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Isolation and Identification of *Salmonellas* from Different Samples

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1. Introduction

Salmonella causes various infections in humans. Contamination of people by *Salmonella* may be caused by infected persons, animals and direct contact of those with fluids *Salmonella* also has an important role in producing pathogens that cause food poisoning. *Salmonellas* act as primary reservoir for foods such as chicken meat, milk and milk products, eggs and meat products etc. Some of microorganisms such as Coliform bacteria have same features with *Salmonella*. For that reason, isolation and identification of *Salmonella* from clinical and other samples are important.

2. *Salmonella*

Efforts related with classification of these bacteria since first *Salmonellas* has been found also continues recently. When *Salmonella* bacteria are examined by DNA/DNA hybridization trials which are performed among bacteria, all *Salmonellas* should be accepted as one species including Arizona species added to them. According to this, subgeneruses that Kauffman has created among *Salmonellas* according to genetic and other characteristics (subgenus 1, subgenus 2, subgenus 3= Arizona and subgenus 4) and subgenus 5 which was added by Le Minor should be accepted as sub-species instead of subgenus.

Up to the present, *Salmonella* bacteria were named according to their pathology, their host and the city where they have been found first and an attention was paid to use an individual name for every bacteria within the same antigen structure in Kauffman-White classification. These bacteria which were accepted as individual serovars were classified as separate species. It is known that these characteristics of bacteria are not appropriate and sufficient to determine a species and none of the methods that has been used up to the present is scientific in terms of taxonomy. Furthermore, international enterobacteriaceae subcommittee which is the most reliable organization about this subject have not performed a scientific guidance about various *Salmonellas* serovars classification. While studies continue, this committee has suggested as follows:

To protect validity of Kauffman white classification without having a bias related with definition of this species; to keep names of bacteria in *Salmonellas* subgenus 1 (like individual species names) by continuing a normal tradition in medicine, clinic and microbiology up to the present; when new bacteria which comply to this subgenus is found, to classify them

individually; to keep names of bacteria which have been found up to the present in other subgenus similarly, but in case of finding new bacteria which comply to this genus, to classify them only by antigenic formulas.

The idea which has arisen lately is that all *Salmonellas* including Arizona are single species and to classify this species as *Salmonella enterica*. Discriminations related with antigenic, biochemical, host and geographical distribution which are seen among bacteria has been depended on differentiation of this single species. Six subgroups were detected as a result of researches performed by DNA hybridization methods.

These are; *Salmonella*: 1 subgroup including *s. enterica* subspecies; *Salmonella* : 2 subgroup including *salamae* subgroup; *Salmonella*: 3a and *Salmonella*: 3b subgroups including *arizonae* and *diarizonae* subspecies; *Salmonella*: 4 subgroup including *hautenae*; *Salmonella*: 5 subgroup including *bongori* subspecies and *Salmonella*: 6 subgroup including *indica* subspecies.

In practice, bacteria are named as serovar names and for example, to use only *Salmonellas* serovar typhimurium, even *Salmonellas* serovar typhimurium name is preferred instead of long names such as *Salmonella enterica* subsp., *Enterica* serovar typhimurium.

2.1 Appearance and staining characteristics

Salmonella bacteria are asporogenic, capsule-free, motile via peritrichous cilium (*Salmonella gallinarum* or *Salmonella pulorum* are immotile), rod-shaped bacteria with an approximate length of 2,0-5,0 μm , width of 0,7-1,5 μm . They are stained well with bacteriologic stains and they are gram-negative (Figure 1). Most of them have type 1 (mannose sensitive (ms), hemagglutinating); *S. Gallinarum* and some origins have type 2 fimbriae. *S. paratyphi* As do not have fimbriae.

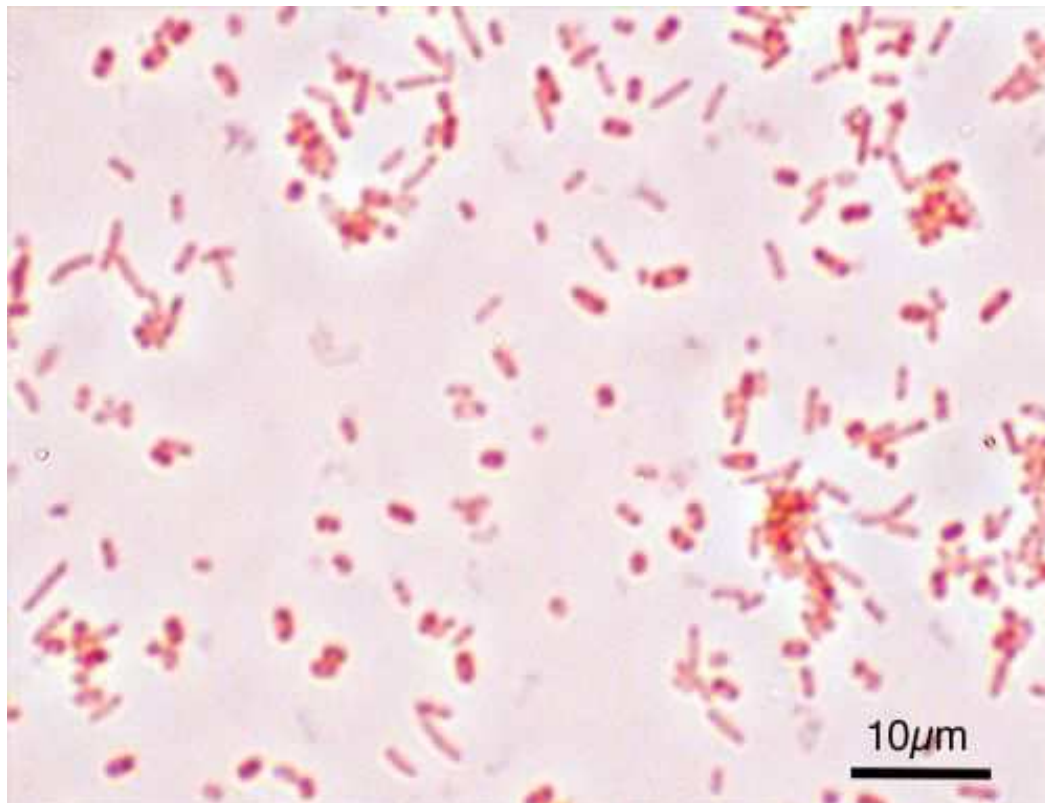


Fig. 1. Microscopic View of *Salmonella*

2.2 Reproduction and biochemical characteristics

Salmonella bacteria reproduce in many ordinary mediums. They are aerobe and facultative anaerobe. Their reproduction temperature limit is very wide even they reproduce at 37° C best. (20°C- 42°C). This is extremely important for reproduction of *Salmonellas* which cause food intoxication at room temperature. They like to produce at average pH of 7,2. They make homogenous turbidity in bouillon and similar liquid medium. They make round, slab sided, mostly tumescent colonies with a diameter of 2-3 mm, regular surface. In colonies of various *Salmonellas*, some differences may exist in terms of size, protuberance, surface and side. *Salmonella typhi* may also make gnome colonies which may reach to 0,2-0,3 mm diameter within the first 24 hours. Biochemical characteristics of bacteria which are obtained from these colonies are same as normal colonies; and they are agglutinated with O serums only antigenically and they differ from bacteria in S colonies in terms of not reacting with anti H, anti Vi serums. If they are reproduced in mediums including sulfurous compounds, sulfates and tiosulfates which may be assimilated, normal colonies occur from bacteria that make gnome colonies.

Some of *Salmonellas*, *S. Schottemuelleri* (s. paratyphi) in particular and some others form M colonies in appropriate mediums. It is detected that these bacteria have M antigens and agglutination is prevented by anti O and anti H serums. Furthermore, R colonies are formed by *Salmonella* which reproduce in inappropriate mediums (Figure 2).

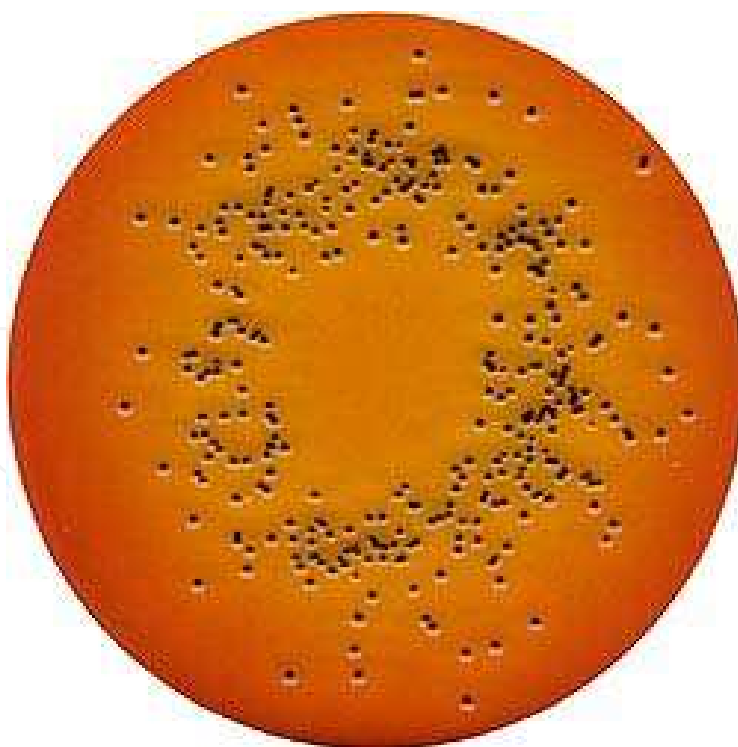


Fig. 2. *Salmonella* colonies

Salmonellas are not effective on lactose. This characteristics is important in first differentiation from *Escherichias*. As these bacteria which are planted in a separator plaque medium (endo, EMB) including lactose and an appropriate reagent are not effective on lactose, they make colorless colonies; however those effective on lactose make dark red, black, greenish bright colonies (Figure 3).

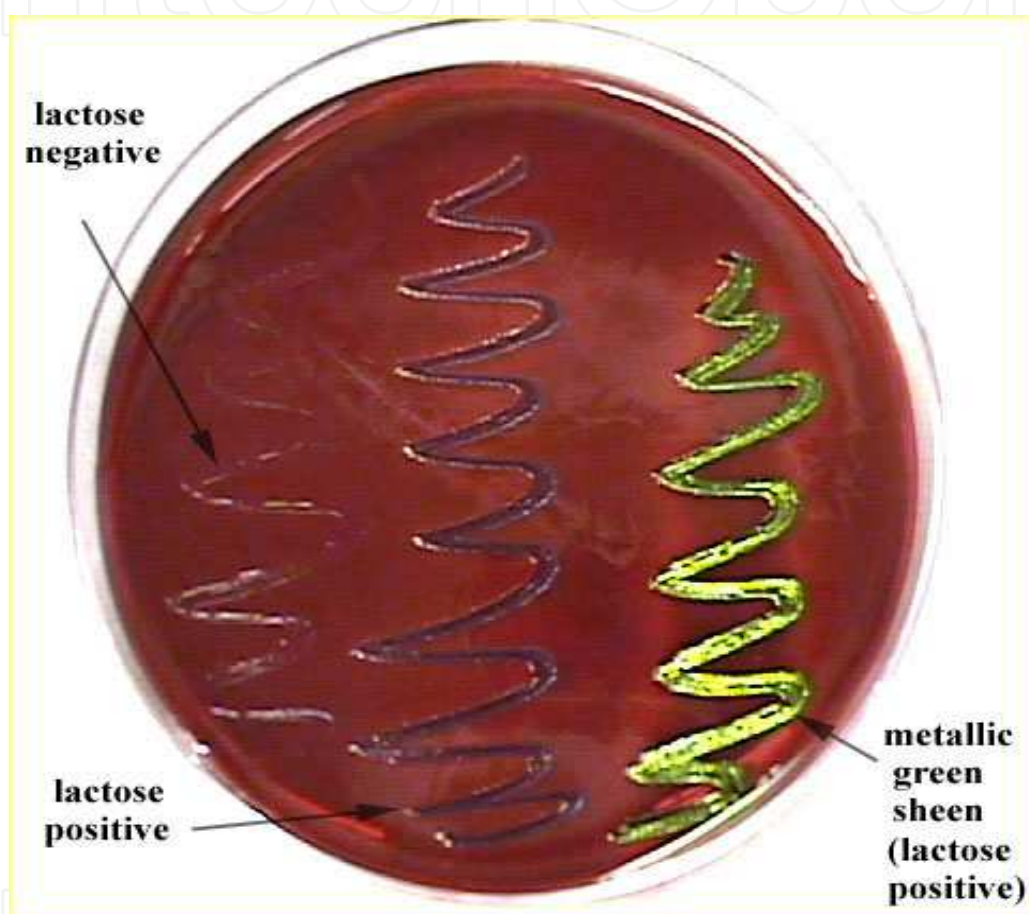


Fig. 3. View of *Salmonella* and Lactose Positive Colonies

Salmonellas do not effect on sucrose, adonitole and salicin in usual other than lactose. They digest glucose, mannite and maltose by producing acid and gas except *Salmonella typhi* and *S. gallinarum*; and *Salmonella typhi* and *gallinarum* digest them by producind acid only. They produce H₂S in general (except *S. paratyphi A*); they are indole negative, methyl red positive, Vogesproskauter negative and they reproduce in citrated mediums (Simmon), they do not digest urea. They could not be produced in KCN (potassium cyanide 0,5%) mediums. ONPG (orthonitro phenyl galactopyranoside) assay is negative. (They do not have beta galactosidase enzymes that may digest lactose). This assay is positive in Arizona. Biochemical characteristics of *Salmonellas* were shown in Table 1.

Motility	+
Indole	-
H2S	+
Oxidase	-
Urease	-
Nitrate reduction	+
Citrate Utilization	+
MR	+
VP	-
Lysine decarboxilation	+
Ornithine decarboxilation	+
Phenylalanine deamination	-
Malonate Utilization	-
Lactose	-
Sucrose	-
Salicine	-
Inositol	-
Amygdalin	-
Gas Production from glucose	+
β-galaktosidase (ONPG Test)	-
Reproduction in KCN	-
(-) Negative (+) Positive	

Table 1. Biochemical characteristics of *Salmonellas*

3. Important terms in taking examination sample

As delay in the diagnosis of acute infection is unhealthful in terms of delay in the treatment and it is also dangerous that other persons may be infected due to more contact. To obtain rapid and correct etiological diagnosis is possible to take the examination material appropriately, to send it to the laboratory rapidly and to examine them in the laboratory well. Inability to produce the active germ is due to faulty examination material taking in general. Examination material should be taken by persons who know the purpose of such procedure and by being careful in the following subjects.

It should be especially noted that examination material should be taken before administration of any antibiotics or other chemotherapeutic medications. Pathogen bacteria may not exist in the purulence even 24 hours after antibiotic administration. If bacteria that chemotherapeutic substance inhibit can not be produced from the examination material during the administration, it may be produced several days after discontinuation of the medication.

Examination material should be taken from the place where suspicious germ may exist. While taking sterile materials in normal such as urine, cerebrospinal fluid, special attention should be paid for contamination with outer germs. Sometimes, falling of several bacteria from outside causes not to obtain a result from the culture. The orifice of the tube or bottle should be singed when it is opened or before closing after material is put. The cap or seal of the tube or the bottle should be closed after removal without contacting any where. Although an extreme care is not necessary for materials including normal flora such as phlegm and stool, it is appropriate to take materials carefully by avoiding contamination from outside.

To obtain a successful result from the culture of the examination material, it should be taken within an appropriate period of the disease. In intestinal infections, bacteria may exist more in diarrhea period. It is more possible to isolate them within this period.

Furthermore, to take the examination material within certain times of the day may be important. To obtain a positive result by a culture made with the blood which was taken in febrile period is more likely. To obtain the phlegm in the morning is easier. The patient may expectorate more with a less effort.

No disinfecting or antiseptic are added into the examination material. If upper surface of the lesion is desired to be cleaned, a swab immersed into sterile saline or sterile cotton which is held by a sterile holder is used. The material is taken after cleaned area dried.

Examination material should be sufficient to perform the examinations completely. It is not necessary to take excessive material. For example, when materials such as phlegm or stool are put more, they smear outside of the container. Particularly, a janitor who does not know the importance of the procedure may be infected by touching these containers. This should be prevented by continuous warnings and trainings.

The examination material should be sent to the laboratory as soon as possible and examined. Sensitive bacteria in the material of which examination is delayed die due to low temperature, effect of enzymes, drying or not being nourished. Saprophyte bacteria which may be present in the examination material reproduce at room temperature; to produce pathogenic bacteria is not possible as their count increases. Therefore, the material is stored in the refrigerator until the examination time when germs which are effected from cold are not searched. By this procedure, saprophytes do not reproduce and pathogens continue their vitality. Sometimes, mediums and required tools are carried near to the patient's bed and examination material is added immediately when taken.

Examination material should be sent to the laboratory early in the day. By this means, examination is possible within working hours of the laboratory. Laboratory should be notified one hour earlier for immediate examination.

3.1 Taking examination material and putting in a suitable container

It is extremely important not to contaminate with external germs for the material of which microbiological examination will be performed. The material which is taken in aseptic conditions should be put in a sterile container immediately and the cap should be closed. When germs in the examination material kept in hot conditions, they may reproduce and die in the cold. Therefore, the material which is taken to a sterile container should be implanted to the medium as soon as possible.

3.2 Stool

The stool that culture will be performed should be taken into a sterile container. By this means, contamination with bacteria in containers that stool was put before. There are special

containers to put stool. These are tubes made of glass which were closed by cork. A small spoon made of metal was put on the edge of cork seal. Purulence or mucous parts of stool is taken by this spoon and put into the tube and cork of the tube is closed. To send to far distances, this special tube is put into a cylinder shaped box and this box is put into a wooden box. Also, scoop shaped small spoons made of metal are used to take stool. The stool is taken by deep side of this spoon which is sterilized by wrapping to a paper and the spoon is put into a screw cap bottle. Especially, bottles with wider orifices are suitable to put stool. Stool may be taken into glass like cardboards directly. If culture of the stool will delay for several hours after taking, 1 g stool is mixed with 10 cm³ 30% neutral glycerol solution in 6% saline buffer. It is seen that reaction is alkaline by pink-violet color after addition of phenol red. If this mixture is kept for a period, reaction becomes acid and indicator shows yellow color; so this mixture is not used anymore. Glycerol solution prevents saprophyte bacteria to reproduce and mask pathogen bacteria.

The solution is prepared as follows:	
6% saltwater	70 cm ³
Glycerol	30 cm ³
Na ₂ HPO ₄	1 g
0,02% phenol red is added into this.	

Prepared solution is divided into bottles as 10 cm³. The stool is taken into this bottle. Stool may be sent to a far laboratory by a filter paper to search *Salmonella* bacteria. For this, stool is applied on a filter or blotter as a thin layer and left for drying in the room. Two edges of the paper is folded by superimposing by a holder, stool is kept between the folded part. The paper is placed into a plastic envelope. Various samples which were prepared by this manner may be sent with a package prepared accordingly for mail. Such paper is cut into three parts in the laboratory. Suspension is made with salt water and it is smeared on appropriate medium in petri plate. Other parts are put into selenite and tetra thionate bouillon, they are smeared on the petri plate after reproduction. Stool sample which was taken into sterile containers or stool sample which was taken by swab rectally, the sample taken from bedpan or diaper of unconscious patient, and stool sample taken from baby diapers should be examined within at least one hour. If the sample is not examined within one hour, it may be stored in the refrigerator for three to four hours. Because, coliform bacteria which are dominant on the stool reproduce more in the room temperature and acidize the environment. This prevents reproduction of *Salmonella* bacteria as well as causes death. Bean sized sample is taken into any of selenite or tetra thionate bouillon mediums and processed to pre-enrichment at 37°C for 3 to 4 hours in the incubator, then it is planted on SS, EMB, endoagar plates according to dilution planting technique and identification process is started by considering lactose negative colonies.

3.3 Microbiological examination of the urine

Kidneys, ureter, bladder and most of the urethra are sterile areas in healthy human. Urethra includes a bacteria flora as far as 1,5-2 cm inside from the orifice both in women and men. In normal, the urine coming from upper sterile areas always contaminates more or less while

passing from this region of the urethra. But this contamination is not over a certain bacteria count in a healthy person. By considering this feature of the urinary system and the urine, some criteria should be followed before taking the urine sample, while taking the sample and sending it to the laboratory for microbiological examinations.

Generally, urinary antiseptics and antibiotic drugs and excessive water should not be given to persons before taking the sample.

The form so called middle flow is the most common method applied in all hospitalized and polyclinic patients who are cooperated for taking urine sample. The most important subject that patients should know and should be informed in this method is genital region cleaning which is required to be performed before taking the urine sample. Because, bacteria reproduce over 10^5 criteria in urine samples taken from persons who do not care about genital area hygiene and cleaning, frequently in women and urinary infection is diagnosed inaccurately. No infection is found in urine samples of these persons after a well cleaning is performed. Soap and water are sufficient for cleaning. Persons should wash their hands first, and clean glans penis and orifice in men and genital areas, labias and the orifice of women from front to back and from up to down. The person will start to urinate just after this cleaning and take the urine which is in the middle of the urination stage, not the first or the last urine into a sterile tube or sterile bottle with a wide opening (as 5 to 10 ml) and close the cap immediately. The urine taken should be transmitted to the laboratory as soon as possible. If this is not possible, the urine should be stored in the refrigerator for a certain period.

The urine sample brought to the laboratory should be stored in the refrigerator or examined within 1 hour.

The other process that will be done with the urine sample is culturing.

The method used in almost every microbiology laboratory is to plant with a washing bottle or loop. Loops used for planting are fabrication products which are calibrated and they are expensive. Apart from this, they should be controlled whether their calibration was deteriorated by special methods. The most suitable for us is 1 ml serological pipettes with 0,1 calibrated.

The urine brought is planted as 0,1 ml on every petri plate (blood agar- EMB, endo, Mac Conkey etc.) by a pipette after mixing well and smeared to the plate by a sterile glass rod.

Plates put into the incubator (35-37°C) are removed after an incubation of 18 to 24 hours, and colony count is performed. The colony amount which is counted in every plate is multiplied with 10 and it is expressed as colony amount in ml. According to the values obtained, colony count between -10,000 is accepted as NORMAL, between 10,000-100,000 as SUSPICIOUS and over 100,000 as URINARY INFECTION.

Except these criteria, lactose negative colonies are evaluated and identified in EMB or ENDO AGAR; and *Salmonella* is diagnosed. In this case, this is accepted as an infectious agent without considering 100,000 ml/cfu bacteria count as we accepted as infectious agent.

3.4 Cerebrospinal fluid

Bacteriological examination of cerebrospinal fluid is performed in cases that meningitis is suspected. Examination material taken under sterile conditions is sent to the laboratory as soon as possible. If the material will not be examined instantly, it is stored in the incubator to examine within one hour. Germs obtained from cerebrospinal fluid most:

Neisseria meningitidis, *Diplococcus pneumoniae*, *Haemophilus influenzae*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Salmonella escherichia*,

Proteus, Pseudomonas, Mimeae, Flavobacterium, Bacteroides, Listeria monocytogenes, Pasteurella multocida, Leptospira, Cryptococcus, Neoformans viruses.

While CSF is taken, the area where puncture will be performed should be cleaned with iodine and alcohol to avoid contamination with flora bacteria on the skin. Sufficient material should be taken to be able to perform cellular, chemical and microbiological examinations. To take material, to put sterile screw cap tubes in lumbar puncture sets is more appropriate instead of cotton or plastic seal tubes. In cases that sufficient CSF can not be obtained, only required examinations should be requested first from the laboratory. CSF should be transmitted to the laboratory immediately and examined instantly. As CSF is regarded as materials that planting should be performed near the patient, if examinations can not be done within a very short period it should be stored in the incubator. The sample taken to isolate *Salmonellas* should be taken into selenite f medium and it is kept under pre-incubation at 37°C for 3 to 4 hours, then planted on mediums such as SS, EMB, MAC KONKEY by dilution planting technique and lactose negative colonies are identified.

3.5 Autopsy material

Examination material is taken from ileum in suspicion of typhoid fever. Intestinal wall is cauterized by superheated spatula. A sterile swab is inserted from the hole opened and moved on the mucosa by rotating. The swab is removed and immersed into tetrathionate bouillon or selenite bouillon. The swab is rotated by pressing on the wall of the tube and a clouded fluid is obtained. Planting is performed from this fluid to Endo, SS, bismuth sulphite agar mediums which are used to produce *Salmonella* bacteria. Material is taken from the bladder by the same way.

3.6 Purulence

The infectious agent from lesions such as abscess, wound and fistula are searched by bacteriological examination.

Taking Examination material:

- The purulence is taken from open lesions by a swab or a loop which is superheated and cooled.
- If there is a closed abscess, upper surface is cleaned with tincture of iodine and alcohol and drying is waited. Abscess is opened, the first purulence is removed, the purulence appeared is taken by a swab, it is put into the tube without contacting the skin. Or, purulence is taken from the abscess by a sterile syringe or a Pasteur pipette and put into a sterile tube. Small pustules are pierced with a sterile needle after the upper surface is cleaned and the fluid appeared is taken by a Pasteur pipette, swab or a loop and examined.

3.7 Serous liquids

Liquids accumulated in cavities of pleura, pericardium and peritoneum and synovial and hydrocele liquids are sent to the laboratory to search for the bacteriological agent in case of infection.

Serous liquids should be taken under sterile conditions and out into a sterile container. To prevent the coagulation by putting some of the liquid into a citrate solution or a beaded bottle is appropriate.

3.8 Microbiological examination of wound and abscess materials

Wound infections and abscess appear as a complication of surgical interventions and traumas or contamination of any infectious disease to the skin, mucosa, tissues and organs. In general, agents in the wound and abscesses are closely associated with the flora in the region. However, open wounds, ulcers and fistulas are contaminated from the flora or air and microorganisms coming from objects according to their region. Therefore, a cleaning should be performed before taking a material from these lesions. To take material from dry lesions is impossible and useless many times.

To isolate the agent in acute wounds is easy, however, to isolate the agent is quite difficult as number of microorganisms in chronic wounds decreased very much.

3.9 Vomitus

The vomit is examined when food poisoning occurs. Bacteria such as *Salmonella*, shigella, *Staphylococcus aureus* and *Clostridium perfringens* (welchii) are searched. Food poisoning also occurs when food contaminated with many other bacteria is eaten. In case of epidemic, the type of pathogen bacteria which is reproduced too many should be thought as the agent.

Examination substances are planted into 2 blood agar and put into aerobe and anaerobe conditions. Selective medium is used for *Salmonella* bacteria. It is possible to obtain *Salmonellas* from the stool in food poisonings. Negative culture does not remove possibility of food poisoning with bacteria.

4. *Salmonella* isolation from non-clinical samples

Bacteria are present everywhere in the nature. Many of them are harmless. Some of them may infect humans and animals. These bacteria may reproduce only under certain circumstances. Dissemination may be from human to human, from animal to human or from human to animal. Dissemination may be either directly or indirectly. Dissemination via food takes an important place. Bacteria which cause disease by food may pass to human as well as some bacteria which may reproduce on food may cause food poisoning.

Salmonella and Shigella bacteria are present in stools of sick human, animal and porters. These bacteria may pass from person to person by contact when hygiene rules are ignored and *Salmonella* bacteria which causes food poisoning may pass to food.

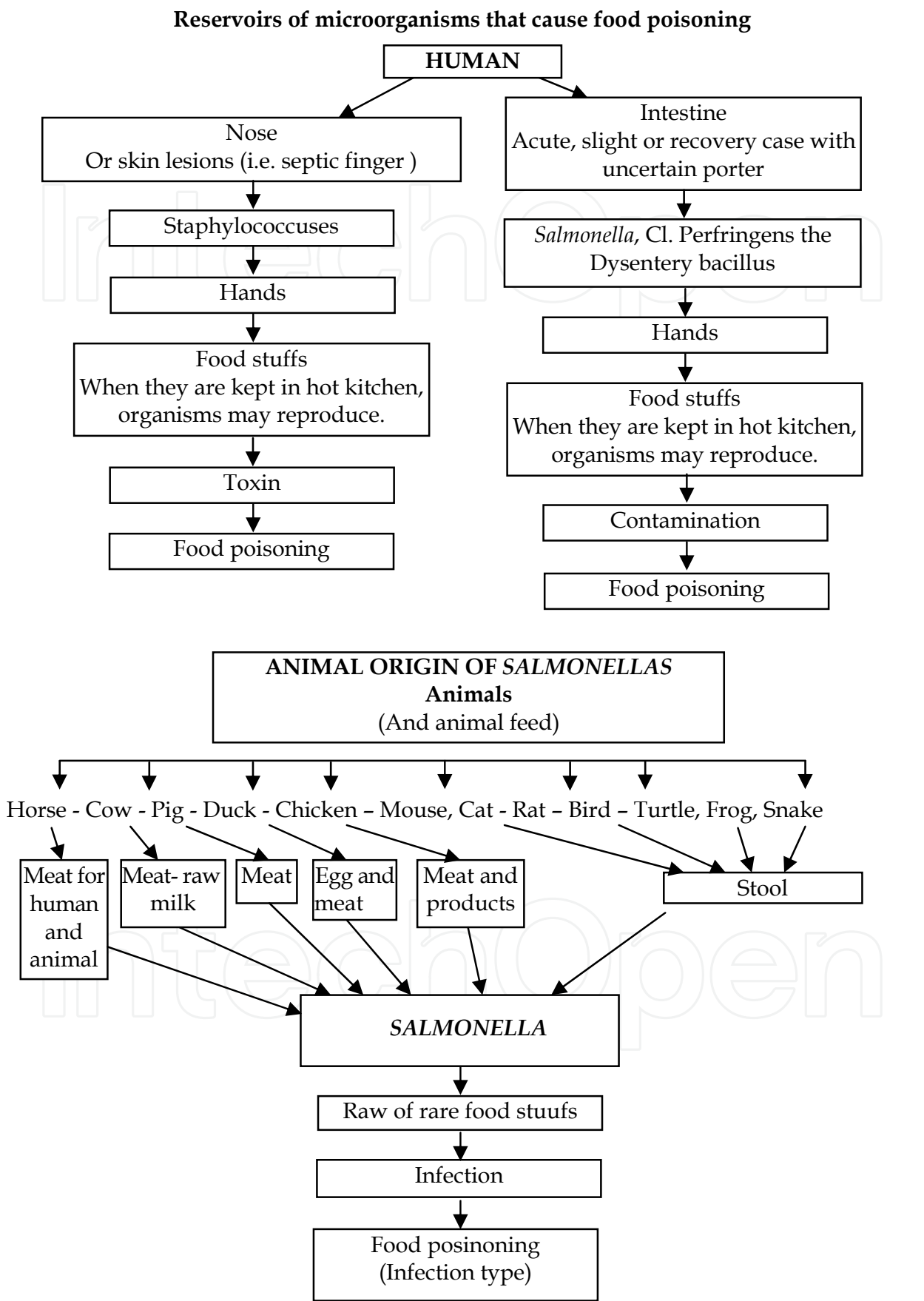
Also, cats and dogs kept at homes may reveal *Salmonella* without any symptom. Livestocks also may be infected with *Salmonellas* and spread them to the environment.

Salmonellas appeared with stools of pets and livestock may directly contaminate to food. However, if hygiene rules are ignored in places where livestock is kept, everything belonging to the animal may cause contamination.

In some places, flies play an important role in spreading of the infection. In case of spoiling of stools on the road in settlements where humans and animals live together, flies are effective in spreading if they were not controlled. If bacteria such as typhoid, dysentery etc. are endemic in these regions, flies create a big problem.

With less possibility, bees, spiders and ants play role for spreading harmful bacteria in places where environmental conditions are not hygienic.

Inorganic objects such as towels, pens, door handles, toilet (WC) equipment, containers may play a role in spreading of the infection from human to human or from human to food.



Methods which is being used to detect pathogen microorganisms in the food are ineffective if such pathogenic microorganisms diffused rarely or the food is severely contaminated with other microorganisms. For these reasons, various indicator microorganisms are used for various purposes.

4.1 Sample taking

To take samples from the foods for microbiological analysis and to bring this to the laboratory by “protecting all microbiological criteria at the moment of sampling” are quite difficult. Subjects such as how much and how many sample will be taken, bringing to the laboratory, opening and preparation for planting should be overemphasized.

4.2 Sample amount

Many national and international standards give the food quantity to be analyzed as 10 g (mL). This value is valid for quantitative analyses only. Pathogens are usually analyzed by present/absent test in 25 grams of food.

While 10 grams of sample is sufficient for a standard analysis, usually 25 grams of food is required for every additional pathogen test in accordance with special homogenization requirements as mentioned above. According to this, at least 60 grams of sample including 10 grams for total bacteria, coliform group bacteria, yeast and mold and staphylococcus and 25 grams of each for *Salmonella* and *Listeria* analysis should be brought to the laboratory.

The requirement that how many items should be taken from a sample mostly causes confusion. In daily controls performed in food industry, only 1 sample is sufficient.

More samples should be taken from foods that have high pathogenic risk such as *Salmonella*, *Listeria* etc.

4.3 Bringing the sample to the laboratory and acceptance

The sample taken from the enterprise should be brought to the laboratory as cleared from all conditions that will increase or decrease the single microorganism count as soon as possible and analyzed.

In microbiologically stable products such as sterile, dry and humid resistant packages, no cooling is required during transportation. On the other hand, unprocessed, cooled, pasteurized, spoiled foods should be transported between 0°C and +4°C and frozen products should be transported at -18°C and those bulged (or having a risk of bulge) should be packaged separately against explosion and leakage and brought to the laboratory.

First, the sample should be accepted to be analyzed by the laboratory. For this acceptance, laboratory personnel should control whether the sample is brought to the laboratory under required conditions; if such personnel is sure that the sample was brought under standard conditions, she/he should accept the sample for the analysis; otherwise she/he should either reject or write all negativities related with this to the acceptance form.

For the sample which has come to the laboratory and accepted, date of acceptance, time of acceptance, all information related with the product (date of production, package features, batch number, shift number, time of sampling, temperature of sampling, temperature of arrival etc. if required) should be recorded according to the features of the laboratory.

Frozen liquid products are not accepted for microbiological or somatic cell analysis. If chemical tests will be performed, sub-samples should be separated for microbiological analysis first.

4.4 Opening the sample

The sample which has come for analysis should be analyzed within the shortest period. If there is a necessity to wait for a while;

- Microbiologically stable products should be analyzed before the expiry date and as soon as possible.
- Fresh and cooled products should be analyzed within 24 hours after the acceptance. If a longer storage period is necessary, the product should be frozen immediately and stored under -18°C . As the frosting process will affect the microbial flora in the product, this situation should be specified in the analysis report exactly.

Frozen products should be thawed in $+4^{\circ}\text{C}$ refrigerator temperature. It should be considered that big particle products will thaw within a longer period than small particle products and psychrophile bacteria may develop within the thawing period, therefore the food should be frozen with portions not more than 50 grams within bounds of possibility. If the sample is frozen by weighing before, it may be thawed by transferring into homogenization solution directly.

Parallel of the sample which has come to the laboratory and accepted should be protected as witness of which features will not change until the termination of the analysis.

Before opening the closed package, the place and its surroundings should be disinfected via 76% (v/v) alcohol or any appropriate chemical agent and if the package is appropriate, it should be singed. Packages that can not be singed (paper etc.) should be removed by cleaning with disinfecting sterile water after chemical disinfection and such disinfection should not be contacted with the food sample anyhow. Otherwise, negative result may be taken. Materials which will be used to open the sample such as scissors, tin opener, bottle opener etc. should have been disinfected or sterilized in the drying oven or autoclave by wrapping to an appropriate package (paper, kitchen type aluminum folio).

Liquid samples may be analyzed directly. Solid foods should be pre-processed such as weighing, homogenization etc.

Weighing to a certain weight (10 g, 25 g, etc.) in solid sample should be performed under aseptic conditions. To weight in vertical type planting cabinet is the most reliable method. The container that weighing will be performed should have been sterilized and should be in the size to take pre-enrichment medium like *Salmonella*.

If solid food consists of particles which may create a problem during weighing in terms of size and qualification, it should be divided into suitable sizes.

It should be remembered that this application is valid for weighing which is more than aimed weight and weighing over 5% should not be performed as far as possible. If microorganisms such as *Salmonella* was weighed as 26 grams instead of 25 grams in present/absent tests, to use a 234 ($=225+9$) mL medium instead of 225 mL of pre-enrichment medium is not a condition. Because, the process performed here by using 25 grams of food + 225 mL medium is not a dilution, but using 9 mL medium per 1 gram food. Tolerance of $\pm 5\%$ is always accepted. The deviation in this sample is only 4%.

Generally, it is the process to make solid and semi-solid foods homogenous in a homogenization solution. Liquid foods show a homogenous distribution in anyway. The purpose of homogeneity here is to distribute all microorganisms in the food to all mass to be analyzed. Homogenization process is performed as 1:9 in general whether it is used for counting or present/absent tests. According to this, 1 part food is homogenized by 9 part solution. In present/absent tests, 1 part food is homogenized by 9 parts of medium. If counting will be performed, 1:9 homogenization is also used as 10^{-1} dilution. Therefore, amount of the food and homogenization solution should be cared about.

4.5 Homogenization and dilution solutions

Although “normal saline” (0.85% NaCl; Merck 1.06404) has been used as diluting solution for general purpose, “Maximum Recovery Diluent (Merck 1.12535) which has the formula of Normal Saline (0,85%) + Peptone (0,1%) in accordance with ISO 6887 is used recently. This solution is also referred as “peptone-saline”. Furthermore, 0.1% peptone solution (Merck 1.07214) and “Buffered Peptone Water” (Merck 1.07228) which is used in *Salmonella* analysis are used for dilution in accordance with ISO 6887.

4.6 Water

To drink and to use waters contaminated with stool or sewage leakages is very important because they may cause infections such as typhoid, dysentery, cholera. In contaminated water, typhoid germ may exist together with intestinal bacteria. However, number of these bacteria is very less in general and it is impossible to obtain. On the other hand, commensal bacteria such as coliform bacteria, *Streptococcus faecalis* and *Clostridium perfringens* (welchii) are always obtained in contaminated water easily. Such water containing these bacteria means that the water was contaminated with the stool and this may contain typhoid germ. Coliform bacteria shows the contamination with the stool with the safest manner. The most important of them is *Escherichia coli* which is main commensal bacteria of the intestine. Existence of spore forming anaerobe bacteria in the absence of other bacteria shows an old contamination with the stool.

Coliform bacteria also exists in water contaminated with stool of various animals. But they are less. More bacteria are present in the water contaminated with sewage water. It is important to detect the bacteria count in the water to determine the level of the contamination.

Detection of alive bacteria count gives information about quantity and type of organic substances. The trial is performed both at 37°C and 22°C. Bacteria which mix from human and animal origin organic substances reproduce at 37°C in particular. Those which reproduce in lower temperature are saprophyte bacteria that mix from the soil and plants or exist in the water normally.

4.6.1 Taking the water sample

The water to be examined should be taken into colorless, preferably glass cap bottles with a volume of 250 cm³. Orifice and cap of the bottle are wrapped with separate papers and sterilized in the autoclave. If water will be taken from running water, the orifice of the tap is burned with spirit flame and the water is put into the bottle after leaving the water run for five minutes. The cap of the bottle is closed by caring the sterility conditions. To take water from the creek or river, the bottle is held from the bottom, it is immersed into the water upside down to 30 cm deep. The orifice of the bottle is turned to the flow direction and water is filled with water without touching. To take water from dead water, lakes and depots, the bottle that a ballast was hung to the bottom and bounded with ropes from the neck and the cap is immersed into the water with a desired depth. The rope is pulled and the cap is opened, after the bottle is filled with water, it is pulled to up and the cap is closed. If a period more than 3 hours will pass from taking the water sample until the examination, the bottle should be kept in ice. It may be sent to far places only in ice.

If sample will be taken from chlorinated water, chlorine should be neutralized immediately. For this, one sodium thiosulphate crystal is put into the bottle or 0,2 cm³ from 1 g of crystallized sodium thiosulphate solution which was dissolved in 100 cm³ sterile water before sterilization.

To mix the water well before the assay, the bottle that water sample is taken is shaken.

4.6.2 Approximate assay for coliform bacteria count

To produce coliform bacteria, water is planted into Durham tubes including 2% peptone, 0,5% sodium taurocholate, 1% lactose and bromthymol blue (or bromeresol purple) as indicator. Amount of the water planted varies between 0,1 cm³ and 50 cm³. Concentrated medium is used if more water will be planted. The least water amount including coliform bacteria is detected by assays and contamination degree of the water is determined. Generally, 20 cm of 15 tubes included medium is taken. 0,1-1 cm³ water is put into 5 tubes; 1 cm³ water is put into 5 tubes; and 10 cm³ water is put into 5 tubes. Tubes are left at 37°C for 48 hours. After 24 and 48 hours, tubes are controlled and acid and gas formation is controlled. If gas is produced, it is examined that such gas has filled 1/10 of the small tube in Durham tube. Formation of gas which will occupy 1/10 of the tube after 24 hours shows possibility of *Escherichia coli* reproduction. If no gas has formed within 24 hours or the gas occurred is less than 1/10 of the small tube, tubes are left at 37°C for 48 hours. Any gas formation is considered as suspicious. No gas formation after 48 hours shows no reproduction of *Escherichia coli*.

To confirm whether reproduced bacteria are *Escherichia coli*, planting is performed from tubes with least amount of water that reproduction was observed to eosine-methylene blue agar by decreasing method. Petri dishes are left at 37°C for 24 hours and examined and it controlled whether the bacteria reproduced is *Escherichia coli*.

4.6.3 Searching for *Salmonella* bacteria

Same amount of water is added into tubes including selenite f medium which was prepared with one portion concentrate. After waiting at 37°C for 24 hours, planting is performed from every tube to SS agar. Furthermore, one cm³ each from tubes are taken and mixed with bismuth sulphite agar and poured into petri plates. When reproduction occurs, it is searched whether it is from *Salmonella* group. (551,602,611)

4.7 Meat

Meat may include many germ types such as *Bacillus subtilis*, *Escherichia*, *Proteus*, *Salmonella*, *Staphylococcus* species and fungus and especially anaerobe spore forming bacteria show fundamental change. These bacteria cause formation of bad odor by making putrefaction in proteins. It is not very possible to decide on the status of the meat by bacteria on it.

To detect bacteria, one gram is weighed, it is meshed in mortar with sand. 1000, 10.000, 100.000 dilutions are prepared. Colony count is performed with these in the petri plate. Diagnose of bacteria is performed if required.

4.8 Egg

Fresh egg is not always sterile. Gram (+) coccus, gram (-) bacillus and some fungus species may be present in the egg. Sometimes, egg may include *Salmonella* bacteria. (617, 618)

The shell of the egg has a porous structure. Gases and microscopic particles may pass through these pores. Bacteria always exist on the shell of the egg. *Escherichia coli* is present almost on all shells. Humidity causes bacteria to pass through pores in humid and dirty eggs. Therefore, eggs which will be stored for a while must not be washed.

The egg which will be examined is cleaned well by washing with brush, water and soap. It is kept in 0,1% sublimated solution for 30 minutes. It is taken from here by a sterile spoon and put into 200 cm³ of sterile water. It is kept in the water for 10 minutes and put into 95% alcohol and left for 5 minutes. The egg is taken from the alcohol and left for drying and

broken from the wide edge by a sterile holder. The content is transferred into a sterile beaded jar. Egg white and yolk are mixed by shaking well. Microorganisms are searched by planting into various mediums.

4.9 Milk

There are various methods for bacterial analysis in the milk. The most used methods include alive bacteria count in petri plate, direct microscope count, coliform bacteria detection, methylene blue assay, phosphatase assay, turbidity assay and assay for special bacteria search. These methods are applied to various milks.

The milk which will be drunk should not include bacteria more than 30,000 and coliform bacteria in 0,1 cm³. 0,01 cm³ of pasteurized milk should not include any coliform bacteria and its pasteurization should be understood by phosphatase assay. Turbidity assay should be positive in sterilized milk.

The milk which was milked in the morning is kept waiting 9 to 11 hours in the shadow and the milk which was milked at night is kept waiting until the next day. Other samples are tried immediately when they come to the laboratory. Milk that analysis is not performed immediately may be stored at refrigerator for 24 hours at most.

4.10 Microbiological examination of fruit juices and other soft drinks

Main soft drink is water. These beverages may be classified as follows.

1. Water (mineral water etc.)
2. Fruit juices (fruit juices including alcohol less than 5 g/l and SO₂ less than 10 mg/l)
3. Fruit juice concentrates (concentrates which were condensed and partially canned and is drunk by diluting, basic substances and essence)
4. Beverages including carbonic acid (lemonades, soda pops, cokes)

Mineral water including carbonic acid is microbiologically safe water. They last for a long time. Some microorganisms may exist in them.

As fruit juices are acidic and sugared foods; yeasts, molds and milk acid bacteria cause spoiling. Microbiological spoiling in fruit juice concentrates is very less. Abovementioned microorganisms also spoil concentrates. Yeasts (*Candida saccharomyces*, *torulopsis* species), milk acid bacteria (*leuconostoc*, *lactobacillus* species) in lemonades, sodas and cokes cause spoiling.

Examination of the samples are performed according to the following steps.

PROCESS

1. BEVERAGE BOTTLES ARE TAKEN UNDER ASEPTIC CONDITIONS. Bottle of beverages with carbonic acid is opened 1 hour before, it is heated slightly and the gas moves out.
2. Dilutions are prepared if necessary. (from intense textured beverages and concentrates)
3. Samples are planted into or on the medium as 1,0 ml or 0,1 ml.
4. If there are membrane filters, 100 ml of sample is filtered via water squinch and the membrane filter is placed on the medium.
5. Plates are incubated at 30-32°C for 3 to 5 days and evaluated.
6. Same amount of sample is added into tubes including selenite f medium which was prepared with one portion concentrate. After waiting at 37°C for 24 hours, planting is performed from every tube to SS agar. Furthermore, one cm³ each from tubes are taken and mixed with bismuth sulphide agar and poured into petri plates. When reproduction occurs, it is searched whether it is from *Salmonella* group. (551,602,611)

5. Culture and identification methods of *Salmonellas*

For identification of various samples, methods which alternate and support each other. These are:

- Culture methods
- Invic test
- Triple tube method
- Api method
- Full automatic bacteria identification device
- Serological tests
- Grubul Widel

5.1 Culture methods

Culture method in identification of *Salmonellas* is conducted with pre-enrichment and selective medium planting.

Identification studies are same regardless from the source of the culture. Variety of mediums used in the culture may depend on characteristics of the sample examined. Especially when number of *Salmonellas* are less and other organisms are more, very careful study is required. If extra clinical samples are processed such as heating, drying and radiation or they are frozen or kept for a long time or pH level is low although clinical samples are examined as fresh, non-selective pre-enrichment culture is applied. Because these processes weakened *Salmonellas* and made them semi-selective. The purpose is to provide this kind of bacteria to their normal reproduction period before contacting inhibitor substances. Because selective substances may make a toxic effect for “weakened” *Salmonellas*. While enrichment bouillon culture facilitates reproduction of *Salmonellas*, it also provides inhibiting or decreasing effect for reproduction of other organisms. Accompaniment organisms mainly include coliforms, proteus species and pseudomonas. As the proportion of these organisms is more than *Salmonellas* in particular, selective enrichment process gains importance. However, there are differences between *Salmonella* types in terms of inhibitor substance sensitivity. Therefore, it is impossible to say which selective enrichment bouillon is the most suitable definitely for today. Selective agar mediums generally include inhibitor substances and an inhibitor system. Indicator system either changes the color of colonies or the color of agar area around the colony changes. Thus, it helps to identify suspicious *Salmonella* colonies.

The following Agar Mediums are used in various countries.

Brillant green agar Brilliantgreen Sulphadiazine agar Brilliant Green Mac Conkey agar Desoksicholate Citrate agar *Salmonella* -Shigella Agar,(SS) Bismuth Sulphite agar EMB AGAR ENDO AGAR

Samples are taken into non-selective enrichment medium (lactone bouillon) according to their clinical or extra clinical sample characteristics and incubates at 35-37 °C for 24 to 48 hours, then 1 ml from them is taken and taken into selective enrichment medium and (Selenite F, tetrathionate bouillon) A and incubated at 35-37 °C for 24 hours.

5.1.1 Non selective enrichment

1. Clinical and other samples are taken into lactose bouillon with appropriate amounts. If the sample food is also solid, it is mixed in the blender. Ot is transferred into 500 ml of erlenmayer flask or flasks.
2. It is incubated at 35-37°C for 48 hours and passed to selective enrichment.

3. At this time, a loop full of the sample is taken and planting to selective agar medium is performed.

5.1.2 Selective enrichment

1. 1 ml of non selective enrichment medium culture is taken and it is transferred into a tube including 10 ml of selenite cystine. 1 ml is planted into one of 5 tetrathionate medium of 10 ml.
2. Tubes are incubated at 35-37 °C for 24 hours.

5.1.3 Planting to selective agar medium

1. Two selective agar medium plate is prepared by drying. One of them may be enrichment and the other may be selective medium.
2. A loop from every enrichment culture with a diameter of 5 mm is taken and planted to provide single colony.
3. Plates are incubated by reversing at 35-37°C for 24 hours. If typical colonies are not observed at the end of 24 hours, incubation is extended to 48 hours.
4. Suspicious two colonies are selected from every selective agar medium and identification is directed.
5. If agar plates are completely full of coliforms, 1/1000 dilution of enrichment culture is prepared and kept at the room temperature or in the refrigerator.
6. Selective agar plates are kept at 5-8°C until completion of identification tests.

Appearances of *Salmonella* colonies in various mediums after incubation are as follows.

5.1.3.1 Appearance of typical *Salmonella* colonies in Brilliant green agar and Brilliant green sulphadiazine agar

It is colorless, pink, semi transparent or opaque. The color of medium which surrounds the colony has become pink or red. Some *Salmonella* colonies make semi-transparent green colonies when lactose or sucrose fermentating organisms are present around them. Lactose or sucrose fermentating colonies make yellow to green colonies (Figure 4).



Fig. 4. Appearance of typical *Salmonella* colonies in Brilliant green agar

5.1.3.2 Appearance of typical *Salmonella* colonies in mac conkey agar

It is colorless and transparent. Coliform organisms precipitate bile salts in the medium. *Salmonella* colonies reproduced near coliforms dissolve precipitated area (Figure 5).



Fig. 5. Appearance of typical *Salmonella* colonies in Mac Conkey agar

5.1.3.3 Appearance of *Salmonella* colonies in *Salmonella* – *Shigella* agar (SS)

Typical *Salmonella* colonies are colorless or very light pink, opaque or semi-transparent. Some of *Salmonellas* make colonies of which the centre is black (Figure 6).



Fig. 6. Appearance of *Salmonella* colonies in *Salmonella* – *Shigella* agar (SS)

5.1.3.4 Appearance of *Salmonella* colonies in desoxyholate citrate agar

Salmonella colonies are colorless or very light pink, opaque or semi-transparent. Some of *Salmonellas* reproduce as black or gray in the middle and colorless on the sided (Figure 7).



Fig. 7. Appearance of *Salmonella* colonies in desoxyholate citrate agar

5.1.3.5 Appearance of *Salmonella* colonies in bismuth sulphite agar

Salmonella colonies appear as brown, gray or black. Sometimes they show a metallic brightness. The medium which surrounds the colony is brown at first. It becomes black when incubation period extends. Some strains make green colonies and they make the surrounding medium to black very less or they do not make blackness (Figure 8).

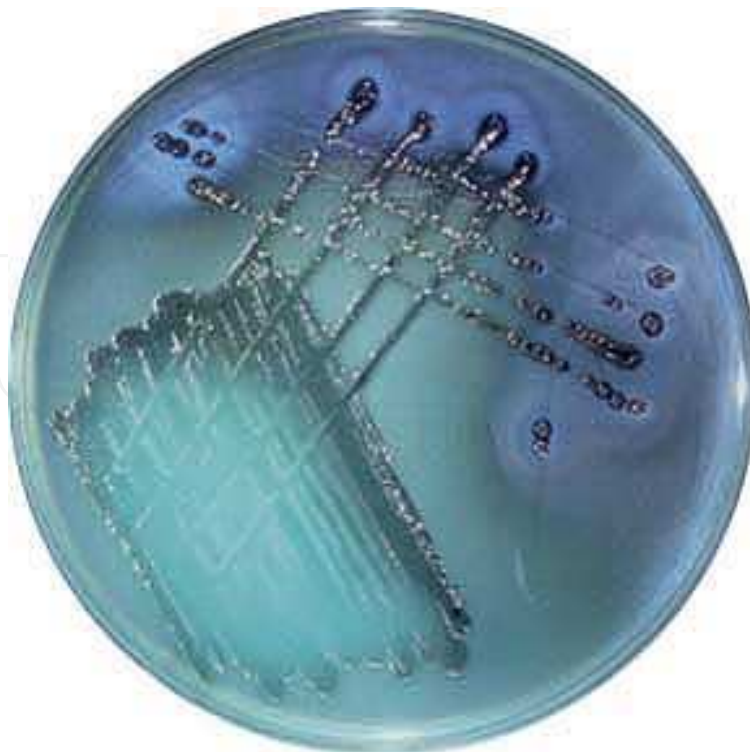


Fig. 8. Appearance of *Salmonella* colonies in Bismuth sulphite agar

5.1.3.6 Appearance of bile-salts-jelatin lactose agar (Tahsin Berkin agar) (BS) L AGAR)

Salmonella colonies make cyclamen colored colonies with a diameter of 1 to 3 mm. These colonies are bright, swollen (s) type colonies.

5.1.3.7 Appearance of *Salmonella* colonies in EMB AGAR

Salmonella colonies make transparent, colorless colonies with a diameter of -4 mm (Figure 9).



Fig. 9. Apperance of *Salmonella* colonies in EMB AGAR

5.2 Identification of *Salmonellas*

Identification of suspicious colonies among *Salmonellas* are performed in three steps.

1. Biochemical examination of suspicious colonies,
2. Serological tests (test with polyvalent H and O group antiserums and H pools)
3. Test with bacteriophages

Salmonellas uspicious colonies in mediums are examined by staining with gram method. Other detection methods are used for colonies where gram negative bacillus were observed.

Although several biochemical tests may be used for identification of *Salmonellas* , sufficient information may be obtained with some of them. Gillen medium 1 and 2 (urea, indole and H₂S formation is controlled by fermentation of motility, glucose, mannite, sucrose and salicine). Triple Sugar Iron Agar (Triple Sugar Iron Agar shows H₂S formation by fermentation of Sucrose, Lactose and Glucose). It is used common in laboratories.

Suspicious colonies in terms of *Salmonella* are controlled by polyvalent H and O antiserums following biochemical tests. These antiserums include antibodies collectively against most of *Salmonellas* . Cultures that has given positive reaction with polyvalent antiserums are then examined with O group and H pool antiserums. These antiserums include antibodies of *Salmonellas* including the groups in Kauffmann-White sceheme. These groups are classified from A to I alphabetically. Positive agglutination presents the group of the culture. Specific H and O antiserums are required for den, itite typing.

5.3 Biochemical tests for identification of *Salmonellas*

5.3.1 Purification of colonies selected

Colonies selected are purified. If time is restricted, this purifying process may be ignored. The following procedure is applied for purification process.

- Every colony selected is planted as to allocate single colony to a separate Mac Conkey agar plate.
- Reversed plates are incubated at 35 to 37 °C for 24 hours.
- Salmonella* colonies appear as transparent and colorless in Mac Conkey agar. Sometimes the centre appears as pink. If there are many organisms that ferment the lactose, the precipitated area around *Salmonella* colonies which are next to them becomes transparent.
- Planting is performed from typical colonies to normal slant agars. Cultures are incubated at 35 to 37°C for 24 hours.
- Preparation is prepared from slant agar cultures and stained with gram method. If cultures are pure, the following mediums are used for passages.

A passage specified in the following is performed from 24 hours, purified slant agars to Gillies medium 1 and Gillies medium 2.

5.3.2 Planting to Gillies medium 1 and assessment

- It is immersed to the bottom by a loop and then it is planted into slanted part. It is incubated at 35 to 37°C for 24 hours.
- Urease reaction, glucose and mannitol fermentation and gas formation are recorded. Cultures with positive urease reaction convert the medium to a dark purple color. Mannitol fermentation is characterized by bottom part turning into yellow; and gas formation is characterized by appearance of gas bubbles in the agar. *Salmonellas* are urease negative. On the other hand, they ferment glucose and mannitol with or without forming gas.
- Urease positive cultures that do not ferment glucose or mannitol are assessed as negative in terms of *Salmonella*.

5.3.3 Planting to Gillies medium II and assessment

- The tube is held vertically and planting is performed by immersing to 2 cm depth with a loop.
- Tubes are incubated at 35 to 37 °C for 24 hours vertically. If reactions are not significant, they are waited for another 24 hours.
- Sucrose and salicine fermentation, motility, H₂S and indole formation are recorded. Color change in the agar, conversion from original blue-green color to yellow shows that sucrose or salicine or both are fermented. Darkening of the lead acetate paper indicates H₂S formation and indole paper becoming red indicates indole formation. Typical *Salmonellas* are motile, indole positive, Sucrose and Salicine negative.

5.3.4 Planting to TSI AGAR and assessment

- Suspicious single colony is taken from purified culture or selective agar medium and inoculation is performed by immersing to the bottom with a loop of triple sugar iron agar (TSI) or by drawing to the slant part.
- Cultures are incubated at 35 to 37 °C for 18 to 24 hours.
- Cultures that do not give reactions specific to *Salmonellas* are not taken into the assessment. Typical reaction in TSI agar is red color on the slant part, "alkaline reaction"

and the bottom is yellow. "Acid reaction, glucose fermentation) H₂S and gas is positive or negative. H₂S reaction manifest itself with blackening of the medium. Typical reactions of *Salmonella* and Arizone species in Lysine-Iron Agar medium are purple colored sloped and red "alkaline reactions", vertical part. They produce H₂S and sometimes gas (Figure 10).

Serological tests are continued with positive *Salmonella* cultures.

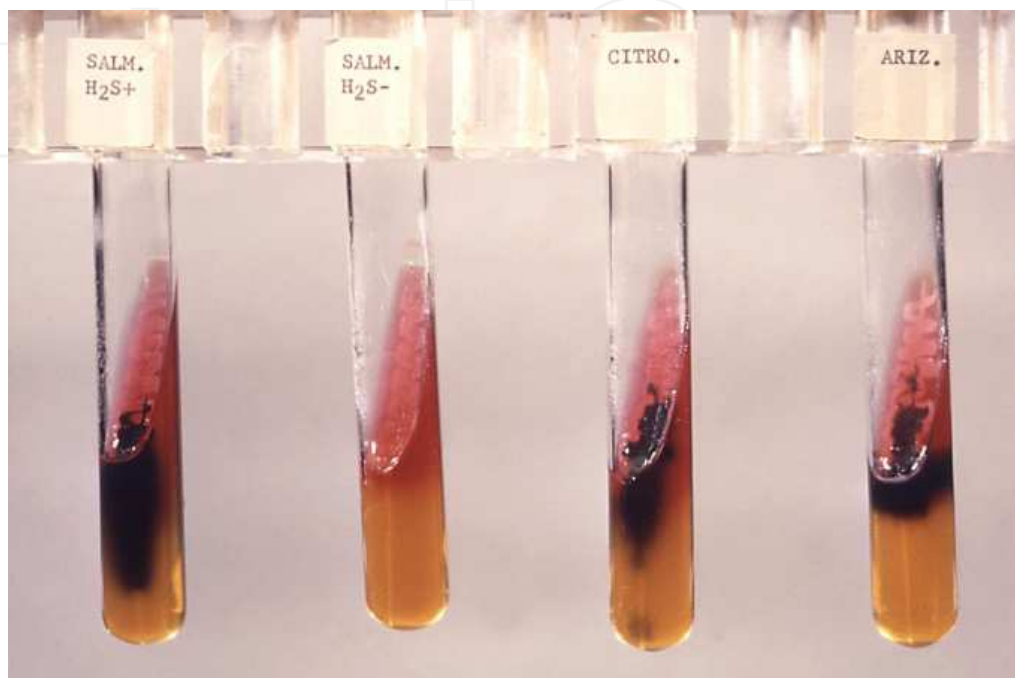


Fig. 10. Appearance of *Salmonella* in TSI Agar

5.3.5 Imvic test

(I=indole, M=methyl red, V=voges pros cover, C=citrate)

INDOL= Planting is performed into tryptophan medium. After incubation at 37°C for 24 hours, 0,2 to 0,5 cc of Kovac indicator is dripped into indole medium. Red circle formation is positive (Figure 11).

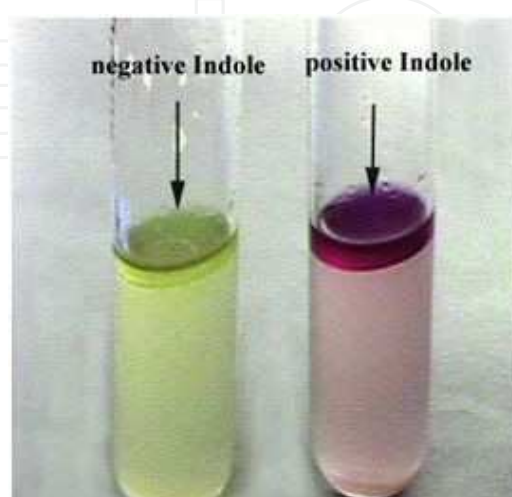


Fig. 11. Indole Test

METHYL RED= Planting is performed into BGB (buffered glucose bouillon) or peptone medium. This test shows pH change in 0,5% buffered glucose medium. 5-6 drops of methyl red indicator is dripped on 1 cc. of medium. If pH drops under 4,2, red color occurs and the result is positive. If there is no color change, it is negative (*Figure 12*).

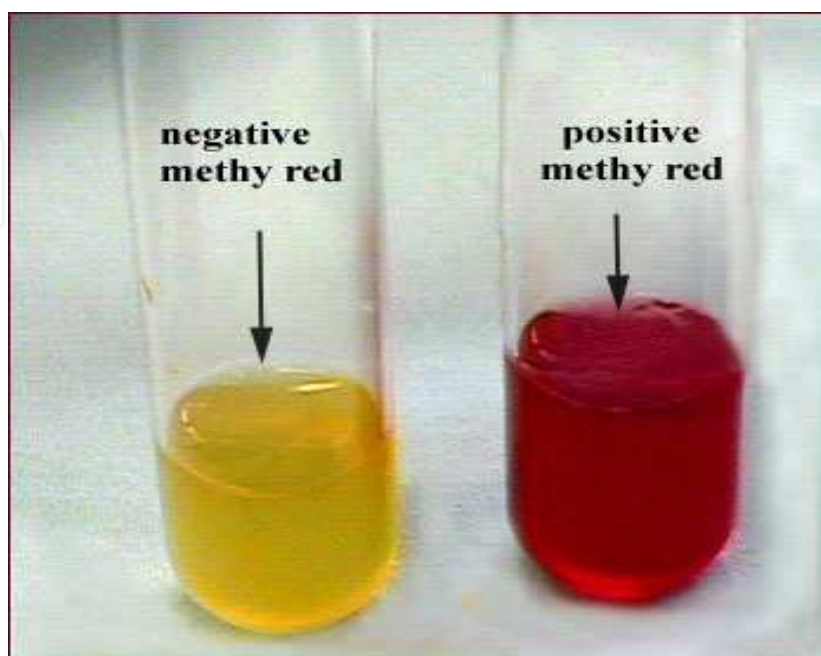


Fig. 12. Methyl RED Test

VOGES PROS COVER= 0,2 cc 40% KOH is dripped into 1 cc BGB medium. Then, 0,6 cc of alpha naphtol indicator is added. Test results 20-30 minutes after.

If red circle appears, the result is positive. In positive cases, acethyl-methyl-carbinol, final catabolism product of glucose occurs. If there is no color change, it is negative (*Figure 13*).

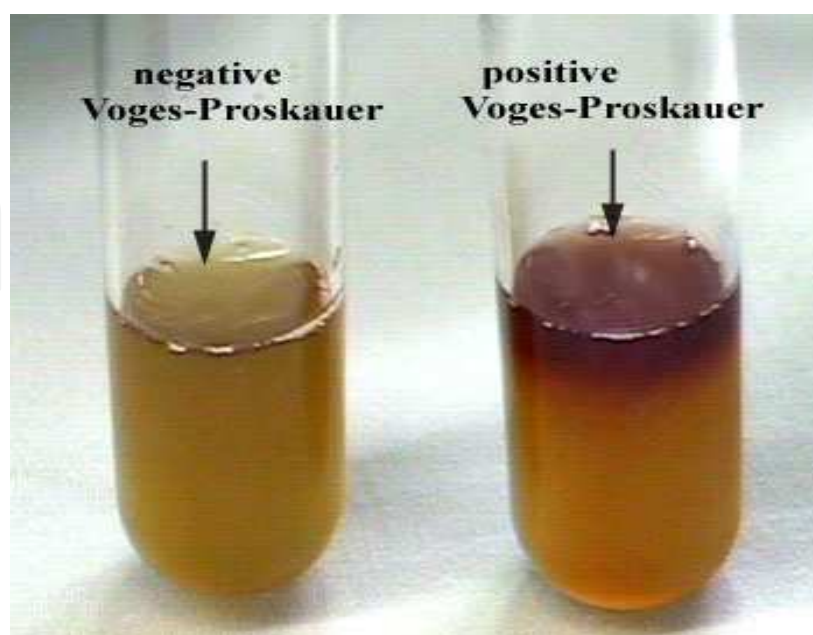


Fig. 13. Voges Pros Cover test

CITRATE=Simmons citrate; line style planting is performed to citrate medium. If the bacteria used citrate as a carbon source, the color of the medium will turn from green into blue. The test is positive (Figure 14).

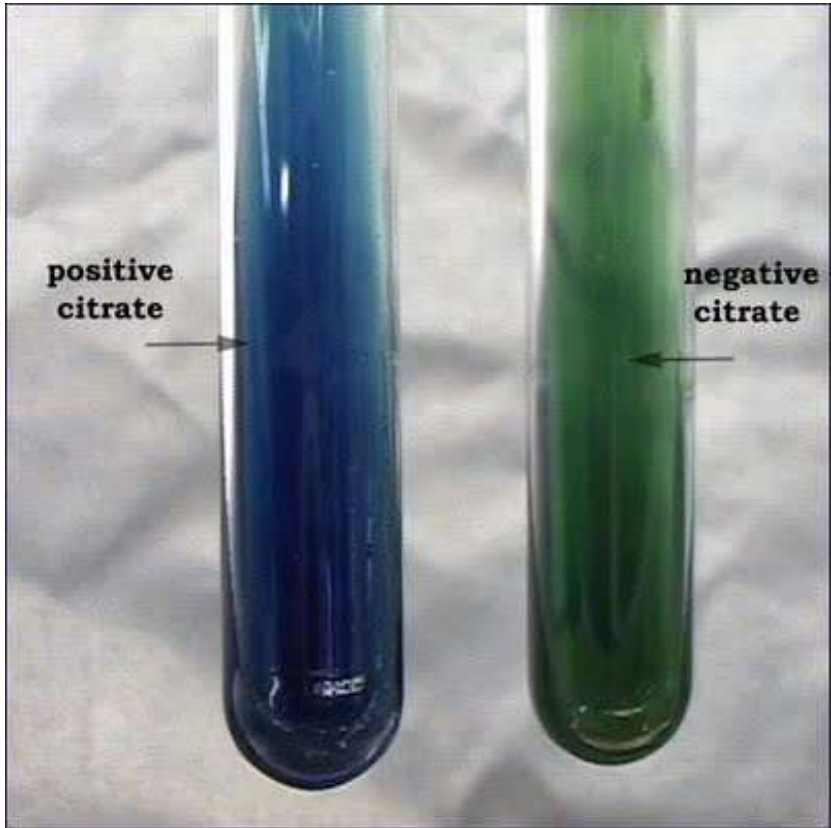


Fig. 14. Citrate test

IMVIC test results for *Salmonella* were given in Table 2.

Reaction	<i>Salmonella</i>
Indole	negative
Methyl Red	positive
Voges Pros Kover	negative
Citrate	positive

Table 2. IMVIC Test Results

5.3.6 Triple tube method

Single colony that identification is desired is made suspension in the bouillon or a 3rd tube. Incubation is performed at 37°C for 3 to 4 hours if required. Plantings are performed to 2nd and 1st tubes. It is left for incubation at 37°C for 18-22 hours.

1. Matters that we may observe in the tube:

- a. Glucose fermentation: It is understood by turning of the bottom of the tube to yellow color.
 - b. Lactose fermentation: The color of sloped surface of the medium turns from orange red into yellow.
 - c. H2S formation: It is understood by formation of black color in the medium.
 - d. Lyzine decarboxylase: 4 ml of 4N NaOH and 2 ml of Chloroform are added on the culture. It is kept at room temperature for 15 minutes, 1 ml from chloroform layer is taken by Pasteur pipette. Equal quantities of ninhydrin (from 0,1% solution in chloroform) is added and kept at room temperature for 10 minutes. Formation of violet color at the end of this period shows that the test is positive.
 - e. Gas formation: It is understood by biodegradation of the medium and occurrence of gas bubbles.
 - f. ONPG Test: Loop full culture which was taken from the surface of the medium is dispersed with 0,25 ml of physiological saline. 0,25 ml ONPG solution is added on this and it is kept in the drying oven at 37°C for 30 minutes. Formation of fixed yellow color at the end of this period was evaluated as positive.
 - g. Other tests: Beta galactosidase
 Phennyaline deaminase
 Oxidase
2. Matters that we may observe in the tube:
- a. Mannitol fermentation: It is understood by conversion of the color from red into yellow.
 - b. Motility: It is smeared through the middle and it is reproduced to right and left alon the planting line.
 - c. Nitrate reduction: 4 drops each from indicators A and B are dripped.
3. Matters that we may observe in the tube:
- a. UREASE formation is observed by conversion o the medium into red color.
 - b. Indole: 0,5 ml of Kovacs indicator is added from the side of the tube slowly. Red color indicates that the test is positive.
 - c. Tryptophane deaminase: 5 drops of medium is transferred into a sterile agglutination tube via a pipette before addition of Kovacs indicator to the medium. 1 drop of 10% FeCl3 is added on it. If the color turns into red tile color within 3 to 5 minutes, test is positive.

Mediumd used in triple tube method

1. TUBE: klikler I A, or TSI agar are used.

Peptone	20 g
Lactose	10 g
Glucose	1 g
Sodyum thiosulphate	0,2 g
Ferroammonium sulphate	0,3 g
NaCl	6 g
Agar	17 g
Phenol red(0,2 %)	12.5 ml
Distilled water	1,000 ml
Ph	7

The mixture is distributed into screw tubes as 7 to 8 ml after boiling. It is sterilized in the autoclave at 121°C for 15 minutes. Tubes are frozen as oblique.

2. TUBE:

Peptone (casein)	5 g
Neopeptone	5 g
Mannitol	2 g
Potassium nitrate	1,7 g
Phenol red(0,2 %)	20 ml
Distilled water	1,000ml
Agar	2,5 g

The mixture is distributed into screw tubes as 5 to 6 ml after boiling. It is sterilized in the autoclave at 121°C for 15 minutes. Ph is adjusted to 7.

3. TUBE:

L-Tryptophane	0,3 g
Potassium dihydrogen phosphate (KH ₂ PO ₄)	0,1 g
Dipotassium hydrogen phosphate (K ₂ HPO ₄)	0,1 g
NaCl	0,5 g
Urea	2 g
Ethanole (95%)	1ml
Phenol red(0,2 %)	1.25 ml
Distilled water	1,000 ml
Ph	6,5

The mixture is sterilized by the filter after dissolving. It is distributed into sterile tubes. If there is no filter, it is sterilized by tyndalisation method (Table 3).

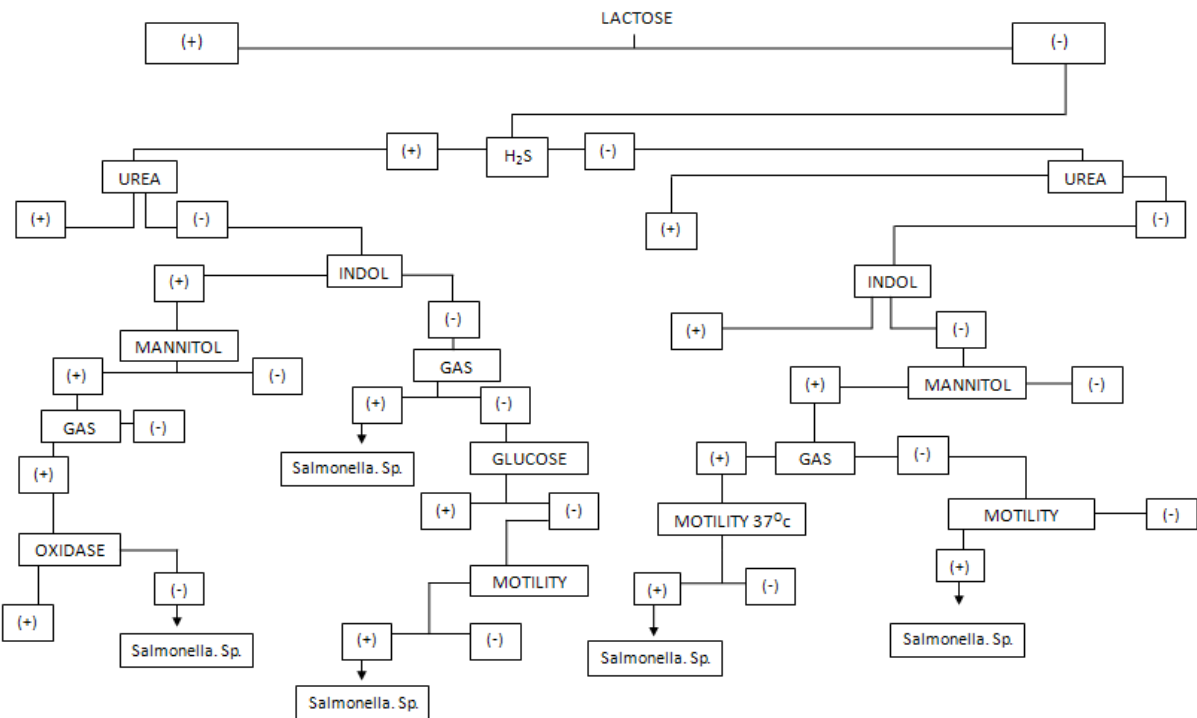


Table 3. Identification Schedule of *Salmonellas* According to triple Tube Method

5.3.7 API method

The API-20E test kit for the identification of enteric bacteria (bioMérieux, Inc., Hazelwood, MO) provides an easy way to inoculate and read tests relevant to members of the Family *Enterobacteriaceae* and associated organisms. A plastic strip holding twenty mini-test tubes is inoculated with a saline suspension of a pure culture (as per manufacturer's directions). This process also rehydrates the dessicated medium in each tube. A few tubes are completely filled (CIT, VP and GEL as seen in the photos below), and some tubes are overlaid with mineral oil such that anaerobic reactions can be carried out (ADH, LDC, ODC, H₂S, URE).

After incubation in a humidity chamber for 18-24 hours at 37°C, the color reactions are read (some with the aid of added reagents), and the reactions (plus the oxidase reaction done separately) are converted to a seven-digit code which is called the Analytical Profile Index, from which name the initials "API" are derived. The code can be fed into the manufacturer's database via touch-tone telephone, and the computerized voice gives back the identification, usually as genus and species. An on-line database can also be accessed for the identification. The reliability of this system is very high, and one finds systems like these in heavy use in many food and clinical labs.

Note: Discussion and illustration of the API-20E system here does not necessarily constitute any commercial endorsement of this product. It is shown in our laboratory courses as a prime example of a convenient multi-purpose testing method one may encounter out there in the "real world."

In the following photos:

- Note especially the color reactions for **amino acid decarboxylations** (ADH through ODC) and **carbohydrate fermentations** (GLU through ARA).
 - The amino acids tested are (in order) arginine, lysine and ornithine. **Decarboxylation** is shown by an **alkaline** reaction (red color of the particular pH indicator used).
 - The carbohydrates tested are glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose. **Fermentation** is shown by an **acid** reaction (yellow color of indicator).
- Hydrogen sulfide production (H₂S) and gelatin hydrolysis (GEL) result in a black color throughout the tube.
- A positive reaction for tryptophan deaminase (TDA) gives a deep brown color with the addition of ferric chloride; positive results for this test correlate with positive phenylalanine and lysine deaminase reactions which are characteristic of *Proteus*, *Morganella* and *Providencia*.

In the first set of reactions:

- Culture "5B" (isolated from an early stage of sauerkraut fermentation) is identified as *Enterobacter agglomerans* which has been a convenient dumping ground for organisms now being reassigned to better-defined genera and species including the new genus *Pantoea*. This particular isolate produces reddish (lactose +), "pimpley" colonies on MacConkey Agar which exude an extremely viscous slime as may be seen here; this appearance is certainly atypical of organisms identified as *E. agglomerans* or *Pantoea* in general.
- Culture "8P44" is identified as *Edwardsiella hoshinae*. The CDC had identified this culture (in 1988) as the ultra-rare Biogroup 1 of *Edwardsiella tarda* which may not be in the API-20E database (Figure 15). This system probably would not be able to differentiate between these two organisms. Note that 8P44 shows H₂S production

which is probably typical of *Edwardsiella tarda* Biogroup 1. Clinical laboratories usually run this test in Triple Sugar Iron Agar in which the organism's fermentation of sucrose (with consequent high acid production) tends to negate the H₂S reaction, and – as a result – the organism is mis-characterized throughout the literature as H₂S negative even though it shows a positive reaction in KIA and other H₂S-detecting media.

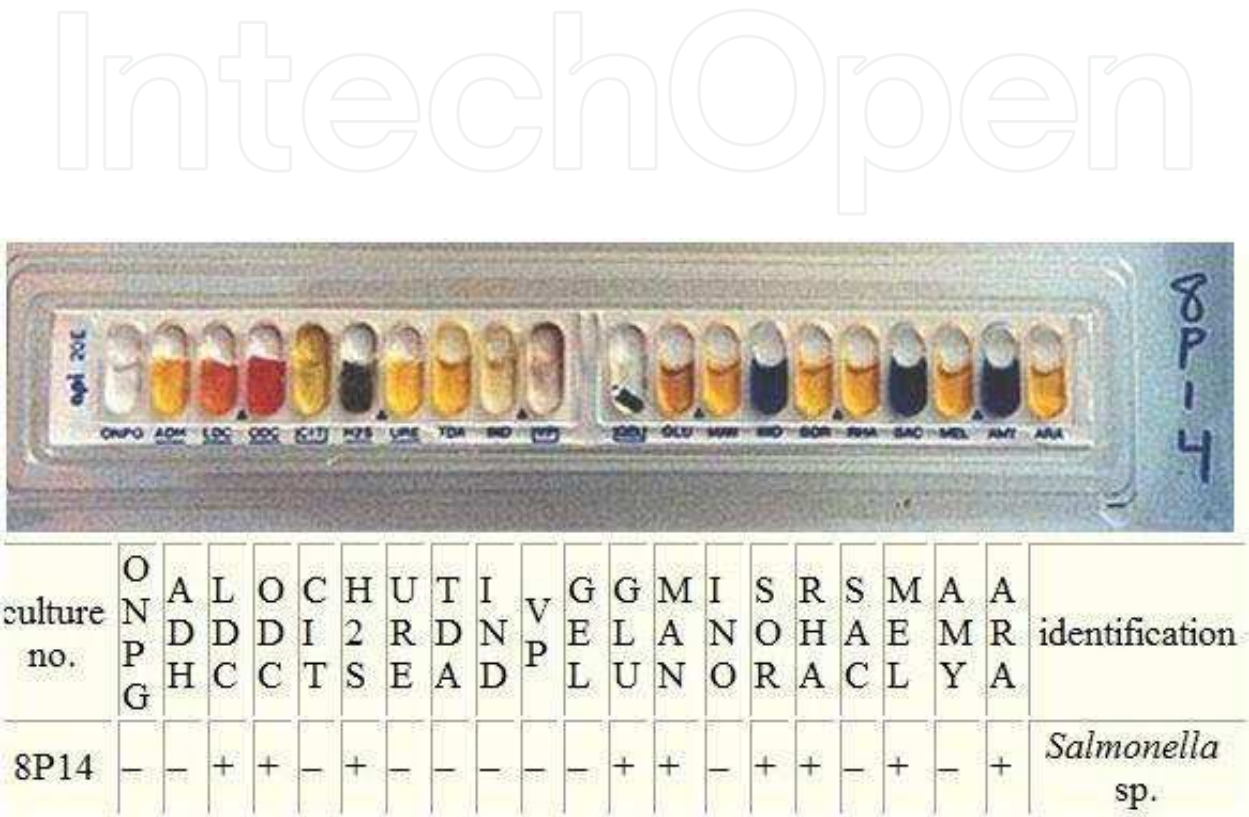


Fig. 15. API Test Result for *Salmonella*

5.3.8 Full automatic bacteria identification device

Some amount of material is taken and transferred into selenite F bouillon (bio-Merieux SA-France). It is incubated at 37°C for 16 to 24 hours in the drying oven. Single colony planting is performed to *Salmonella* -Shigella agar (bio-Merieux SA-France) after the period has passed. It is incubated at 37°C for 16 to 24 hours in the drying oven. Bacteria is made suspension to provide 0,40-0,60 McFarlandt turbidity from reproduced suspicious colonies to Phoenix ID broth.

1 drop of Phoenix AST indicator (Phonex AST Indicator solution, BD Sparks, Benex Limited, Shannon, Ireland) to Phoenix AST broth (Phonex AST borth, BD Sparks, Benex Limited, Shannon, Ireland). 25 µl ID broth is taken and pipetted into AST Broth (BD Phoenix NMIC/ID-82 Sparks- USA). ID and AST broths are transferred to ID and AST pplate. Identification and antibiogram process are performed as full automatically in Phoenix-100 (BD Sparks-USA).

5.4 Serological tests

Antiserums which are used in serological tests of *Salmonellas* can be classified as follows.

Pure *Salmonellas*uspicious culture reproduced in TSI agar, agar agar or bouillon and culture of *Salmonellas* erotypes reproduced in normal or oblique agar can be classified as follows to be used in serological tests:

- a. *Salmonella* polyvalent 0 (somatic) antiserum: 1,16,19,22,23,24,25 and vi in the least,
- b. *Salmonella* individual (0) somatic group anti-serums: A,B,C1,C2,D E (E1,E2,E3,E4), F,G,H,I, vi,
- c. *Salmonella* polyvalent H (flagella) antiserum: It will include agglutinins of a,b,c,d,en,enx,fg,tgt,gm,gms,gp,gpu,gq,gst,gt,i,k,lv,lw,lz13,lz28,mt,r,y,z,z4z23,z4z24,z4z32,z6,z10,z29,1,2,1,5,1,6 ve 1,7 antigens.
- d. *Salmonellas* picer-Edward's H (flagella) antiserums:

It is consisted of seven antiserums and it gives a reaction as follows:

- d₁) *Salmonella* H antiserum Spicer –Edwards 3: Gives reaction with a, d, eh, k, z, z4z23, z4z32 ve z29 antigens.
- d₂) *Salmonella* H antiserum Spicer –Edward 4: Gives reaction with b, d, fg, fgt, gm, gms, gmt, gp, gpu, gq, gst, ms, mt, k, r, z, ve z10 antigens.
- d₃) *Salmonella* H antiserum e,n complex: It gives reaction with enx and enz 15 antibodies.
- d₄) *Salmonella* H antiserum L complex: Gives reaction with 1v,1w,1zl3,lz28 antigens.
- d₅) *Salmonella* H antiserum 1 complex: Gives reaction with 1,2; 1,5 1,6 1,7 ve z6 antigens.

5.4.1 Lame test for polyvalent 0 (somatic) antigen

1. First, reliability of antiserum should be detected. For this, various dilutions of the antiserum should ne prepared and controlled with a certain culture.
2. The lame is divided into two by a glass cutter.
3. A small amount of culture a taken and put on both parts of the lame. (cultures in normal oblique agar and 24 hours cultures of TSI agar should be used.).
4. One drop each from 0,85% sodium chloride solution is put on drops above and it is emulsified well.
5. One drop of *Salmonella* polyvalent (0) antiserum is put into the first section and mixed well.
6. The mixture is mixed by moving to the front and back for one minute and it is examined on a dark base. Positive reaction is rapid and strong reaction.

5.4.2 Polyvalent H (flagella) antigen agglutination test

1. Various dilutions of antiserum is prepared and it is controlled that whether the antiserum is safe with a known culture.
2. 5 ml of 24 hours H bouillon culture is taken, 5 ml of 0,6% formalin physiological saline is added on it. It is kept for one hour. Formalin bouillon is kept for a couple of days at 5 to 8°C if required.
3. A small serological tube (10 X 75 mm or 13X 100 mm) is taken. 0,02 ml (one drop) is put from H (flagella) antiserum which was diluted appropriately and 1 ml of formalin bouillon culture (antigen) is added on it.

4. If formalin culture includes granular particles or thin membrane or sediment, control is performed by adding formalin salty water instead of antiserum (salty water control). For this, 0,02 ml Formalin salty water is put into the tube with the same length and 1 ml of formalin bouillon culture is added on it.
5. Antigen-serum mixture and antigen-salty water mixture are incubated at 50°C warm water bath for one hour. It is controlled by 15 minutes of interval first and the final result is read after one hour.
6. Polyvalent H test is assessed as follows.
 - a. If agglutination is present in culture+formalin salty water + serum mixture and agglutination is absent in culture- formalin salty water mixture, reaction is positive.
 - b. If there is no agglutination in culture+ formalin salty water + serum mixture, reaction is negative.
 - c. If there is agglutination in both mixtures, reaction is non-specific.
7. Immotile *Salmonella* cultures or *Salmonella* polyvalent H (flagella) negative cultures are assessed according to Edwards and Ewing.

5.4.3 “0” antiserum groups test

This test is performed to determine the “0” group that the culture belongs to.

1. Various dilutions of antisera are performed and it is processed with a known culture and reliability of antiserum is controlled.
2. The test is applied as told in section II. But, “0” group antisera are used in here instead of 0 polyvalent. (Including Vi).
3. Intense suspensions of cultures which give positive reactions with Vi antisera in 1 ml physiological saline and it is heated in boiling water for 20-30 minutes and left for cooling. The test is repeated by using D, C and Vi antisera of 0 group with these heated cultures. Vi positive cultures which react with Somatic D group antisera are likely *Salmonella* typhi. Vi positive cultures which react with Somatic C1 group antiserum are probably *Salmonella* paratyphi C. If Vi positive cultures heated which does not react with any of = group antiserum continues to give positive reaction after heating, they are not probably *Salmonella*. They belong to *Citrobacter* group.
4. The culture is accepted as belonging to the group that the culture reacted positively with which of “0” group antisera. Cultures that do not react positively with any of 0 group antisera are accepted as negative.

5.4.4 Spicer-Edwards H (flagella) test

These tests may be used instead of polyvalent H test which was specified in 4.4.2. It is used in determination of H antigens.

1. Various dilutions of antisera are prepared and it is processed with a known culture and reliability of antiserum is detected.
2. Every seven Spicer-Edwards H antiserum is processed with each of them. This examination is as told in section III. Spicer-Edwards H antisera are used instead of polyvalent H antiserum.
3. Positive agglutination shows presence of H antigen. Antigen is detected according to Spicer-Edwards antiserum agglutinins which was shown in the following table 3.

H antigen	Positive reaction with Spiecer Edward <i>Salmonella</i> H antiserum
a	1, 2, 3
b	1, 2, 4
c	1, 2
d	1, 3, 4
eh	1, 3
G complex	1, 4
i	1
k	2, 3, 4
r	2, 4
y	2
z	3, 4
z ₄ complex	4
z ₁₀	4
z ₉	2, 3
enx, enz ₁₅	en complex
1v, 1w, 1z ₁₃ , 1z ₂₈	1 complex
1,2; 1,5; 1,6; 1,7; z ₆	complex

Table 3.

5.5 Gruber-Widal reaction

Gruber Widal reaction is used to reveal infections caused by *Salmonella* group bacteria. *Salmonella* typhi, *Salmonella* paratyphi B bacteria are used in the reaction, because they are most common species in our country. *Salmonella* paratyphi A is added for many times. Bacteria such as *Salmonella* paratyphi C are also important in another countries. Both O and H antigens of bacteria are used separately in the reaction. Because, only O or G agglutinins occur especially in first episode of the disease. O agglutinins appear before H agglutinins generally in typhoid fever. As O antigen fractions of *Salmonella* typhi are present in *Salmonella* paratyphi B, they give common agglutination. H agglutination is more valuable as it is not common.

Gruber Widal reaction is performed quantitatively, because serum titration is important. It is impossible to put definite rules to improve various titrations. Reaction is improved by considering other findings. It should be considered that in which day of the disease serum has been taken, whether an infection appeared previously, whether protective vaccination is performed and normal antibody level in healthy persons in the population.

Agglutinins may exist normally in the serum. H agglutinins of *Salmonella* typhi and *Salmonella* paratyphi B may be 1/40 titration and O agglutinins may be as 1/50 titration. Therefore, reaction is started as final dilution of the serum in the first tube will be as 1/50. If a non-specific antigen such as fimbria antigen is present in the bacteria suspension, false positive Gruber-Widal reaction occurs with an agglutinin in the human serum.

Specific antibodies occur in serums of those who had typhoid-paratyphoid vaccination and Gruber-Widal reaction is positive in them. If previously vaccinated persons have a pyretic disease, agglutination titration elevates. When vaccinated persons are examined after months, it is seen that they have H agglutinins mainly. Therefore, high H agglutinin titration of those who was vaccinated is meaningless. However, if more than six months has passed from the vaccination date and titration of O agglutination is more than 1/100 and titration elevates in continuous assays, such result is insignificant in the diagnosis.

Presence of both O and H agglutinins in persons who had undergone the infection may last long.

Detection of O antibodies in the serum shows that a new infection was experienced. H agglutinins are 1/400 or higher in those who had new infections and they stay for a long time, sometimes for years. Serum titration elevates during an pyretic disease (anamnestic reaction). O agglutinins decrease rapidly in the blood and it may be shown rarely after one year. High O titration is not seen with anamnestic reaction.

1/100 positive O agglutination and 1/200 positive agglutination are valuable in persons who was not infected and vaccinated. 1/200 O agglutination and 1/400 H agglutination in persons who was infected and vaccinated before is valuable in terms of diagnose of a new infection. Only 1/100-1/200 positive H agglutination shows an undergone infection, new vaccination or anamnestic reaction.

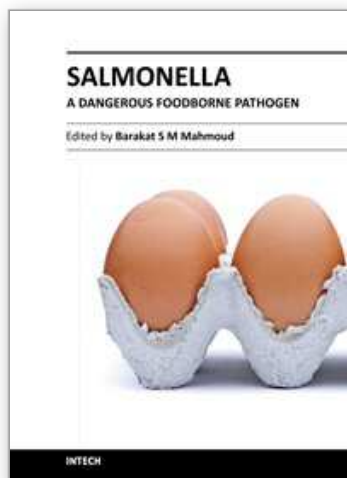
Making the Assay;

6 series of tubes are taken, 6 tubes are put in every series as 1/50, 1/100, 1/200, 1/400 serum dilutions, antigen control and serum control. To make serial dilution, 0,5 cm³ salty water except 1st tube and last serum control tube and 0,5 cm³ diluted immunized serum is put into the last tube. Immunized serum which was generally obtained from rabbits as immunized serum is used by diluting 100 or 1000 times and adding 0,5% phenol. First Tube 7.2 cm³ and 0.3 cm³ of saline placed in serum 1 / 25 dilution of each series after the 1st and 2 tube is added 0.5 cm³. Serum was diluted as 1/25 in the 1st tube. Same amount of salty water was diluted as much in the 2nd tube and dilution has become 1/50.

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Salmonella - A Dangerous Foodborne Pathogen

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More than 2,500 serotypes of Salmonella exist. However, only some of these serotypes have been frequently associated with food-borne illnesses. Salmonella is the second most dominant bacterial cause of food-borne gastroenteritis worldwide. Often, most people who suffer from Salmonella infections have temporary gastroenteritis, which usually does not require treatment. However, when infection becomes invasive, antimicrobial treatment is mandatory. Symptoms generally occur 8 to 72 hours after ingestion of the pathogen and can last 3 to 5 days. Children, the elderly, and immunocompromised individuals are the most susceptible to salmonellosis infections. The annual economic cost due to food-borne Salmonella infections in the United States alone is estimated at \$2.4 billion, with an estimated 1.4 million cases of salmonellosis and more than 500 deaths annually. This book contains nineteen chapters which cover a range of different topics, such as the role of foods in Salmonella infections, food-borne outbreaks caused by Salmonella, biofilm formation, antimicrobial drug resistance of Salmonella isolates, methods for controlling Salmonella in food, and Salmonella isolation and identification methods.

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