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# A review of the International Organization for Standardization (ISO) guidelines for the detection of *Salmonella* from faeces

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## ABSTRACT

Adherence to the guidelines of The International Organization for Standardization (ISO) for the isolation of *Salmonella* from faeces is important to laboratory personnel, researchers and epidemiologists to ensure effective diagnosis, maximal recovery and high sensitivity/specificity of laboratory tests. The aim of this study is to review the ISO guidelines for the detection of *Salmonella* from faeces using standards published by ISO and other internationally recognized bodies. The ISO 6579 of 2002 described the process for the detection of *Salmonella* in faeces. About 25g of faecal samples is collected, mixed together and specimen taken from different parts to ensure maximum recovery of the organism. Processes for the identification of the organism were prescribed by the standard. In the amendment made to ISO 6579 (Annex D) it was recommended that Modified Semisolid Rappaport Vasiliadis (MSRV) be used as selective enrichment medium. Further amendment made to this standard was in 2007 where clause 4 described non-selective pre-enrichment, selective enrichment, use of 2 selective solid media and biochemical tests as the 4 stages of the isolation of *Salmonella* in faeces. The amendment of ISO 6579 in 2017 combined the guidelines for the isolation of *Salmonella* species, *S*. Typhi/Paratyphi from milk/milk-products and faeces. It proposed the use of selenite cysteine broth in addition to Rappaport Vasiliadis (RVS) and Muller-Kauffmann Tetrathionate-Novobiocin (MkTTn) and BSA in addition to Xylose Lysine Deoxycholate (XLD) Agar for the isolation of *S. typhi* and *paratyphi*. It recommended the performance of methyl-red, Simmon-citrate, $H_2S$, motility and urease biochemical tests, deleted Vogues Proskaer test while indole and β-Galactosidase (ONPG) tests were made optional. This standard has evolved over the years to ensure efficient recovery of *Salmonella* from faeces based on real-time result and feedback received by the organization from laboratories all over the world.

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## INTRODUCTION

*Salmonella* species are gram-negative rod-shaped bacteria belonging to the *Enterobacteriaceae* family, they are ubiquitous, hardy and can survive several weeks in dry environment and several months in water **(WHO, 2022).** This group of bacteria possess peculiar biochemical and genetic characteristics that are used in their detection **(Percival and Williams, 2014).**

*Salmonella* is an important foodborne zoonotic pathogen with relevance to global public health **(Oludairo *et al*., 2013a, Gomes *et al*., 2022).** It had been estimated to cause 115 million human infections and 370,000 deaths per year globally **(Qin *et al*., 2022).** The World Health Organization (WHO) estimated that each year, approximately 1 in 10 people become ill with this foodborne infection and millions of years of healthy life are lost due to the disease **(Lee and Yoon, 2021, WHO, 2022).**

*Salmonella* species are spread mainly through contaminated faeces, foodborne illness and transmission of salmonellosis therefore occur when feed and water are contaminated by *Salmonella*-contaminated stools or through direct faecal oral route making faeces important reservoirs of *Salmonella* serovars **(Kumar *et al*., 2009, Nesa *et al*., 2011, Oludairo *et al*., 2013, Ehuwa *et al*., 2021b, EAIDG, 2022, Atlaw, 2022).** Faeces of humans and animals are the primary sources of *Salmonella* from which isolation of *Salmonella* for epidemiological surveys are made **(WHO, 2010a, Liu *et al*., 2012, Drozdz *et al*., 2022).** Several published articles have reported isolation of *Salmonella* from faeces of humans and animals **(Oludairo *et al*., 2013b, Oludairo *et al*., 2013c, Kasumba *et al*., 2021, Dieye *et al*., 2022, Wottlin *et al*., 2022, Shittu *et al*., 2022).**

International Organization for Standardization (ISO) is an independent, non-governmental organization that was established in 1946 with a membership of 167 national standard bodies with central secretariat in Vernier, Geneva, Switzerland (**Fisher, 2016, ISO, 2022a).** The abbreviation, ISO, arrived at by the founders of the organization was derived from the Greek word 'isos' which means 'equals'; the same standard of abbreviation no matter the language or country **(ISO, 2022a).** The organization as at June, 2022 has 24,347 international standards covering virtually all aspect of technology and manufacturing, about 100 standards every month and 808 technical committees and subcommittees to take care of standard developments **(ISO, 2022a).**

There is need for standardization of the methods of isolation of bacteria for the purpose of worldwide procedure uniformity. The organization specified standards for the isolation of *Salmonella*, this includes ISO 3565 of 1975 for the detection of *Salmonella* from meat and meat products, ISO 6579 of 1981, 1990, 1993 and 2002 on the general guidance on the methods for the detection of *Salmonella* **(ISO, 2022b).** The standard for the detection of *Salmonella* from faeces is ISO 6579 of 2002, 2012 and 2017 **(ISO, 2022c).** The aim of this study is to review the International Organization for Standardization's (ISO) methods for the detection of *Salmonella* spp. in faeces.

## MATERIALS AND METHODS

The standard published by the International Organization for Standardization was used as the main resource for the study. Other international and national published standards like that of the World Health Organization (WHO), United States Food and Drug Administration (FDA) and Health Promotion Administration (HPA) that were derived from the general ISO guidelines were used as reference points in the study. Other peer reviewed published work with explanatory notes on ISO guidelines for the detection of *Salmonella* in faeces were retrieved online after thorough search using the google search engine and used for this study.

### Faecal sample collection

Sterilized materials should be used to collect faecal samples on the field or from patients. If containers are used, they should be clean, sterilized and without soap or disinfectant residue **(FDA, 2022).** At least 25g should be collected. In cases where only small faecal samples and swabs are collected, they may be placed on transport media (ISO 2002). The samples must be kept cold and transported within eight hours of collection to the laboratory. If the samples cannot reach the laboratory within eight hours, it should be frozen at < - 70 ˚C or stored on dry ice **(WHO, 2010b).**

### Sample analysis

Personal protective equipment (PPE) should always be used in dealing with faecal samples. Field observation of the texture of the stool should be noted and recorded **(ISO, 2002).** It is empirically believed by some health care providers that cholera is indicated if the stool is watery while bloody stool is thought to be dysentery. This is however, not scientific and not definitive as diarrhoea caused by *Shigella* is bloody only in 50% of the time and there are other aetiological agents that could cause diseases with watery diarrhoea **(WHO, 2002).** Maximal recovery is usually obtained by using enrichment medium. Enrichment media for *Salmonella* are usually very selective and may inhibit certain serotypes of *Salmonella* particularly *S*. Typhi **(WHO, 2002).** *Salmonella* species may not be evenly distributed within a sample; therefore specimen can be mixed prior to testing and should be obtained from several locations within the sample **(WHO, 2002; HPA, 2008).**

### Recovery of *Salmonella* species from faecal specimens

### Day 1

### Non-selective pre-enrichment

Weigh 1 g faeces in to 9 mL buffered peptone water to obtain 1 part sample in 9 part buffer. Mix and incubate at 36˚C (+/- 1˚C) for 16-20 hours. Twenty - five grams of faeces can also be inoculated into 225 mL of buffered peptone water or 20 g faecal material into 180 mL buffer **(HPA, 2007).** This is because the volume of the faeces sampled determines the sensitivity of detection. It is therefore generally advised that large quantity of the sample should be used **(WHO, 2010b).** Pre enrichment will also allow stressed and injured *Salmonella* to recover before exposure to selective media **(Andrews *et al*., 2001).**

### Day 2

Prepare selective enrichment I and II (Tetrathionate broth and Rappaport Vassiliadis (RVS) broth or selenite F broth). Inoculate 1 mL and 0.1 mL respectively into 10 mL each of the selective

enrichment. Incubate the inoculated tetrathionate broth at 37 ˚C and the Rappaport Vassiliadis broth or selenite F broth at 42˚C for 24 hours **(HPA, 2007).** Tetrathionate broth provides good control for *Proteus* and *Pseudomonas* **(WHO, 2010b).**

**Day 3**

Streak 10 µl loop full from the inoculated and incubated tetrathionate broth and RVS broth or selenite F broth on Xylose Lysine deoxycholate (XLD) and Brilliant Green Agar (BGA) and incubate at 37˚C for 24 hours **(WHO, 2010b).**

**Day 4**

Examine the growth on the XLD plates for slightly transparent red halo and a black centre, a pink-red zone may be seen in the media surrounding the colonies **(HPA, 2007; WHO, 2010b).** Examine the BGA plates for red/pink colour. Other enteric agents usually appear green or yellow **(WHO, 2010b).** Afterwards plate suspect colonies from XLD agar and BGA onto non-selective media (e.g. nutrient agar) for biochemical confirmation and serotyping **(ISO, 2002, Brenner and McWhorter-Murlin, 1998; WHO, 2010b).**

**Day 5-7**

**Biochemical identification and serotyping**

As typical of all *Enterobactericeae*, salmonellae are Gram negative, oxidase negative, facultative anaerobes, Voges-Proskauer negative, methyl red positive and reduce nitrate to nitrite without production of gas. They are typically indole and urease negative, although rare indole and urease positive strains may be encountered. They are usually motile, although non motile ones may be encountered including the host adapted avian pathogen *Salmonella* serotypes pullorum and Gallinarum which are always non motile **(WHO, 2003; HPA, 2008).**

## Recovery of *S.* Typhi from faecal specimens

**Day 1**

Maximum recovery of *S.* Typhi from faeces is achieved by using enrichment broth although isolation from acutely ill persons may be possible by direct plating **(WHO, 2002).** Though most enrichment broth used for *Salmonella* isolation will inhibit certain serotypes of *Salmonella* especially *S.* Typhi. The selective medium used to isolate *S.* Typhi therefore is selenite broth **(WHO, 2002).** Gram Negative (GN) broth may also be used for enrichment of *S.* Typhi that can then be incubated for 14-16 hours at 37$^0$C.

**Day 2**

The incubated broths are then streaked to selective agar like Bismuth Sulfite Agar (BSA), deoxycholate agar (DCA), *Salmonella Shigella* agar (SSA) or xylose lysine deoxycholate agar (XLD) **(WHO, 2002).** Other standard enteric media could also be used and these include hektoen enteric agar (HE) and Mac Conkey agar (MAC). However, BSA is the preferred medium for the isolation of *S.* Typhi and should be used if resources permit **(WHO, 2002, ISO, 2002).** Incubation should be done for 48 hours at 37$^0$C. Well-isolated colonies on BSA appear black surrounded by a black or brownish black zone with a metallic sheen. Streaking should be carefully done to prevent crowdedness of colonies, which does not allow *S.* Typhi to produce typical blackening on BSA **(WHO, 2012).** Colonies of *S.* Paratyphi A, B and C and most other *Salmonella* serotypes have similar appearances to *S.* Typhi on MAC, BSA, HEA, DCA and XLD agar (Fig.2) **(WHO, 2002).** In developing countries, typhoid fever is frequently diagnosed solely on clinical grounds; however, isolation of the causative agent is necessary for a definitive diagnosis and the performance of antimicrobial testing for accurate treatment **(WHO, 2003).**

## ISO 6579 of 2002 Guideline for the Isolation of *Salmonella*

The standard method for detection of *Salmonella* in food and animal feed is described in ISO 6579 **(ISO, 2002).** This ISO procedure is less applicable to the detection of salmonellosis in animal faeces. For this reason, another selective enrichment medium was selected: Modified Semi Solid Rappaport Vasilliadis (MSRV). The full procedure for the use of this new medium is as described in the annex of ISO 6579, Annex D **(ISO, 2007; Veenman *et al*., 2007; PHE, 2014).** The MSRV is recommended for the isolation of *Salmonella* in products with high background flora for example animal faeces and environmental samples in areas of primary production such as dust **(ISO, 2002, ISO, 2007; PHE, 2013).** The medium is intended for the detection of motile salmonellae and is not appropriate for the detection of non-motile salmonellae **(De Smedt *et al*., 1986; ISO, 2007).**

The number of existing non motile *Salmonella* are usually low, the most common serotypes are *S.* gallinarum and *S.* pullorum which appear not to survive long and as a result are rarely detected in faeces and environmental samples like dust regardless of the method used **(ISO, 2007).** Many previous studies that have reported very low *Salmonella* detection rates could have been because the biovar of *Salmonella* could not survive long in faeces or environmental samples **(ISO, 2007).** It could also be because only selective media were used which may not be the recommended MSRV leading to the reportage of high number of false negatives **(De Smedt *et al*., 1986; ISO, 2007).**

## Amendment to ISO 6579 of 2007 Guideline for the Isolation of *Salmonella*

The 2007 Annex D amendment, clause 4 to the ISO, 2002 protocol described four stages in the detection of *Salmonella* in animal faeces (Fig. 1) **(ISO,**

**2007).** Pre-enrichment is done in non-selective liquid medium like buffered peptone water (BPW); it is inoculated with the test portion at ambient temperature and incubated at 37+/-1 ˚C for 18+/-24 hours (**ISO, 2007).** This is followed by enrichment of the pre-enriched test samples on selective semi solid medium, which is the Modified Semi Solid Rappaport Vassiliadis (MSRV) agar plates and incubated at 41.5 ˚C+/-1 ˚C for 24 hours +/- 3 hours.

Plates that are negative after 24 hours are further incubated for 24h +/- 3h **(ISO, 2007; Veenman *et al*., 2007).** Further tests and identification are done after using two selective solid media, XLD agar and any other solid selective media complementary to XLD agar like BGA, SSA or Brilliance *Salmonella* agar. The second selective media is incubated according to Manufacturer's instructions **(ISO, 2007).** Presumptive *Salmonella* are then plated out/sub cultured and their identity confirmed by means of appropriate biochemical and serological tests **(ISO, 2007).**

The MSRV should be allowed to equilibrate at room temperature if storage was done at lower temperature, they should then be inoculated with 3 drops (total of 0.1 mL) of incubated BPW culture and should be placed separately and equally spaced on the surface of the medium **(Veenman *et al*., 2007).** Samples from the BPW should not be mixed, shaken or swirled, inoculum should be extracted from largest volume of free fluid nearest the interphase between container and surface of the culture, but it is advisable to go deeper if there are particles floating on the surface **(ISO, 2007).**

Positive plates will show grey-white, turbid zones extending out from the inoculated drop; the turbid zone is characterized by a white halo with a clearly defined edge **(ISO, 2007).** To sub culture positive MSRV plates, determine where the furthest point of spread of opaque growth from the inoculation point is and dip a 1 µl loop just inside the border of the opaque growth, withdraw the loop ensuring no large lumps of MSRV are extracted. Inoculate the surface of an XLD plate so that well isolated colonies will be obtained, use a new sterile loop to repeat the same procedure for the second selective plating medium **(ISO, 2007; Veenman et *al*., 2007).** Incubate the XLD plates at 37 ˚C +/-1 ˚C for 24 h+/-3 h and perform the selective plating procedure if after 48 hours of incubation, other MSRV plates become positive **(ISO, 2007; Veenman *et al*., 2007).**

Typical colonies of *Salmonella* on XLD agar have a lightly transparent zone of reddish colour and black centre while the *Salmonella* $H_2S$ negative variants (e.g. *S*. Paratyphi A) are pink with a darker pink centre while lactose positive *Salmonella* on XLD agar are yellow with or without blackening (Table 1 **(ISO, 2007; HPA, 2008).** For confirmation, it is prescribed that isolated colonies from the selective plating media be streaked on nutrient agar before performing the biochemical confirmation **(ISO, 2007).** This extra cultural step may not be necessary if well isolated colonies of pure cultures are available on the selective plating media **(ISO, 2007).**

## ISO 6579 of 2017 Guideline for the Isolation of *Salmonella*

In 2017, the new guideline/standard for the isolation and identification of *Salmonella* was published (ISO, 2017). This incorporated the methods of isolation from milk and its products in ISO-6785 and guidelines for the collection of samples from animal faeces in the environment and detection of *S*. Typhi and Paratyphi **(ISO, 2001; ISO, 2002; Veenman, 2007; ISO, 2017).** For the isolation of *S*. Tyhpi and Paratyphi, it was proposed that selenite cysteine (SC) broth additional to Rappaport Vassiliadis (RVS) and Muller –Kauffmann tetrathionate-Novobiocin broth (MkTTn) be used for selective enrichment while BSA, additional to XLD be used for selective plating (Fig.2) **(Mooijman, 2014).**

The publication **(ISO 2017)** is less specific about the type of Buffered Peptone Water (BPW) to be used for the pre-enrichment of collected samples as opposed to ISO-6579 and ISO-6579-1 of 2002 and 2007, respectively which specify the BPW to consist of 'enzymatic digest of casein' **(ISO, 2002; ISO, 2007; ISO, 2017).** The range of incubation temperature for the non-selective pre-enrichment media is now broader. While ISO 6579 indicates 37˚C+/-1 ˚C, the new publication proposes the incubation temperature to be between 34 ˚C and 38 ˚C. Only MSRV is recommended for samples from primary production as mentioned in Annex D of ISO 6579 of 2007. While the first selective enrichment could either be done with RVS or MSRV and the second selective enrichment should be done with MkTTn for samples from other sources **(Mooijman, 2014, ISO, 2017).**

The new guideline further recommended that the pH of the MkTTn should be between 7-8 while the incubation period for selective enrichment media has been retained as 24 h except for dried milk/cheese and samples from primary production stage (on MSRV), which may be incubated for 48 h, if necessary **(Carrique and Davies, 2008; Mooijmann, 2016; ISO, 2017).** In the plating out and confirmation, XLD is retained as the mandatory isolation medium, confirmation can also be done based on only one suspect colony instead of the colonies of the two plating media, this allows for the performance of parallel biochemical testing/purity check. The use of non-selective medium for purification has been left to the researcher's choice **(ISO, 2017).** For confirmation of isolates, two tests; β-Galactosidase (ONPG) and indole reaction have become optional while Voges-Prokauer reaction test has been deleted (**Mooijmann, 2014; ISO, 2017).**

Some other biochemical tests that could be carried out on presumptive isolates include methyl red, Simmons citrate, $H_2S$, motility and urease tests; for which triple sugar iron agar (TSI), kliger iron agar (KIA), urea broth, Simmons citrate agar, MRVP broth, SIM agar could be used according to Manufacturer's instructions. Reagents like Kovac's, methyl red and Barritt's reagents A (alpha-naphthol and B (KOH) will be needed after incubation to complete some of the tests.

Table 1: Characteristics of *Salmonella* growth on different agar media:

| Selective agar* | Colour of colonies* | Size of colonies* |
|---|---|---|
| Bismuth sulfite agar (BSA) | Black, surrounded by a black or brownish zone with a metallic sheen | 1 – 3 mm |
| MacConkey agar (MAC) | Transparent or colourless opaque | 2 – 3 mm |
| Hektoen enteric agar (HE) | Blue-green (with or without black centres) or yellow with black centres | 1 – 2 mm |
| Xylose lysine desoxycholate agar (XLD) | Red (with or without black centres) or yellow with black centres | 1 – 2 mm |
| *Salmonella-Shigella* (SS) agar | Colourless | 1 – 2 mm |
| Desoxycholate citrate agar (DCA) | Colourless | 1 – 2 mm |

* Most *Salmonella* serotypes appear similar to *S.* Typhi on these media; therefore, confirmatory testing is necessary. Source: WHO, 2003.

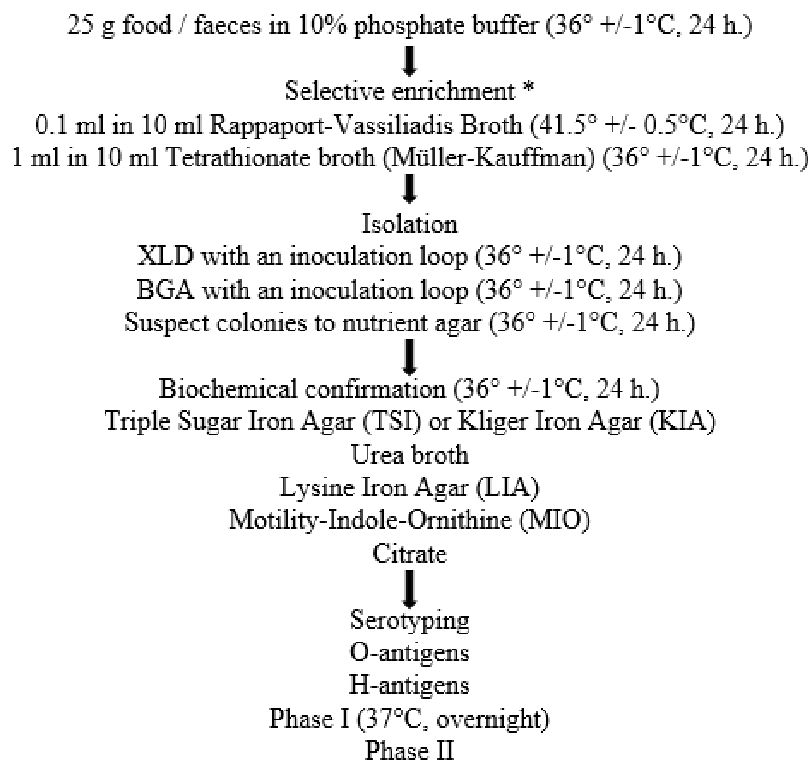
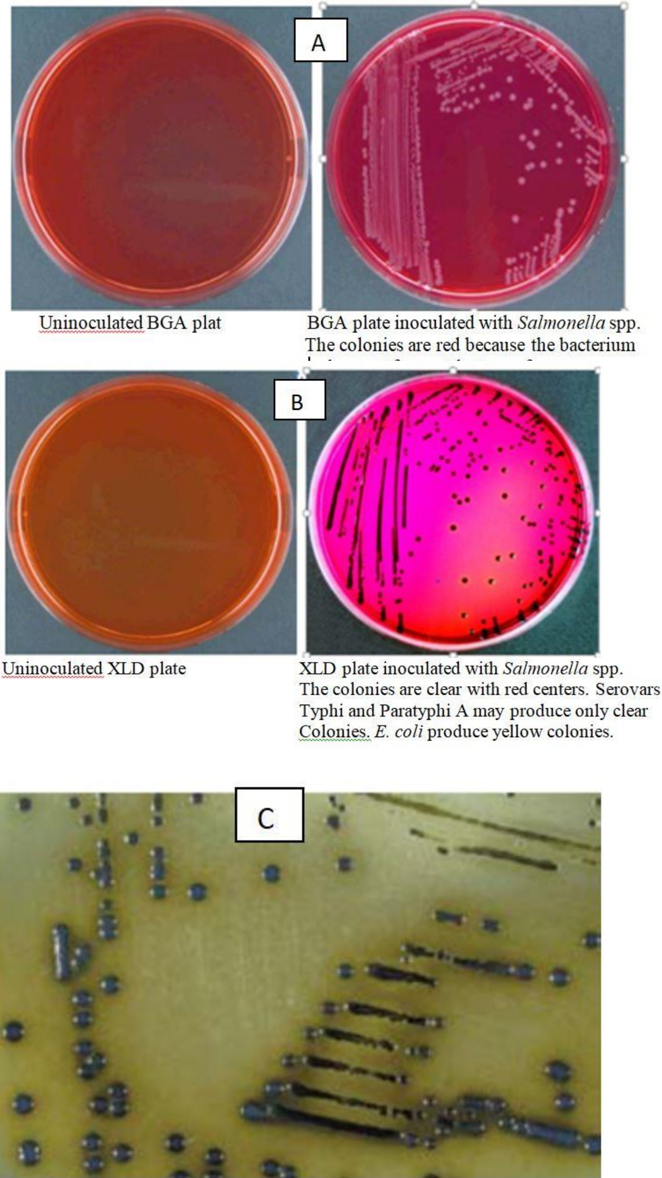



Fig. 1: Flow diagram for isolation/identification of *Salmonella* from Food / Animal Faeces **(WHO, 2010b).**




*Salmonella* ser Typhi colonies on Bismuth sulphite (BS) agar

Fig.2: Uninoculated plates and growth of *Salmonella* on BGA (A), XLD (B) and BS (C) agar Plates **(WHO, 2010b).**

## CONCLUSION

### The guideline for the isolation of *Salmonella* is described in ISO 6579

For the detection of *Salmonella* spp. in faeces, clean, sterilized materials should always be used to collect about 25g of faecal materials. Small faecal samples or swabs should be placed on transport media. If sample analysis cannot commence within 8 hours of collection, it should be stored at -70ºC or on dry ice until ready for analysis. Maximum recovery of *Salmonella* is obtained if enrichment media are used. Faeces should be mixed before testing is done while sample can be taken from different parts of the specimen. Non-selective pre-enrichment, selective enrichment could be done before plating in solid media for good recovery of *Salmonella* serovars. This should then be followed by biochemical identification and serotyping of presumptive isolates.

For the isolation of *S*. Typhi from faecal samples, the recommended enrichment broth for maximum recovery is selenite F or Gram-negative broth. Faecal samples from actively ill persons may be plated directly on the recommended enteric solid media; Bismuth Sulfite Agar (BSA) for possible isolation. The effective isolation of *S*. Typhi which is the causative agent of typhoid fever is important for the definitive diagnosis of the disease.

Annex D of the 2002 ISO 6579's guideline for the isolation of *Salmonella* from faeces recommended the use of Modified Semisolid Rappaprt Vasiliadis (MSRV) as selective enrichment media for motile *Salmonella*. The amendment to this guideline was done in 2007 where clause 4 described the 4-stage protocol for the isolation of the organism which are; pre-enrichment using BPW, enrichment using MSRV, 2 selective media – XLD and BGA/SSA and biochemical tests on the colonies from the 2 solid media. The 2017 amendment contained procedures for the isolation of *Salmonella* from milk/milk products and faeces including guideline for the isolation of *S*. Typhi and Paratyphi. Selenite cysteine broth was proposed to be used for the isolation of *S*. Typhi and Paratyphi in addition to RVS and MkTTn while BSA is to be used in addition to XLD. Other amendments include; carrying out confirmation of isolates based on only colonies of one plating medium instead of the 2, leaving use of non-selective media for organism purification to researcher's discretion, recommendation of methyl-red, Simmon-citrate, $H_2S$, motility and urease tests as biochemical tests, deletion of Voges-Prokaer test including making indole and β-galactosidase (ONPG) tests optional.

## RECOMMENDATION

Researchers and laboratory personnel need to be conversant with the recommended methods for the isolation of *Salmonella* so as to comply with best standard practices. Laboratory protocol for the detection of *Salmonella* should be designed in line with the guidelines of the international organization for standardization. Adherence to the ISO standard in the isolation of *Salmonella* will enhance uniformity, reproducibility and fair basis for the comparison of results of research work world over. Protocols and methods of isolation of *Salmonella* should be updated in line with reviews and amendments of the International Organization for Standardization as published by the organization from time.

## Conflicting Interests

The authors declare that there is no conflict of interest.

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