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Application Note

Salmonella Detection in the Food Chain: EN ISO 6579-1 Will Give Testing Labs Greater Flexibility

Scope of revised standard to be expanded to milk and milk products, animal feces and environmental samples from the primary production stage.

In early 2016, the International Organization for Standardization (ISO) plans to publish the EN ISO 6579-1 standard, which specifies a horizontal method for the detection of *Salmonella* spp. in the food production chain. Like the preceding version, EN ISO 6579:2002/Amd 1:2007, it will cover products intended for human consumption, animal feeding and environmental samples in food production and handling. But its scope will be broader: it will include milk and milk products (until now described in ISO 6785 I IDF 93, which is planned to be withdrawn) as well as samples from the primary food production stage, such as animal feces, dust and boot swabs.

EN ISO 6579-1 will form part one of the three-part EN ISO 6579, titled "Microbiology of the food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella*". The complementary EN ISO/TS 6579-2, a procedure for *Salmonella* enumeration, and EN ISO/TR 6579-3, a guidance for serotyping of *Salmonella*, have already been published. This white paper is based on the contents of the EN ISO 6579-1 FDIS:2015 (Final Draft International Standard), which in its technical content is likely to resemble the final standard.

New EN ISO 6579-1 at a glance

- Part one (detection) of three-part EN ISO 6579 replaces EN ISO 6579:2004+Amd 1:2007
- ISO 6785 I IDF 93 (dairy products) method details incorporated
- Samples from primary production stage added to scope
- Incubation temperature range for non-selective media extended to 34° – 38°C
- Choice of RVS or MSRV agar for food, animal feed and environmental samples
- Directions for choice of second isolation medium in addition to XLD
- Only one suspect colony needed for confirmation (if negative, 4 more to be tested)
- Optional: biochemical confirmation on well-isolated colony direct from selective plate
- β-galactosidase and indole tests optional, Voges-Proskauer reaction omitted
- Detection of S. Typhi and S. Paratyphi described
- Performance testing for the culture media in-depth included

Outline of the detection procedure

Based on the flowchart in Annex A, figure 1 gives an outline of the Salmonella detection procedure that EN ISO 6579-1 stipulates for food, animal feed and environmental samples from the food production area. It leads through four stages: non-selective pre-enrichment, selective enrichment, plating out and confirmation. This white paper goes through the stages one by one, describing the most important changes that simplify, substantiate or standardize testing requirements. For primary production samples the workflow is very similar. It differs in the selective enrichment step, in which only the usage of MSRV (modified semi-solid Rappaport-Vassiliadis) agar is mandatory.

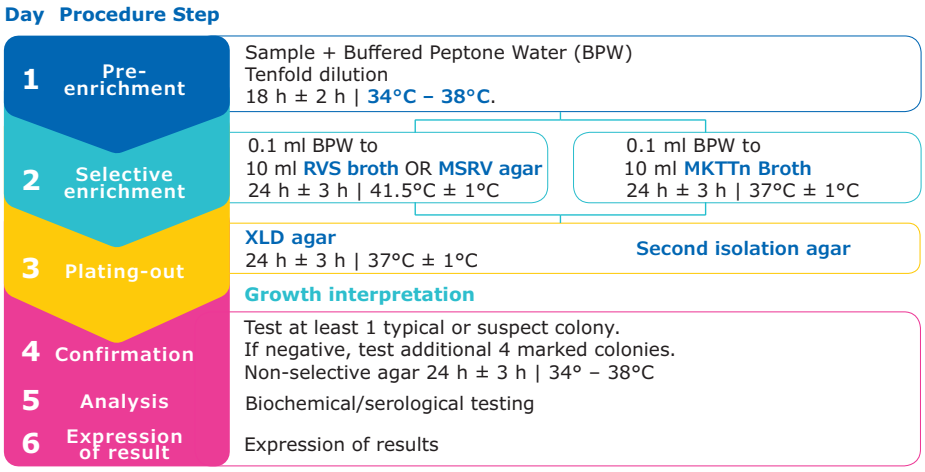


Figure 1: Procedure for Salmonella spp. detection in food production samples according to EN ISO 6579-1 FDIS:2015.

Sample preparation and non-selective pre-enrichment: Greater flexibility and consistency with other ISO standards

For sample preparation, the earlier EN ISO 6579:2002 with Amd 1:2007 contained sets of instructions on how to prepare initial suspensions from the various sample types. Many of these have been omitted from EN ISO 6579-1 FDIS:2015. References have instead been made to different parts of EN ISO 6887 ("Microbiology of the food chain – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination") to match the evolving system of standards relating to microbiology of the food chain. For preparing initial suspensions of milk and milk products, EN ISO 6579-1 will contain a newly added Annex F. In case of discrepancies between the procedures in Annex F and in ISO 6887-5, those in Annex F should be followed. For all sample types, Buffered Peptone Water (BPW) remains the diluent and pre-enrichment medium. One of the most important changes to give labs greater flexibility is the expanded temperature range for incubation. The new lower and upper limits will be 34°C and 38°C – without further tolerance.

MSRV for earlier presumptive positive results

Considerable changes have been made to the selective enrichment stage. For food production samples, EN ISO 6579:2002 had prescribed the selective enrichment in both RVS (Rappaport-Vassiliadis with soya) and MKTTn (Muller-Kauffmann tetrathionate/novobiocin) broths. EN ISO 6579-1 now offers the option to use MSRV agar instead of RVS for such samples. MSRV agar is a semi-solid modification of RVS with a detection principle based on the motility of salmonellae to migrate into the semi-solid medium, forming visible halos due to their swarming. MSRV is intended for the detection of motile Salmonella and is not appropriate for the detection of non-motile ones (e.g. S. Pullorum).

MSRV is inoculated directly from the incubated pre-enrichment medium by transferring 0.1 mL to between one and three equally spaced spots on the surface of the medium. Like RVS, the MSRV plates are incubated at 41.5°C (tolerance: +/- 1°C) for 24 hours +/- 3 hours. Positive MSRV plates will show a grey-white, turbid zone extending out from the inoculated drop. From the positive growth obtained on the agar, the furthest point of opaque growth from the inoculation points is determined. To inoculate an XLD plate, a 1 µL loop is dipped just inside the border of the opaque growth and subsequently withdrawn, ensuring that no large lumps of MSRV are extracted.

Using MSRV instead of RVS simplifies the workflow in the lab, saving time and resources. Presumptive positive results are delivered in 24 hours and thus an entire day earlier than with RVS and subsequent plating on selective agars.* Performance characteristics of MSRV from interlaboratory validation studies have been added to Annex C for informational purposes.

Since RVS and MSRV are comparable media, selective enrichment must additionally be performed in MKTTn broth if food, animal feed and environmental samples from the food production area are tested. The inoculated MKTTn broth is incubated for 24 hours +/- 3 hours at 37°C (tolerance: +/- 1°C). For animal feces and environmental samples from the primary production stage, only MSRV may be used forselective enrichment. For such samples there is no need to enrich in MKTTn.

* For some products, e.g. for dried milk and cheese samples, Salmonella may be sublethally injured. The selective enrichment media from these products have to be incubated for an additional 24 +/- 3 hours.

Plating out: Complementary medium to XLD

EN ISO 6579-1 also provides greater flexibility in the plating-out step of Salmonella detection. The procedures for the inoculation of the isolation medium have been made less prescriptive. This allows lab technicians to use the techniques to which they have become accustomed and which work best in the given laboratory setting.

Another important amendment is that for the second isolation medium, which must be used in addition to XLD (Xylose Lysine Deoxycholate) agar, more detailed directions for the choice are given. Annex E of EN ISO 6579-1 lists several selective culture media from which testing laboratories may choose. This second medium should be complementary to XLD, i.e. be based on different diagnostic characteristics to balance the disadvantages of XLD. One such disadvantage is that lactose-positive Salmonella cannot always be reliably detected on XLD, which may lead to false negative results. Another is that Proteus spp. and the coliform Citrobacter spp. show a tendency to mimic Salmonella spp. on this agar, causing an elevated level of false-positive results. However, several of the listed additional media (e.g. Hektoen Enteric and XLT4 agars) primarily test for lactose fermentation and H_2S production in the same way that XLD agar does. A culture medium that differs significantly from the XLD principle is the chromogenic Rambach® agar1, which tests for β-galactosidase activity and acid production from propylene glycol. This allows Salmonella, Proteus and coliforms to be clearly distinguished by colony color (see figure 2). Due to their different underlying principles, using XLD agar in combination with Rambach® agar (both are available from EMD Millipore as dehydrated and ready-to-use media) is suitable for the identification of the overwhelming majority of Salmonella isolates.

Figure 2: Mixed culture of *Salmonella typhimurium* (WDCM 00031; red colonies), Escherichia coli (WDCM 00013; dark colonies), and *Proteus mirabilis* ATCC 14153; small pale colonies)

Confirmation: Simpler and more flexible

The confirmation step, in which biochemical and serological test results indicate whether an isolate belongs to the genus Salmonella, has seen the most far-reaching improvements for testing laboratories. Only one typical or suspect colony must now be selected for confirmation and subcultivation instead of one such colony from each isolation plate, as EN ISO 6579:2002 had stipulated. This saves time, effort and material resources in the lab. If an isolate tests negative for Salmonella, four more suspect isolates representing different combinations of pre-enrichment and isolation media must be selected for further confirmation. The colonies are subcultivated on a non-selective medium, e.g. Nutrient agar, to generate well-isolated colonies.

In addition to this simplification, a convenient alternative to the above procedure has been included in EN ISO 6579-1: if well-isolated colonies of a pure culture are available on the selective plates, it is now permitted to perform biochemical confirmation directly on such a suspect colony. The purity check on the non-selective medium can then be performed in parallel, which gives testing labs some extra flexibility by freeing up time.

1 Rambach, A. (1990): New Plate Medium for Facilitated Differentiation of Salmonella spp. from Proteus spp. and Other Enteric Bacteria. Applied Environmental Microbiology 56: 301-303.

Biochemical and serological testing requirements amended

Biochemical testing itself has become less prescriptive: the β-galactosidase and indole confirmation tests are now optional and the Voges-Proskauer reaction has been omitted. As EN ISO 6579-1 now also covers primary production samples, the Table for the interpretation of the biochemical tests has been expanded to include two Salmonella strains that are relevant to poultry farming. Serological testing has been allocated to the appropriate parts of EN ISO 6579: serological confirmation to serogroup level is described in EN ISO 6579-1, whereas guidance on serotyping to serovar level is given in EN ISO/TR 6579-3.

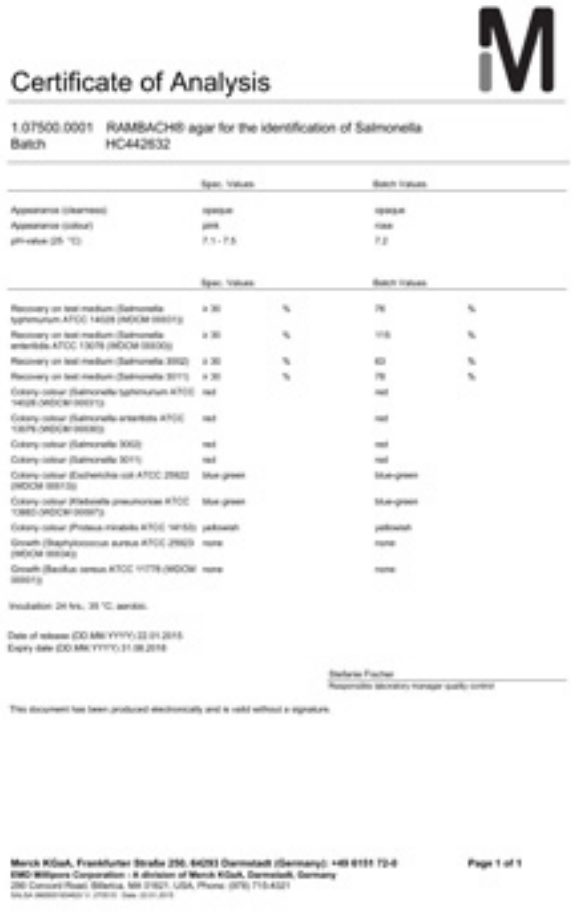
Adapted methods for S. Typhi and S. Paratyphi

To accommodate for the fact that the general EN ISO 6579-1 method does not consistently detect S. Typhi and S. Paratyphi, adaptations are described in the new Annex D that must be followed when these serovars are of specific concern. In the selective enrichment stage, SC (Selenite Cystine) broth must be used as a third medium in addition to RVS broth and MKTTn broth. For plating-out of the two strains, BS (Bismuth Sulphite) agar is the second medium that must be used in addition to XLD agar.

Performance testing details included

While EN ISO 11133:2014 is the general standard describing the methods for quality assurance of culture media to be used in food and water testing, the performance testing of the specific culture media in EN ISO 6579-1 has been added to Annex B. Testing labs that produce their own media and conduct performance testing in-house must follow these specifications meticulously. Labs that instead use ready-to-use culture media can rely on the performance tests that their supplier has conducted, as long as the transport conditions are observed and the supplier's QC test has been performed according to EN ISO 11133:2014 and EN ISO 6579-1 requirements. Details are stated in the quality control certificate, a supporting document that the manufacturer provides to the end user. It discloses the test organisms used, the acceptance criteria of the performance tests and the test results of the batch.

An example of such a certificate from EMD Millipore is shown in figure 3. It also states the WDCM numbers of the test organisms. EMD Millipore has recently updated all the certificates of analysis of its full range of Salmonella detection culture media for compliance with EN ISO 6579-1 requirements.


Certificate of Analysis

1.07500.0001 RAMBACH® agar for the identification of Salmonella
Batch HC442632

Figure 3: EMD Millipore certificate of analysis in accordance with new EN ISO 6579-1 and EN ISO 11133:2014.

Ask-the-expert

As a worldwide leading provider of a broad range of dehydrated granulated and ready-to-use culture media for food, beverages and water microbiology, EMD Millipore closely tracks and supports the development of relevant standards aimed at increasing consumer confidence and safety. EMD Millipore has implemented all the requirements that the new EN ISO 6579-1 demands of its product portfolio. The author of this whitepaper, Barbara Gerten, is an Application Training Scientist at EMD Millipore who can look back on many years of experience in regulatory matters. She is a member of the Technical Committee ISO/TC 34 (Food products), Subcommittee SC 9 (Microbiology), and as such was involved in preparing the first edition of EN ISO 6579-1. As part of EMD Millipore's ask-the-expert setup, Barbara Gerten is available to take questions from customers on this new standard at: www.emdmillipore.com/regulatory-expertise.

How to purchase EN ISO 6579

EN ISO 6579-1 FDIS:2015, as well as EN ISO/TS2 6579-2 and EN ISO/TR 6579-3, can be acquired from www.iso.org and the national standardization bodies. After approval by the ISO members, EN ISO 6579-1 will be published and made available for purchase, probably in early 2016.

References

ISO International Standardisation Organisation:

1. Microbiology of the food chain – Horizontal method for the detection, enumeration and serotyping of Salmonella – Part 1: Horizontal method for the detection of Salmonella spp. EN ISO 6579-1 FDIS:2015.
2. Microbiology of food and animal feed – Horizontal method for the detection, enumeration and serotyping of Salmonella – Part 2: Enumeration by a miniaturized most probable number technique. EN ISO/TS 6579-2:2012.
3. Microbiology of the food chain – Horizontal method for the detection, enumeration and serotyping of Salmonella – Part 3: Guidelines for serotyping of Salmonella spp. EN ISO/TR 6579-3:2014.
4. Microbiology of food, animal feed and water – Preparation, production, storage and performance testing of culture media. EN ISO 11133:2014.

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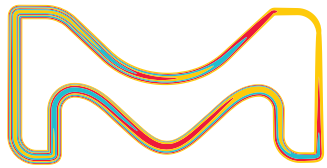

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