrichment stage in buffered-peptone water to enhance viability, followed by selective enrichment in liquid media such as selenite or tetraphionate broths or Rappaport-Vassiliadis medium (57). Standard plating media include brilliant green agar and its various modifications and desoxycholate agar. Bacteria are identified biochemically and serologically.

3. PROGRESS IN DIAGNOSIS - CULTURAL METHODS

Improvements in culture methods are continually being made, both to accelerate the process of isolation and identification of *Salmonella* organisms in feed and poultry samples, and to make more accurate cultural identification on solid media. Thus xylose lysine desoxycholate (XLD) medium (66), originally developed for use with *Shigella*, has been useful for identification of *Salmonella* and *Klebsiella*. However, *Citrobacter* strains may produce colonies resembling *Salmonella*. Not all *Salmonellae* produce H₂S and thus would not produce colonies with black centres. Rambagh agar (51) has been developed, based on the ability of many *Salmonella* strains to ferment propylene glycol, which produces red colonies with neutral red. A chromogen indicator for β-galactosidase activity can be included to differentiate lactose fermenters. *Citrobacter* species may again cause problems. Garrick and Smith (36) recently found that 23/25 *Salmonella* strains produced typical reaction and morphology on Rambagh agar while 17/25 did so on XLD.

Many developments in enrichment culture have been made for the food industry but these are also relevant for analysing poultry feed components (for review, see 17). Attempts have been made to reduce incubation times in selective enrichment medium to 6-8 hours, but reports of success have been conflicting. Some authors report results comparable with those produced by conventional methods, while others find a high number of false negative results. A membrane filtration method has been developed with a 6-hour enrichment stage (34), a portion of which is filtered through a hydrophobic grid membrane which is then incubated on EF-18 agar (33, 59). The hydrophobic membrane reduces the high level of background flora but colony purification is sometimes necessary. Nevertheless, this method has received Association of Analytical Chemists (AOAC) International official first action status (1).

Enrichment serology has been developed in an attempt to combine specificity and sensitivity. Enhancement of flagella production by incubation for 24 h in M-broth, containing D-mannose, prior to examination with polyvalent flagellar antisera, produced 95% of the total positive *Salmonella* isolations obtained by conventional plating (18). In addition *Salmonella*-specific antibodies may be coupled to coloured latex particles. However, at least 10⁷-10⁸ colony forming units (CFU)/ml are required to induce agglutination (22), and *Citrobacter freundii* can also produce false positive reactions. Several kits are available commercially in Europe and the USA, most of them with the ability to detect 75-88% of the samples found positive by conventional culture.

Rappaport-Vassiliadis (RV) medium in semi-solid form has been used. Drops of pre-enrichment culture are transferred onto the medium. If migration occurs, slide agglutination is performed (30). The use of anti-flagella serum on a disc incorporated in the agar to specifically identify swarming *Salmonellae* followed by plating on Rambagh agar was found to give reliable results within 48 hours of culture starting (28).

Rapid methods have also been developed to accelerate identification, by using a combination of biochemical characteristics and conductance. Conductance methods have been increasingly found to produce results which are comparable with standard culture methods (37). A method involving pre-enrichment in buffered peptone water containing lysine and glucose, with sub-culture to selenite-based media also containing lysine or dulcitol whose conductance was then measured, has been found to produce results comparable with those produced with standard methods and has been adopted as an AOAC International first action method (2).
A number of immunoassays have been developed for the detection of Salmonellae in pre-enrichment or selective-enrichment media (17). A number of different systems have been developed in which polyclonal or monoclonal antibodies have been used, largely in antigen-trap-type assays. A solid surface, frequently a microtitre plate, has specific antibodies bound to the well surface. These then trap the organisms in the culture. A reaction is then produced by a further layer of conjugated antibody with a chromogenic substrate. A number of commercial kits are available which under laboratory conditions perform well. Sensitivity is generally limited to a bacterial density of not less than $10^3$-$10^5$ CFU/ml. With field samples false negative results occur, but the bigger problem is false positives (<50%) produced by antigenically similar organisms (41). Other solid surfaces such as dip-sticks, magnetic particles or polystyrene beads can also be used. Thus the problem of this method is the relatively poor sensitivity and specificity.

Gene probes may be used for the detection of Salmonella organisms in carcasses or feed. A number of probes developed over the last 10 years have been developed from labelled DNA fragments from S. typhimurium. Good results have been reported with selective enrichment cultures but not with pre-enrichment cultures (35). The specificity can be high, but exhaustive surveys of related organisms have not been carried out. Sensitivity is limited by the amount of DNA or organisms present in the sample. For samples with small amounts of bacteria the amount of target DNA must be increased either by choosing ribosomal DNA (50) as the target (since this is present in high copy number), or by culture, or by the polymerase chain reaction (PCR). The advantage of this latter system is its great sensitivity. However, it cannot differentiate between dead or living organisms, so that an additional stage of pre-enrichment coupled with a comparison of the reaction with standards is necessary to produce a semi-quantitative test. It works well with some biological samples such as chicken skin (44), but not with faeces which usually contain inhibitors for the Taq polymerase. Two or more probes may be used simultaneously to increase specificity.

In many of the methods described above the time saved may amount to several hours, but for samples such as feeds preliminary pre-enrichment is still required. The cost of many commercial kits may be prohibitive and initial capital expenditure may be required. In the long term PCR technology seems to offer the greatest potential for rapid, specific and sensitive assays, but the method is a long way from being routine laboratory technology.

4. PROGRESS IN DIAGNOSIS - SEROLOGICAL METHODS

Progress in the serological detection of Salmonella infection in poultry has also been made over the last few years. As with earlier technology based on agglutination methods, the newer techniques are applicable mainly to those serotypes and strains that are invasive and induce the production of circulating IgG, including S. enteritidis, S. typhimurium, S. arizonae, S. berta and S. gallinarum-pullorum. It is fortuitous that these are also major public and animal health problems.

With increasing interest in poultry meat- and egg-derived Salmonella infections in man, national and international legislation has been introduced (23) requiring control involving frequent monitoring of breeding flocks. Bacteriological methods were the standard procedures. However, following increasing international interest in monitoring such infections by ELISA (14, 57), the European Commission has sanctioned the use of ELISA technology for this purpose.

The advantages of serology are that circulating IgG is persistent and avoids the sampling problems arising from intermittent faecal excretion. The major disadvantage is that soon after infection serum IgG concentrations may be low whereas faecal excretion will be high. However, use of the ELISA as a flock test should minimise this problem. Non-invasive serotypes will also not be detected. The advantages of the ELISA over agglutination and microantiglobulin techniques relate to ease of procedure and cost.

There are two basic systems available, the indirect ELISA (6) and the competitive "sandwich-type" ELISA (71).

The indirect ELISA involves the use of a detection antigen coated onto the wells of a microtitre plate. After the application of a blocking reagent to reduce non-specific binding, test samples are applied to the wells. Specifically bound antibody in the sample is detected by an antibody-enzyme conjugate. A variety of antigens, including LPS, flagella, SEF14 fimbriae and cruder antigen preparations have been used.

The competitive-sandwich ELISA employs a specific mechanism of coating antigen to wells, namely monoclonal antibody. This is then followed by a pure or crude antigen preparation. Test samples are applied,
followed by conjugated monoclonal antibody, which will not bind to the antigen if the test sample contained antibodies with the same specificity. The assay can be shortened by adding both test sample and conjugate together. Monoclonal antibodies have been prepared for flagella, LPS and SEF14 for *S. enteritidis* (67, 71).

There are advantages and disadvantages to both systems. The indirect assay is simpler and reagents are available applicable to all *Salmonella* serotypes in chickens, turkeys and ducks. The competitive ELISA can be applied to all animal species and in general shows higher specificity. However, reagents are not commercially available for all serotypes. There are also some affinity problems and it may be less sensitive than the direct assays. In the field both systems have been known to produce false positive reactions.

A number of detecting antigens have been used. Lipopolysaccharide (LPS) is the most frequently used antigen. A certain degree of discrimination can be made between sera from chickens infected with different serogroups such as B, C1, C4, D, E1 and E4 (21, 40). However, varying degrees of cross-reaction occur in the case of groups B and D due to the common 12 antigen (21). The problem can be reduced by adjustment of the test sample dilution (6) or by mild periodate treatment of group D LPS, which destroys cross-reacting epitopes while preserving the 09 specificity (70).

Flagellar antigens have also been used to avoid the problem of cross-reactions. Unfortunately no information is currently available on the cross-reactions produced by *in vivo* expression of phase 1 and phase 2 flagellar antigens. Under experimental conditions using *S. enteritidis* infections (3) or *S. typhimurium* infections (40), specific immune responses can be demonstrated. However, in the field confusing cross reactions seem to occur. The former authors also report that flagella-specific IgG is not as persistent as LPS-specific IgG, detectable quantities disappearing within 4 months, and that the titres peak before those of LPS-specific antibody. Not all chickens produce high titres of flagella-specific antibody (68). One area where flagellar antigen can already be used with success is to differentiate infection caused by flagellate and non-flagellate serotypes, particularly *S. enteritidis* from *S. gallinarum/pullorum*. Timoney *et al.* (68) were able to differentiate these infections in this way. Barrow *et al.* (1992) found similar results by ELISA and by immunoblotting. Birds in a Brazilian flock, in which clinical typhoid was observed, were identified by high IgG titres to group D LPS and by low gm-H specific IgG titres. Thorns *et al.* (67) have used indirect and capture ELISAs using the *S. enteritidis* fimbrial SEF14 antigen. Specific IgG was detectable by one week post-infection and persisted for at least eight weeks when the experiment was terminated. No cross reactions were observed with sera from *S. typhimurium* or *S. gallinarum*-infected birds. A capture ELISA was used to examine field sera and was compared with an indirect ELISA using flagella or heat-extract antigen. The SEF14 antigen ELISA was as sensitive as the heat-extract antigen ELISA and as specific as the flagella ELISA.

Egg yolk may be used in place of sera (6, 9, 27, 45, 46, 48). There is some indication that a standard indirect ELISA might be more appropriate for analysing egg yolk than a competitive (blocking) sandwich ELISA. The low dilution of the samples used in the latter ELISA may lead to interference and to reduced sensitivity and false negative reactions (69).

Serum or blood may be dried onto absorbent paper and eluted at a later date with no loss of titre. Antibiotic therapy of recent infections may result in lower antibody titres. Vaccination of birds could also induce production of cross-reacting antibodies.

There is increasing evidence that ELISAs are useful for screening birds in the field for infection with *S. typhimurium*, *S. enteritidis* and *S. gallinarum/pullorum* (11, 24, 29, 31, 45, 47, 49). However, a number of problems remain. These relate both to the choice of a cut-off optical density value whereby sera from infected flocks may be differentiated from those from uninfected flocks, and also to the number of samples that should be taken.

Selection of a cut-off point (optical density, OD) from the values obtained with sera from uninfected specific pathogen-free birds may result in a proportion of field sera appearing positive. The use of commercial birds, free of infection, to provide negative control sera should avoid this problem. However, another approach is to choose a cut-off point after observation of the distribution of OD values from infected and uninfected flocks. For *S. enteritidis* at least, the profiles are very different (8), and it is clear that at least for this serotype, and

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probably also for *S. gallinarum* and *S. pullorum* (Berchieri and Barrow, unpublished data), a much higher cut-off OD value may be chosen to avoid false positive reactions. The number of reactions taken at each time should ideally be higher than the number (60) mentioned in legislation. A number such as 300 samples at each time would produce greater confidence in detecting lower infection prevalences.

Thus ELISAs have been shown to be very useful for large scale screening of poultry flocks. This can be done with very little capital outlay. The ELISA has been accepted both by the European Union and by WHO for use with *S. typhimurium* and *S. enteritidis*, and there is no reason why it should not also be used to replace the rapid slide agglutination test for *S. pullorum* and *S. gallinarum*, wherever possible.

### 5. RECENT PROGRESS IN CONTROL

There have recently been a number of reviews (7, 72) which have covered *Salmonella* control in livestock very well. They make clear the necessity for a comprehensive package of measures including control of the three major sources of infection recognised by the World Health Organisation (53, 54, 55) and by the international community (63, 64, 65), namely stock themselves, contaminated feed, and the immediate environment (housing).

Control of fowl typhoid and pullorum disease by serological test and slaughter of reactors has been recognised for many years as one of the means to break the cycle of vertical, and thus horizontal, transmission of these pathogens. Improvements in hygiene and management must be integral parts of the disease control procedure. However, this can present problems for laying and breeding flocks in countries where the ambient temperature is high and where open-sided accommodation is a not uncommon feature. Under these conditions, environmental contamination can be heavy, and other procedures should be introduced. These could include vaccination, and a number of vaccines are available including the 9R strain developed in the 1950s (60). More recently (4) a rough, virulence-plasmid-cured derivative of a virulent strain has been developed which, like *aroA* strains (38) may be suitable for young birds of more susceptible lines, perhaps followed by vaccination with a less attenuated strain shortly prior to onset of lay. Unless considerable mortality is experienced with fowl typhoid, antibiotics should not be used. The development of resistance to furazolidone is an indication that excess usage of any antibiotic will induce the development of resistance (39). Incorporation of volatile fatty acids (see below) in feed, although developed largely for controlling feed-borne infection, may have a role to play in reducing environmental infection.

Preferred methods for controlling food-poisoning *Salmonella* serotypes change according to changes in public perception, new problems arising or the results of research. However, production of poultry with a greatly reduced incidence of infection must be a central aspect of control. The recent worldwide problem of *S. enteritidis* has resulted in a number of countries increasing their awareness of the problem and of the need for an integrated approach to control. Prior to this problem a small number of countries, which typically have small poultry industries (73), have used comprehensive control involving a combination of legislation, financial incentives, slaughter and biological approaches (competitive exclusion) to attempt to eradicate *Salmonella* from food. The cost of such schemes prevents their wholesale adoption by many other countries. In some other countries attempts are being made to control infection at the breeding flock level (23). Monitoring, either bacteriological or serological, is followed by control if infection is found. The options available are vaccination, the combined use of antibiotics (generally enrofloxacin) followed by competitive exclusion, or slaughter. This should at least provide useful information on the efficacy of such approaches, although the extensive use of quinolone antibiotics is bound to be counterproductive in the long term. Other countries, aware that salmonellosis is a perennial problem, have been more cautious in adopting expensive comprehensive measures and have resorted to combinations of codes of practice, used in many countries, together with pilot studies for control to ascertain their likely success if applied to larger area of the industry. The majority of other countries which submit information to OIE have either increased their sampling to allow a more extensive collection of information or have done nothing. The latter group may not have the resources to apply expensive monitoring or control measures and their major problems may be limited to the economic losses caused by *S. gallinarum* and *S. pullorum*. 

### 6

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For those countries which are attempting some degree of control, elimination or reduction in the level of infection in poultry will not provide a long term solution unless reinfection is prevented. In the United Kingdom, codes of practice advocate measures to reduce contamination during the production of animal feeds (52) and legislation to improve the situation has been introduced (62). Improvements in the quality of the raw materials together with extension of bacteriological monitoring to all organic feed components have been advocated (7). There are now also a number of reports indicating that incorporation of organic acids, particularly formic acid, in animal feed can reduce the rate of infection of poultry with feed-borne Salmonella (42, 43). As mentioned above, it may be possible to extend this approach to controlling infection from environmental sources since there is evidence that formic acid is antibacterial both in the feed and in the crop.

After depopulation of an infected flock, disinfection of contaminated housing is a major problem and there is evidence that many disinfectants, including iodophores and even phenol-based preparations, may be ineffective at killing S. enteritidis when it is dried into organic material such as feed or faeces (Berchieri and Barrow, unpublished findings). An improvement in the quality of housing to prevent entry of vermin and wild birds and also to facilitate cleaning is regarded as a priority (56). This is valid because major breeding companies and research institutes already use much housing which allows poultry to be kept clear of several infectious agents for many months.

A number of biological procedures may also be used to reduce Salmonella infection.

There has been a recent increase in interest in the use of live vaccines against S. typhimurium and S. enteritidis. Although bacterins have been used on an ad hoc basis for many years, it is generally believed that they are not very effective. Of the various attenuations used for Salmonella, aroA mutants appear to be the most promising in terms of combined avirulence for birds and degree of protection. While aroA mutants do not induce the same degree of immunity as do fully virulent Salmonellae (10), they do produce reasonable protection when birds are infected by environmental challenge (25). Other auxotrophic attenuations have been used without extensive testing and one of these vaccines is now used for extensive vaccination in Germany. A number of outstanding problems remain to be resolved. The vaccines must be as avirulent for man as are the commensal Escherichia coli population also present on chicken carcases. This has not yet been tested thoroughly for the aroA vaccines. Live vaccines could conceivably cause confusion during bacteriological or serological monitoring. This could be overcome by the use of positive or negative markers. Other criteria are also important in selecting vaccine strains (5).

Competitive exclusion continues to be used and the quality control of an undefined product does not appear to be a problem at the moment. In the future, simpler systems with a similar effect may become available. Thus, it is known that pre-colonisation of newly-hatched chicks with a Salmonella strain will almost completely prevent colonisation by a second strain given some hours later (12). The basis of this phenomenon is not completely understood, but a similar effect can be demonstrated in vitro (15). The use of a single bacterial strain which would have identical colonisation characteristics to wild-type Salmonella strains and could prevent it from establishing in the gut, and which could be attenuated, has a number of attractions. Live, attenuated Salmonella vaccines could, therefore, conceivably be administered soon after hatching and might display a dual protective function of colonisation inhibition in young birds (thereby reducing the severity of the consequences of hatching infection), while simultaneously stimulating the development of a true immunity to infection.

Competitive exclusion using undefined flora is being used increasingly in association with antibiotics. Originally used with tetracyclines or furazolidone, it is now being used with the newer quinolone enrofloxacin. Unfortunately quinolones rapidly select for resistance by chromosomal mutation. It is likely therefore that extensive use of this chemotherapeutic agent will result in increasing incidence of resistant mutants. There is experimental evidence that resistant E. coli mutants develop quickly in response to exposure to enrofloxacin in chicks (Barrow, unpublished data).

Other systems are being explored. These include the use of fermentable carbohydrates in the drinking water (26), the elimination of Salmonella from the gut by the administration of lytic bacteriophage (16), and the breeding of poultry which are more resistant to salmonellosis (19, 20). The potential value of such new ideas must be ascertained.
Some of these new developments may turn out to be of little value in the long term but it does indicate the need for continued work in new areas, particularly relating to the ability of *Salmonella* to colonise the alimentary tract. Work in this area is under way (11).

Whether or not any of these additional options become available in the future, it is salutary to remind ourselves that *Salmonella*-free poultry can be produced with current knowledge and technology. Tentative steps are being taken towards a top-down scheme of control within the European Union and it will be interesting to assess its effect in a few years time. A comprehensive programme of control, not only at breeder level but also in broilers and layers, which many involved in the public health side of the industry set as a goal, could cause great disruption to national poultry industries, unless proper support is given for the changes desired. Slaughter policies and improvement in housing can be very expensive and governments have to be certain of their ultimate goal, whether it be eradication of major serotypes or some degree of control. The gross expenditure required for extensive control could result solely in the import of poorer quality meat from countries unwilling or unable to do the same.

It is likely therefore that microbiological measures such as vaccination and competitive exclusion will be valuable supports for comprehensive control measures for the foreseeable future.

**REFERENCES**


