SNI

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Indonesian National Standard

Methods for testing microbial contamination in meat, eggs and milk, as well as their processed products

ICS 67.050

National Standardization Agency

BSN

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Preface

Standard microbial contamination testing methods include *Total Plate Count* (TPC), *Coliform, Escherichia coli, Staphylococcus aureus, Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes* in meat, eggs and milk, and their by-products,

This standard is a revision and refinement of most of the scope of testing in SNI 01-2897-1992 Test methods for microbial contamination, except for testing for Enterococci, Clostridium perfringes, and Vibrio cholerae, as well as the addition of testing for Campylobacter spp. and Listeria spp. which is adapted to the current development of science and technology (IPTEK). This standard was prepared and formulated after going through test validation in a veterinary public health laboratory.

This standard was prepared and formulated by the Technical Committee 67-03 Livestock and Livestock Products. This standard was discussed in a technical meeting on September 10, 2007, and was last agreed at a consensus meeting on October 30, 2007 in Bogor which was attended by members of the Technical Committee and other related parties. This standard has also been through the polling stage on April 7, 2008 to June 7, 2008, but to reach a quorum, it was extended until July 7, 2008 and immediately approved as RASNI.

This SNI is prepared to support the applicable laws and regulations of the Republic of Indonesia in the field of food safety of animal origin.

Methods for testing microbial contamination in meat, eggs and milk, as well as their processed products

1 Scope

This standard stipulates the method of testing for microbial contamination of *Total Plate Count* (TPC), *Coliform, Escherichia coli, Staphylococcus aureus, Salmonella* spp., *Campylobacter* spp., and *Listeria monocytogenes* qualitatively and quantitatively in meat, eggs and milk, as well as their processed products.

2 Terms and definitions

2.1 microbial

contamination of micro-organisms/microbial contaminants in meat, eggs and milk, as well as their processed products which can damage products and or endanger human health

2.2

meat
Skeletal muscle parts of livestock/animal carcasses that are safe, fit and commonly consumed by humans, can be in the form of fresh meat, cold fresh meat, or frozen meat

2.3

processed
meat that has undergone processing

2.4

eggs
produced by poultry that have not undergone processing and incubation for human consumption

2.5

eggs
processed eggs that have undergone processing

2.6

milk
liquid derived from the udder of healthy and clean dairy cattle, which is obtained by proper milking in accordance with applicable regulations, whose natural content is not reduced or added by anything and has not received any treatment except the cooling process

2.7

dairy milk
that has undergone processing

2.8

Most Probable Number
(MPN) an estimate (estimate) of the number of microbes in a food, by fertilizing at a level of dilution into three or five tubes filled with liquid media

2.9
Total Plate Count (TPC)
how to calculate the number of microbes contained in a product that grows on agar media at a specified temperature and incubation time

3 Abbreviations

a) ***BPW 0,1%:Buffered Pepton Water 0,1%;*** **b)** ***BGLBB :Brilliant Green Lactose Bile Broth;*** **c)** ***BHI :Brain Heart Infusion;*** **d)** ***BHIB: Brain Heart Infusion Broth;*** **e)** ***BPA :Baird-Parker Agar;*** **f)** ***BSA :Bismuth Sulfite Agar;*** **g)** ***CFU : Colony Forming Unit;***

h) ***EDTA :Ethylene Diamine Tetraacetic Acid;*** **i)** ***ECB :Escherichia Coli Broth;*** **j)** ***E.coli :Escherichia coli;*** **k)** ***LEMBA : Levine Eosin Methylene Blue Agar;*** **l)** ***FBS : Fetal Bovine Serum;*** **m)** ***HEA :Hektoen Enteric Agar;*** **n)** ***IMViC: Indole, Methylred, Voges Proskauer dan Citrate;*** **o)** ***KCN :Potassium Cyanide;*** **p)** ***KCNB : Kalium Cyanide Broth;***

q) ***LIA :Lysine Iron Agar;*** **r)** ***LDB :Lysine Decarboxylase Broth;*** **s)** ***LB :Lactose Broth;*** **t)** ***LSTB :Lauryl Sulfate Tryptose Broth;***
u) ***MPN :Most Probable Number;*** **v)** ***MR-VP : Methyl Red-Voges Proskauer;*** **w)** ***PCA :Plate Count Agar;*** **x)** ***RV :Rappapport Vassiliadis;*** **y)** ***S.aureus : Staphylococcus aureus;*** **z)** ***SCB :Selenite Cystine Broth;*** **aa)** ***TPC :Total Plate Count;*** **bb)** ***TSIA :Triple Sugar Iron Agar;***

cc) ***TTB : Tetra Thionate Broth;***
dd) ***TSTB :Trypticase Soy Tryptose Broth;***
ee) ***TB : Tryptose Broth;*** **ff)** ***XLDA:Xylose Lysine Deoxycholate Agar;*** **gg)** ***KCB : Koser Citrate Broth;*** **hh)** ***SCA : Simmons Citrate Agar.***

4 Test method

4.1 Pengujian Total Plate Count (TPC)

4.1.1 Principle

Total Plate Count (TPC) **is intended to indicate the number of microbes contained in a product by counting bacterial colonies grown on agar media.**

4.1.2 Media and reagents

a) *PCA;*
b) *BPW* 0,1%.

4.1.3 Equipment

a) petri dish; b) test tubes; c) volumetric pipette; d) micropipette; bottles; e) *colony counter;* f) scissors; g) tweezers; h) inoculation needle (ose); i) *stomacher;* j) Bunsen burner; k) *stomacher bags;* scales; m) *magnetic stirrer;* n) tube shaker (vortex); o) incubator; p) water bath; q) autoclave; r) sterilization set (refrigerator); t) *freezers.*

4.1.4 Sample setup

a) Weigh solid and semi-solid samples as much as 25 g or measure liquid samples as much as 25 ml aseptically, then put in a sterile container.

b) For examples of meat, eggs and milk
Add 225 ml of sterile 0.1% ***BPW*** solution **into a sterile bag containing the sample, homogenize with *a stomacher* for 1 minute to 2 minutes (except for liquid milk samples). This is a solution with a dilution of 10-1 .**

4.1.5 Test method

a) Transfer 1 ml of the suspension at the **10-1** dilution **with a sterile pipette into the 9 ml *BPW*** solution to obtain a **10-2 dilution .**

b) Make a dilution of 10-3 , 10-4, 10-5 and so on in the same way as at point a), as needed.

c) Next, put 1 ml of suspension from each dilution into a petri dish in duplicate.

d) Add 15 ml to 20 ml of ***PCA*** **which has been cooled to a temperature of 45 °C ± 1 °C in each cup that already contains the suspension. So that the sample solution and *PCA*** media **are completely mixed, rotate the cup back and forth or form a figure eight and let it sit until it becomes solid.**

e) Incubate at a temperature of 34 °C to 36 °C for 24 hours until 48 hours by placing the cup upside down.

f) Especially for dairy products, incubate at a temperature of 32 °C ± 1 °C for 24 hours up to 48 hours by placing the cup upside down.

4.1.6 Counting the number of colonies

Count the number of colonies in each dilution series except for the petri dish containing *spreader colonies.* Choose a dish that has a colony count of 25 to 250.

4.1.7 Interpretation of results

4.1.7.1 Cups with less than 25 colonies

If duplicate plates from the lowest dilution produce less than 25 colonies, count the number of plates in each dilution.
Average the number of colonies per plate and multiply by the dilution factor to determine the *TPC value.*
Mark the *TPC* value with an asterisk (Table 1 number 3) to indicate that the count is outside 25 colonies up to 250 colonies per cup.

4.1.7.2 Cups with more than 250 colonies

If the number of colonies per plate is more than 250, count the colonies on the plate to give a representative picture of the colony distribution. Flag *TPC* calculation with an asterisk to indicate that the count is outside of 25 colonies up to 250 colonies per cup (Table 1 number 4).

4.1.7.3 Spreaders

Colonies that spread *(spreaders)* are usually divided into 3 forms:

a) Colony chains are not clearly separated due to the disintegration of the bacterial clump. b) Formation of a layer of water between the agar and the bottom of the cup. c) The formation of a layer of water on the side or surface of the agar.

When the cup prepared for the example is more overgrown by the *spreader* like (a), and the total area that exceeds 25% and 50% of the growth is reported as a *spreader cup.*

Average the number of colonies from each dilution, then report the number as *TPC* (Table 1 number 5).
In addition to 3 (three) forms of *spreader,* it can be counted as one colony growth.
For type a) if there is only one chain, count as a single colony. If one or more chains are visible from other sources, count each of those sources as a colony, including for types b) and c) also counted as colonies.
Combine colony calculations and *spreader* calculations to calculate *TPC.*

4.1.7.4 Beaker without colonies

If petri dishes of all dilutions yield no colonies, report the *TPC* as less than 1 times the lowest dilution used. Mark the *TPC* with an asterisk that the count is outside of 25 colonies up to 250 colonies (Table 1 number 6).

4.1.7.5 Duplo cups, one cup with 25 colonies up to 250 colonies and the other cup with more than 250 colonies

If one plate produces between 25 and 250 colonies and the other has more than 250 colonies, count both plates in the *TPC* calculation (Table 1 number 7).

4.1.7.6 Duplo plates, one cup from each dilution with 25 colonies up to 250 colonies

If 1 plate of each dilution yields 25 colonies to 250 colonies, and another plate has fewer than 25 colonies or produces more than 250 colonies, count all four in the *TPC* calculation (Table 1 number 8).

4.1.7.7 Duplo plates, two plates from one dilution with 25 colonies up to 250 colonies, only 1 plate with more than 25 colonies up to 250 colonies and from another dish with 25 colonies up to 250 colonies

If both plates from one dilution yield 25 colonies up to 250 colonies, count the four plates including those with less than 25 or more than 250 colonies in the *TPC* calculation (Table 1 number 9).

4.1.8 Reporting results

a) Round the number to 2 appropriate numbers, if the third number is 6 or above, then the third number becomes 0 (zero) and the second number increases by 1 digit, for example 456 becomes 460 (4.6 x 102).

b) If the third digit is 4 or below, then the third digit becomes 0 (zero) and the number both remain, for example 454 becomes 450 (4.5 x 102).

c) If the third number is 5, then the number can be rounded to 0 (zero) and the second number is an even number, for example 445 becomes 440 (4.4 x 102).

d) If the third digit is 5, then that number can be rounded up to 0 (zero) and the second number is increased by 1 digit, for example 455 to 460 (4.6 x 102).

Table 1 - TPC calculation instructions

No 10 $^{-2}$	10-3	10-4	TPC per ml or gram (5)	Information
(1) (2)	(3)	(4)		(6)
1 === ===	175 208	16 17	190.000	if only one dilution is within the appropriate limits, calculate the average of the dilutions. if there are two dilutions that are within appropriate
2 === ===	224 225	25 30	250.000	limits, calculate the amount of each of the dilutions before averaging the actual amount.
3 18 14	2 0	0 0	1.600*	The number of colonies is less than 25 colonies at the lowest dilution, count the number and multiply by the dilution factor and put a * (excluding the number of colonies from 25 to 250).
4 === ===	==== ====	523 487	5.100.000	The number of colonies is more than 250 colonies, count the colonies that can be counted or which represent a sign * (excluding the number of colonies from 25 to 250).
5 === ===	245 230	35 spreader	290.000	If there are two dilutions between the number of colonies of 25 to 250, but there is a *spreader,* count the number and multiply by the dilution factor, but for the *spreader* not calculated.
6 0 0	0 0	0 0	100*	If the plate is without colonies, the number of TPC is less than 1 times the lowest dilution used, and put a * The number of colonies is 25
7 === ===	245 278	23 20	260.000	to 250, and the other is more than 250 colonies, count the two petri dishes including those with more than 250 colonies, and the average number .
8 === ===	225 255	21 40	270.000	If one dish has 25 colonies up to 250 colonies from each dilution, count the number of each dilution including those less than 25 colonies, then average the actual number.
9 === ===	220 240	18 48	260.0000	If only one plate deviates from each dilution, count the number of each dilution including those less than 25 colonies or more than 250 colonies, then average the actual number.
=== ===	260 230	30 28	270.000	

4.2 Pengujian Most Probable Number (MPN) Coliform

4.2.1 Principle

The *Most Probable Number (MPN)* method **consists of a *presumptive*** test **(estimator) and a confirmation test (confirmation), using liquid media in a test tube and based on the number of positive tubes. Positive tube observations can be seen by the emergence of gas in the *Durham tube.***

4.2.2 Media and reagents

**a) larutan *BPW* 0,1 %; b)
BGLBB; c) *LSTB.***

4.2.3 Equipment

**a) Durham tube; b)
test tubes; c) pipette
size 1 ml, 2 ml, 5 ml, 10 ml; d) media bottles; e)
scissors; f) tweezers; g) inoculation
needle (ose); h) *stomachers;* i) Bunsen
burner; j) thermometer; k) analytical scale; l) shaker
(vortex); n) incubator; o) water bath;
p) autoclave; q) sterile cabinet *(clean
bench);* r) s)
magnetic stirrer)
refrigerator (refrigerator);
*freezer.***

4.2.4 Sample setup

a) Weigh solid and semi-solid samples as much as 25 g or measure liquid samples as much as 25 ml aseptically then put in a sterile container.

b) For examples of meat, eggs and milk
Add 225 ml of sterile 0.1% ***BPW*** solution **into a sterile bag containing the sample, homogenize with *a stomacher* for 1 minute to 2 minutes (except for liquid milk samples). This is a solution with a dilution of 10-1 .**

4.2.5 Test method

Testing using a series of 3 tubes.

4.2.5.1 Test of conjecture

a) Transfer 1 ml of the 10-1 dilution solution **with a sterile pipette into 9 ml of 0.1% *BPW*** solution to obtain a **10-2 dilution. In the same way as above, a 10-3** dilution was made .

b) Pipette 1 ml each of each dilution into 3 series of *LSTB* tubes **which**
contains a ***Durham* tube .**

c) Incubation at a temperature of 35 °C for 24 hours to 48 hours.

d) Note the presence of gas formed in the *Durham tube.* Test results declared positive when gas is formed.

4.2.5.2 Confirmation test (affirmation)

a) The test is always accompanied by a positive control.

b) Transfer the positive culture from 4.2.5.1 d) by using the inoculation needle from each *LSTB* tube **into the *BGLBB*** tube **containing the *Durham tube.***

c) Incubate at a temperature of 35 °C for 48 hours ± 2 hours.

d) Note the presence of gas formed in the *Durham tube.* Test results declared positive when gas is formed.

e) Then use the *Most Probable Number (MPN)* table **to determine the value of *MPN* based on the number of** positive ***BGLBB*** tubes **as the number of coliforms per milliliter or per gram.**

4.2.6 Interpretation of results

The number of coliforms in the test sample is interpreted by matching the combination of the number of tubes that show positive results, based on the table of *MPN* values.
(Appendix A). The combination is taken, starting from the highest dilution which still produces all positive tubes, while in the next dilution there are negative tubes. The combination taken consisted of three dilutions. The sample ***MPN*** value **is calculated as follows:**

$$MPN\ MPN\ value\ MPN\ (\text{ml or g}) = MPN \quad \frac{\text{sample table}}{100} \text{ x middle dilution factor}$$

4.3 Escherichia coli . MPN Testing

4.3.1 Principle

The tests were carried out by predicting, confirming and isolating-identification through the *Indole, Methyl red, Voges-Proskauer* and *Citrate (IMViC) biochemical tests.*

4.3.2 Media and reagents

a) *BPW* 0,1 %;
b) *BGLBB;* c)
***LSTB;* (d) the**
ECB;* (e) *L-
EMBA;* f) *MR-
VP;* (g) *PCA;
(h) *KCB;* i)
SCA;* (j) *Covas
***reagent;* (k)**
Voges-Proskauer (VP)
reagent.

4.3.3 Equipment

a) Durham tube; b) Petri dish; c) test tubes; d) pipette size 1 ml, 2 ml, 5 ml, 10 ml; e) media bottles; f) scissors; g) tweezers; h) inoculation needle (ose); i) *stomacher*; j) Bunsen; k) *magnetic stirrer;* n) tube shaker (vortex); o) incubator; p) water bath; q) autoclave; s) refrigerator (refrigerator); t) *freezers.*

4.3.4 Sample setup

a) Weigh solid and semi-solid samples as much as 25 g or measure liquid samples as much as 25 ml aseptically then put in a sterile container.

b) For examples of meat, eggs and milk
Add 225 ml of 0.1% ***BPW*** solution **into a sterile bag containing the sample, homogenize with *a stomacher* for 1 minute to 2 minutes (except for liquid milk samples). This is a solution with a dilution of 10-1 .**

4.3.5 Test method

The test uses a series of 3 tubes, isolation-identification test, and biochemical test.

4.3.5.1 Series 3 tube

4.3.5.1.1 Test of conjecture

a) Transfer 1 ml of the 10-1 dilution solution **with a sterile pipette into 9 ml of 0.1% *BPW*** solution to obtain a **10-2 dilution. In the same way as above, a 10-3** dilution was made .

b) Pipette 1 ml of each dilution into 3 series of *LSTB* tubes **containing *Durham tubes.***

c) Incubate at a temperature of 35 °C for 24 hours to 48 hours.

d) Note the presence of gas formed in the *Durham tube.* Test results declared positive when gas is formed.

4.3.5.1.2 Confirmation test (affirmation)

a) Tests should always be accompanied by the use of positive controls. b) Transfer positive cultures from 4.3.5.1.1 d) using inoculation needles from each *LSTB* tube into the *ECB* tube containing the *Durham tube.*

c) Incubate the ***ECB*** **at 45.5°C for 24 hours ± 2 hours, if the result is negative, incubate it again for 48 hours ± 2 hours.**

d) Note the presence of gas formed in the *Durham tube.* Test results declared positive when gas is formed.

e) Then use the *Most Probable Number (MPN)* table **to determine the value of *MPN* based on the number of** positive ***ECB*** tubes **containing gas in the *Durham*** tubes **as the number of *E. coli* per milliliter or per gram.**

4.3.5.1.3 Interpretation of results

The number of coliforms in the test sample is interpreted by matching the combination of the number of tubes that show positive results, based on the table of *MPN* values. **(Appendix A). The combination is taken, starting from the highest dilution which still produces all positive tubes, while in the next dilution there are negative tubes. The combination taken consisted of three dilutions. The** sample ***MPN*** value **is calculated as follows:**

$$MPN\ MPN\ value\ (MPN\ /ml\ or\ /g) = MPN\ \frac{\text{sample table}}{100}\ x\ middle\ dilution\ factor$$

4.3.5.2 Isolation-identification

a) Make a streak on *L-EMBA* or *VRBA media* from a positive ***ECB*** **tube , incubate at a temperature of 35°C for 18 hours to 24 hours.**

b) Colonies suspected of *E. coli* are 2 mm to 3 mm in diameter, black or dark in color at the center of the colony, with or without a shiny greenish metallic on *L-EMBA media.*

c) Take the presumed colonies from each *L-EMBA* medium **using a loop, and transfer them to** an **inclined *PCA* . Incubate** inclined ***PCA*** **at a temperature of 35 °C for 18 hours to 24 hours for biochemical tests.**

4.3.5.3 Biochemical test by IMViC test.

4.3.5.3.1 Indole production test

a) Inoculate colonies from ***PCA*** **tubes on *TB* and incubate at 35°C for 24 hours ± 2 hours.**

b) Add 0.2 ml to 0.3 ml of *Kovac's reagent.*

c) The positive reaction results are indicated by the formation of a red ring on the top layer of the media, while the negative reaction results are indicated by the formation of a yellow ring.

4.3.5.3.2 Uji Voges-Proskauer (VP)

a) Take the culture from *PCA* media and inoculate it into a tube containing 10 ml of *MR* media *VP* and incubated at 35 °C for 48 hours ± 2 hours.

b) Transfer 5 ml *of MR-VP* to a test tube and add 0.6 ml of -naphthol and solution 0.2 ml KOH 40 %, then shaken.

c) Positive reaction results are indicated by the presence of a pink eosin color within 2 hours.

4.3.5.3.3 Uji Methyl Red (MR)

a) Take the culture from *PCA* media and inoculate it into a tube containing 10 ml of *MR* media *VP* and incubated at 35 °C for 48 hours ± 2 hours.

b) Add 2 drops to 5 drops of the *MR* indicator in the tube.

c) A positive test result is indicated by a red color and a negative test result is indicated by the presence of yellow.

4.3.5.3.4 Uji citrate

a) Inoculate colonies from ***PCA* agar slanted into *KCB media,* and incubate at 35°C for 96 hours.**

b) A positive test result is indicated by the formation of turbidity in the media.

4.3.5.3.5 Interpretation of biochemical test results

Classification of *E. coli* is
***IMViC* reaction with + + - - or - + - - pattern (Table 2),**

Table 2 - Results of the reaction of Indole, Methyl Red, Voges- Proskauer, Citrate (IMViC) against E. coli

Tipe Organisme *E.*	Indo	MR	VP	Citrate
***coli* spesifik *E. coli* non**	+	+	-	-
spesifik *Typical intermediate*	-	+	-	-
Atypical intermediate Typical	N/A	+	-	+
Enterobacter aerogenes	-	+	-	+
Atypical Enterobacter aerogenes	-	-	+	+
	+	-	+	+

4.3.6 Interpretation of final results

The number of *E. coli* was stated based on the results of *MPN,* Isolation - identification, and biochemical tests.

4.4 Testing the number of Staphylococcus aureus

4.4.1 Principle

The method used is to count the plates spread on the surface of the media.

4.4.2 Media and reagents

a) *BPA;*
b) *Egg yolk tellurite emulsion;*
c) *BHIB;* d) *TSA;* e) coagulase plasma alone *(coagulate rabbit plasma* with 0.1ÿ% *EDTA);* f) *BPW* 0.1 %.

4.4.3 Equipment

a) petri dish; b) test tubes; c) pipettes, size 1, 10 ml; d) media bottles; e) beaker; f) inoculation needle (ose); i) *stomachers;* j) Bunsen burner; k) pipette; l) vortex shaker (vortex); o) incubator; p) water bath; q) autoclave; r) sterile cabinets *(clean bench);* s) refrigerator (refrigerator); t)

4.4.4 Sample setup

a) For milk sample (liquid)
The samples tested started with a dilution of 100 (sample without dilution), then a series of 10-1, 10-2, 10-3 dilutions were made and so on.

b) For example meat, eggs and milk (solid and semi-solid)
Weigh the solid and semi-solid samples as much as 25 g or measure the liquid sample as much as 25 ml, aseptically then put in a sterile container.

c) Add 225 ml of sterile *BPW* solution into a sterile bag containing the sample, then homogenize with *a stomacher* for 1 minute to 2 minutes. This is a solution with a 10-1 dilution, then 10-1, 10-2 , 10-3, and so on are made.

4.4.5 Test method

a) The test is always accompanied by using a positive control.

b) Transfer 1 ml of the sample from 100 to a solution of 9 ml of *BPW* to obtain a dilution of 10-1. In the same way, dilutions of 10-2, 10-3, and so on are made.

For samples of liquid milk, start with a dilution of 10-0 , while for examples of meat, eggs, and milk (solid and semi-solid) start with a dilution of 10-1 .

c) Pour 15 ml to 20 ml of *BPA* media which has been added with *egg yolk tellurite emulsion* (5 ml into 95 ml of *BPA media)* in each cup to be used and allow it to solidify.

d) Pipette 1 ml of suspension from each dilution, and inoculate 0.4 ml, 0.3 ml, and 0.3 ml, respectively, in 3 petri dishes containing the media in letter c above.

e) Spread the sample suspension on the surface of the agar medium using a rod glass *(hockey stick),* and leave until the suspension is absorbed.

f) Incubate at a temperature of 35 °C for 45 hours to 48 hours at position backwards.

g) Choose a petri dish containing the number of colonies from 20 to 200. If the petri dish at the lowest dilution contains < 20 colonies and or > 200 colonies, then continue counting the colonies in the petri dish with the higher dilution.

h) Colonies of *S. aureus* are characterized by round, smooth and smooth, convex, diameter 2 mm to 3 mm, gray to black in color, surrounded by an opaque zone, with or without a bright outer zone *(clear zone).* The edges of the colony are white and surrounded by bright areas. Colony consistency is like butter or fat when touched by the *ose.* The non-lipolytic strains had the same colony characteristics as above, but were not surrounded by an opaque zone and a bright outer zone.

i) Record the number of each colony that has the same characteristics as in h).

j) Take one or more colonies of each growing form and do the test identification.

4.4.6 Identification test

4.4.6.1 Gram Painting

Take one or more colonies from each of the growing colonies and apply *Gram stain.* The results of Gram staining will show bacteria in the form of purple cocci *(Gram* positive), clustered like grapes or visible only one bacterium.

4.4.6.2 Coagulase test

a) Take one or more colonies suspected of *Staphylococcus aureus* and insert them into the in 0.2 ml to 0.3 ml *BHIB* and homogenize.

b) Take a full loop (3.0 mm diameter) of suspension from *BHIB* and *scratch it* on Agar TSA machines .

c) Incubate *BHIB* and *TSA* agar **at a temperature of 35 °C for 18 hours until with 24 hours.**

d) Add 0.5 ml of rabbit plasma *coagulase* containing *EDTA* into the incubated ***BHIB*** suspension **and then homogenize.**

e) Incubate the tube at 35°C for 6 hours and observe hourly for clot formation.

f) A positive ***Staphylococcus aureus*** **coagulase test result is indicated by the presence of clumping.**

4.4.7 Calculation

a) Count the colonies from petri dishes that show typical colonies of *Staphylococcus aureus* and show a positive coagulase test result, then multiply by the dilution factor.

b) Results are reported as the number of *Staphylococcus aureus* per milliliter or per gram.

4.5 Testing for Salmonella spp.

4.5.1 Principle

Salmonella **growth on selective media with pre-enrichment *(pre-enrichment),* and enrichment *(enrichment)* followed by biochemical tests and serological tests.**

4.5.2 Media and reagents

a) *LB;*
b) *SCB;*
c) *TTB;* d)
***RV;* e)**
***XLDA;* f)**
***HEA;* g)**
***BSA*, h)**
***TSIA;* i)**
LIA;
j) *LOB;* k)
***KCNB;* l)**
***MR-VP;* m)**
SCB;* n) *TB;
o) *TSTB;* p)
***SIM;* q)**
Reagen
***Kovac;* r)**
BHI;
s) *Urea Broth;* t)
***Malonate Broth;* u)**
***Phenol Red Lactose Broth;* v)**
***Phenol Red Sucrose Broth;* w)**
kristal keratin;

x) *Larutan Bromcresol Purple Dye 0,2 %;* y) *Larutan Physiological Saline O,85 %;* z) *Larutan Formalinized Physiological Saline;* aa) *Salmonella Polyvalent Somatic (O) Antiserum A-S; bb) Salmonella Polyvalent Flagellar (H) Antiserum Fase 1 Dan 2; cc) Salmonella Somatic Grup (O) Monovalent Antisera : Vi.*

4.5.3 Equipment

a) petri dish; b) test tubes; c) serology tube measuring 10 x 75 mm; d) pipettes measuring 1 ml, 2 ml, 5 ml, 10 ml; e) media bottles; f) scissors; g) tweezers; h) inoculation needle (ose); i) *stomacher;* j) scale; m) *magnetic stirrer;* n) tube shaker (vortex); o) incubator; p) water bath; q) autoclave; r) sterile cabinet (*laminar flow*); s) refrigerator (refrigerator), freezer.

4.5.4 Test method

Each testing process is always accompanied by the use of positive controls.

4.5.4.1 Pre-enrichment

a) Weigh solid and semi-solid samples as much as 25 g or measure as much as 25 ml of the sample melt aseptically then put in a sterile container.

b) For example meat, eggs and milk
Add 225 ml of *LB* solution into a sterile bag containing the sample, homogenize with *a stomacher* for 1 minute to 2 minutes (except for liquid milk samples).

c) Transfer the suspension to an *Erlenmeyer* or sterile container.

d) Incubate at a temperature of 35 °C for 24 hours ± 2 hours.

4.5.4.2 Enrichment

a) Gently stir the pre-enrichment culture then take and transfer 1 ml each into 10 ml *TTB media,* while for *RV* media transfer 0.1 ml into 10 ml *RV media.*

b) Examples with suspected contamination of *Salmonella* spp. high *(high microbial load).*
Incubate the *RV* medium at a temperature of 42 °C ± 0.2 °C for 24 hours ± 2 hours.
Meanwhile, *TTB* media was incubated at a temperature of 43 °C ± 0.2 °C for 24 hours ± 2 hours.

c) Examples with suspected contamination of *Salmonella* spp. low *(low microbial load).*
Incubate the *RV* medium at a temperature of 42 °C ± 0.2 °C for 24 hours ± 2 hours.
Meanwhile, ***TTB* media was incubated at a temperature of 35 °C ± 2 °C for 24 hours ± 2 hours.**

4.5.4.3 Isolation and identification

a) Take two or more colonies with a *ose* needle from each enrichment medium that has been incubated, and inoculate them on *HE, XLD* and *BSA media.* Incubate at 35 °C for 24 hours ± 2 hours. For *BSA* , if it is not clear, it can be incubated again for 24 hours ± 2 hours.

b) Observe the ***Salmonella* colonies** on the ***HE* medium which appear bluish green with or without black dot (H2S).**

c) On *XLD* media the colonies look pink with or without shiny dots or almost all black colonies are seen.

d) On *BSA* media the colonies look grayish or blackish, sometimes metallic, the media around the colonies is brown and the longer the incubation time will turn black.

e) Identify by taking the suspected colonies from the three media.
Inoculate into *TSIA* and *LIA* by piercing into the base of the agar media, then scratching on the agar media at an angle.

f) Incubate at a temperature of 35 °C for 24 hours ± 2 hours. Observe specific colonies *Salmonella* with the reaction results as listed in Table 3.

Table 3 - Samonella sp test results on TSIA and LIA

Media	To tilt (Slant)	Agar base (buttom)	H2S	Gas
NO	Alkaline / K (red)	Acid / A (yellow)	Positive (black)	Negative/ positive
HIS	Judge / K (ungo)	Judge / K (ungo)	Positive (black)	Negative/ Positive

4.5.4.4 Biochemical test

4.5.4.4.1 Uji urease

a) Inoculate colonies from positive *TSIA* with *ose* to *Urea Broth.*

b) Incubate at a temperature of 35 °C for 24 hours ± 2 hours.

c) The result of the *Salmonella* specific test was negative for the *urease test.*

4.5.4.4.2 Uji indole

a) Inoculate colonies from *TSIA* media on *TB* and incubate at 35°C for 24 hours ± 2 hours.

b) Add 0.2 ml to 0.3 ml of *Kovacs Reagent.*

c) Positive test results are indicated by the presence of a red ring on the surface of the media. d) A negative test result is indicated by the formation of a yellow ring.

e) The result of the *Salmonella* specific test was negative for the *indole test.*

4.5.4.4.3 Uji Voges-Proskauer (VP)

a) Take the culture from the *TSIA medium* with *ose* and then inoculate it into a tube containing 10 ml of *MR-VP* media and incubate at a temperature of 35 °C for 48 hours ± 2 hours.

b) Transfer 5 ml *of MR-VP* into a test tube and add 0.6 ml of -naphthol solution and 0.2 ml of 40% KOH, then shake until mixed and allowed to stand.

c) To speed up the reaction add creatine crystals. Read the results after 4 hours.

d) The test result is positive if there is a change in color from pink to ruby red.

e) Generally *Salmonella* gives a negative result for the *VP* test (no color change in the media).

4.5.4.4.4 Uji Methyl Red (MR)

a) Take the culture from the *TSIA medium* by *inoculating* it into a tube containing 10 ml of *MR-VP* media and incubate it at a temperature of 35 °C for 48 hours ± 2 hours.

b) Add 5 drops to 6 drops of *Methyl Red* indicator in the tube.

c) A positive test result is indicated by the diffusion of red color into the media.

d) Negative test results are indicated by the occurrence of a yellow color in the media.

e) Generally *Salmonella* gives a positive result for the *MR* test .

4.5.4.4.5 Uji citrate

a) Inoculate colonies from *TSIA* into *SCA* with *ose.*

b) Incubate at a temperature of 35 °C for 96 hours ± 2 hours.

c) A positive test result is indicated by the growth of colonies followed by a color change from green to blue.

d) A negative test result is indicated by the absence of colony growth or very little growth and no color change.

e) Generally *Salmonella* gives a positive result on the *citrate* test .

4.5.4.4.6 Uji Lysine Decarboxylase Broth (LDB)

a) Take one colony loop from *TSIA and* inoculate it into *LDB.*

b) Incubate at a temperature of 35 °C for 48 hours ± 2 hours and observed every 24 hours.

c) *Salmonella* gave a positive reaction marked by the formation of a purple color in the entire medium and the result of a negative reaction gave a yellow color.

d) If the result of the reaction is doubtful (not purple or not yellow) add a few drops 0.2% *bromcresol purple dye* and observe the color change.

4.5.4.4.7 Potassium Cyanide (KCN) Test

a) Inoculate one culture loop from *TSIA to TB* media .

b) Incubate at a temperature of 35 °C for 24 hours ± 2 hours.

c) Take a colony loop from *TB* and inoculate it into *KCNB .*

d) Incubate at a temperature of 35 °C for 48 hours ± 2 hours.

e) A positive test result is indicated by the presence of growth which is indicated by turbidity.

f) Negative test results are indicated by the absence of growth in the media.

g) *Salmonella* gave a negative result on the *KCN test.*

4.5.4.4.8 Test candy

a) Phenol red dulcitol broth atau purple broth base dengan 0,5 % dulcitol

- Take colonies from *TSIA* and inoculate them in *dulcitol broth medium.*

- Incubate at a temperature of 35 °C and observed every 24 hours for 48 hours ± 2 hours.

- Most *Salmonella* give a positive reaction characterized by the formation of gas in the *Durham* tube **and a yellow color (acid pH) on the media.**

- The result of a negative reaction was indicated by the absence of gas in the *Durham* tube **and a red color (alkaline pH) was formed in the media for the *phenol red*** indicator **or purple for the *bromcresol purple indicator.***

b) Uji malonate broth

- Transfer one *ose* from *TB* to *malonate broth.*

- Incubate at a temperature of 35 °C and observed every 24 hours for 48 hours ± 2 hours.

- A positive test result is indicated by a color change to blue.

- *Salmonella* gives a negative reaction which is indicated by the presence of a green color or no color change.

c) Uji phenol red lactose broth

- Inoculate the colony from *TSIA* oblique into *Phenol red lactose broth.*

- Incubate at a temperature of 35 °C and observed every 24 hours for 48 hours ± 2 hours.

- Positive reaction results are indicated by the production of acid (yellow color) with or without gas

- *Salmonella* gives a negative reaction, which is indicated by no color change and gas formation.

d) Uji phenol red sucrose broth

- Inoculate colonies from *TSIA* oblique into *Phenol red sucrose broth.*

- Incubate at 35 °C for 48 hours ± 2 hours and observed every 24 hours.

A positive test result is indicated by a color change (yellow) and with or without gas formation.

- *Salmonella* gives a negative test result marked by no color change and gas formation.

4.5.4.5 Serological test

4.5.4.5.1 Uji polyvalent somatic (O)

a) Place a colony loop from *TSIA* or *LIA* in a glass preparation and add one drop of sterile physiological saline (NaCl 0.85%) and mix with culture.

b) Give one drop of *Salmonella polyvalent somatic* (O) antiserum in addition to the suspension colony.

c) Mix the colony suspension into the antiserum until completely mixed.

d) Tilt the mixture left and right with a dark background while an agglutination reaction was observed.

e) Prepare control by mixing physiological saline and antiserum.

f) Perform the somatic test (O) of the antisera Vi monovalent group as above.

4.5.4.5.2 Uji polyvalent flagelar (H)

a) Colonies from *TSIA* with negative *urease* test results were inoculated into *BHIB* and incubated at a temperature of 35°C for 4 hours to 6 hours or into *TSTB* and incubated at a temperature of 35°C for 24 hours ± 2 hours.

b) Add 2.5 ml of ***formalinized* physiological saline solution *saline)* into 5 ml of one of the above cultures.**

c) Pipette 0.5 ml of *Salmonella Polyvalent flagellar* (H) antisera solution and put it in a 10 x 75 mm serology tube.

d) Add 0.5 ml of the antigen to be tested.

e) Prepare a control physiological saline solution by mixing 0.5 ml of formalinized physiological saline solution with 0.5 ml of *formalinized antigen.*

f) Incubate the two mixtures in a water bath at a temperature of 48 °C up to 50 °C.

g) Observe for clots every 15 minutes for 1 hour.

h) A positive test result is indicated by the presence of clots, while in the control it is not clumping occurs.

4.5.5 Interpretation of results for Salmonella spp.

The interpretation of the results of the biochemical tests of *Salmonella* spp. can be seen in Table 4, while the criteria for determining non *Salmonella spp* can be seen in Table 5.

Table 4 - Salmonella biochemical reactions

No	Substrate test	Reaction result		
		Positive	Negative	Salmonella
1	Glucose (TSI)	Yellow stab	Red prick	+
2	Lysine Decarboxylase(LIA)	Purple stab	Yellow stab	+
3	H2S (TSI dan LIA)	Black	Not black	+
4	Urease	Pink to red Stay yellow		-
5	Lysine Decarboxylase *Broth*	Purple	Yellow	+
6	*Phenol Red Dulcitol Broth*	Yellow color and or with gas	Without changing color and without forming gas	a)
7	*Broth* Industrial Park	There is growth	There is no growth	-
8	Malonat *Broth*	Blue	Do not change color	b)
9	Test indole	Surface color red	Yellow color surface	-
10	Uji *Polyvalent flagelar*	Agglutination	No agglutination	+
11	*Polyvalent Somatic* Agglutination Test		No agglutination	+
12	*Phenol Red Lactose Broth* with/without gas	Yellow	Does not form gas and does not change color	-
13	*Phenol Red* Sukrosa *Broth*	Yellow color with/ without gas	Does not form gas and does not change color from pink to red	-
14	Uji *Voges-Proskauer*	Does not change color		-

Table 4 – Advanced

No	Substrate test	Reaction result		
		Positive	Negative	Salmonella
15	Uji *Methyl Red*	Red spread	Yellow color spreads	+
16	*Simmon's* sitrat	Blue color growth	No growth and no change	IN

DESCRIPTION :

[a]) Majority of S. arizonae cultures were negative b)
The majority of S. arizonae cultures were positive
V Variable

Table 5 - Criteria for determining non-Samonella spp

No	Substrate test	Results
1	Urease	Positive (pink – red)
2	Lysine Decarboxylase (LIA)	Negative (Clear)
3	Lysine Decarboxylase *Broth*	Negative (Clear)
4	*Broth* Industrial Park	Positive (there is cloudy growth)
5	Indole test	Positive (red color on the surface)
6	Uji *Polyvalent flagelar*	Negative (no clumping)
7	Uji *Polyvalent Somatic*	Negative (no clumping)
8	*Phenol Red Lactose Broth*	Positive (yellow color with/no gas)
9	*Phenol Red* Sukrosa *Broth*	Positive (yellow color with/no gas)
10	Uji *Voges-Proskauer*	Positive (pink to red)
11	Uji *Methyl Red*	Negative (spread yellow color)

DESCRIPTION:

a. The Malonate broth test is further positive to see if the culture is *Salmonella Arizona.* b. Do not discard positive culture if the LIA shows a reaction characterized by *Salmonella,* further test to see if it is *Salmonella* species

4.6 Campylobacter spp.

4.6.1 Principle

***Campylobacter* growth on selective media through the stages of pre-enrichment, enrichment, isolation and identification and confirmation.**

4.6.2 Media and reagents

a) *Campylobacter enrichment broth*
***Bolton broth base* 1000 ml plus 50 ml *lysed horse blood* plus 2 vials** of ***Bolton* antibiotics;**
b) to isolate *Campylobacter*

Abeyta-Hunt-Bark **(AHB) and modified** ***Campy blood-free Agar*** **(mCCDA); c) confirmation media** ***Campylobacter broth (broth enrichment,*** **free of antibiotics), with or without** ***Foetal Bovine Serum*** **(FBS); d)** ***heart infusion agar ;*** **e) peptone 0.1%; f) brucella agar, semi-solid; g) TSIA; h)** ***oxidative fermentation test media;*** **i)** ***Mac Conkey Agar;*** **j) glycine; k) sodium and** ***cysteine chloride;*** **l) potassium nitrate; m) nitrite detection reagent; n) sodium hippurate reagent; o)** ***Ninhydrin*** reagent ; **p) Pb acetate strip paper; q) strip filter paper containing** ***Malonic acid*** **and** ***Catalase broth,*** **r) Hydrogen peroxide 3%, s)** ***Oxidase*** **reagent; t) Gram** staining reagent **(S6) with 5%** ***carbol fuchsin*** **(S4) as dye. w)** ***Campylobacter*** **spp isolate for positive control.**

4.6.3 Equipment

a) 10 x 75 mm tube; b) Erlenmeyer 250, 500 ml and 50 ml; c) object glass and cover; d) 250 ml centrifuge bottles and 50 ml sterile centrifuge tubes or the like; e) glass gas shaker system (Erlenmeyer ***vacuum system);*** **f)** ***stomachers;***

g) 100x magnification and oil immersion microscope; h) mechanical blender/jar blender; i) scales; j) refrigerated centrifuge; k) ***sterile tongue depressor*** **and swab; l)** ***anaerobic jars*** **with** ***gas generating envelopes;*** **m)** ***shaking gas flask system/ gas tank system*** **(5 % O2, 10 % CO2, 85 % N2); n) incubator: 25 °C; 30 °C; 35 °C to 37 °C and 42 °C; o) 0.65 m, 47 mm** ***(millipore/ genex) filter paper;*** **p) disc paper; q) long-handled forceps;**

4.6.4 Sample setup

a) Weigh and grind the meat sample, as much as 25 g and add 100 ml of 0.1% peptone, then centrifuge at 16,000 rpm for 15 minutes, then discard the supernatant.

b) Measure the milk sample as much as 25 ml then centrifuged cold 20,000 rpm for 40 minutes, then filtered and discarded the supernatant.

c) Weigh the egg yolk sample as much as 25 g and add 100 ml of ***enrichment broth (Bolton broth base*** **1,000 ml plus 50 ml** ***lysed horse blood*** **plus 2 vials** of ***Bolton***

antibiotics) then homogenized.

d) Transfer 3 ml of precipitate a) or b) into a sterile centrifuge bottle containing 100 ml of *enrichment broth (1,000 ml of Bolton broth base* plus 50 ml *of lysed horse blood).*
plus 2 vials of *Bolton* antibiotics)

4.6.5 Test method

4.6.5.1 Pre-enrichment (Park and Humphrey's modified method)

Incubate suspension 4.6.4 c) or d) at 37 °C for 4 hours under microaerobic conditions with a *Gas Tank System.*

4.6.5.2 Enrichment

Increase the incubation temperature of the suspension 4.6.5.1 to 42 °C for 23 hours to 24 hours, and 48 hours for the milk sample.

4.6.5.3 Isolation

a) Suspension 4.6.5.2 is made at a dilution of 1: 100 (0.1 ml put into 9.9 ml of peptone 0,1 %).

b) Scratch 2 oses each of suspension *4.6.5.2* and 4.6.5.3 a) on *AHB* or *mCCDA agar medium.*

c) Incubate at a temperature of 42 °C for 24 hours to 48 hours in a *Gas Tank System.* Observe growth at 24 h incubation.

4.6.5.4 Identification

a) Colonies are round or irregular in shape with smooth edges and translucent white with diffuse growth.

b) Select at least 1 colony from each Petri dish and prepare it on the object glass. See the shape of bacterial cells under a microscope with oil immersion. *Campylobacter* cells appear comma-shaped or curved, 1.5–5 m long and in a *zigzag formation.*
Campylobacter is generally motile and 20% *of C. jejuni* is non-motile. Cells that are old and injured *(injured)* will experience decreased motility and a change in shape becomes round.

c) The test is always accompanied by a positive control.

4.6.5.5 Biochemical confirmation and test

Each test is always accompanied by a positive control.

4.6.5.5.1 Catalase test

a) Take a positive *Campylobacter* colony then drop 3% *hydrogen peroxide* and when a bubble appears, it is a *Campylobacter positive colony.*

b) If the colony was positive in the catalase test, it was transferred to two *Campylobacter confirmation broth* media (Formulation 1 and Formulation 2). One using *FBS* and one without *FBS.* Inoculate the colony into the two media above, and incubate at

temperature of 42 °C ± 1 °C for 24 hours under microaerobic conditions *(Gas Tank System)* until both look cloudy.

c) If the growth is sufficient, use culture *broth* without *FBS* for biochemical tests next.

4.6.5.5.2 Antibiotic sensitivity test

a) Inoculate the culture evenly on the surface of the *Heart Infusion Agar* medium containing 5% blood and 0.35% *FBS* using a sterile swab.

b) Place the *Cephalothin* and *Nalidixic acid* disc paper on the media.

c) Incubate the media at a temperature of 42 °C ± 1 °C for 24 hours to 48 hours under microaerobic conditions.

d) Look for the zone of inhibition surrounding the paper disc. The presence of a zone indicates the sensitivity of the bacteria to the antibiotic. *Campylobacter jejuni* very sensitive to *nalidixic acid* and resistant to *cephalothin.*

4.6.5.5.3 Gram Painting

On Gram staining , *Campylobacter spp .* is comma-shaped and is *Gram* negative.

4.6.5.5.4 Uji hippurate hydrolysis

a) Create an emulsion with *a* diameter of 2 mm between colonies grown from the *Heart Infusion Agar* medium used in the antibiotic sensitivity test by adding 0.4 ml of *hippurate solution* in a 10 x 75 mm tube.

b) Incubate at 37 °C for 2 hours in a water bath.
Add 0.5 ml of *Ninhydrin* reagent while stirring. Incubate again for 10 minutes. See immediately the changes to the tube.

c) Positive reaction when there is a clear change in the medium to violet. *Campylobacter jejuni* showed a positive reaction on the *hippurate hydrolysis test.*

4.6.5.5.5 Test with TSIA

a) Inoculate the *TSIA medium* from the culture broth by piercing the uprights and scratch on the oblique part.

b) Incubate at a temperature of 35 °C to 37 °C for 5 days under microaerobic conditions in a *Gas Tank System. Campylobacter jejuni* on *TSIA* media
upright and sloping sections are red (alkaline) with no H2S production.

4.6.5.5.6 Uji glucose utilization

a) Inoculate 2 tubes of *oxidative-fermentative* test media that have been inoculated with culture. One tube contains glucose, and one without glucose.

b) Incubate at a temperature of 35 °C to 37 °C for 4 days under microaerobic conditions in a *Gas Tank System. Campylobacter spp* does not use glucose or other sugars, characterized by the absence of changes in the media in the tube.

4.6.5.5.7 Catalase-oxidase test

a) Inoculate the culture from the broth medium into the inclined ***Heart Infusion Agar*** medium , **and incubate at a temperature of 35 °C to 37 °C for 48 hours.**

b) Take the colonies that grow on the agar slanted and drop 3% ***hydrogen peroxide .***

c) Add the oxidizing reagent to the slanted ***Heart Infusion*** **Agar media culture. If the medium turns purple, then the culture is positive for the oxidation test.** ***Campylobacter jejuni*** **was positive on** ***catalase and oxidase tests.***

4.6.5.5.8 Uji growth temperature tolerance

a) Mix 2 ml of culture broth with ***Peptone*** **0.1% with a turbidity level using** ***McFarland*** standard **Number 1. Scrape the media into 3 Petri dishes of** ***Heart Infusion–Blood–FBP*** **Agar media.**

b) Inoculate on each Petri dish. Incubate one cup at a temperature of 25 °C, one cup at a temperature of 35 °C to 37 °C and the other at a temperature of 42 °C for 3 days under microaerobic conditions in a ***Gas Tank System.***

c) A positive test was indicated by the presence of turbidity in the inoculum. ***Campylobacter jejuni*** **did not grow at a temperature of 25 °C, but grew well at temperatures of 35 °C to 37 °C and 42 °C.**

4.6.5.5.9 Test growth on MacConkey Agar

Scratch one colony on ***MacConkey Agar,*** **incubate at 37 °C for 3 days under microaerobic conditions in a** ***Gas Tank System. Campylobacter jejuni*** **grows well on** ***MacConkey Agar.***

4.6.5.5.10 Growth test

This test uses ***Brucella broth*** **with 0.18% agar. Pipette 7 ml of the medium per tube, drop 0.1 ml of the culture, and spread it over the surface of the semi-solid medium.**
Incubate at a temperature of 35 °C to 37 °C for 5 days.

a) Growth test on glycine 1%

Inoculate the culture on 1% glycine medium, ***Campylobacter jejuni*** **grows (positive).**

b) Growth test on NaCl 3.5%

Inoculate the culture on 3.5 ***% NaCl media, Campylobacter jejuni*** **did not grow (negative).**

c) H2S test **of cysteine**

Inoculate the culture on ***cysteine media.*** **Colonies of** ***Campylobacter jejuni*** **were black and shiny.**

d) Reduction of
nitrate Inoculate the culture on nitrate media. After 5 days add nitrite reagents A and B.

Campylobacter jejuni **showed a positive reaction with the formation of a red color.**

The biochemical reactions of ***Campylobacter jejuni*** **and** ***Campylobacter spp*** **can be seen in Table 6.**

Table 6 - Comparison of Campylobacter species characteristics

Characteristics	*C. fasting*	*C. coli*	*C. laridis*	*C. fetus fetus*	*C. cinaedi*	*C. fennelliae*	*C. cyraerophila*	*C. hyointestinal*	*C. upsaliensis*
Catalase	+	+	+	+	+	+	+	+	
Resistance to Cephalothin	R	R	R	S	S	S	S	S	S
Resistance to Nalidixic acid	S	S R		R	S	S	S	R	S
Hippurate hydrolysis	+	+	.	.	.	.	.	.	.
H2S from TSIA	.	d	.	.	.	.	.	+	.
Glucose	.	.	.	.	.	.	.	.	.
Catalase Oxidase	+	+	+	+	+	+	+	+	+
Grow on									
25 C	.	.	.	+	.	.	+	d	.
35 C-37 C	+	+	+	+	+	+	+	+	+
42 C	+	+	+	d	d	d	.	+	+
MacConkey		+	+	+	.	.	.	+	.
Motility	+	+	+	+	+	+	+	+	+
Grows on 1% glycine	+	+	+	+	+	+	.	+	+
NaCl 3,5 % -		.	.	.	.	.	.	.	.
H2S from cysteine	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	.	+	+	+

DESCRIPTION (+) is positive; (-) is negative; (d) is positive or negative; R is resistance; S is sensitive.

4.7 Testing Listeria monocytogenes

4.7.1 Principle

This test method is based on the isolation and identification of ***Listeria monocytogenes .*** bacteria **by culturing on selective media.**

4.7.2 Media and reagents

a) *BPW* 0,1 %; b) *Buffered Listeria Enrichment Broth (BLEB);* c) *Listeria Enrichment Broth Base (LEBB);* d) *Selective Enrichment Supplement (SES);* e) *Listeria Selective Agar (LSA);* f) *Listeria Selective Supplement LSS);* g) *Oxford Agar (OXA);* h) *PALCAM Agar (PALCAM);* i) *Modified Oxford Agar (MOX);* j) *Lithium chloride-Phenylethanol-Moxalactam (LPM) agar;* k) *Trypticase Soy agar* dengan *Yeast extract (TSAy);* l) antibiotik *Cycloheximide* atau *Natamycine;* m) agar darah domba/kuda; n) media semisolid; o) pereaksi H2O2 3 %; p) Manitol; q) Xylosa; r) Rhamnosa; s) Pengecatan *Gram;* t) *Staphylococcus aureus* ATCC 25923; u) *Rhodococcus equi* ATCC 6939.

4.7.3 Equipment

a) petri dish; b) *Craigie's tube;* c) test tubes; d) volumetric pipette; e) *Erlenmeyer;* f) media bottles; g) scissors; h) tweezers; i) inoculation needle *(ose);* j) *stomachers;* k) microscope; l) Bunsen burner; m) pH meter; n) scales; o) *magnetic stirrer;* p) tube shaker *(vortex);* q) incubator temperature 30 °C ± 1 °C; r) incubator temperature 37 °C ± 1 °C; s) water bath; t) autoclave; u) sterile cabinet *(clean bench);* v) refrigerator *(refrigerator);* w) *freezers.*

4.7.4 Sample setup

a) Weigh solid and semi-solid samples as much as 25 grams or measure liquid samples as much as 25 ml aseptically, then put them in a sterile container.

b) For example meat and eggs (solid and semi-solid)
Add 225 ml of ***Buffered Listeria Enrichment Broth*** **into a sterile bag containing the sample, homogenize with *a stomacher* for 1 minute to 2 minutes.**

c) For milk samples (liquid)
Add 225 ml of ***Buffered Listeria Enrichment Broth*** **into a sterile bag or container containing the sample, homogenize.**

4.7.5 Test method

The test is always accompanied by a positive control.

4.7.5.1 Pre-enrichment

Incubate suspension 4.7.4 b) or c) at 30 °C for 4 hours.

4.7.5.2 Enrichment

Continue incubation of suspension 4.7.5.1 for up to 48 hours by adding the antibiotic *Cycloheximide* or *Natamycine* at a concentration of 25 mg/liter.

4.7.5.3 Isolation

a) After being incubated for 24 hours and 48 hours, each smear 1 *ose* of suspension 4.7.5.2 on *OXA* or *PALCAM* or *MOX* or *LPM.*

b) Incubate the *OXA, PALCAM,* and *MOX* media **at a temperature of 35 °C for 24 hours to 48 hours, while the *LPM*** media are **incubated at a temperature of 30 °C for 24 hours to 48 hours.**

c) On *LPM* media, ***Listeria* colonies appear bluish-gray or white.**

d) In *OXA* and *PALCAM* black colonies surrounded by a clear zone.

e) On sheep/horse blood agar with *Listeria monocytogenes bacteria,* it looks concave in the middle, bluish-gray in color, surrounded by a clear zone (because of the hemolytic properties of *Listeria monocytogenes).*

4.7.5.4 Identification

Grow *Listeria* colonies on ***TSAy* media , then incubate at 30 °C for 24 hours to 48 hours.**

4.7.5.4.1 Gram . stain test

Listeria monocytogenes* is a *Gram positive **bacterium that shows violet colored cells on microscopic examination.**

4.7.5.4.2 Motility test

a) Using semi-solid media in a tube in which there is a *Craigie tube.*
The isolates were tested and inoculated in *Craigie tubes.*

b) Incubate at 37 °C for 24 hours.

c) Positive motility test, if there is *Listeria monocytogenes* bacteria **on the surface of the semi-solid agar outside the *Craigie tube.***

4.7.5.4.3 Test candy

a) Take colonies from sheep/horse blood agar media suspected of *Listeria monocytogenes,* and inoculate them on media containing prepared carbohydrates (glucose, maltose, mannitol, rhamnose, and xylose).

b) Incubate at 37 °C for 24 hours.

c) A positive test result when the candy media above changes color from red to yellow.

d) Negative test results when the candy media remains red.

4.7.5.4.4 Catalase test

a) Mix the colonies suspected of *Listeria monocytogenes* with one drop of 3% **H2O2** reagent **on a glass slide until blended.**

b) The catalase reaction is positive if bubbles form in the cell mixture.

c) The catalase reaction is negative if no bubbles are formed in the cell mixture.

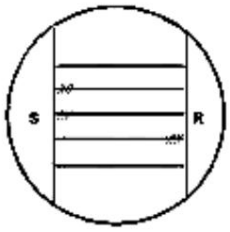
4.7.5.5 Confirmation test

a) To confirm the hemolytic activity of *Listeria moncytogenes* (ß-hemolise), performed with the *CAMP test.*

b) Inoculate colonies suspected of *Listeria monocytogenes* on sheep/horse blood agar media using *Staphylococcus aureus and Rhodococcus equi* cultures **as shown in Figure 1.**

c) Incubate at 35 °C for 24 hours and 48 hours.

d) The ***CAMP test* is positive for *Listeria monocytogenes,* if the hemolysis zone is around the *Staphylococcus aureus scratch.***



KETERANGAN S = *Staphylococcus aureus,* R = *Rhodococcus equi*
~~III~~ = ÿ hemolisa

Gambar 1 - CAMP test Listeria monocytogenes

The interpretation of ***Listeria monocytogenes*** **test results can be seen in Table 7.**

Table 7 - Interpretation of Listeria monocytogenes test results

No.	Test Type	Test results	Information
a	***Gram*** **Painting**	**Positive Short rod Positive**	
b	**Motility**	**There is** ***L. monocytogenes*** bacteria **on the surface of the semi-solid agar outside the** ***Craigie*** tube	
c	**Glucose**	**Positive Yellow color Positive**	
d	**Maltose**	**Yellow color Negative Yellow color**	
and	**MANNITOL**	**Positive Yellow color Negative**	
f	**Rhamnosa**	**Yellow color Positive Positive**	
	Xylose	**bubble Formation of hemolysis**	
	Calatase test	**zone around** ***Staphylococcus*** Streak	
write the	***CAMP test***		***aureus***

Appendix A
(normative)
MPN draw three tubes

The **three-tube series *MPN* table calculations** with 95% confidence intervals for the combination of positive results and tube dilutions used (0.1 ml; 0.01 ml and 0.001 ml) (BAM 2001 or 2006) are as per Table A.1.

Table A.1 - MPN three-tube series

Number of positive tubes (3 tubes) 0.1 g 0.01 g 0.001 g			MPN / g <	Confidence limit 95% Lower
0 0 0 0 0 1 0 1 0 0 1 1 1 1 2 0 0 1 2 1 0 1 1 2 0 1 2 2 0 1 0 2 0 1 1 2 1 0 2 1 1 2 1 2 1				Upper 9.5 9.6 11 18 18 18 38 38
2 2 2 0 2 2 1 2 2 2 2 3 1 0 2 2 1 2 3 1 0 3 3 2 0 1 3 2 1 2 3 3 2 0 3 1			3,6 3 3	20 38
3 3 1 3 3 2 3 3 3 3			6,1	0,15 42 42
			6,2	0,15 38 42
			9,4 3,6	1,2 42 42
			7,2 11	1,2 42 94
			7,4 11	3,6 42 94
			11 15	0,17 94 94
			16 9,2	1,3 94 94
			14 20	3,6 110
			15 20	1,3 180
			27 21	3,6 180
			28 35	3,6 200
			29 36	4,5 420
			23 38	4,5 420
			64 43	1,4 420
			75	3,6 420
			120	4,5 430
			160	3,7 1,000
			93 150	4,5 1,000
			210	8,7 2,000
			290	4,5 4,100
			240	8,7
			460	8,7
			1.100	8,7
			>	8,7
			1.100	4,6
				8,7
				17
				9
				17
				37
				40
				18
				37
				40
				90
				42
				90
				180
				420

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