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AMENDMENT 2
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**Microbiology of food and animal
feeding stuffs — Horizontal method
for the enumeration of coagulase-
positive staphylococci (*Staphylococcus
aureus* and other species) —**

**Part 1:
Technique using Baird-Parker agar
medium**

**AMENDMENT 2: Inclusion of an
alternative confirmation test using RPFA
stab method**

*Microbiologie des aliments — Méthode horizontale pour
le dénombrement des staphylocoques à coagulase positive
(*Staphylococcus aureus* et autres espèces) —*

Partie 1: Technique utilisant le milieu gélosé de Baird-Parker

*AMENDEMENT 2: Ajout d'un essai alternatif de confirmation utilisant
la méthode de piqure sur RPFA*



Reference number
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Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase- positive staphylococci (*Staphylococcus aureus* and other species) —

Part 1:

Technique using Baird-Parker agar medium

AMENDMENT 2: Inclusion of an alternative confirmation test using RPFA stab method

Clause 2

Add the following normative reference.

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

Clause 5

Add the following text.

5.6 Rabbit plasma fibrinogen agar (RPFA) medium

5.6.1 General

Commercially available media, in accordance with this document, can be used. Nevertheless, considering the established variability of manufactured lots of the supplement, it is recommended that each batch of bovine fibrinogen/rabbit plasma solution be tested before use, by running positive and negative controls.

5.6.2 Base medium

Prepare the base medium as stated in 5.3.1, with the exception of the distribution of the base medium, in quantities of 90 ml per flask or bottle.

5.6.3 Solutions

5.6.3.1 Potassium tellurite solution

Prepare the potassium tellurite solution as indicated in 5.3.2.1.

5.6.3.2 Bovine fibrinogen solution

5.6.3.2.1 Composition

Bovine fibrinogen	5 g to 7 g ^a
Sterile water	100 ml

^a Depending on the purity of the bovine fibrinogen.

5.6.3.2.2 Preparation

Under aseptic conditions, dissolve the bovine fibrinogen in the water just prior to use.

5.6.3.3 Rabbit plasma and trypsin inhibitor solution

5.6.3.3.1 Composition

Rabbit plasma with EDTA for coagulase (EDTA coagulase plasma)	30 ml
Trypsin inhibitor	30 mg

5.6.3.3.2 Preparation

Operating under aseptic conditions, dissolve the components in the water, just prior to use.

5.6.4 Complete medium

5.6.4.1 Composition

Base medium (5.6.2)	90 ml
Potassium tellurite solution (5.6.3.1)	0,25 ml
Bovine fibrinogen solution (5.6.3.2)	7,5 ml
Rabbit plasma and trypsin inhibitor solution (5.6.3.3)	2,5 ml

5.6.4.2 Preparation

Melt the base medium, then let it cool down to 47 °C to 50 °C in a water bath (6.4) because at a lower temperature, this medium solidifies.

Under aseptic conditions, add the three solutions previously warmed to 48 °C ± 1 °C in a water bath. Mix thoroughly after each addition by rotation to minimize foaming.

Use the complete medium **immediately after its preparation**, in order to avoid any precipitation of the plasma.

WARNING — If a commercially available solution of bovine fibrinogen/rabbit plasma is used, follow with great care the manufacturer's instructions for the preparation of this solution and of the complete medium (in particular the temperature of the base medium). Otherwise, the medium can completely lose its activity.

5.6.5 Preparation of RPFA plates

Place the appropriate quantity of the complete medium into sterile Petri dishes in order to obtain an agar thickness of about 4 mm, and allow to solidify.

The prepared RPFA plates may be stored, prior to drying, at $5\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$ for a given period of time before use if the stability of the medium is proven for this period.

For plates prepared commercially, the instructions of the manufacturers should be followed.

Before use, dry the plates, according to the conditions of ISO 7218.

5.6.6 Performance testing for the quality assurance of the culture medium

Table 1 shows the performance testing for the quality assurance of RPFA medium used as confirmation medium.

For the definition of productivity, selectivity and specificity, refer to ISO 11133. In general, follow the procedures for performance testing described in ISO 11133.

Table 1 — Performance testing for the quality assurance of the RPFA medium

Medium	Type	Function	Incubation	Control strain	WDCM number ^a	Method of control	Criteria ^c	Characteristic reaction
RPFA	Se	Productivity	(24 ± 2) h to (48 ± 2) h / (37 ± 1) $^{\circ}\text{C}$	<i>Staphylococcus aureus</i>	00034 ^b 00032	Qualitative ^f	Good growth (2)	Black or grey colonies with opacity halo
		Selectivity	(48 ± 2) h / (37 ± 1) $^{\circ}\text{C}$	<i>Escherichia coli</i> ^d	00012 or 00013	Qualitative	Total inhibition	—
		Specificity	(24 ± 2) h to (48 ± 2) h / (37 ± 1) $^{\circ}\text{C}$	<i>Staphylococcus saprophyticus</i> <i>Staphylococcus epidermidis</i>	00159 ^b 00036	Qualitative	—	Black or grey colonies without opacity halo

^a Refer to the reference strain catalogue on at <http://www.wfcc.info> for information on culture collection strain numbers and contact details; WDCM: World Data Centre for Microorganisms.

^b Strain to be used by the user laboratory (minimum).

^c Growth is categorized as: 0: — no growth; 1: — weak growth; 2: — good growth (see ISO 11133).

^d Strain free of choice, one of the strains has to be used as a minimum.

^e S: solid medium.

^f In case of both quantitative and qualitative use for the RPFA medium, only results of the quantitative tests are required.

NOTE *Proteus* or *Bacillus* may also grow but appear as brown colonies on Baird-Parker Agar.

6.4

Replace paragraph 6.4 with the following text.

6.4 Water baths, or similar apparatus, capable of being maintained between $47\text{ }^{\circ}\text{C}$ to $50\text{ }^{\circ}\text{C}$.

6.7

Replace paragraph 6.7 with the following text.

6.7 Straight wire, (see ISO 7218) or Pasteur pipette.

9.5

Replace subclause 9.5 with the following text.

NOTE The text for the replacement 9.5.2 is identical to the previous 9.5 "Confirmation (coagulase test)".

9.5 Confirmation

9.5.1 General

The confirmation of coagulase positive staphylococci is undertaken by a tube test (9.5.2). Alternatively, it can be undertaken by a plate test using RPFA (9.5.3) (see References [9], [10] and [11]).

9.5.2 Coagulase tube test

From the surface of each selected colony (9.4), remove an inoculum with a sterile wire (6.7) and transfer it to a tube or bottle of brain-heart infusion broth (5.4).

Incubate at 35 °C or 37 °C[5] for 24 h ± 2 h.

Aseptically add 0,1 ml of each culture to 0,3 ml of the rabbit plasma (5.5) (unless other amounts are specified by the manufacturer) in sterile haemolysis tubes or bottles (specified in 6.5), and incubate at 35 °C or 37 °C[5].

By tilting the tube, examine for clotting of the plasma after 4 h to 6 h of incubation and, if the test is negative, re-examine at 24 h of incubation, or examine at the incubation times specified by the manufacturer.

Consider the coagulase test to be positive if the volume of clot occupies more than half of the original volume of the liquid.

As an uninoculated control, for each batch of plasma add 0,1 ml of sterile brain-heart infusion broth (5.4) to the recommended quantity of rabbit plasma (5.5) and incubate without inoculation. For the test to be valid, the control plasma shall show no signs of clotting.

9.5.3 Plate test using RPFA

From the surface of each selected colony (9.4), take an inoculum with a sterile wire (6.7) and stab inoculate on RPFA plates (5.6.4).

Several colonies in addition to one positive control strain and one negative control strain, can be inoculated onto one plate such that the individual colonies have well separated precipitation zones.

NOTE As a guidance, up to ten colonies can be inoculated onto one plate.

Incubate the plates at 35 °C or 37 °C for 24 h to 48 h.

After incubation, the staphylococci form black or grey or even white, small colonies surrounded by a halo of precipitation, indicating coagulase activity.

Before reading, the plates can be stored in the refrigerator at 5 °C ± 3 °C for 24 h to 48 h.