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(54) Titre : MILIEU DE CULTURE POUR LE CRIBLAGE OU L'ENRICHISSEMENT DE S. AUREUS RESISTANT A LA METHICILLINE
(54) Title: A CULTURE MEDIUM FOR SCREENING OR ENRICHMENT OF METHICILLIN-RESISTANT S. AUREUS

(57) **Abrégé/Abstract:**

The invention relates to culture medium for screening or enrichment of methicillin-resistant *Staphylococcus aureus* (MRSA), which medium comprises a combination of at least two cephalosporins.

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(54) Title: A CULTURE MEDIUM FOR SCREENING OR ENRICHMENT OF METHICILLIN-RESISTANT *S. AUREUS*

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A culture medium for screening or enrichment of methicillin-resistant *S. aureus*

The invention relates to the rapid identification or enrichment of methicillin-resistant *Staphylococcus aureus* (MRSA).

5

BACKGROUND OF THE INVENTION:

Staphylococcus aureus is one of the most commonly identified pathogens in human medicine and is a major cause of nosocomial infections and community-acquired infections. Resistance to methicillin, reported for the first time in 1961, is now widespread
10 in hospitals all over the world. The rapid and reliable identification of methicillin-resistant *Staphylococcus aureus* (MRSA) has become essential for appropriate patient care, and control of strain spreading.

A specific medium is optimal when it promotes the growth of the expected species and inhibits the growth of non-expected species. For the detection or enrichment of MRSA, it is
15 critical to get all MRSA and to inhibit all MSSA, because both micro-organisms share identical phenotypic activities. For such a detection or enrichment medium, both sensitivity and specificity must be excellent.

The standard procedure for identifying MRSA is based on cultures using selective agar media (Cherkaoui et al, 2007, J. Med. Microbiol., 56:500-503).

20 International patent applications WO2004/027086 and WO2004/063391 disclose chromogenic agar media containing a β -lactam antibiotic, in particular a cephalosporin.

Several chromogenic media for the screening of MRSA are commercially available. Cherkaoui et al, *supra*, compared the performance of four of them: oxacillin-resistance screening agar base (ORSAB; Oxoid), MRSA ID (BioMérieux); Chromogen oxacillin *S. aureus*
25 (Axon Lab), and MRSASelect (Bio-Rad). Stoakes et al, 2006, J. Clin. Microbiol., 44(3):637-639) compared MRSASelect to CHROMagar MRSA (Becton-Dickinson), and mannitol –salt medium supplemented with oxacillin or cefoxitin (MSA-OXA and MSA-CFOX, Oxoid).

However these media are not devoid of drawbacks.

Their stability is not warranted, since cephalosporins are known to be very unstable at
30 room temperature or at higher temperature. For a commercial standpoint, it is important that the performances of the plates or tubes do not decrease during the shipping or delivery that can take several days usually at room temperature.

Furthermore, most of these media give a result after 18-24h of incubation. To improve the sensitivity, the user often needs to extend the incubation time (generally 48h), which leads to a delayed diagnosis and generally to a decreased specificity, particularly when the medium is not stable enough. It is also often recommended for the user to perform confirmation tests, such as antimicrobial susceptibility testing, latex agglutination or PCR, which leads to an increased cost of the analysis and, once again, a delayed diagnosis.

Therefore there is a continuing need to provide a MRSA detection medium with higher performance than the current chromogenic media in terms of sensitivity and/or specificity. Preferably such a medium should allow a rapid detection (within 18-24h) and exhibit a good stability.

SUMMARY

The present application relates to a culture medium that aims to meet these needs.

A subject of the application is thus a culture medium for screening or enrichment of methicillin-resistant *Staphylococcus aureus* (MRSA), comprising a combination of cephalosporins selected from the group consisting of cefotetan (CTT) and ceftriaxone (CRO); cefotetan (CTT) and cefpodoxime (CPD); cefsulodin (CFS) and ceftriaxone (CRO); cefsulodin (CFS) and cefepime (FEP); and cefotetan (CTT) and ceftiofur (XLN), and wherein each cephalosporin is present in a concentration of between 1.0 mg/l and 3.5 mg/l.

In a preferred embodiment, the medium further comprises a chromogenic agent, which may be e.g. 6-chloro-3-indoxyl-phosphate.

In a preferred embodiment the culture medium is an agar culture medium. However it may also be a liquid culture medium, i.e. a broth.

In a particular embodiment, the medium comprises two cephalosporins, which may be e.g.: cefotetan (CTT) and ceftriaxone (CRO); CTT and cefpodoxime (CPD); cefsulodin (CFS) and CRO; CFS and cefepime (FEP); CTT and ceftiofur (XLN) or any other combination thereof.

2a

Another subject of the application is a method for screening or enriching methicillin-resistant MRSA in a culture medium, which method comprises (i) inoculating the culture medium defined above with bacteria inoculum suspected of including MRSA bacteria, and (ii) culturing said bacteria in said culture medium. In a particular embodiment, the method
5 comprises identifying the presence of MRSA strains, which appear as colored colonies on the medium when the medium comprises a chromogenic agent.

In another particular embodiment, said bacteria inoculum is in the form of a clinical sample *i.e.*: a nasal swab, a wound sample, a blood sample or any other form of clinical sample.

The application further provides a method for screening or enriching methicillin-resistant
10 *Staphylococcus aureus* (MRSA) in a culture medium or broth, which method comprises (i) inoculating the culture medium defined herein, with bacteria to test, (ii) culturing said bacteria in said culture medium, and (iii) optionally identifying the presence of MRSA strains, which appear as colored colonies on the medium when the medium comprises a chromogenic agent.

15 In a particular embodiment, the bacteria to test are in the form of a clinical sample, such as a nasal swab.

DETAILED DESCRIPTION OF THE INVENTION:

The inventors hypothesized that in the long run stability of an MRSA culture medium comprising a cephalosporin could be improved by adding a second cephalosporin in the medium. Surprisingly enough, they found out that adding another cephalosporin made it possible not only to indeed improve stability of the culture medium, but also to increase its specificity in the long run.

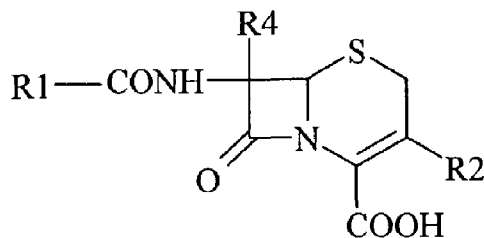
For a commercial point of view it is important that the performances of the plates or tubes do not decrease during the shelf life of the product due to shipping/delivery conditions that can take several days. The mix of cephalosporins unexpectedly increased the robustness or stability of the medium as observed by the performances obtained after thermal stress (when plates were placed on an incubator for 3 days at 37°C).

The mix of cephalosporins further limited the growth of false-positive colonies keeping an optimal sensitivity.

A subject of the invention is a culture medium for screening or enrichment of methicillin-resistant *Staphylococcus aureus* (MRSA), which medium comprises a combination of at least two cephalosporins.

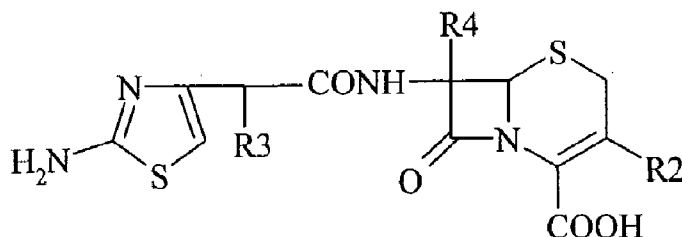
Examples of cephalosporins include, but are not limited to, any first, second, third and fourth generation cephalosporin.

The expression "second or third generation cephalosporin" is intended to denote the antibiotics of the cephalosporin family having a formula derived from formula (I) below:



in which R2 is an H group, an acetoxymethyl group, a methylthiotetrazol group, a dimethylaminoethylthio-tetrazol group, a triazine group, an acetaminopyridine (pyridinium) group, or a pyridinium group substituted with a carbamoyl group, a cyclopentopyridinium group or a thiomethylacetoxithiazol group, R1 is an amino-2-thiazole heterocycle, an alpha-piperazinedione or an alpha-sulfophenyl, and R4 is an H group or an alpha-methoxy radical.

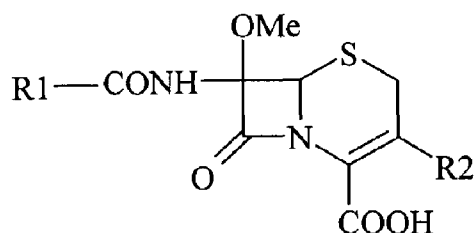
In particular, the compounds having the formula below:



- 5 in which R3 is an H group or an alpha-methoxyimino group, are intended to be denoted.

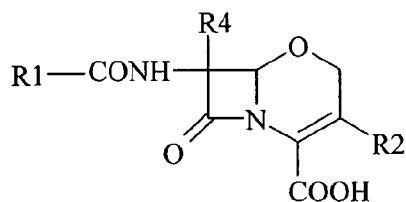
In a particular case, the R4 group is hydrogen.

Cephameycins are compounds in which the R4 group is an alpha-methoxy radical, protecting the beta-lactam ring against hydrolysis by beta-lactamases, and correspond to the formula below:



10

Oxacephems are compounds in which the sulfur atom of the cephem ring is replaced with an oxygen atom, and are considered to be derivatives of formula (I) given above.



In general, for these compounds, the R4 group is an [alpha]-methoxy.

- 15 A definition of the cephalosporins thus envisioned can be found in Binger "Mécanisme d'Action des Bêta-lactamines, (de la structure bactérienne à la structure de la molécule)" 1986, Roussel (Paris) publisher, chapter III, pages 47-62, and chapter IV, pages 63-68), and in the work by Richmond (Beta-lactam antibiotics (the background to their use as therapeutic aents), Hoechst Aktiengesellschaft, D-6230 Frankfurt (Main) 80 publisher, 20 1981, chapter 3, pages 55-65).

Among the second and third generation cephalosporins, mention may be made of: loracarbef, cefaclor, cefuroxime, cefprozil, cefoxitin (cefoxitan), cefamandole, cefotian, cefotetan, cefmetazole, cefocinide, ceforanide, cefpodoxime, cefixime, cefotaxime, ceftizoxime, ceftriaxone, ceftazidime, cefmenoxime, cefodizime, cefoperazone, cefepime
5 (sometimes classified as a fourth generation cephalosporin), cefpirome, cefsulfonide, cefetamete, ceftibutene, moxalactam (latamoxef) and flomoxef, in particular in the form of salts (e.g. sodium salts).

In particular, cephalosporins may be chosen from the group of cephamycins (including e.g. cefoxitin, cefotetan, cefmetazole, cefbutperazone, cefminox) and of oxacephems
10 (including e.g. moxalactam or flomoxef).

Preferably said cephalosporins are selected from the group consisting of cefotetan (CTT), ceftriaxone (CRO), cefuroxime (CXM), cefsulodin (CFS), ceftibuten (CTB), cefepime (FEP), cefoperazone (CFP), cefpodoxime (CPD), cefoxitine (FOX), flomoxef, cefmetazole (CMT),
15 moxalactam (MOX) and ceftiofur (XLN), or salts thereof. Hereinafter, reference is made either to the complete name of the cephalosporin or the 3 letter abbreviation.

More preferably, the medium comprises a combination of cephalosporins selected in the group consisting of:

- 20 cefotetan and ceftriaxone;
- cefotetan and cefoxitin;
- cefotetan and cefpodoxime;
- cefpodoxime and cefsulodin;
- cefuroxime and cefsulodin;
- 25 cefsulodin and cefotetan;
- cefsulodin and ceftriaxone;
- ceftiofur and cefsulodin;
- cefsulodin and cefepime;
- moxalactam and cefoxitin;
- 30 moxalactam and cefpodoxime;
- cefotetan and ceftiofur;
- cefpodoxime and ceftriaxone;

cefoperazone and ceftiofur;
cefoperazone and cefsulodin;
ceftriaxone and cefoperazone;
cefoperazone and cefuroxime;
5 cefotetan and ceftibuten;
ceftriaxone and ceftibuten;
and
ceftriaxone and cefepime.

- 10 A “culture medium” as described herein is a nutritive agar medium or a liquid medium (i.e. a broth) that contains nutrients allowing *Staphylococcus aureus* to grow. Culture media for *S. aureus* are commonly known, and generally contain meat extracts and peptone, as well as salts (see for instance WO2004/027086).

- The term “screening” or “screening MRSA” refers to the detection and/or identification of MRSA, and involves promoting the growth and distinguishing MRSA from other *S. aureus*
15 and other bacteria.

The term “enrichment” or “enriching MRSA” refers to the selective culture of MRSA and involves promoting the growth of MRSA over the growth of from other *S. aureus* and other bacteria.

- 20 The culture medium is inoculated with bacteria to test or directly with a biological or environmental sample. The bacteria to test may be from human clinical sources or from any source including but not limited to food, living or dead animal or plant tissue, water, air, other inanimate environmental surfaces. If the source of the sample is solid, the sample may be suspended in a liquid diluent prior to inoculation of the culture medium.
- 25 Also, a liquid or liquefied sample may be diluted prior to inoculating the culture medium. In certain embodiments, the sample may be serially diluted and the serial dilutions used to inoculate a plurality of culture media in order to obtain a more precise enumeration of *S. aureus* in the original sample. In a preferred embodiment, the bacteria to test are in the form of a clinical sample, such as a nasal swab, a wound sample or a blood culture.

- 30 The term “stability” refers to the ability of the medium according to the invention to allow the selective screening or detection of MRSA for a given period of time, with the same reliability throughout said period of time.

The inoculated culture medium is incubated under conditions that permit the growth of staphylococci. In one embodiment of the method of the present invention, the inoculated culture medium is incubated at 37° C for about 24 hours. However, in other embodiments, the inoculated culture medium may be incubated from about 18 hours to about 48 hours at about 30° C to about 42° C. In particular, the incubation temperature is from about 33°C to 39°C. Although supplementation with additional NaCl, up to a concentration of about 5%, produce optimal results, concentrations as low as 2.5 % NaCl also perform well. Concentrations as low as 0.01% may also provide satisfactory results.

The concentration of each cephalosporin in the medium according to the invention is preferably between 0.01 and 50 mg/l, preferably 0.5 and 30 mg/l, in particular 0.5 and 15 mg/l, still preferably between 0.5 mg/l and 10mg/l. In a particular embodiment, the culture medium comprises from 1 to 2 or 2.5 mg/l of cefotetan and from 1.5 to 2.5 mg/l of ceftriaxone, preferably 1.75mg/l, 2mg/l or 2.4 mg/l cefotetan and 2.4 mg/l ceftriaxone.

In another particular embodiment, the culture medium comprises from 1.5 to 2.5 mg/l of cefotetan and from 1.5 to 2.5 mg/l of cefpodoxime, preferably 2mg/l cefotetan and 1.5 mg/l cefpodoxime.

The culture medium preferably comprises a chromogenic agent, and the cultured microorganisms that survive produce a detectable colored colony in or on the medium, indicating the microorganisms' growth and permitting the specific detection of *S. aureus* (the methicillin resistant *Staphylococci epidermidis* growing on the medium as white colonies).

The media according to the present invention preferably contain from 0.01 to 0.50 g/l, in particular from 0.05 to 0.40 g/l of chromogenic agent that allows the coloration of the strains of *Staphylococcus aureus*. The chromogenic agent is preferably 6-chloro-3-indoxyl phosphate. Other examples of chromogenic agents that can be used in the medium according to the invention include, but are not limited to, 5-bromo-6-chloro-3-indoxyl phosphate, 5-bromo-4-chloro-3-indoxyl-phosphate, 5-bromo-3-indoxyl-phosphate, 3-indoxyl-phosphate, 6-bromo-3-indoxyl- α -D-glucoside, 5-bromo-4-chloro-3-indoxyl- α -D-glucoside, 5-bromo-4-chloro-3-indoxyl-N-methyl- α -D-glucoside, 6-Chloro-3-indoxyl- α -D- glucoside.

In a preferred embodiment, in addition to the chromogenic agent mentioned above the medium according to the invention comprises at least one additional chromogenic agent

that allows the coloration of other microorganisms that could be present in the inoculum, such as 5-bromo-4-chloro-3-indoxyl glucuronide ("X-glucuronide") and/or 5-bromo-4-chloro-3-indoxyl glucoside ("X-glu"), 5-bromo-4-chloro-3-indoxyl galactoside ("X-gal"). The medium according to the invention may also comprise a fluorogenic agent such as 4-Methylumbelliferyl- α -D-glucoside.

The culture medium of the invention may comprise other antibiotics, such as vancomycin, teicoplanin, avoparcin. Preferably the culture medium does not comprise oxacillin and/or does not comprise cefoxitin.

- 10 The culture medium of the invention is very sensible. It allows for identification of weakly resistant MRSA strains and provides a good specificity.

Preferably the medium comprises any of the following combinations of cephalosporins (it is to be understood that each concentration can be applied at $\pm 10\%$):

- 15 CTT 2 mg/l + CRO 2 mg/l
 CTT 2.4 mg/l + CRO 2.4 mg/l
 FOX 4.5 mg/l + CTT 2 mg/l
 CTT 2 mg/l + CPD 1.5 mg/l
 CTT 2.4 mg/l + CPD 1.8 mg/l
 20 CPD 1.5 mg/l + CFS 3.5 mg/l
 CXM 0.75 mg/l + CFS 3 mg/l
 CXM 0.75 mg/l + CFS 3.5 mg/l
 CFS 3.5 mg/l + CRO 2 mg/l
 XLN 0.75 mg/l + CFS 3 mg/l
 25 CFS 3 mg/l + FEP 2 mg/l
 MOX 6 mg/l + FOX 4.5 mg/l
 MOX 4 mg/l + CPD 1 mg/l
 CTT 2 mg/l + XLN 0.75 mg/l
 CTT 1.75 mg/l + CXM 0.75 mg/l
 30 CPD 2.5 mg/l + CRO 3.5 mg/l
 CPD 2 mg/l + CRO 4 mg/l
 CFP 1.75 mg/l + XLN 2 mg/l

CFP 2.25 mg/l +XLN 1.25 mg/l

CFP 1.5 mg/l +CFS 3 mg/l

The media of the invention show an excellent performance in terms of sensitivity and
5 specificity. Optimal media were shown to be both sensitive and specific when tested within
24h. Some others performed better when tested within 48h. However the delay in
diagnosing was balanced by an excellent stability.

The media of the invention showed a good selectivity. Indeed, the growth of other
staphylococci (i.e. non -aureus), mainly methicillin resistant *S. epidermidis*, can be an issue,
10 because some of them are likely to give a false-positive reaction and then be classified as
MRSA. The mix of cephalosporins according to the invention makes it possible to inhibit
these undesired species or to limit their coloration.

In a preferred embodiment, the culture medium according to the invention comprises a
15 compound that inhibits the growth of *Staphylococcus epidermidis* without inhibiting
Staphylococcus aureus. Preferably, said compound is deferoxamine, which is preferably
used in a concentration of 0.01 to 0.10 g/l. Preferably said compound is cefoperazone.
Indeed the inventors have shown that the addition of cefoperazone in a medium
containing already at least one other cephalosporin (to select the MRSA and inhibit the
20 MSSA) advantageously reduces the coloration or inhibits the growth of methicillin resistant
S. epidermidis, reducing the risk of false positive results. Preferably, cefoperazone is used
at a concentration from 0.1 to 3 mg/l.

In still a preferred embodiment, the culture medium comprises three cephalosporins,
including cefoperazone.

25 In a most preferred embodiment, the culture medium comprises cefotetan, ceftriaxone
and cefoperazone.

The below examples illustrate the invention without limiting its scope.

EXAMPLES**Example 1: Comparison of performance of a culture medium using a cephalosporin vs. cephalosporins in combination**

5

Materials & Methods

The basal medium comprises peptone, salts, antifungal and antibacterial agents to inhibit the growth of Gram negative bacteria and fungi. The basal medium is chosen to promote the growth of staphylococci, in particular *Staphylococcus aureus*.

- 10 Several strains of MRSA (methicillin resistant *S. aureus*) and MSSA (methicillin susceptible *S. aureus*) have been chosen from a collection of strains. Strains were cultivated first on a non selective medium. The colonies are used to perform a "heavy inoculum or HI", corresponding to approximately 10^8 bacteria/mL inoculated as a spot on a small portion of the plate, and a "light inoculum or LI", corresponding to approximately 10^5 bacteria/mL streaked on a large portion of the plate.. Plates were incubated for 48h at 35-37°C. The
- 15 reading was performed at 24 and 48h of incubation time.

Abbreviations and legends to the Tables:

- cefotetan (CTT)
- ceftriaxone (CRO)
- 20 cefuroxime (CXM)
- cefsulodin (CFS),
- Cefepime (FEP)
- Cefoperazone (CFP)
- cefpodoxime (CPD)
- 25 moxalactam (MOX)
- cefoxitin (FOX)
- cefmetazole (CMT)
- ceftiofur (XLN)

- G: growth (if nothing else is written, "G" means growth with HI and LI at 24h); Imp G: heavy growth; AG: absence of growth; cfu: colony forming unit; μ cfu: pinpoint colonies;
- 30 SENS: Sensitivity (%); SPE: Specificity (%); MRSA: methicillin-resistant *Staphylococcus*

aureus; MSSA: methicillin-sensitive *Staphylococcus aureus*; MRSE: methicillin-resistant *Staphylococcus epidermidis*; MSSE: methicillin-sensitive *Staphylococcus epidermidis*;

Results

- 5 The below Tables show the performance of various formulas, containing a single cephalosporin or a mixture of cephalosporins. The best media provide growth and coloration of MRSA without growth of MSSA.

Table 1: Performance of a medium with a single cephalosporin (antibiotic concentration in mg/L)

Strains	CTT, 15 mg/L	CTT, 17 mg/L
Experiment #	17	18
MRSA 653130502	G	G
MRSA 599190802 R	G	G of colorless cfu
MRSA 1.1.8	G	G
MRSA 5030	G with HI at 24h G with LI at 48h	Trace at 24h ; G only with HI at 48h
MRSA ATCC 43300	G	G
MRSA FOX 684 RDC 77	G with HI + 1 cfu with LI at 24h	G only with HI at 24h; with HI+LI at 48h
MSSA 1.1.2	1cfu at 24h; G at 48h	AG
MSSA M366806	G	AG
MSSA M366930	AG	AG
MSSA 4007	G	G at 48h
MSSA96019	G	AG
MSSA ATCC 25923	AG	AG
MSSA 3890	AG	AG
MSSA 93096	AG	AG
Sensitivity (24h)	100	67
Specificity (24h)	50	100

Strains	CTT, 15 mg/L	CTT, 17 mg/L
Sensitivity (48h)	100	100
Specificity (48h)	50	88
SENS+SPE 24h	150	167
SENS + SPE 48h	150	188
TOTAL	300	355

CTT, 17 mg/L, was chosen as the best concentration to obtain the most satisfying, albeit not ideal, balance in sensitivity and specificity. For each molecule used alone, the best compromise between sensitivity and specificity has been chosen.

5

Table 2: Performance of CRO + CTT

Strains	CRO 5 mg/L	CTT 17 mg/L	CRO 2 mg/L + CTT 2 mg/L
Experiment #	18	18	18
MRSA 653130502	G	G	G
MRSA 599190802 R	G of colorless cfu	G of colorless cfu	G
MRSA 1.1.8	G	G	G
MRSA 5030	G only with HI at 24h ; G at 48h	Trace at 24h ; G only with HI at 48h	G
MRSA ATCC 43300	G of colorless cfu	G	G
MRSA FOX 684 RDC 77	G only with HI at 24h ; HI + LI at 48h	G only with HI at 24h; with HI+LI at 48h	G
MSSA 1.1.2	G at 48h	AG	AG
MSSA M366806	AG	AG	AG
MSSA M366930	AG	AG	AG
MSSA 4007	1 CFU at 48h	G at 48h	AG
MSSA96019	G at 48h	AG	AG
MSSA ATCC 25923	AG	AG	AG
MSSA 3890	G at 48h	AG	AG

Strains	CRO 5 mg/L	CTT 17 mg/L	CRO 2 mg/L + CTT 2 mg/L
MSSA 93096	AG	AG	AG
Sensitivity (24h)	67	67	100
Specificity (24h)	100	100	100
Sensitivity (48h)	100	100	100
Specificity (48h)	50	88	100
SENS+SPE 24h	167	167	200
SENS + SPE 48h	150	188	200
TOTAL	317	355	400

With CRO used alone, the MRSA colonies were colorless, and MSSA were able to grow.

With CTT used alone, MSSA strains grew, while some MRSA did not grow optimally.

With the mix of CRO and CTT, all MRSA were able to grow, and no growth of MSSA was

5 observed (100% sensitivity and 100% specificity at 24h and 48h).

Table 3: Performance of CTT + CPD

Strains	CTT 17 mg/L	CPD 6 mg/L	CTT 2 mg/L + CPD 1.5 mg/L
Experiment #	18	18	18
MRSA 653130502	G	G	G
MRSA 599190802 R	G of colorless cfu	G	G
MRSA 1.1.8	G	G	G
MRSA 5030	Trace at 24h ; G only with HI at 48h	AG at 24h ; 4 CFU with HI at 48h	1 cfu with HI at 48h
MRSA ATCC 43300	G	G	G
MRSA FOX 684 RDC 77	G only with HI at 24h	G only with HI at 24h	G
MSSA 1.1.2	AG	G at 48h	AG
MSSA M366806	AG	AG	AG

Strains	CTT 17 mg/L	CPD 6 mg/L	CTT 2 mg/L + CPD 1.5 mg/L
MSSA M366930	AG	AG	AG
MSSA 4007	G at 48h	AG	AG
MSSA96019	AG	AG	AG
MSSA ATCC 25923	AG	AG	AG
MSSA 3890	AG	AG	AG
MSSA 93096	AG	AG	AG
Sensitivity (24h)	67	83	83
Specificity (24h)	100	100	100
Sensitivity (48h)	100	100	100
Specificity (48h)	88	88	100
SENS+SPE 24h	167	183	183
SENS + SPE 48h	188	188	200
TOTAL	355	371	383

With CTT used alone, MSSA strains grew, while some MRSA did not grow well.

With CPD used alone, MSSA strains grew, while some MRSA did not grow optimally. No optimal concentration could be found.

- 5 With the mix of CTT and CPD, all MRSA were able to grow at 48h, and no growth of MSSA was observed (100% sensitivity and 100% specificity at 48h).

Table 4: Performance of CXM + CFS

Strains	CXM 2 mg/L	CFS 6 mg/L	CXM 0.75 mg/L + CFS 3 mg/L
Experiment #	17	17	17
MRSA 653130502	G	G	G
MRSA 599190802 R	G	G of pinpoint colourless cfu	G
MRSA 1.1.8	G	G of pinpoint colourless cfu	G
MRSA 5030	AG	G with HI at 24h Trace with LI at 24h ; G	AG

Strains	CXM 2 mg/L	CFS 6 mg/L	CXM 0.75 mg/L + CFS 3 mg/L
		at 48h	
MRSA ATCC 43300	Trace of colourless pinpoint cfu at 24h	G	G
MRSA FOX 684 RDC 77	G only with HI	Trace with HI, 1 colorless cfu with LI	G only with HI
MSSA 1.1.2	G	1cfu at 24h ; G at 48h	1cfu at 24 or 48h
MSSA M366806	AG	G at 48h	AG
MSSA M366930	AG	1cfu	AG
MSSA 4007	AG	G at 48h	AG
MSSA96019	G	G at 48h	AG
MSSA ATCC 25923	AG	AG	AG
MSSA 3890	G at 48h	AG	AG
MSSA 93096	AG	G at 48h	AG
Sensitivity (24h)	67	50	83
Specificity (24h)	75	75	87
Sensitivity (48h)	83	100	83
Specificity (48h)	63	25	87
SENS+SPE 24h	142	125	170
SENS + SPE 48h	146	125	170
TOTAL	288	250	340

With CXM or CFS used alone, MSSA strains grew, while some MRSA did not grow optimally. No optimal concentration could be found.

With the mix of CXM and CFS, all MRSA but one were able to grow, and only one cfu of MSSA was observed.

Table 5: Performance of CRO + CFS

Strains	CRO 5 mg/L	CFS 6 mg/L	CRO 2 mg/L + CFS 3.5 mg/L
Experiment #	18	18	18
MRSA	G	G	G

Strains	CRO 5 mg/L	CFS 6 mg/L	CRO 2 mg/L + CFS 3.5 mg/L
653130502			
MRSA 599190802 R	G	G	G
MRSA 1.1.8	G	G colorless cfu	G
MRSA 5030	G only with HI at 24h ; G at 48h	G with HI & trace with LI at 24h ; G at 48h	Trace with HI at 24h G with HI+LI at 48h
MRSA ATCC 43300	G	G	G
MRSA FOX 684 RDC 77	G with HI at 24h ; G at 48h	Only 1cfu with HI at 24h G with HI + 2 cfu only with LI at 48h	G with HI at 24h ; G with HI+LI at 48h
MSSA 1.1.2	G at 48h	G at 48h	AG
MSSA M366806	AG	G at 48h	AG
MSSA M366930	AG	G at 48h	AG
MSSA 4007	1 CFU at 48h	G	AG
MSSA96019	G at 48h	G at 48h	AG
MSSA ATCC 25923	AG	AG	AG
MSSA 3890	G at 48h	G at 48h	AG
MSSA 93096	AG	G at 48h	AG
Sensitivity (24h)	67	83	83
Specificity (24h)	100	88	100
Sensitivity (48h)	100	100	100
Specificity (48h)	50	13	100
SENS+SPE 24h	167	171	183
SENS + SPE 48h	150	113	200
TOTAL	317	284	383

With CRO used alone, MSSA were able to grow.

With CFS used alone, MSSA strains grew, while some MRSA did not grow optimally. No optimal concentration could be found.

With the mix of CRO and CFS, all MRSA were able to grow, and no growth of MSSA was observed.

Table 6: Performance of FEP + CFS

Strains	FEP 5 mg/L	CFS 6 mg/L	FEP 2 mg/L +CFS 3 mg/L
Experiment #	18	18	18
MRSA 653130502	G colorless cfu	G	G
MRSA 599190802 R	G white cfu	G	G
MRSA 1.1.8	G	G colorless cfu	G
MRSA 5030	G only with HI	G with HI & trace with LI at 24h ; G at 48h	2 pinpoint cfu with HI at 24h Growth with HI at 48h
MRSA ATCC 43300	G colorless cfu	G	G
MRSA FOX 684 RDC 77	AG at 24h ; G 48h	1cfu with HI at 24h G with HI + 2 cfu only with LI at 48h	Trace with HI at 24h ; G 48h
MSSA 1.1.2	G	G at 48h	AG
MSSA M366806	AG	G at 48h	AG
MSSA M366930	AG	G at 48h	AG
MSSA 4007	AG	G	AG
MSSA96019	G	G at 48h	AG
MSSA ATCC 25923	AG	AG	AG
MSSA 3890	G	G at 48h	AG
MSSA 93096	AG	G at 48h	AG
Sensitivity (24h)	33	83	67
Specificity (24h)	63	88	100
Sensitivity (48h)	100	100	100
Specificity (48h)	63	13	100
SENS+SPE 24h	96	171	167
SENS + SPE 48h	163	113	200
TOTAL	259	284	367

With FEP used alone, some MRSA did not grow well, while MSSA strains grew. No optimal concentration could be found.

With CFS used alone, MSSA strains grew, while some MRSA showed difficulty to grow. No optimal concentration could be found.

- 5 With the mix of FEP and CFS, all MRSA were able to grow at 48h, and no growth of MSSA was observed.

Table 7: Performance of CTT + XLN

Strains	CTT 17 mg/L	XLN 2 mg/L	CTT 2mg/L+XLN 0.75 mg/L
Experiment #	18	18	18
MRSA 653130502	G	G of pinpoint colorless cfu	G
MRSA 599190802 R	G of colorless cfu	G of pinpoint white cfu	G
MRSA 1.1.8	G	G	G
MRSA 5030	Trace at 24h ; G only with HI at 48h	Trace pinpoint colorless cfu at 24h; G with HI at 48h+1cfu with LI	3 colored cfu on the spot at 24h ; G with HI at 48h
MRSA ATCC 43300	G	G of colorless cfu	G
MRSA FOX 684 RDC 77	G only spot at 24h; with HI+LI at 48h	No growth at 24h (trace); G with HI 9cfu at 48h with LI	G
MSSA 1.1.2	AG	Trace at 24h ; little G at 48h	AG
MSSA M366806	AG	AG	AG
MSSA M366930	AG	AG	AG
MSSA 4007	G at 48h	AG	AG
MSSA96019	AG	G at 24h	AG
MSSA ATCC 25923	AG	AG	AG
MSSA 3890	AG	AG	AG
MSSA 93096	AG	AG	AG
Sensitivity (24h)	67	17	100

Strains	CTT 17 mg/L	XLN 2 mg/L	CTT 2mg/L+XLN 0.75 mg/L
Specificity (24h)	100	100	100
Sensitivity (48h)	100	100	100
Specificity (48h)	88	88	100
SENS+SPE 24h	167	117	200
SENS + SPE 48h	188	166	200
TOTAL	355	283	400

With CTT used alone, MSSA strains grew, while some MRSA did not grow enough to be detected.

With XLN used alone, MSSA strains grew, while some MRSA did not grow. No optimal
5 concentration could be found.

With the mix of CTT and XLN, all MRSA were able to grow at 48h, and no growth of MSSA was observed (100% sensitivity and 100% specificity).

Table 8: Performance of CXM + CTT

Strains	CXM 2 mg/L	CTT 17 mg/L	CXM 0.75 mg/L + CTT 1.75 mg/L
Experiment #	18	18	18
MRSA 653130502	G	G	G
MRSA 599190802 R	G of colorless cfu	G of colorless cfu	G
MRSA 1.1.8	G	G	G
MRSA 5030	Trace at 24h ; G only with HI at 48h	Trace at 24h ; G only with HI at 48h	AG
MRSA ATCC 43300	AG	G	G
MRSA FOX 684 RDC 77	G only spot at 24h	G only spot at 24h	G
MSSA 1.1.2	Trace at 24h ; G at 48h	AG	AG
MSSA M366806	AG	AG	AG

Strains	CXM 2 mg/L	CTT 17 mg/L	CXM 0.75 mg/L + CTT 1.75 mg/L
MSSA M366930	AG	AG	AG
MSSA 4007	AG	G at 48h	AG
MSSA96019	Trace at 24h ; G at 48h	AG	AG
MSSA ATCC 25923	AG	AG	AG
MSSA 3890	AG	AG	AG
MSSA 93096	AG	AG	AG
Sensitivity (24h)	50	67	83
Specificity (24h)	100	100	100
Sensitivity (48h)	83	100	83
Specificity (48h)	75	88	100
SENS+SPE 24h	150	167	183
SENS + SPE 48h	158	188	183
TOTAL	308	355	366

With CTT used alone, MSSA strains grew, while some MRSA did not grow well.

With CXM used alone, MSSA strains grew, while some MRSA did not grow. No optimal concentration could be found.

- 5 With the mix of CTT and CXM, all MRSA but one were able to grow at 48h, and no growth of MSSA was observed.

Example 2: Stability studies

- 10 When two cephalosporins are added, the stability of the formula is increased. Due to the fact that the molecules are unstable, growth of MSSA often appears after stress. The inventors have mimicked the stress due to transportation of the Petri dishes to the customer's lab, in summer, by placing the plates for 3 days at 37°C before inoculation and incubation 24 or 48h. The basal medium composition was the same as described in Example 1. Abbreviations have the same meaning.

15

The optimal concentration was chosen for Fox and Mox. Then inhibition of MSSA was observed (Table 9A).

Table 9A: Stability of FOX + MOX (24h of incubation)

Strains	FOX 6 mg/L	MOX 14 mg/L	FOX 4.5 mg/L +MOX 6 mg/L	FOX 6 mg/L STRESSED	MOX 14 mg/L STRESSED	FOX 4.5 mg/L +MOX 6 mg/L STRESSED
Experiment #	17	17	17	18	18	18
MRSA 653130502	G	G	G			
MRSA 599190802 R	G	G	G			
MRSA 1.1.8	G	G	G			
MRSA ATCC 43300	G	G	G			
MRSA FOX 684 RDC 77	G	G	G			
MSSA 1.1.2	AG	AG	AG	Imp G	AG	Trace
MSSA M366806	AG	AG	AG	G	Imp G	G
MSSA M366930	AG	AG	AG	Imp G	Imp G	AG
MSSA 4007	AG	AG	AG	AG	Imp G	AG
MSSA96019	AG	AG	AG	G	G	AG
MSSA ATCC 25923	AG	AG	AG	AG	AG	AG
MSSA 3890	AG	AG	AG	AG	AG	AG
MSSA 93096	AG	AG	AG	AG	AG	AG

- 5 With an optimal concentration of FOX, the molecule being used alone, 4 MSSA strains grew at 24h after stress.

With an optimal concentration of MOX used alone, 4 MSSA strains grew at 24h after stress.

With FOX + MOX combined, 1 MSSA only grew at 24h after stress.

The inventors herein show that the growth of MSSA is still inhibited after stress and is less important with a mix of cephalosporins when compared to a cephalosporin used alone.

Additional experiments were performed with a combination of CTT and CPD (Table 9B), or
5 a combination of CTT and CRO (Table 9C).

CTT, 22 mg/L, was eventually chosen as an optimal concentration to obtain the most satisfying, albeit not ideal, balance in sensitivity and specificity, when used alone.

CPD, 4 mg/L, was eventually chosen as an optimal concentration to obtain the most satisfying, albeit not ideal, balance in sensitivity and specificity, when used alone.

10 CRO, 4.5 mg/L, was chosen as the best concentration to obtain the most satisfying, albeit not ideal, balance in sensitivity and specificity, when used alone.

In tables 9B and 9C,

LG means « limited growth » for MRSA, i.e. growth only with a heavy inoculum (HI) ; no
15 growth with the light inoculum (LI)

Table 9B: Stability of CTT +CPD (24h of incubation)

Strains	CTT 22 mg/L	CPD 4 mg/L	CTT 2.5 mg/L + CPD 1.8 mg/L	CTT 22 mg/L STRESSED	CPD 4 mg/L STRESSED	CTT 2.5 mg/L + CPD 1.8 mg/L STRESSED
Experiment #	71	71	71	71	71	71
MRSA 653130502	G	G	G	G	G	G
MRSA 599190802 R	G	G	G	G	G	G
MRSA 1.1.8	G	G	G	G	G	G
MRSA 5030	LG	LG	LG	G	LG	LG

Strains	CTT 22 mg/L	CPD 4 mg/L	CTT 2.5 mg/L + CPD 1.8 mg/L	CTT 22 mg/L <i>STRESSED</i>	CPD 4 mg/L <i>STRESSED</i>	CTT 2.5 mg/L + CPD 1.8 mg/L <i>STRESSED</i>
MRSA ATCC 43300	G	G	G	G	G	G
MRSA FOX 684 RDC 77	LG	LG	LG	LG	LG	LG
MSSA 1.1.2	AG	G	AG	G (colorless)	G	AG
MSSA M366806	AG	AG	AG	G	AG	AG
MSSA M366930	AG	AG	AG	G	AG	AG
MSSA 4007	G	AG	AG	G	AG	AG
MSSA96019	AG	G	AG	G	G	AG
MSSA ATCC 25923	AG	AG	AG	AG	AG	AG
MSSA 3890	AG	AG	AG	G (colorless)	AG	AG
MSSA 93096	AG	AG	AG	G	AG	AG

In control plates with CTT or CPD only, not submitted to any stress, all MRSA grew, when few strains of MSSA were able to grow at 24h.

In plates combining CTT and CPD, but not submitted to stress, all MRSA grew and no MSSA was able to grow.

All MRSA grew, and the majority of MSSA strains was also able to grow at 24 h in plates with CTT submitted to stress (for 3 days at 37°C before inoculation). There was a dramatic decrease in specificity in comparison with the control (not stressed) plates.

All MRSA grew, and two MSSA strains were also able to grow at 24 h in plates with CPD submitted to stress (for 3 days at 37°C before inoculation).

In plates combining CTT and CPD, submitted to stress, all MRSA grew and no MSSA was able to grow.

The mixture of antibiotics thus gave the best performance and showed the best stability after stress.

Table 9C: Stability of CTT + CRO (24h of incubation)

Strains	CTT 22 mg/L	CRO 4.5 mg/L	CTT 2.4 mg/L + CRO 2.4 mg/L	CTT 22 mg/L <i>STRESSED</i>	CRO 4.5 mg/L <i>STRESSED</i>	CTT 2.4 mg/L + CRO 2.4 mg/L <i>STRESSED</i>
Experiment #	71	71	71	71	71	71
MRSA 653130502	G	G	G	G	G	G
MRSA 599190802 R	G	G	G	G	G	G
MRSA 1.1.8	G	G	G	G	G	G
MRSA 5030	LG	G	LG	G	G	G
MRSA ATCC 43300	G	G	G	G	G	G
MRSA FOX 684 RDC 77	LG	LG (	LG	LG	LG	LG
MSSA 1.1.2	AG	AG	AG	G (colorless)	G	AG
MSSA M366806	AG	AG	AG	G	AG	AG
MSSA M366930	AG	AG	AG	G	Nc	AG
MSSA 4007	G	AG	AG	G	Nc	G
MSSA96019	AG	G	AG	G	G	AG

Strains	CTT 22 mg/L	CRO 4.5 mg/L	CTT 2.4 mg/L + CRO 2.4 mg/L	CTT 22 mg/L <i>STRESSED</i>	CRO 4.5 mg/L <i>STRESSED</i>	CTT 2.4 mg/L + CRO 2.4 mg/L <i>STRESSED</i>
MSSA ATCC 25923	AG	AG	AG	AG	AG	AG
MSSA 3890	AG	AG	AG	G (colorless)	AG	AG
MSSA 93096	AG	AG	AG	G	AG	AG

In control plates with CTT or CRO only, not submitted to any stress, all MRSA grew, when one strain of MSSA was able to grow at 24h.

In plates combining CTT and CRO, but not submitted to stress, all MRSA grew and no MSSA
5 was able to grow.

All MRSA grew, and the majority of MSSA strains was also able to grow at 24 h in plates with CTT submitted to stress (for 3 days at 37°C before inoculation). There was a dramatic decrease in specificity in comparison with the control (not stressed) plates.

10

All MRSA grew, and four MSSA strains were also able to grow at 24 h in plates with CRO submitted to stress (for 3 days at 37°C before inoculation). There was a decrease of specificity in comparison with the control (not stressed) plates.

15 In plates combining CTT and CRO, submitted to stress, all MRSA grew and only one MSSA was able to grow at 24h.

The mixture of antibiotics thus gave the best performance and showed the best stability after stress.

20 **Example 3: Inhibition of other species**

Sensitivity, specificity as well as selectivity of a medium comprising CFP + CRO + CTT was studied.

The basal medium composition was the same as described in Example 1. Abbreviations have the same meaning.

Table 10: Inhibition of *Staphylococcus epidermidis*

Strains	CFP 3.5 mg/L	CRO 2 mg/L CTT 1.75 mg/L	CRO 2 mg/L CTT 1.75 mg/L CFP 0.5 mg/L	CTT 2 mg/L CPD 1.5 mg/L	CTT 2 mg/L CPD 1 mg/L CFP 1 mg/L
Experiment #	7	7	7	18	18
MRSE SDP 1589	AG	G pinkish	G white	G pink	G white to slightly pinkish
MRSE SE 44476	AG	G pinkish	Little G white	G pinkish	G less colored
MRSE SE 49704	AG	G white	G white with smaller cfu	G pinkish	G less colored and smaller cfu
6 strains of MRSA	4 AG ; 2 G	6 G	1 AG ; 5 G	1 AG ; 5 G	1 AG ; 5 G

- 5 Adding CFP in a medium containing already another cephalosporin reduces the coloration or inhibits the growth of methicillin resistant *S. epidermidis*, reducing the risk of false positive results.

Furthermore higher sensitivity and specificity was observed against MSSA and MRSE.

CLAIMS

1. A culture medium for screening or enrichment of methicillin-resistant *Staphylococcus aureus* (MRSA), comprising a combination of cephalosporins selected from the group consisting of cefotetan (CTT) and ceftriaxone (CRO); cefotetan (CTT) and cefpodoxime (CPD); cefsulodin (CFS) and ceftriaxone (CRO); cefsulodin (CFS) and cefepime (FEP); and cefotetan (CTT) and ceftiofur (XLN) and wherein each cephalosporin is present in a concentration of between 1.0 mg/l and 3.5 mg/l.
2. The culture medium of claim 1, which further comprises a chromogenic agent.
3. The culture medium according to claim 2, wherein the chromogenic agent is 6-chloro-3-indoxyl phosphate.
4. The culture medium of any one of claims 1 to 3, which is an agar culture medium.
5. The culture medium of any one of claims 1 to 3, which is a broth.
6. The culture medium of any one of claims 1 to 5, wherein said combination of cephalosporins is cefotetan (CTT) and ceftriaxone (CRO).
7. The culture medium of any one of claims 1 to 5, wherein said combination of cephalosporins is cefotetan (CTT) and cefpodoxime (CPD).
8. The culture medium of any one of claims 1 to 5, wherein said combination of cephalosporins is cefsulodin (CFS) and ceftriaxone (CRO).
9. The culture medium of any one of claims 1 to 5, wherein said combination of cephalosporins is cefsulodin (CFS) and cefepime (FEP).

10. The culture medium of any one of claims 1 to 5, wherein said combination of cephalosporins is cefotetan (CTT) and ceftiofur (XLN).

11. The culture medium of anyone of claims 1 to 5, which comprises:

- a) 1 to 2.5 mg/l of cefotetan and 1.5 to 2.5 mg/l of ceftriaxone;
- 5 b) 1.75 mg/l cefotetan and 2.4 mg/l ceftriaxone; or
- c) 2.4 mg/l cefotetan and 2.4 mg/l ceftriaxone.

12. The culture medium of anyone of claims 1 to 5, which comprises:

- 2 mg/l cefotetan and 2 mg/l ceftriaxone,
- 10 2.4 mg/l cefotetan and 2.4 mg/l ceftriaxone,
- 2 mg/l cefotetan and 1.5 mg/l cefpodoxime,
- 2.5 mg/l cefotetan and 1.8 mg/l cefpodoxime,
- 3.5 mg/l cefsulodin and 2 mg/l ceftriaxone, or
- 3 mg/l cefsulodin and 2 mg/l cefepime.

15

13. The culture medium of any one of claims 1 to 10, wherein each cephalosporin is present in a concentration of between 1.25 mg/l and 3.5 mg/l.

14. The culture medium of any one of claims 1 to 10, wherein each cephalosporin is present in a concentration of between 1.1 mg/l and 3.5 mg/l.

20

15. The culture medium of any one of claims 1 to 14, which further comprises cefoperazone.

25

16. The culture medium of claim 15, wherein said cefoperazone is in a concentration of between 0.1 mg/l to 3 mg/l.

17. A method for screening or enriching methicillin-resistant *Staphylococcus aureus* (MRSA) in a culture medium, which method comprises (i) inoculating the culture

medium defined in any one of claims 1 to 16, with bacteria inoculum suspected of including MRSA bacteria, and (ii) culturing said bacteria in said culture medium.

5 18. The method of claim 17, further comprising (iii) identifying the presence of MRSA strains, which appear as colored colonies on the medium when the medium comprises a chromogenic agent.

19. The method of claim 17 or 18, wherein the bacteria inoculum is in the form of a clinical sample.

10 20. The method of claim 19, wherein the clinical sample is a nasal swab, a wound sample or a blood sample.