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Qualitative evaluation of causes for routine *Salmonella* monitoring false-positive test results in adult poultry breeding flocks in the Netherlands

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Background

Commissioned by the Ministry of Agriculture, Nature and Food Quality (LNV), the Statutory Task Unit - Infectious Animal Diseases of Wageningen Bioveterinary Research (WBVR) in Lelystad, the Netherlands, has made an analysis of the **discrepancy** between **test positive results of the routine *Salmonella* monitoring sampling by the poultry farmer** (according to article 2.2.2.1 of EU Commission regulation 200/2010) and **the test negative results of a retest by the Netherlands Food and Consumer Product Safety Authority** (NVWA) (based on new samples collected by the NVWA shortly after the initial routine monitoring test positive results were reported; according to article 2.2.2.2.c, and executed according to article 2.2.2.1. of EU Commission regulation 200/2010) because of a **suspicion of a false positive test result of the routine monitoring sampling** in adult breeding flocks of *Gallus gallus*.

The prevalence of *Salmonella* infections in adult breeding flocks of *Gallus gallus* is quite low, which is also the target of the European Union as referred to in article 4(1) of Regulation (EC) nr. 2160/2003. Based on the epidemiological principles and interpretation behind sampling with a diagnostic test (see Appendix 1), the **predictive value of a test result** is an important parameter (Dohoo et al., 2003). This is important because it is a reflection of the way in which the test result is used in the field. For in many cases the question in the field is: **given the fact that a subject is tested positive, what is the probability that the subject actually is infected?** The *Salmonella* monitoring program asks for repeated sampling in order to ascertain progress in achievement of the European Union target (see introduction of EU Commission regulation 200/2010). According to the Regulation, routine sampling consists primarily of i) pooled fresh faeces samples, or ii) boot swabs and/or dust samples. In practice, almost 100% of samples collected in the Netherlands on this type of poultry farms is with boot swabs.

In principle, the **laboratory diagnostic test characteristic** of the *Salmonella* test (which is culture) has a specificity of 100%, meaning that if there are no *Salmonella* bacteria in the sample, you will get a negative test result. However, there are several steps taken between sampling at the farm and diagnostic testing at the laboratory that might introduce contaminations and therefore reduces the **system-specificity**.
Even a small loss of **system-specificity** may have an enormous effect on the diagnostic accuracy when the

Salmonella prevalence is very low, entailing an erroneous identification of a Salmonella negative flock as positive. As an example, when a farmer steps into the boot swabs with boots that are *Salmonella*-contaminated by use of the boots in the environment outside the poultry house, the boot swab sample might turn up with a test result that is positive. In reality, the flock is not *Salmonella*-infected, but the farmer might have introduced *Salmonella* from outside the barn by contaminated boots into the boot swab sample.

Therefore, one can define such a **diagnostic system result** as **false positive**, because it does not reflect the status of the flock, but is the result of detection of a contamination from outside the barn and poultry.

Objective

The objective of this evaluation is to get insights into a population estimate of false positive diagnostic results during *Salmonella* surveillance of Dutch adult breeding flocks. Furthermore, we aim to identify and assess factors that could be associated with the probability of leading to the discrepancy between test positive results of the routine *Salmonella* monitoring sampling by the poultry farmer and the test negative results of a retest after resampling by the NVWA (based on new samples collected by the NVWA).

Material and Methods

Data

We analyzed data on all *Salmonella* monitoring samplings (results of initial routine sampling by farmers) for the period 2015-2019. The data consisted of: 1) flock category: Parent Stock (PS) or Grand Parent Stock (GPS); 2) lab identification number, indicating the private lab that has processed the samples from the initial routine sampling by the farmers; 3) unique identification number of the poultry farm; 4) postal code of poultry farm; 5) type of sample (boot swab, or other); 6) poultry house identification from which sample originate; 7) birth date of the flock in the poultry house; 8) date of sampling; 9) date of reporting the diagnostic test result by private lab; 10) diagnostic test result (*Salmonella* detected/*Salmonella* not detected); 11) *Salmonella* serotype if *Salmonella* was detected.

In addition, we also analyzed the data on *Salmonella* resampling and retesting by the NVWA. NVWA collected new samples at the farm after test positive results of the routine *Salmonella* monitoring were reported by AVINED[#]/private lab to the NVWA, using the same collection method as the poultry farmers (boot swabs). Samples were collected by the NVWA on average 1 day (min: 0, max: 3) after they received a report on the routine monitoring positive test result and on average 4-6 days after the routine monitoring sampling performed by the poultry farmer. The NVWA data we received consisted of: 1) unique identification number of the poultry farm; 2) postal code of poultry farm; 3) type of sample (boot swab or other); 4) date initial routine monitoring sampling by poultry farmer; 5) date of re-sampling (new samples collected) by NVWA; 6) date of reporting the NVWA retest test result; 7) NVWA retest test result (*Salmonella* detected/*Salmonella* not detected); 8) *Salmonella* serotype if *Salmonella* was detected. We limited our investigation to detections of *Salmonella enterica* serotypes listed in EU Regulation 200/2010: Enteriditis, Infantis, hadar, Typhimurium and Virchow.

[#] AVINED has been established to continue part of the activities of the former Product Board for Poultry and Eggs (PPE); it has a role as provider of information, acts as a consultation platform for the poultry sector and spokesperson towards the Dutch government

Qualitative assessment of the probability of false results

For this evaluation, we assume that the possible false outcomes in a *Salmonella* test arise from factors/actions taken between the sample collection and the processing at the laboratory. The assessment of the factors' association with a possible false outcome was based on conversations with stakeholders, bacteriologists, laboratory personnel and a veterinary practice with a large number of poultry farmer clients and extensive experience of *Salmonella* sampling of poultry breeding flocks by poultry farmers.

We provide an overview of possible explanations for the observed discrepancy between routine *Salmonella* monitoring positive results and the results of resampling and retesting by the NVWA. We distinguish between two main hypotheses:

1) *The routine monitoring positive result is incorrect*:

This means that, on the basis of the test results, it is incorrect concluding that the poultry breeding flock is infected with Salmonella. False positive routine tests could potentially arise from six factors/actions: i) (Cross-) Contamination during sampling by the poultry farmer; ii) (Cross-)Contamination during transport of sampled boot swabs; iii) Contamination of samples in the laboratory before or during diagnostic testing; iv) Test

2) *The negative retest (confirmatory testing by the NVWA) result is incorrect*:

This means that, on the basis of the retest result, it is incorrect concluding that the breeding flock is free from Salmonella. False negative confirmatory tests could arise from six factors/actions: i) lack of sensitivity of the sampling performed; ii) lack of sensitivity of laboratory testing (i.e., Salmonella concentration around or below the detection limit of the test); iii) inactivation of Salmonella during transport to the laboratory; iv) intermittent Salmonella excretion; v) treatment of poultry with antibiotics; and vi) acidification of drinking water after initial positive sampling.

Each of the identified factors was qualitatively assessed. The probability of their association with the specific assessed hypothesis was expressed qualitatively based on a scale used by EFSA (2006), which is an adaptation of OIE (2004). The assessment probability scale has six levels: very high, high, medium, low, very low, negligible.

Assessment of the post-test probability of infection of a poultry breeding flock following a monitoring routine positive test

To assess the positive predictive value (PPV) of a routine *Salmonella* monitoring test, four scenarios of failure (f) were created accounting for one cross-contamination out of 100, 1,000, 10,000, and 100,000 collected and processed samples. The specificity (Sp) is the complement of the failure (i.e., Sp=1-f). The sensitivity (Se) used is 99%, and the prevalence p=0.02%, and p=0.04%, correspond the frequencies of monitoring and retest positive samples, respectively (see Table 1). The PPV is calculated according to Greiner & Gardner (2000): $PPV=(Se*p)/[(Se*p)+(1-Sp)*(1-p)]$.

Questionnaire

A questionnaire about the potential for cross-contamination of samples was set out among poultry veterinarians of the Poultry Veterinary Study Group of the European Union (www.pvsgeu.org). Members of this group are selected expert poultry veterinarians from the following 22 European countries: Austria, Belgium,

Bulgaria, Cyprus, Denmark, Finland, France, Germany, Great Britain, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Norway, Poland, Portugal, Romania, Spain, Sweden, and Switzerland. The following questions were asked: In your experience as a poultry practitioner: Q1) do you think that it is possible to get a false positive Salmonella result from a farm because samples taken by the farmer have become contaminated through cross-contamination during sampling? Q2) do you think that it is possible to get a false positive Salmonella result from a farm because samples taken by the farmer have become contaminated through cross-contamination during transport of the samples from the farm to the laboratory? Q3) do you think that it is possible to get a false positive Salmonella result from a farm because samples have become contaminated through cross-contamination in the process of handling and isolation in the lab? Q4) Do you think that, given the existence of false positive Salmonella results in practice, it is important that all initial positive Salmonella results from a farm are confirmed by resampling and retesting from the same barn and farm? For each answer by a responder, the likelihood of the chosen answer (yes or no) is asked on a scale from 0 (not sure) to 10 (very sure). The last question was: If you have answered questions Q1 to Q3 with "Yes", what would be your ranking of the likelihood of the appearance of false positives? The details of the questionnaire are depicted in Appendix 2.

Results

Data summary and exploration

The Netherlands had approximately 300-400 adult breeding flocks in the period 2015-2019, and the number of breeding flocks is declining over the years (Table I). A total of 44 routine monitoring positive samplings were resampled and retested by the NVWA in the 5 year period, of which 21 samplings (**48%**) retested negative and therefore judged **false positive**. From the routine monitoring positive samplings that were confirmed as positive in the resampling and retest by the NVWA, the serotypes of the *Salmonella* strains isolated were identical. The proportion of routine monitoring positive samplings was significantly lower (Mantel-Haenszel Chi-square test (stratified by year): $p < 0.05$) in Grand Parent Stock (GPS) compared to in Parent Stock (PS). This is an indication for differences in the way biosecurity is applied at the breeding farms, because it can be assumed that biosecurity measures are more strict in GPS compared to in PS.
Furthermore, four breeding farms showed **recurrent occurrence** of routine monitoring positive samplings that were resampled and retested negative by the NVWA: at the same farm in the same flock (same birth date) or in a different flock with a different birth date. This might be an indication of **a systematic problem** with respect to introducing contamination during the sampling process.

Table I Routine *Salmonella* monitoring positive samplings, NVWA-retest positive samplings and NVWA-resampling and retest negative samplings in relation to the total number of *Salmonella* samplings in Dutch adult breeding farms by year.

Year	Breeding Stock[$]	Number of farms[*]	Number of *Salmonella* samplings[**]	Number of routine monitoring positive samplings	*Salmonella* serotype	Number of retest positive samplings	Number of retest negative samplings (% false positive samplings within initial positive samplings)
2015	PS	347	13,175	**14** (0.1 %)	**12** *S.* Enteritidis **2** *S.* Typhimurium	**9** (0.07 %)	**5 (36 %** = 5/14)
2015	GPS	50	1,533	1 (0.065 %)	**1** *S.* Enteritidis	1 (0.065 %)	**0**
2016	PS	330	17,196	**13** (0.08 %)	**13** *S.* Enteritidis	**7** (0.04 %)	**6 (46 %** = 6/ 13)
2016	GPS	35	1,145	**0** (0 %)	-	-	
2017	PS	321	20,465	**1** (0.005 %)	**1** *S.* Typhimurium	**0** (0%)	**1 (100 %** = 1/1)
2017	GPS	35	2,783	**0** (0 %)		-	
2018	PS	293	19,481	**4** (0.02 %)	**1** *S.* Enteritidis **3** *S.* Infantis	**0** (0%)	**4 (100 %** = 4/4)
2018	GPS	31	3,267	**0** (0 %)	-	-	
2019	PS	260	16,983	**11** (0.06 %)	**6** *S.* Enteritidis **5** *S.* Infantis	**6** (0.035 %)	**5** (**45 %** = 5/11)
2019	GPS	29	3,405	**0** (0 %)	-	-	
Total		1,731	99,433	**44** (0.04%)		**23** (0.02%)	**21** (**48 % =** 21/44)

[$] GPS: Grand Parent Stock; PS: Parent Stock

* Farms are sampled on average every 3 weeks. These values are the expected total number of *Salmonella* evaluations made on the farm population per year.

** A *Salmonella* sampling is defined by a unique combination of poultry farm, sampling date, birth date of the flock and the average number of poultry houses within the farm that were sampled. This represent the yearly total number of samples (poultry houses) tested.

Qualitative assessment of the likelihood of routine monitoring positive result is incorrect

(Cross-)Contamination during sampling by the poultry farmer

Conversation with a knowledgeable veterinary practice indicated that in some cases there are doubts about taking the correct precautions during sampling by poultry farmers. Comparison of the protocol on how to perform sampling with boot swabs provided by the poultry industry for poultry farmers (see Appendix 3) with the one used by the NVWA (see Appendix 4), suggests that the protocol from the poultry industry maybe improved to prevent contamination with Salmonella from outside the poultry house during the sampling process. Differences between both protocols are for instance the use of disposable gloves (as is the instruction of the NVWA sampling protocol, see Appendix 4) before unpacking the boot swabs from the package and putting the boot swabs on, versus hand washing as described in the protocol of the poultry industry. Another difference is the use of plastic disposable over-boots before putting on the boot swabs as is the instruction of the NVWA sampling protocol (see Appendix 4), but not in the protocol from the poultry industry. In summary, the sampling protocol and hygienic procedures followed by farmers may not be enough to avoid cross-contamination of the sample. Therefore the probability assessment for this factor was rated **high**.

(Cross-)Contamination during transport of sampled boot swabs

There were interview reports of incidences in which boot swab samples were delivered at the lab in non-sealed, simple plastic bags (non-designated packaging materials (UN 3373 safety seal bags)) and/or use of non-designated methods of transport (UN 3373 conditions).

This could occasionally lead to contamination along the way from other poultry samples or other possible sources during transport. These reports led to the probability assessment for this factor to be rated **medium**.

Contamination of samples in the laboratory before or during diagnostic testing

In principle, a laboratory can be regarded as a much better controlled environment compared to a poultry breeding flock, farm or transport vehicle. Moreover, laboratories that have been designated by the Dutch competent authorities for performing diagnosis for the national Salmonella monitoring program, have to demonstrate that they operate competently and generate valid results. This approval demands amongst others an ISO 17025 accreditation for detection of Salmonella according to ISO 6579-1, serotyping of these strains according ISO 6579-3. Additionally, in the NVWA-designated laboratories, labs are rated/reviewed by the National Reference Laboratory on their proficiency test performances. Results from recent Salmonella proficiency testing show that participating laboratories occasionally report false-positive results. An analysis of results of proficiency tests performed in the period 2019-2020, both from NVWA-designated laboratories and laboratories in other countries (e.g. a combination of laboratories from Italy, Belgium, Germany, Spain, Portugal, UK, Eire, Greece, Cyprus, Malta, Serbia, Croatia, Turkey, Morocco, Canada, USA, South Africa, Botswana, Malaysia, India, Brazil, Thailand, Brunei, Singapore, Chile), shows that the proportion of false positive test results is, on average, 2.3 % for the NVWA-designated laboratories and 2.7 % for laboratories in other countries (Table II and Table III). Such false positive test results may be caused by incorrect identification of other bacteria as Salmonella, or through cross-contamination in the lab. The culture method followed by serotyping has been shown to be an extremely specific test.

Table II *Salmonella* proficiency test results of NVWA-designated laboratories in the period 2019 – 2020, based on results from VETQAS proficiency tests (source: National Reference Laboratory for *Salmonella*, RIVM, Bilthoven, The Netherlands)

Year	**2019**				**2020**				
Quarter	**1st**	**2nd**	**3rd**	**4th**	**1st**	**2nd**	**3rd**	**4th**	**Overall**
Number of negative samples in proficiency test	2	1	1	2	3	1	3	2	
Number of positive samples in proficiency test	3	4	4	3	2	4	2	3	
Number of laboratories	24	23	23	23	23	21	24	24	
Number of false positives	2	0	1	2	1	0	1	1	8
Total number of negative samples tested (labs x negative samples)	48	23	23	46	69	21	72	48	350
% false positives	**4.17**	**0.00**	**4.35**	**4.35**	**1.45**	**0.00**	**1.39**	**2.08**	**2.29**

Table III ***Salmonella*** **proficiency test results of laboratories from several countries in the period 2019 – 2020. (source: VETQAS, proficiency testing for veterinary laboratories; Animal and Plant Health Agency, UK)**

Year	2019				2020				
Quarter	1st	2nd	3rd	4th	1st	2nd	3rd	4th	Overall
Number of negative samples in proficiency test	2	1	1	2	3	1	3	1	
Number of positive samples in proficiency test	3	4	4	3	2	4	2	3	
Number of laboratories	94	110	87	93	88	100	74	97	
Number of false positives	2	3	3	9	1	2	11	3	34
Total number of negative samples tested (labs x negative samples)	188	110	87	186	264	100	222	97	1254
% false positives	**1.06**	**2.73**	**3.45**	**4.84**	**0.38**	**2.00**	**4.95**	**3.09**	**2.71**

However, serotyping is not routinely performed in every laboratory, and proficiency test results may be entered as Salmonella spp., potentially lowering the specificity of the method. Also levels of Salmonella in proficiency samples may be relatively high compared to field samples, increasing the risk for cross-contamination compared to field samples. Nevertheless, the results suggest that a false positive result might also be generated during daily laboratory practice. The probability assessment for this factor as explanation for false positive results in the Dutch monitoring program was rated as **medium**. It should be taken into consideration, that even if cross-contamination is a rare event, that if the number of samples tested are high (as in the case in Salmonella monitoring) this will inevitably lead to a number of false positive test reports.

Based on the number of false positive (fp) and the total number of negative samples tested (to) during proficiency testing in tables II (for the NVWA-designated laboratories) and III (from several countries) we used a beta distribution to describe the uncertainty around the estimate of probability of false positive (pp): pp~Beta(fp+1; to-fp+1) (Vose, 2008). For the NVWA-designated laboratories, the mean probability of false positive testing during proficiency testing by a lab is 0.025 ranging from 0.011 to 0.044 (95% confidence interval). For other countries, it is 0.027 ranging from 0.019 to 0.037 (95% confidence interval). False positive probabilities higher than 0.05 and lower than 0.003 are rare in both the NVWA-designated laboratories and in other countries (Fig. 1).

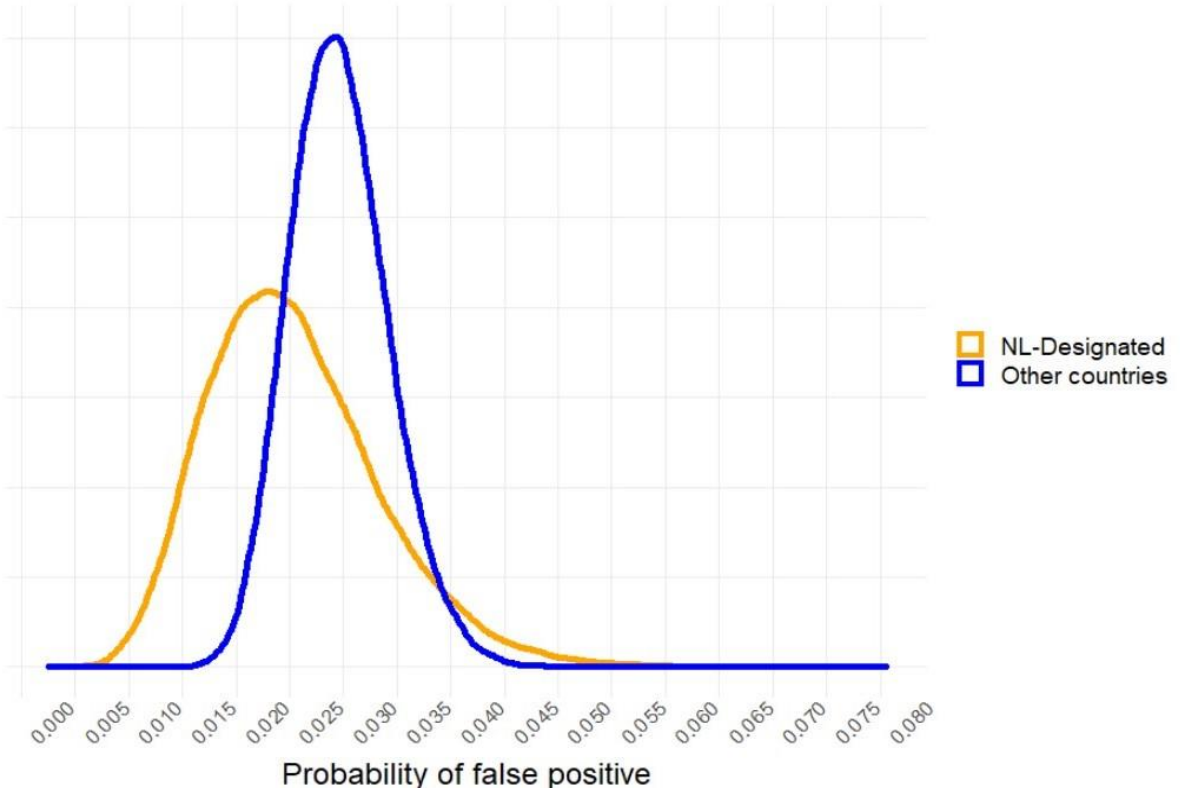

Fig. 1 Uncertainty density distribution for the probability of false positive given a misclassification of a negative Salmonella sample at the lab based on the Salmonella proficiency tests results in the period 2019 – 2020.

Test characteristics

The Salmonella culture method, performed according to ISO 6579-1, is considered a test with a 100% specificity, i.e. when Salmonella is isolated from a sample, it can be assumed that Salmonella was actually present in the sample. According to EU regulation 2160/2003, it is allowed to use an alternative test method instead of culture, provided it has been validated in accordance with EN ISO 16140-2 (validation alternative methods). To our knowledge, there is at least one commercially available polymerase chain reaction (PCR) test that complies with this and is also used by a number of NVWA approved laboratories. Since this PCR test cannot distinguish between Salmonella serovars, this PCR test is always followed by bacteriological examination of the samples and serotyping of the isolated Salmonella if the test result is positive. Based on this, the probability of a false positive test result is very unlikely.

At the laboratory incidental errors such as sample changes, contamination during testing, administrative errors and/or other actions during a test can occur. Since the laboratories approved by the NVWA for participation in the Salmonella monitoring program meet a certain quality standard, it may be assumed that these errors occur at a minimum. The probability assessment for this factor was rated **very low to negligible**.

Contamination of the poultry house without infection of the chickens

This could, for example, be a contamination entering the poultry house, whereby the Salmonella is unable to colonize the animals, but where the Salmonella is demonstrable (for a short time). Based on expert opinion, the probability assessment for this factor was rated **very low**.

Vaccination

For prevention of Salmonella in poultry, vaccination is considered a valuable additional measure by increasing the resistance of birds against infection and to decrease shedding of Salmonella. In Europe, both live and inactivated vaccines are available. Of the available Salmonella live vaccines, Salmovac® offers the possibility of differentiation from field strains by means of a PCR test (performed by GD Animal Health Service, Deventer, The Netherlands). In other vaccines, the vaccine strains are unable to grow on the selective media used, so a culture method here should not give rise to false positive test results. Sources from the field indicate that the majority of broiler breeders flocks in the Netherlands are vaccinated, during the rearing period, mostly with live vaccines and sometimes (also) with an inactivated vaccine. However, there are still some questions remaining about the influence of vaccination on the possibility of low or intermittent excretion of Salmonella, and so a temporary positive flock. Based on the available information, the probability assessment for this factor was **rated low**.

In Table IV, a summary is given of the probability assessment and the sources for the assessment as well as identified limitations in the assessment concerning an incorrect test result during initial routine Salmonella monitoring.

Table IV Summary of assessed factors/actions considered to be associated with an incorrect positive test result during routine *Salmonella* monitoring

Factor/action	Probability assessment	Sources for assessment and limitations
(Cross-)Contamination during sampling by the poultry farmer	High	**Assessment:** Poultry industry sampling instruction compared with NVWA sampling protocol; interview with a veterinary practitioner with extensive experience in *Salmonella* sampling of his clients. **Limitation:** No documented follow-up of the procedures have been made so far.
(Cross-)Contamination during transport of sampled boot swabs	Medium	**Assessment:** Interview with a veterinary practitioner with extensive experience in *Salmonella* sampling of his clients. **Limitation:** No documented follow-up of the procedures have been made so far.
Contamination of samples in the laboratory before or during diagnostic testing	Medium	**Assessment:** ISO 17025 accreditation and results of proficiency testing by (VETQAS). **Limitation:** It lacks some specific evidence about the probability of contamination of samples at the lab.
Test characteristics	Very low to negligible	**Assessment:** Laboratories follow the internationally validated reference method ISO standard ISO 6579-1 for *Salmonella* isolation and identification. **Limitations:** No audits of laboratories are performed by NRL (RIVM); no overview of the diagnostic tests that are used and how these tests are deployed.
Contamination of the poultry house without infection of the chickens	Very low	**Assessment:** Expert opinion **Limitation:** No specific evidences could be found and the assessment is based on expert opinion.
Vaccination	Low	**Assessment:** Expert opinion and literature evidences about vaccination. **Limitation:** Degree of *Salmonella* vaccination in the broiler breeding sector is unknown. Also, the effect on *Salmonella* shedding is still not clear.

Qualitative assessment of the negative retest (confirmatory testing by the NVWA) result is incorrect

Lack of sensitivity of the sampling performed

This would mean that the Salmonella bacteria present would not be picked up during the sampling of the NVWA. According to ISO 6579-1:201, for boot stock samples the limit of detection (LOD_{50}) was determined to be 3.8 cfu/sample (3.2-4.4 95% confidence interval). The sensitivity (Se) of the boot swab method for detection of infection at the flock level is dependent on the within-flock prevalence; when this within-flock prevalence is high (e.g. >50%), the Se is expected to be very high (99%), provided that the sampling protocol is properly followed. However, if the within-flock prevalence is low, the flock Se and the repeatability of the diagnostic system may be compromised. This means that with a low within-flock prevalence, two repeated samples from

the same flock (taking within a short period of time) could give contradictory results (flock negative = all five boot samples negative; positive = at least one boot sample positive). However, subsequent sampling of such a flock (when prevalence is likely to be higher) would suggestively confirm a positive test result. Retesting is carried out by the NVWA protocol with supposedly a limited risk of sample contamination. This is confirmed by the historic results: subsequent routine monitoring samplings in the months after the (negative) retest by the NVWA confirmed the negative status of flocks. The probability assessment for this factor was rated **very low**.

Lack of sensitivity of laboratory testing (i.e., Salmonella concentration in samples around or below the detection limit of the test)

If the number of Salmonella bacteria in the sample is close to the detection limit of the test, stochastic probability processes will play a role and samples from the same "population" return conflicting results. If a flock is infected with Salmonella, assumably sufficient Salmonella bacteria will be present in the sample to be detected in the lab (13). The five boot swab samples should be taken from different places in the poultry house, with each pair of boot swabs representing 20% of the floor area of the house. In the routine Salmonella positive samplings that were retested by the NVWA and tested positive, in 70% of those cases 5 out of 5 pairs of boot swabs tested positive, indicating that if infection is present, it will be easily detected. The probability assessment for this factor was rated **negligible**.

Inactivation of Salmonella during transport to the laboratory

Samples of the NVWA are conditioned according to the NVWA protocol and transported according to fixed protocols. Compared to the protocol and procedures followed by the farmers, the procedures followed by the NVWA are very strict and likely to minimize risks of loss of viable Salmonella in sample material. The probability assessment of this event to happen resulting in a false negative results is therefore rated **very low**.

Intermittent Salmonella excretion

This is a possible factor at the animal level, as it is known that Salmonella can be excreted intermittently. At the flock level, the more animals are infected, the more constant the excretion pattern will be over time. However, with a low excretion of Salmonella in a limited number of animals, the chance of detection may be lower (low flock system-sensitivity), and therefore be more variable over time. The lack of positive lab results in subsequent samplings after a negative retest (as mentioned under Lack of sensitivity of the sampling performed), and the very short time between routine sampling and retesting, in combination with the results mentioned above (generally a positive retest shows Salmonella in almost all sampled boot swabs), the probability assessment for this factor was rated **very low to negligible**.

Treatment of poultry with antibiotics

If antibiotics would be administered to a flock in the event of a positive Salmonella result, the flock will be declared infected without further sampling and testing. In theory, an antibiotic treatment could be applied by the poultry farmer, without this being officially stated. During the resampling process by the NVWA (after initial positive test by poultry farmer), five random chickens per flock are selected and tested at the lab for antibiotic residues. Results were at all times negative. The probability assessment for this factor was rated **negligible**.

Acidification of drinking water after initial positive sampling

The bacteria that are already present in the house are probably not inactivated by it, but this may lead to a reduction in the excretion by infected chickens, or loss of viability of excreted Salmonella possibly resulting in a negative culture result. This means that the probability of detection by the acidification in the relatively short period between the two samples will probably only be influenced to a very limited extent. The probability assessment for this factor was rated **negligible**.

In Table V, a summary is given of the probability assessment and the sources for the assessment as well as identified limitations in the assessment concerning an incorrect retest result.

Table V Summary of assessed factor/actions which could be associated with an incorrect retest negative test result

Factor/action	Probability assessment	Sources for assessment and limitations
Lack of sensitivity of the sampling performed	Very low	**Assessment:** Sensitivity of the sampling and testing is dependent on within-flock prevalence. If prevalence is higher than 10%, a high sensitivity of boot swabs method is expected. **Limitation:** No documented follow-up of the procedures have been made so far.
Lack of sensitivity of laboratory testing (i.e., *Salmonella* concentration in samples around or below the detection limit of the test)	Negligible	**Assessment:** Sensitivity of *Salmonella* may be hampered when the sample has a low concentration. It is expected that the concentration is high for positive flocks, which is supported by the NVWA retesting results of the routine *Salmonella* monitoring samplings testing positive. **Limitation:** No specific data to support the concentration of *Salmonella* in positive samples.
Inactivation of *Salmonella* during transport to the laboratory	Very low	**Assessment:** Samples collected by NVWA are conditioned and transported according to a strict NVWA protocol (presumed the golden standard description in appendix III). **Limitation:** No documented follow-up of the procedures have been made so far.
Intermittent *Salmonella* excretion	Very low to negligible	**Assessment:** There is a short time (maximally a few days) between routine sampling and retesting; with a low excretion of *Salmonella* in a limited number of chickens, the chance of detection may be lower. **Limitation:** More data about the length of excretion an interval between shedding cycles should be available.
Treatment of poultry with antibiotics	Negligible	**Assessment:** Five random chickens are included in every retest by the NVWA. (after initial positive test by poultry farmer) and tested for antibiotic residues; results were at all times negative. **Limitation:** Small limitation
Acidification of drinking water after initial positive sampling	Negligible	**Assessment:** There is a very short time (maximally a few days) between routine sampling and retesting, which limits a hypothetical effect of acidification of drinking water. **Limitation:** Small limitation

Assessment of the post-test probability of infection of a breeding broiler flock following a monitoring routine positive test

Four specificity loss scenarios were considered which assumed that the false positive rate, compared to the observed apparent false positive rate of 0.02% (Table 2), was 5 to 50 times higher (0.1% (Sp = 99.9%) to 1% (Sp = 99.9%)) or 2 to 20 times lower (0.01% (Sp = 99.99%) to 0.001% (Sp = 99.999%)). The post-test probability of infection or positive predictive value of the routine Salmonella test tends to decrease sharply at low prevalence (Fig. 2).

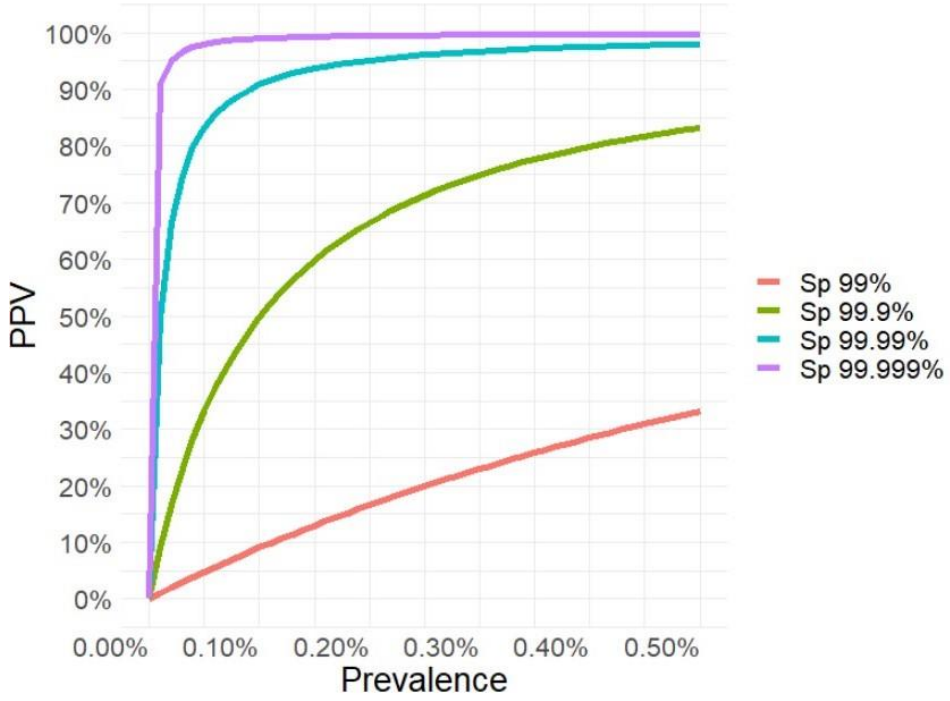

Fig. 2 Relationship between the positive predictive value (PPV) of a positive test result and prevalence of a disease. For calculations, test specificities (Sp) from 99% to 99.999% and a test sensitivity (Se) of 99% - when the within-flock prevalence is higher than 10% - were used.

The predictive value of the initial positive test result (PPV) at a 0.04% prevalence of infected farms would range, therefore, between 3.8% and 97.5% for Sp values ranging from 99% to 99.999%, respectively (Table VI). Considering the prevalence observed in retesting samples (0.02%) the positive predictive values range between 1.9% and 95.2% (Table VI).

Table VI Results of the four scenarios set to assess the positive predictive value (PPV) of the *Salmonella* routine testing in poultry breeding flocks in the Netherlands. The prevalences used are 0.02% and 0.04%

Scenario (failure rate)	Sp	PPV (prevalence 0.02%)	PPV (prevalence 0.04%)
1 – (1/100)	99%	1.9%	3.8%
2 – (1/1,000)	99.9%	16.5%	28.4%
3 – (1/10,000)	99.99%	66.4%	79.8%
4 – (1/100,000)	99.999%	95.2%	97.5%

Questionnaire

A total of 97 poultry veterinarians from 22 European countries were sent the on-line questionnaire by e-mail and 65 poultry veterinarians from 21 European countries (Austria, Belgium, Bulgaria, Cyprus, Denmark, Finland, France, Germany, Great-Britain, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Poland, Portugal, Romania, Spain, Sweden, and Switzerland), responded to the targeted questions in the questionnaire (response rate: 67%). About 90% of the responding poultry veterinarians indicated that they believe it is possible that cross-contamination during sampling by the farmer can lead to a false positive Salmonella test result. That statement was done with an average certainty of 79% (Figure 3).

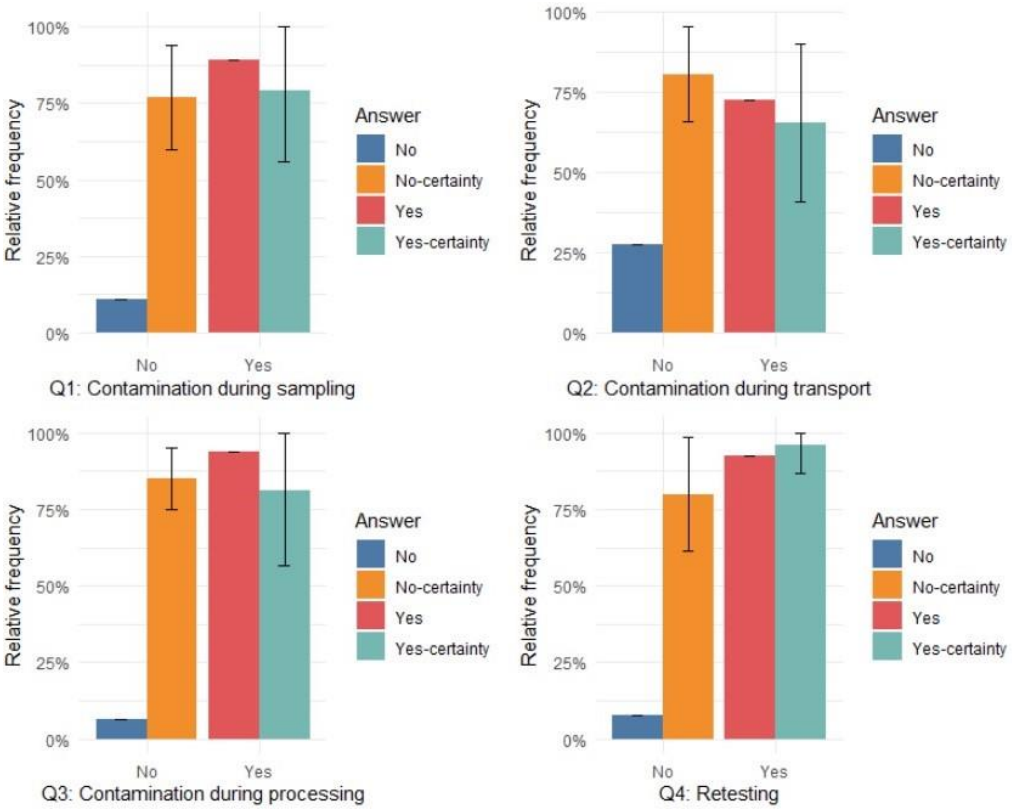

Fig. 3 Poultry veterinarians (N=65) responding to questions (Q1 - Q3) on source of contamination of Salmonella samples in poultry breeding flocks leading to false positive routine Salmonella monitoring results and the need for making resampling and retesting possible of routine Salmonella monitoring positive results (Q4)

Furthermore, 70% of the responders indicated that contamination may happen during the transport of samples from the farm to the laboratory, with an average certainty of 63%. A total 94% of the responders indicated that they consider cross-contamination at the laboratory possible, with an average certainty of 81%. Ranking the possible sources of cross-contamination, respondents ranked first sampling at the farm, followed by cross-contamination at the lab and then cross-contamination during transport (Figure 4).

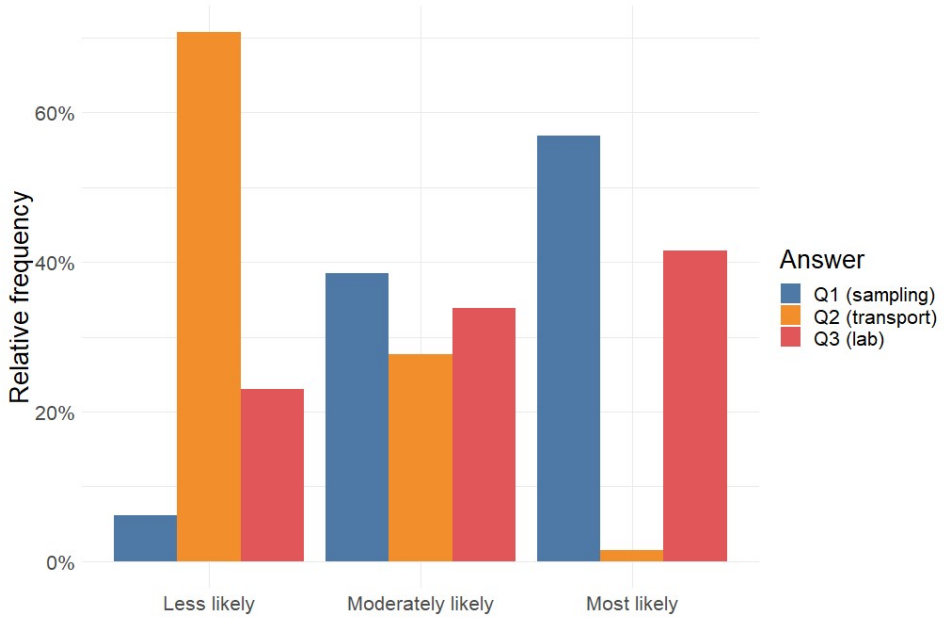

Fig. 4 Poultry veterinarian (N=65) opinions on the less, moderate and most likely source of contamination of Salmonella samples in poultry breeding flocks leading to false positive routine Salmonella monitoring results.

A total of 92% of the responders 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 done with an average certainty of 96%.

Conclusions

Salmonella prevalence in poultry breeding flocks in the Netherlands is low, and the PPV of an initial positive test is also low, which, in line with the re-test findings of the NVWA, justifies an official resampling and retesting by the competent authorities. It is unethical to cull a poultry breeding flock in the Netherlands based on a routine Salmonella monitoring sampling positive test result, because in only about 50% of the cases this reflects the true Salmonella status of the flock.

Various possible explanations for obtaining false positive results during routine *Salmonella* samplings have been provided and their probable contribution toward obtaining a false result assessed. In addition, we received response to a questionnaire on the subject of false positive Salmonella test results of poultry breeding flocks from experienced European poultry veterinarians. Taken together, the results of our assessment and those of the questionnaire, we conclude that the most likely cause of the considerably high occurrence of false positive samplings (48% of initial positive samplings) is cross-contamination during the sampling process by the poultry farmer. Cross-contamination of samples during transport from the farm to the laboratory and/or false positive testing in the laboratory is also considered possible.

In the meantime, in response to our evaluation, the Dutch poultry industry commissioned the present routine Salmonella sampling protocol to be redesigned, taking into account the possible flaws mentioned in the evaluation. It is highly recommended that communication of the new protocol by the poultry industry to the farmers is detailed and explicit in wording and visualization using flyers, demonstration workshops performed by experienced veterinarians and possibly making videos available on the website of the poultry industry.

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APPENDIX 1

The epidemiological principles behind sampling with a diagnostic test

(Source: Dohoo I, Martin W, Stryhn H. Screening and diagnostic tests. In: Veterinary Epidemiologic Research. AVC Inc. publishers, Charlottetown, Prince Edward Island, Canada, 2003 (www.upei.ca/ver). pp. 85-120).

The **sensitivity** of a test is the proportion of subjects (individual but may also be a farm) who test positive among all those who actually have the disease/contamination. Mathematically expressed as $P(T+|D+)$, where (T+) stands for positive test and (D+) stands form positive sample.
The **specificity** of a test is the proportion of subjects who test negative among all those who actually do not have that disease/contamination. Mathematically expressed as $P(T-|D-)$, where (T-) stands for negative test and (D-) stands for negative sample.

The predictive values are directly related to prevalence and allow you to clinically say how likely it is that the tested subject has a specific disease. The **positive predictive value** (PPV), or post-test probability of infection, is the probability that following a positive test result, that individual will truly have that specific disease. PPV can be expressed mathematically using the Bayesian theorem:

$$PPV = P(D+|T+) = \frac{P(T+|D+)*(D+)}{P(T+|D+)*(D+)+(1-P(T-|D-)*(D-))},$$

and the **negative predictive value** (NPV) is the probability that following a negative test result, that individual will truly not have that specific disease. Similarly to the PPV, NPV can be expressed mathematically using the Bayesian theorem:

$$NPV = P(D-|T-) = \frac{P(T-|D-)*(D-)}{P(T-|D-)*(D-)+(1-P(T+|D+)*(D+))}.$$

The predictive value of a test result is an important parameter because it is a reflection of the way in which a diagnostic test should be interpreted in practice.. Because, in many cases the question in the field is: given the fact that a subject is tested positive, what is the probability that the subject actually has the disease?
As prevalence of disease decreases, the PPV decreases because there will be more false positives for every true positive. This is because we're looking for a "needle in a haystack" and likely to find lots of other things that look similar along the way – the bigger the haystack, the more frequently one mistake things for a needle. Conversely, as prevalence decreases, the NPV (our degree of confidence that the farm is not infected) increases.

APPENDIX 2 Questionnaire on the potential for contamination of samples during the process of *Salmonella* testing in poultry breeding farms

Name: Country:										
Q1: In your experience as a poultry practitioner, do you think that it is possible to get a false positive *Salmonella* result from a farm because samples taken by the farmer have become contaminated through cross-contamination during sampling?									( ) Yes ( ) No	
How sure are you about your statement regarding Q1 (please indicate on a scale from 1-10)?										
0 Not sure	1	2	3	4	5	6	7	8	9	10 Very sure
Q2: In your experience as a poultry practitioner, do you think that it is possible to get a false positive *Salmonella* result from a farm because samples taken by the farmer have become contaminated through cross-contamination during transport of the samples from the farm to the laboratory?									( ) Yes ( ) No	
How sure are you about your statement regarding Q2 (please indicate on a scale from 1-10)?										
0 Not sure	1	2	3	4	5	6	7	8	9	10 Very sure
Q3: In your experience as a poultry practitioner, do you think that it is possible to get a false positive *Salmonella* result from a farm because samples have become contaminated through cross-contamination in the process of handling and isolation in the lab?									( ) Yes ( ) No	
How sure are you about your statement regarding Q3 (please indicate on a scale from 1-10)?										
0 Not sure	1	2	3	4	5	6	7	8	9	10 Very sure
Q4: Do you think that, given the existence of false positive *Salmonella* results in practice, it is important that all initial positive *Salmonella* results from a farm are confirmed by resampling and retesting from the same barn and farm?									( ) Yes ( ) No	
How sure are you about your statement regarding Q4 (please indicate on a scale from 1-10)?										
0 Not sure	1	2	3	4	5	6	7	8	9	10 Very sure

If you have answered questions Q1 to Q3 with "Yes", what would be your ranking of the likelihood of the appearance of false positives?

	Q1 (Sampling)	Q2 (Transport)	Q3 (Laboratory)
Ranking 1 (most important)			
Ranking 2 (moderately important)			
Ranking 3 (less important)			

APPENDIX 3

Description of sampling protocol by the poultry farmer

(based on a translation from a flyer on AVINED website: https://www.avined.nl/thema/monstername-salmonella-leghennen)

Specific description for boot swab samples

With washed/clean hands, moisten the surface of both specimens of a pair of boot swabs. The boot swabs should be made of absorbent material.

For each poultry house, put a pair of boot swabs over your (rubber) boots and walk around through the entire stable (to be repeated with several pairs of boot swabs).

When leaving the poultry house, put the boot swabs used for sampling in a sterile plastic bag. The bag must be provided with the name and address details of the holder, sampler, farm number, date of sampling, poultry house number, date of birth of flock, farm type, sample type and name and signature of the sender.

Samples must be sent as soon as possible (next day at the latest) to an accredited laboratory.

APPENDIX 4

(Based on a translation of the work instruction VERD PRO 1216v of the Netherlands' Food and Consumer Product Safety Authority (NVWA))

Work instruction handling suspicion of zoonotic *Salmonella* at the poultry farm

The instructions for official samples taken by veterinarians to confirm a case of a positive flock in the Netherlands are divided into three steps: i) preparing the visit, ii) visit at the location, and iii) finishing the visit.

Preparing the visit

Preparation actions	**Considerations**
Receive the notification	**Receive the notification and case order from NVIC by phone or email.**
Print out the information	**Print out the work order and the attachments, bringing it all with you.**
Inform the farmer	**Inform the farmer about the suspected case of *Salmonella* infection and to declare eggs and animals to be officially in isolation until the results of the investigation are known**
Make an appointment with the farmer	**Schedule a date to collect samples from the suspected poultry houses. Ask for the identification of the poultry houses.**
Prepare the sampling and inform NVIC	**Inform the NVIC about the time and location of the sampling.**
Order/require the sample materials	**The containers with boot swabs, including peptone water are delivered to your home via the NVIC. Make sure you have enough large disposable plastic overshoes, disposable plastic gloves, etc.**
Check required PBM's	**Check the hygiene instructions sheet and take it with you.**
Sample form	**Fill an individual sample form per poultry house. Use the RAA number for each barn.**

Visit at location

Preparation actions	**Considerations**
Arrival at the farm, consider hygiene and PPE (private protection equipment) use	**Consult the work instructions for hygiene and occupational health and safety. Also, follow the biosecurity measures from the farm.**

Counting and classification	**Check the number of animals in the poultry house with the data from the farm-system. Register it on the visit report.**
Check the records of antimicrobial use	**Check in the logbook of the farm if antibiotics were used in animals of the suspected flock. If antibiotics were administered, check the information in prescription whether the waiting period has been expired or not. Besides that, 5 individual chickens have to investigated for antibiotic residues. When no antibiotics were administered, inform the farmer that the flock will be cleared from suspicion when the test results of the boot swabs are negative. The antibiotic residue investigation takes about 8-12 days. If antibiotic residues are detected during the investigation, the flock will be declared infected yet.**
Fill in the visit report and the questionnaire	**Note the unique identification number used on the eggs from the suspected poultry house. If eggs are not labeled with a unique identification number per poultry house, this must be stated on the visit report.**
Call the currier	**Warn/call the courier to be prepared to pick up the samples for transport to the lab**
Sampling procedures  	**Boot swabs (5 pairs per suspect poultry house)** **● Put on coveralls provided by the farm. If this is not present, put on a second disposable coveralls.** **● Put on plastic disposable overshoes over your socks before you put on the (rubber) boots provided by the farm.** **● Before taking samples, wash your hands thoroughly with disinfectant soap and put on disposable gloves.** **● 5 pairs of boot swabs are used per suspect poultry house.** **● The boot swabs come in pairs in a jar with peptone water.** **● Put a RAA sticker with a serial number on the outside of each jar. See image.**

	**• Take a large seal bag or clean bucket containing the jars into the poultry house.** **• After sample preparation put on a pair of clean disposable gloves.** **• First, put on a pair of large plastic disposable overshoes in the stable over the farm boots.** **• Put the 5 pairs of boot swabs over each other over the plastic disposable overshoes.** **• Walk slowly through the poultry house over the parts with manure. Walk about a fifth of the area to be sampled, and pull off the outer pair of boot swabs. Fold the pair of boot swabs inside out, so that the adhering manure remains in the boot swab. Put the pair of boot swabs (1 pair) in a jar and close it immediately. Repeat this until the last pair of boot swabs are put in the last jar just before leaving the poultry house. You now have five jars with two boot swabs each.** **• Put the 5 jars with the boot swabs in the supplied seal bag.** **• Stick a label with the case number and poultry house number on the outside of the large seal bag. This poultry house number must match the poultry house numbers on the map.** **If several poultry houses need to be sampled, wash your hands in between with disinfectant soap and use clean PPE.**
Sampling procedure	**5 chickens** **In the suspect poultry house 5 chickens are selected and taken for testing for the presence of antibiotic residues.** **The chickens are sent by courier to the reference lab, the courier will also take the boot swabs for transport.**
Sample forms RAA0144037-XX	**Fill in a digital sampling form per poultry house. Use a new sheet of RAA stickers for each poultry house. State on the chicken sample form "Poultry scan".**

	Also, stick the barcode stickers with the XX code (for both the boot swabs and the chickens) on the "Report site visit".
Packaging of samples	**Boot swabs** **The different jars with boot swabs are put in a large seal bag and closed.** **Chickens** **The chickens are transported alive in poultry crates to the reference lab**
Address of reference lab	**Put the correct address of the reference laboratory on the shipment; contact the reference lab by phone and report the shipment of samples**
Floor plan	**During the visit, describe the place properly or use the empty floor plan provided by NVIC. Cleary indicate the poultry house identificaions and collects the farmer's signature for confirmation.**
To fil in forms	**Make sure that the following items are filled: floor plan, visit report, attendance list, questionnaire for zoonotic *Salmonella* and digital sampling form.**

Finish the farm visit

Preparation actions	Considerations
Results	**Diagnostic results are known 8 to 10 days after the samples arrived at the reference lab. This is the maximum lead time for S. Enteritidis and Typhimurium serovars. If other serovars are suspected, then the isolate must be submitted to Institute of Public Health and the Environment (RIVM) and this investigation may take from 10 to 13 days.**
PPE use	**For the order in which to get out of coveralls, boots etc., please check the work instruction " hygiene and ARBO" (ALG PRO 1001)**
Outer package samples	**Use "Sterilium" to disinfect the outside of the seal bag with samples when leaving the farm.**
Transfer samples to a courier	**Hand over the samples to the courier yourself and sign digitally.**
Sign out at NVIC	**Report to NVIC by phone that you are leaving the farm.**
Return all forms to NVIC	**Scan the forms and send them as soon as possible by email, ultimately the next working day; return all forms to NVWA.**