Confirmatory Testing of Poultry for Salmonella

Date : November 16th 2023

The Poultry Veterinary Study Group Europe (PVSGE) welcomes the opportunity to comment on the issue of confirmatory testing of poultry flocks where a Salmonella of public health significance has been identified in routine testing.

The PVSGE is a formally constituted group of about 90 European specialised poultry veterinarians, with practical responsibility for the health, welfare, production and food safety aspects of most European poultry production. PVSGE has existed for over 50 years and the members are mostly working as private practitioners or are sometimes working for a company (breeding companies, integrations, hatcheries, pharmaceutical companies). Full-time government veterinarians are not eligible for membership. The following 23 countries are currently represented in the PVSGE: Austria, Belgium, Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Great Britain, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Norway, Poland, Portugal, Romania, Spain, Sweden, Switzerland. This document has been prepared by the PVSGE Legislation Working Group.

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1. Summary:

Salmonella National Control Plans required under EU Regulation 2160/2003 and various pieces of subsidiary legislation have been rightly credited with achieving considerable success in reducing the prevalence of *Salmonella* of human health significance in farmed poultry in Europe and hence reducing the burden of an important zoonotic infection. PVSGE members are involved in contributing veterinary expertise to farms and industry bodies and frequently liaise with the competent authorities responsible for delivering these official controls. As with any testing programme there is the potential for both Type 1 (false positive) and Type 2 (false negative) testing results. Although prevalence of these is believed to be low the economic consequences of official controls on affected farms can be devastating. The legislation in place permits the use of confirmatory testing for both breeding chickens and commercial layers and this was frequently carried out prior to 2018. Since then, FVO audits have exerted pressure to avoid confirmatory testing and most Competent Authorities (CA's) now carry out this procedure rarely. This paper reviews the relevant published literature with a view to provide advice applicable to reducing the risk of inaccurate results and minimising the public health and economic impact where they do occur. We offer specific actions applicable to farmers, laboratories and competent authorities and seek the cooperation of the EU Commission to encourage these measures.

2. Legislative and Operational Background

It is about 20 years since the EU set up a system of monitoring and controlling important zoonoses within the framework of the Zoonoses Directive 2003/99 and the Zoonoses Regulation 2160/2003. The implementation of National Control Plans by individual countries has provided a mechanism to deliver improved surveillance and control measures which has, no doubt, contributed to a significant reduction in human health burdens. The system relies mainly on bulk environmental swabbing using overshoes, and then predominantly classical microbiological testing using ISO6579-1 method[1]. The diagnostic specificity of the test itself is considered to be 100%, and in practice it can detect <10 organisms amongst the millions of similar Enterobacteriaceae in a typical sample. This very high sensitivity does, however, mean that small defects in system operation can result in detectable positivity due to cross-contamination, whether of samples at collection, or during the multiple phases of processing. Some laboratories do not routinely carry out detailed serotyping when they obtain a positive result. This can lead to delays in identifying the occurrence of an unusual number of positive samples of a single serotype, which is often the first sign of a problem. Competent authorities will typically apply restrictions to a flock identified as positive as soon as they have confirmed the identity of a submitted isolate as a target serotype. This issue was recognised when the relevant subsidiary EU legislation was drafted (see appendix). This envisages confirmatory testing for layers and breeding chickens, and this was applied routinely in most countries up to 2017. Since then, CA's have become increasingly reluctant to conduct or even, in some cases, to allow confirmatory testing. Good evidence in favour of a Type 1 error can take many weeks to develop. Restrictions in egg and hatching egg use applied due to the positive finding, in contrast, have an immediate financial impact. With the prospect of mounting operational costs (in particular, feed costs) and no saleable product the affected producer is forced to cull a productive, often young, apparently healthy flock. Though this happens only rarely, with the reduction in prevalence of true infection with target serovars the proportion of false positives rises. This has important ethical, economic and practical implications. Every case of an unnecessarily culled flock which is allowed to occur risks damaging the cooperation between the poultry industry and Competent Authorities, on which the substantial progress already achieved has been built.

3. Literature Review

Erroneous results do not commonly get reported in the scientific literature. In the case of Type 1 errors there may be a reluctance to publish details for reasons of client or laboratory confidentiality. Type 2 errors are only likely to be documented where they result in onward spread to other poultry flocks, or a human food safety issue is identified. Increasing use of whole genome sequencing has the potential to link wide-spread individual cases to a source flock, but only once it is confirmed to be positive. De Lappe *et alii* (2009)[2] of the Irish National Reference Laboratory (NRL) carried out a very detailed assessment of the risk of false positive Salmonella results. Over an 8 year period (2000-2008) they identified 23 incidents with 56 separate isolates of *Salmonella* Enteritidis and S.Typhimurium which were attributed to cross-contamination as a result as molecular sub-typing. In this series the probable sources of contamination identified were the laboratory positive control isolate (n = 13), other test isolates (n = 9) or proficiency test samples (n = 1). The subtyping used was phage-typing and, in the case of S.T. multi-locus variance analysis.

In contrast Pedersen *et alii* (2014)[3] reported on an apparent pseudo-outbreak of S.Goverdhan in Denmark in which poultry flocks were not confirmed to be infected on repeat testing This was associated with the use of non-sterile hospital gauze imported from outside the EU to make the overshoes.

Rasschaert *et al.* (2016)[4] showed in a case report that the issue is not restricted to on-farm samples when they investigated the occurrence of *Salmonella* Rissen in exported Belgian chocolate, as reported by a food testing laboratory. They used 3 different sub-typing system to show that the source was in fact the same strain that had been isolated from a fish meal sample in the same laboratory 7 weeks previously.

McMullin (2021)[5] has reported on a series of 8 investigations of suspected false positive testing results from poultry samples in the UK, of which 3 were S.Typhimurium, 2 were S.Enteritidis (including 1 vaccinal) and one each S.Nottingham, S.Poona and S.Newport. Of these it was concluded that the sources of cross-contamination included 4 laboratory controls, 3 field samples and one proficiency test sample. Only 1 of the 8 submissions was of boot swabs, and 5 were bulk table egg samples tested as part of an industry control programme.

The most in-depth recent study on this topic was reported by Costa *et alii* (2021)[6]. This group focussed on results from routine boot-swab sampling of poultry breeding flocks in the Netherlands. In addition to reviewing results of testing of Dutch poultry breeding flocks in a period in which routine confirmatory testing was required they conducted an opinion survey of PVSGE members in relation to the topic. They identified the various points that false positivity (or negativity) could arise and carried out detailed epidemiological analysis of the available data. They did not have access to detailed genotyping which would allow identification of likely source(s) of positivity. They also assessed the specificity of Salmonella testing based on proficiency test results. This showed that the proportion of false positive test results is, on average, 2.3% for the Dutch laboratories and 2.7% for laboratories in other countries. As not all participating laboratories carry out serotyping some of these will be incorrect identification of other organisms as Salmonella. Proficiency test samples have been identified as a source of false positivity in other cases so it may well be that the lack of competing organisms in some proficiency test samples results in high growth of the Salmonellae, facilitating cross-contamination. Nevertheless, these data support the view that there is a significant risk of false positive results, which the authors assess as a medium risk in their environment. They also modelled their effect on the success of the Salmonella control programme. The post-test probability of infection or positive predictive value of the routine Salmonella test tends to decrease sharply at low prevalence, particularly below a prevalence of 0.2%. Supplementary materials were provided which detail a survey of PVSGE members, and sampling methodology of Dutch farmers at that time[7]. A high survey response rate was obtained from PVSGE members (67%) and this covered 21 of the 22 European countries represented in the organisation. Most respondents believed that cross-contamination could occur in the laboratory (94%) or on farm (90%), with fewer believing it could occur in transport (70%).

A total of 92% of the respondents indicated that given the existence of false positive Salmonella results in practice, it is important that all initial positive Salmonella results from poultry farms are confirmed by resampling and retesting; that statement is made with an average certainty of 96%. These authors conclude that Salmonella prevalence in poultry breeding flocks in the Netherlands is low, and the positive predictive value (PPV) of an initial positive test is also low, which, in line with the retest findings of the Dutch competent Authority, justifies an official resampling and retesting by the competent authorities.

4. Conclusions & Recommendations:

It should be our common objective to minimise the occurrence of false reports, but we must also recognise that, given the nature of the samples tested, and the very high sensitivity of the testing methodology, Type 1 errors will occur from time to time. Clear evidence that they have occurred has often taken weeks to develop. Sadly, this has resulted in unnecessary culling of uninfected poultry flocks, with the attendant ethical concerns, economic damage leading to increased cost in the food chain, and stress for all involved with the process.

Our review permits the identification of specific sources of Type 1 errors in relation to Salmonella testing of poultry flocks and guides our proposal of specific measures to mitigate the risks. Table 1 lists issues and recommendations relevant to all samples and those associated with sampling. While sampling is not covered by laboratory accreditation, laboratories have a general duty to document anything which may affect the validity of its results. Table 2 covers, in turn, the issues which have been related to laboratory procedures.

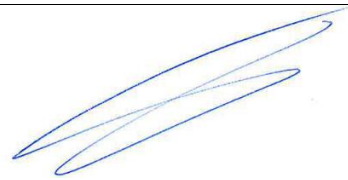
Table 1 Salmonella False positivity: Sources and Mitigations relevant to All Issues, as well as Sampling and Transport

Source	Mitigation	By
All suspected cases of false positivity		
All	Laboratories should take care to document any concern with respect to an unusual result, investigate possible cross contamination, and cooperate with investigators. Having the ability to serotype all controls in use and target serovars rather than relying on third parties will help reduce the risk of multiple farms/samples being affected. Instances which, on the balance of probability, are false positive should be treated as a formal non-conformance and fully investigated with a view to early implementation of corrective measures. Where on-farm sampling, or sample packing may have contributed, the customer should be directed to a suitable source of advice to avoid repetition.	Lab
Farm Issues		
Contaminated sampling materials	Obtain sampling materials as packaged kits from a reputable source. Ensure that they are stored in a clean, dry, dust-free area prior to use. Use sterile diluent, potable from farm service area (not hyperchlorinated or acid treated) or bottled, still water. Sampling materials, in particular diluents, should not be manipulated in laboratory areas where field samples are received or testing is being carried out.	Farm/Lab
Within-farm cross contamination	Follow good sampling practice - see Sampling Protocol V1 2023[8]	Farm
Contamination of sample in transport	Separate packaging of samples from each source. Ensure external surfaces are not contaminated with farm-origin organic material. Laboratory should log instances of poorly-taken or poorly packed samples and request re-sampling as required.	Farm/Lab

Table 2 Salmonella False positivity: Sources and Mitigations -Laboratory specific issues		
Source	**Mitigation**	**By**
Laboratory Issues		
Cross contamination from other samples	Improved laboratory hygiene, and physical separation between culture phases. Special attention to hand hygiene, laboratory clothing, reusable pipettes, routine screening of the laboratory environment, and disposal of waste cultures (all phases)	Lab/Quality Assurance Scheme
Cross-contamination from laboratory controls	As above. Require laboratories to be able to serotype their internal control. Avoid use of target serovars as controls. Develop and provide laboratory controls with specific genetic markers.	Lab/ Competent Authority/ Quality Assurance Scheme
Cross-contamination from proficiency test samples	As above. Extra care is required with these as they commonly include target serovars and grow abundantly. If used for training inexperienced staff extra supervision of hygiene is required. C.A's should endeavour to produce more realistic test samples, and, where target serovars are included ensure that they can be readily identified as such. Require labs to serotype or have serotyped any positives - or at least any samples showing discrepancy with expected result.	Lab/Competent Authority/Quality Assurance Scheme

Based on the above literature review and analysis we are pleased to support calls, from the poultry industry and respected academics, for the EU and Competent Authorities to review their policies with respect to confirmatory testing of poultry flocks for *Salmonella* spp. We understand that at least some CA's already allow confirmatory testing of layers and breeding chickens either in accordance with specified conditions[9], or "on a trial basis"[10]. In principle the options for confirmatory testing laid down in the legislation should remain available in all member states, and in trading partners required to follow EU rules, unless there is clear evidence that the result is accurate and there is an immediate public health risk which would preclude this. We recognise the need to be particularly precautionary with respect to breeding flocks given the potential for vertical transmission of at least some important serovars. If a likely source of cross contamination is identified, particularly where this is within-laboratory and supported by molecular typing of the 'false' isolate and the plausible source isolate, then the need for repeated retesting and follow-up can, and should, be minimal. In other circumstances every effort should be made to avoid unnecessary culling. Sometimes this culling is for sampling large numbers of carcasses, sometimes economics-led premature flock depletions. This must be minimised while still protecting both animal and human health. Achieving this may require both confirmatory testing and ongoing independent monitoring of suspect flocks and associated facilities. Routine environmental sampling of hatcheries and table egg packing stations is also very useful in identifying early indications of below-detection-limit infections in the breeding and egg-supply chains and is to be encouraged. Even where such positives are not linked to a currently affected supply flock, they are helpful in focusing cleaning and disinfection activity to remove such residual contamination and reduce the risk of spread both forward and backward in the production chain.

November 17th, 2023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Appendix - Relevant legislation

Laying flocks (Table eggs)

Detailed sampling and controls based on results were laid down in EU Commission regulation 1237/2007[11]. Competent authorities were required to apply restrictions to flocks where there was evidence from outbreaks in consumers, or from sampling of the flocks that they were infected with Salmonella Enteritidis or Salmonella Typhimurium. It was recognised, however that there was a risk of false positivity in such designation, and that the competent authority may lift restrictions where the flock is not the cause of a food-borne outbreak, and it has been re-tested with negative results. The regulation specifies that one of 3 sampling options be used:

1. Faecal and dust samples (7 in total, with a sub-sample of 25 g being tested), as laid down in Commission Decision 2004/665/EC[12]

2. bacteriological investigation of the caeca and oviducts of 300 birds

3. bacteriological investigation of the shell and the content of 4 000 eggs of each flock in pools of maximum 40 eggs.

In addition to the sampling in point (b), the competent authority was expected verify the absence of the use of anti- microbials, potentially affecting the result of the analyses of the sampling.

Chicken Breeding Flocks

The Salmonella monitoring programmes, as outlined in the EU Commission regulation 200/2010[13], asks for repeated sampling in order to ascertain progress in achievement of the EU target. According to Article 2.2.2.2.c of this regulation, states "In exceptional cases where the competent authority has reason to question the results of the testing (such as false positive or false negative results), it may decide to repeat the testing...". In the various member states, the competent authorities were resampling and retesting all initial positive samplings in breeding flocks for several years because of doubts about false positive initial test results.

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