MICROBIOLOGICAL WATER ANALYSIS VALIDATION CERTIFICATE

We hereby certify that the following method and/or products:

Protocol SEILAGUA for microbiological analyses of water (presentations and references MICROKIT between parenthesis) (PRT-SEILA-002 with 39 pages, with all the specificities for every parameter derivate of PRT-AG-012) and made with the appropriate culture media with the methods and kits for Cetrimide Agar (DMT034, TPL100, RPL010, PPL906 and its version cromogenic broth Pseudomonas P/A: RPL302, FPA903), YEA-NUTRIENT AGAR CROMOGENIC (BCD511, TPL060, RPL106, PPL901), COMPACT-DRY-PLATES –TC (1000166), GVPC Broth (TPL016), GVPC Agar (DMT007+SBL604, RPL018+SBL604, PPL908) **Mannitol Salt Agar** (DMT078, TPL066, RPL023, PPL907 and its version Broth P/A: RPL320, FPA907), **COMPACT-DRY-PLATES®-STAF** (1002960), **SS Broth** (DMT067, TPL401, RPL060, idem concentrate in tubes P/A: RPL331), **CHROMOSALM Agar** (DMT500, TPL402, RPL012, PPL925), **XLD Agar** (DMT142, TPL504Z), **MugPlus Cfs.Vanc.Agar** (DMT400, TPL400, RPL444, PPL902, incl. **COMPACT-DRY-PLATES®-EC** 1000168), **MCC Broth** (DMT900, TPL637 and its version P/A COLICULT: RPL303, FPA900) (See the certificate of validation of **Coliforms and *E.coli***), **Slanetz-Bartley Agar** (DMT115, DMT117+SDA018, PPL909), **Bilis Esculina Azida Agar** (DMT160, TPL002, PPL915 and its version cromogenic Broth **P/A ENTEROCULT**: RPL301, FPA901), **KAA AGAR** (DMT060, TPL190, RPL069), **COMPACT-DRY-PLATES®-ETC** (1002944)**, SPS Agar** (BCD901, TPL089, TPL049, RPL039, RPL062), **TSC Agar** (DMT175, TPL137, PPL905 and its version **P/A CLOSTRICULT**: RPL308, FPA902).

meet the VALIDATION standards of UNE-EN-ISO 16140:2003, and the results are annexed.

The validation is made using comparative methods and certified qualitative and quantitative strains, conform with the official methods of reference (Royal Decrees 1074/2002 y 140/2003, which accomplished the actual Technical Norms ISO/UNE for microbiology of water and also the ISO 11731 for Legionella).

The present certificate for our products is only valid until the date of expiration of the given methods. We give a warranty every three months; it will be renovated when using the comparative method SEILAGUA®. It should be renovated before the five years of its written date of expiration.

This certificate allows the user to validate the methods and the media and kits through the studies of validation/equivalence of MICROKIT® for the internal quality control or for the quality control of methods, media and kits with their own points of reference, the equipment and the workers in their own premises, provided that the methods and products covered by this certification are not mixed with other similar commercial products.

Warranty by: Date: 09-July-2009

Jorge Sanchis Solera
Coordinator SEILAGUA and Quality Director MICROKIT

❖ METHOD OF VALIDATION

Comparison studies were made for a minimum of 20 specimens, for each one of the microbiological parameters, for detection of its presence or absence and their count, following the method MICROKIT® (protocol PRT-SEILA-002 and PRT-AG-001 and PRT-AG-012 of water) with quantitative certified strains HPA, in accordance with the official method (Royal Decrees 1074/2002 and 140/2003, which accomplished the actual Technical Norms ISO/UNE for microbiology of water and also the ISO 11731 for Legionella), using the culture mediums MICROKIT® described in the protocols, which are indicated here.

Please notice that the method MICROKIT for Coliforms and E. Coli have been validated with excellent results, demonstrating its accuracy in our previous validation, published in 2008 during the Congress of Microbiology in Cordoba and in May 2009 in the publication Laboratory Techniques. For that reason, we are considering here, in one of the presentations which accomplished the actual Technical Norms ISO/UNE for microbiology of water and also the ISO 11731 for Legionella). The dates of validation of Salmonella are coming from another exercise, in this case, with the participation of 6 laboratories with 250 compared specimens, published in the XIX Congress of Microbiology in Santiago de Compostela in May 2003 in the Journal "Laboratory Techniques". The Certification is included.

For the Compact-Dry- Plates®-STAF, the SS Broth for Shigella and the Compact-Dry-Plates®-ETC it is too early to give the data. However the actual data, in the three cases, are showing excellent results in relation to the official method, for this reason are included here. The definitive results will be given later on.

The data is compared with the results obtained with identical patterns and inoculations used for the 70 Spanish laboratories participating in the Quality Assessment of SEILAGUA® in the last 7 years, specially the last ones from 2007-2009. It is a total of 28 series, comparing more than 700 identical probes, within the participants, who are using the REFERENCE METHOD and the MICROKIT®, which allows a periodical validation.

Because of the homogenous results written in the publication "12/2007: **Protocols MICROKIT** for microbiological control of water, which were validate during the 6 years of comparative tests SEILAGUA®. See Laboratory Techniques 332, 6/2008 and the VII Meeting of Microbiology for Waters SEM in Bilbao 9/2008". In the assessment, we are not taking into account the data of all the samples, only the ones of the last three years (12 comparative studies between 2007 and 2009) so we will not duplicate statistics, otherwise it will be redundant.

In all the studies of the last 3 years, we used quantitative certified strains obtaining two simultaneous methods of contrast: the original strain and a method of pars for comparison. From the 70 participant laboratories, at least 6 of then hat accreditation for microbiological analyses for the Norm ISO 17025, in accordance with the ISO 17994 for equivalence of microbiological methods.

For the MATRIX we used drinking water, bottled water, water without treatment, purified pharmaceutical water and water used for refrigeration of air conditioners.

Restrictions of use from the protocol, the mediums and the kits of MICROKIT®: In the validation are excluded sea water or salty, water from swimming pools, because there are not enough proof on it. Because we know the kits are apt for used on this type of water, validations can be use later on.

❖ RESULTS

In blue letters, are the results of MICROKIT for detection and counts in any presentation. In black letters are the results of the reference method. They are not third methods to be counted. The participants of SEILAGUA ® who are not strictly applying with the reference methods are not being taking into account.

1. RESULTS OF QUALITATIVE PARAMETERS

PARÁMETER	SENSIBILITY (scarcity of False Negatives)		SPECIFICITY (scarcity of False Positives)	
	% METHOD MICROKIT	% METHOD OF REFERENCE	% MÉTHOD MICROKIT	% METHOD OF REFERENCE
Pseudomonas aeruginosa	P/A: 0 false negatives of 21 specimens 100 % SENSIBILITY >>>	UNE-EN-ISO 12780 Agar Cetrimide: 17 false negatives in 64 specimens 73,4 % SENSITIVITY	P/A: 0 false positives of 12 specimens 100 % SPECIFICITY>>>	UNE-EN-ISO 12780 Agar Cetrimide: 2 false positives of 23 specimens 91,3 % ESPECIFICITY
Legionella pneumophila	Broth GVPC+Agar GVPC 2 false negatives of 12 specimens (1) 83,3 % SENSIBILITY	ISO 11731 Agar GVPC 18 false negatives of 29 specimens 38 % SENSIBILITY (1)	Broth GVPC+Agar GVPC 3 false positives of 9 specimens 66,7% SPECIFICITY (2)	ISO 11731 Agar GVPC 7 false positives of 35 specimens 80 % SPECIFICITY (2)
Staphylococcus aureus	Compact-Dry-Plates® STAF 0 false negatives of 9 specimens 100 % SENSIBILITY (3) - - - - - - - - - - - - - - - - - - P/A: 0 false negatives of 12 specimens 100 % SENSIBILITY	Mannitol Salt Agar 6 false negatives of 15 specimens 60 % SENSIBILITY - - - - - - - - - - - - - - - - - - Agar Baid Parker y RPF 5 false negatives of 17 specimens 71 % SENSIBILITY	Compact-Dry-Plates® STAF 0 false positives of 6 specimens 100 % SPECIFICITY (3) - - - - - - - - - - - - - - - - - - P/A: 1 false positives of 24 specimens 96 % SPECIFICITY	Mannitol Salt Agar 3 false positives of 18 specimens 83 % SPECIFICITY - - - - - - - - - - - - - - - - - - Agar Baid Parker and RPF 7 false positives of 20 specimens 65 % SPECIFICITY
Salmonella spp.	Agar Cromosalm MICROKIT®: 89,47 % SENSIBILITY (1)	ISO 6340, ISO 6579 47,22 % BGA, 62,96 % XLD SENSIBILITY (1)	Agar Cromosalm MICROKIT®: 98,45 % SPECIFICITY	ISO 6340, ISO 6579 60,84 % BGA, 65,38 % XLD, 73,91 % Hektoen, 42,55 % SS Agar, 73,47 % Magenta-Gal SPECIFICITY
Shigella spp.	Broth SS MICROKIT®: 0 false negatives of 3 specimens 100 % SENSIBILITY (3)	Typical methods Salmonella: 2 false negatives of 3 specimens 33 % SENSIBILITY	Broth SS MICROKIT®: 0 false positives of 31 specimens 100 % SPECIFICITY	Typical methods Salmonella: 6 false positives of 28 specimens 79 % SPECIFICITY
Coliforms-*E.coli*	Compact-Dry-Plates® EC 0 false negatives of 21 specimens (Colif.y E.coli) 100 % SENSIBILITY (4)	ISO 9308 Agar Tergitol TTC: 2 false negatives of 87 (Colif.) and 4 of 92 (*E.coli*) 97,7 y 95,6 % SENSIBILITY	Compact-Dry-Plates® EC 0 false positives of 12 specimens (Colif.y E.coli) 100 % SPECIFICITY (4)	ISO 9308 Agar Tergitol TTC: 2 false positives of 87 (colif.) and 4 of 92 (*E.coli*) 94,2 y 94,6 % SPECIFICITY
faecal Enterococci	Compact-Dry-Plates® ETC 0 false negatives of 10 specimens 100 % SENSIBILITY (3) - - - - - - - - - - - - - - - - - - P/A ENTEROCULT: 0 falsos negativos de 24 muestras 100 % SENSIBILIDAD	ISO 7988 Slanetz-Bartley and Bilis Esculina 4 false negatives of 91 specimens 95,6 % SENSIBILITY	Compact-Dry-Plates® ETC 0 false positives of 1 specimens 100 % SPECIFICITY (3) - - - - - - - - - - - - - - - - - - P/A ENTEROCULT : 0 false positives of 10 specimens 100 % SPECIFICITY	ISO 7988 Slanetz-Bartley and Bilis Esculina 16 false positives of 41 specimens 61 % SPECIFICITY
Clostridium perfringens and its spores	P/A CLOSTRICULT: 5 false negatives of 22 specimens (1) 77,3 % SENSIBILITY	R.D.1074/2002 y 140/2003 (m-CP Agar): 18 false negatives of 21 specimens 14,33 % SENSIBILITY (1)) - - - - - - - - - - - - - - - - - - ISO/CD 6461 (TSC Agar) 22 false negatives of 50 specimens 56 % SENSIBILITY(1)	P/A CLOSTRICULT: 0'false positives of 12 specimens 100 % SPECIFICITY	R.D.1074/2002 y 140/2003 (m-CP Agar): 0 false positives o 11 specimens 100 % SPECIFICITY - - - - - - - - - - - - - - - - - - ISO/CD 6461 (TSC Agar) 1 false positives of 24 specimens 95,8 % SPECIFICITY

(1). Sensibilities less than 95%, but greater of the reference methods, are demonstrating the suitability using the MICROKIT for Legionella and for *Clostridium perfringens*, two of the most conflictive parameters. The Clostricult P/A is working better, since his modification in 2008, its sensibility is now close to 100% in both presentations (Bottles with liquid media and ampoules with weighed sterile powder

(2). The specificity is obtained using the full method, we can not blame for bad results of the MICROKIT, which is the reference method, to innovations such as previous incorporation of

revitalizing Broth GVPC , because it is caused more for bad interpretation of the latex, (used or not used) in some of the laboratories.

(3). Should be taking with precaution the Sensibility and Specificity showed for the Compact-Dry-Plates®-STAF and for the Compact-Dry-Plates®-ETC and the Sensibility of the SS Broth for Shigella, for a lack of sufficient comparative studies.

(4). The Compact-Dry-Plates®-EC made with medium MUGPLUS were validated in the testing for Coliforms and E.coli, nevertheless we include here the new specific data of this chart.

Data from the previous study from 2002 to 2006 published in the bibliography:

PARAMETER	DATA 2002 TO 2006 EFFICIENCY: SENSIBILITY + SPECIFICITY (Scarcity of False Negatives and False Positives) % METHOD MICROKIT	% METHOD OF REFERENCE
Pseudomonas aeruginosa	P/A *Pseudomonas aeruginosa* Broth: 95 % EFFICIENCY	UNE-EN-ISO 12780 Agar Cetrimide: 79 % EFFICIENCY
Legionella pneumophila	Broth GVPC+Agar GVPC: 70 % EFFICIENCY	ISO 11731 Agar GVPC: 54 % EFFICIENCY
Staphylococcus aureus	P/A *Staphylococcus aureus*: 70 % EFFICIENCY	Agar Baid Parker and RPF: 62 % EFFICIENCY
Salmonella spp.	Agar Cromosalm MICROKIT®: 100 % EFFICIENCY	ISO 6340, ISO 6579 47,22 % BGA, 62,96 % XLD EFFICIENCIA
Shigella spp.	Caldo SS MICROKIT®: 100 % EFFICIENCY	Métodos típicos Salmonella: 33 % EFFICIENCY
Coliforms-*E.coli*	P/A MCC COLICULT: Coliformes 86,5 % and E.coli 90% EFFICIENCY	ISO 9308 Agar Tergitol TTC: Coliformes: 80% and *E.coli* 78 % EFFICIENCY
faecal Enterococci	P/A ENTEROCULT: 100 % EFFICIENCY	ISO 7988 Slanetz-Bartley y Bilis Esculina: 95,6 % EFFICIENCY
Clostridium perfringens and its spores	P/A CLOSTRICULT: 70 % EFFICIENCY	R.D.1074/2002 y 140/2003 (m-CP Agar): 29 % EFFICIENCY

From the comparative results of past years SEILAGUA® 2002-2006, we are taking the same conclusions, like in the actual validation with quantitative strains, SEILAGUA® 2007-2009. In all these cases the method MICROKIT® is more efficient than the traditional method, especially for its superior Sensibility in all parameters.

The limits of detection are also extraordinarily optimized thorough the protocols of MICROKIT. The data is showing innumerable cases of detection with our methods with a low inoculation, that even the reference method was not able to detect it, in the main laboratory and not either with the participants of the comparative study or survey. We do not have data of what will be happening, using strains that will be between the media of the actual minimum detected with the methods MICROKIT, and the minimum actual detected with the reference methods in this study, because we did not made values of the strain of, for example 25 ufc of *Pseudomonas aeruginosa*; the logic make us to think that the difference between both methods should be not so great. However with the data we have, we can affirm that the revitalization method with selective broths, like the P/A de MICROKIT, are showing the correctness of this protocol for the detection of microorganisms d*iana*, including when they are in very low concentrations and in the presence of innumerable interferences or contaminants:

LIMITS OF DETECTION CONFIRMED FROM *:		
	METHOD MICROKIT	METHOD OF REFERENCE
Pseudomonas aeruginosa	3 ± 1 ufc/100 ml	150-192 ufc/100 ml
Legionella pneumophila	40 ± 10 ufc/litro	1000-10.000 ufc/litro
Staphylococcus aureus	70 ± 5 ufc/100 ml	100-1000 ufc/100 ml
Shigella spp.	Without confirmation	
Coliforms-*E.coli*	5 ± 2 ufc/100 ml (correlation in both parameters *E.coli* as coliform and also in both methods)	
Faecal Enterococci	41 ± 4 ufc/100 ml (correlation with the three methods)	
Clostridium perfringens and its spores	150 ± 10 ufc/100 ml	400-1500 ufc/100 ml

* Please notice that the microbiological uncertainty because of the contagious spreading of microorganisms (Poisson or binomial negative) are not allowing to affirm that two specimens of water recently agitated are identical, therefore we can not assure in a statistical secure form, the limits of detection less than the ones here mentioned, because in one specimen could be present 1 ufc, in another 2 or nothing. We observed that the method MICROKIT, in all the cases, is very near to the limit of the ideal detection (theoretical of 1 ufc /100) compared with the reference method in a very significant statistical form. Because of the vagueness due to the impossibility of improve the homogenous distribution of strains in the samples, we believe that the limit of detection, in reality, is going to be close to the ideal of 1 ufc/100 in 100% of the samples using the optimized methods of MICROKIT.

2. RESULTS OF QUANTITATIVE PARAMETERS

Even if the only quantitative parameter of maximal importance in water is the total aerobic count (because in the other indicators and pathogens, the only important fact is the absence of any ufc), we included also the quantitative data of all otheir parameters (except *Salmonella spp. and Shigella spp*.), in order to satisfy the most orthodox entities.

PARAMETER	ACCURACY (measure in relative recuperation: proximity of the counts to the model value - strain value of reference or accepted value in the survey)		PRECISION (dispersion of results measured in repeatability and reproducibility, capable of present equivalent results) Its depends more from other factors than the media/method.	
	% METHOD MICROKIT	**% MÉTHOD OF REFERENCE**	**% METHOD MICROKIT**	**% METHOD OF REFERENCE**
Aerobic total count at 22 °C	Compact-Dry-Plates® TC: 114 % **** - - - - - - - - - - - - - - - - - - YEA-Cromogenic: 83%	ISO 6222 Agar YEA: 102 % ****	Compact-Dry-Plates® TC: < ± 0,1 log y 0 out of range: OK - - - - - - - - - - - - - - - - - - YEA-Cromogenic: < ± 0,16 log y 3% out of range: OK	ISO 6222 Agar YEA: < ± 0,3 log y 22% of samples out of range: OK
Aerobic total count at 35-37 °C	Compact-Dry-Plates® TC: 106 % **** - - - - - - - - - - - - - - - - - - YEA-Cromogenic: 84 %	ISO 6222 Agar YEA: 108 % ****	Compact-Dry-Plates® TC: < ± 0,1 log y 0 out of range: OK - - - - - - - - - - - - - - - - - - YEA-Cromogenico: < ± 0,1 log y 3% out of range: OK	ISO 6222 Agar YEA: < ± 0,4 log y 19% out of range: OK
Pseudomonas aeruginosa	In recounts is the same method UNE-EN-ISO 12780 Agar Cetrimide: 80% *		In recounts is the same method UNE-EN-ISO 12780 Agar Cetrimide: < ± 1,57 log y 8% of samples out of range: OK	
Legionella pneumophila	ISO 11731 Agar GVPC after revitalization broth GVPC: 63,5 % *	ISO 11731 Agar GVPC 87,6% *	ISO 11731 Agar GVPC after revitalizatonón broth GVPC Not sufficient data	ISO 11731 Agar GVPC < ± 0,45 log y **43**% of samples out of range: POOR
Staphylococcus aureus	Compact-Dry-Plates® STAF: 80% * - - - - - - - - - - - - - - - - - - Mannitol Salt Agar: 42 % *	Baird Parker Agar y/o RPF: 29% **	Compact-Dry-Plates® STAF : < ± 0,1 log y 14% of samples out of range: OK - - - - - - - - - - - - - - - - - - Mannitol Salt Agar:< ± 0,17lg y 17% of samples out of range: OK	Baird Parker Agar and/or RPF: < ± 0,57 log y 12 % of samples our of range: OK
Coliforms-*E.coli* (see more results MUGPLUS in the validation/ equivalence 2008)	Compact-Dry-Plates® EC: Coliforms: 171,4 % **** E.coli: 110,6% ****	ISO 9308 Agar Tergitol TTC: Coliforms: 119 % **** E.coli: 66,8 % *	Compact-Dry-Plates® EC: Colif) < ± 0,47 log, (y 7 % of samples out of range): OK *(E.coltedi) < ± 0,33 (y 3 % of samplesout of range):* OK	ISO 9308 Agar Tergitol TTC: Colif) < ± 0,54 log, (y 15 % of samples out of range): OK *(E.coli) < ± 0,39 (y 12 % of samples out of range):* OK
Faecal Enterococci	Compact-Dry-Plates® ETC: 55% *	ISO 7988 Slanetz-Bartley y Bilis Esculina: 74,4% *	Compact-Dry-Plates® ETC: There is not sufficient data	ISO 7988 Slanetz-Bartley and Bilis Esculina: < ± 0,62 log and 3% of samples out of range OK
Clostridium perfringens and its spores	*It is better not to enumerate and detect the presence o absence with a validated methcd like the vials of powder Clostricult P/A, than to obtain results that are not reliable like the official method*	ISO/CD 6461 (TSC Agar): 9,3 % ** - - - - - - - - - - - - - - - - - - R.D.1074/2002 y 140/2003 (m-CP Agar): 1,9% ***	It can not be data of count in a *method P/A*	ISO/CD 6461 (TSC Agar): < ± 0,52 log y **43**% of samples out of range POOR - - - - - - - - - - - - - - - - - - R.D.1074/2002 y 140/2003 (m-CP Agar): <± **1,97** log y **100**% of samples out of range BAD

* Totally within the statistical standard value of tolerance of ± 2 log, acceptable

** Far from adequate accuracy, even it is within the ± 2 log, it is inacceptable. Only 29 of every 100 ufc presents of *S.aureus are detected with* B.Parker (or with RPC). Only 9 of each 100 ufc of *Cl.perfringens* presents are detected for Filtration with TSC Agar

*** Out of range from the ± 2 log, only two of every 100 ufc are inedetected, therefore we do not recommend the official method of m-CP Agar because it is not valid

**** Those are working better than the strain pattern, because its count is referred to other methods/media, like the Blood-Agar or the EMB Levine for example.

In regards to Precision, it is in all cases within the established statistical limits of ± 2 log, except for the m-CP Agar which is closed. The proportion of the aberrant data out of range, it is also maximal in the m-CP Agar (100%), followed for the BCYE (43%) and for the TSC (43%). It is also remarking the maximal precision of the Compact-Dry-Plates®-TC (0% of aberrant samples) follow for the YEA-Cromogenic (3% of all the samples out of range in both temperatures), while in the reference method with YEA there is less than 22% (at 22 °C) and 19% (at 35-37°C) of samples in the survey which have been discarded precisely for the aberrant results out of range obtained.

In regards with precision, we emphasized that a lot of the detected lack of precision is due to the laboratory's analyst and the component inter-laboratories, more than the component culture media or its format

❖ CONCLUSIONS

1- We observed that all the proposed methods for MICROKIT in their protocols, are equal or even BETTER than the analytical results of the reference methods :

a) The selective broths and/or cromogenic and/or revitalizing: Pseudomonas P/A, broth GVPC previous to the application of the Norm ISO 11731, Staphylococus P/A, Salmonella-Shigella Broth, MCC Colicult P/A, Enterocult P/A and Clostricult P/A.
All of then are showing better sensibility, specificity and the range of detection is also better than the classic methods of reference, as the results of years and decades of integrated and comparative survey work, which gives the maximal efficiency to all of then. The revitalization method with selective broths as the P/A of MICROKIT, are showing the suitability of this protocol to detect the microorganisms *Diana*, inclusive when they are at very low concentrations and in the presence of innumerable interferences or contaminants.

b) The Compact-Dry-Plates® maximize the sensibility, the specificity, the accuracy and precision, because it's save the critical point of the mass inoculation for mixture of agars, even more than in classical methods, since in those are not possible sometimes, due to the excessive heat for the microorganisms *Diana.* The Compact-Dry-Plates®-TC for the anaerobic count were also validated. (In short time, we will wide up the validation with another integrated survey) and also for the Compact-Dry-Plates®-EC for coliforms and *E.coli*.
Those are still in the phase of validation, with excellent perspectives, the Compact-Dry-Plates®-XSA for Compact-Dry-Plates®-XSA and the Compact-Dry-Plates®-ETC for Enterococos fecalis.

c) The Agar Cromosalm, increase in significant form, the sensibility and specificity of the classic agars of Salmonella and the other modern cromogenic media, saving unnecessary laboratory confirmations of microorganisms, which we are not looking for.

2- All the methods MICROKIT here described, in any of its presentations, if they are exactly and properly used with our media and kits are valid, because their reliability in comparison with the used reference method. In some cases they are even better. They detect and/or count, in all cases, the adequate concentrations of all groups of microorganisms studied: Total Aerobios, *Pseudomonas aeruginosa, Legionella pneumophila, Staphylococcus aureus, Salmonella spp., Shigella spp.,* Coliformes, *E.coli*, Enterococcus fecalis and *Clostridium perfringens* and its spores. The sensibility and specificity are showing better results than the

reference method and also it is easy to use and economical, compared with the reference method established in the different laboratories.

3-The scarcity of false positives or greater specificity, allows the laboratories to save time and money for confirmation of false positives, which its necessary when using the reference method. The time saved can be used for performing more analyses. Consequently the implementation of all these optimized techniques, will give more impulse to the global laboratories analyses.

4- The scarcity of false negatives or maximal sensitivity, recommends using these methods for negative screen, especially when it's necessary to analyze a great number of negative samples, confirming with prudence, only the probably positive ones, inoculating in an adequate agar like it was demonstrated in the survey study. The easy use of the method P/A allows processing more samples without the need of filtration equipment, also diminishing the number of critical points of the analyses and therefore increasing its potency.

5- We demonstrated in another similar survey studies, that the method of detection and count of Enterococcus fecalis in water, are very much stronger than the methods of detection of Coliforms and *.E.coli* in water, no matter in which laboratory is done or the screening method used for this indicator of fecal contamination (reference methods or method MICROKIT P/A Enterocult). However in the present validations whit recent data, we do not observe important differences between the qualities of both parameters. The fact of the greater survival of the Enterococcus fecalis in water (Ref: 1-2 weeks compared with 1-2 days of E. coli) are suggesting us to propose the routine search every day, in order to know the real risk status of the water. We consider that the differentiation between Enterococcus fetalis and Streptococcus fecalis is a non practical academic performance, because both are indicators of fecal contamination (especially of human and mostly of animal origin) which is the reason for the search.

6- Because the detection of *Clostridium perfringens* and it's spores are indicators of a possible presence of Enterovirus and protozoan like Cryptosporidium, Entamoeba and Giardia, it can not be settled only because the official method of m-CP and the TSC are less adequate, probably caused for the stress due to the membrane filtration of the strict anaerobics. They are methods much more adequate, like we demonstrated for Clostricult P/A. The use of Broth Costricult P/A increases the sensibility near 63% for detection of *Clostridium perfringens* in difficult samples, in regard to the use of filtration with membrane with Agar m-CP; and more than 21% with the Agar TSC. Therefore closed to a 63% of samples with this pathogen and indicator, could be not detected with the current official method, not even 21% of then with the method most use for the laboratories when they proof for themselves de extreme inefficacy of the agar m-CP.

7- The use of Broth P/A Pseudomonas increases in more than 26% the sensibility of detection of *Pseudomonas aeruginosa* in dificult samples, in regards to the use of membrane filtration with Agar Cetrimida CN. The 26% of samples with this pathogen, couldn't be detected with the current official method.

8-The use of broth GVPC Legionella before the application of ISO 11731, enhance in more that 45% the sensibility in the detection of *Legionella pneumophila* in difficult samples, without the need to use the membrane filtration with Agar GVPC. With the current official method, 45% of samples with this pathogen could be not detected. Also the limit of detection from 1000 until 50 ufc/L is a great advantage for the analyses of this dangerous microorganism.

9-The use of Broth P/A Stafilococcus increases in a 29% the sensibility in the detection of *Staphylococcus aureus* in difficult samples, in regards to the use of membrane filtration with

Agar Mannitol or Baird Parker o RPF. A 29% of samples with this pathogen couldn't be detected with the current official method.

10-The use of Cromosalm Agar increase in more than 26% the sensibility in the detection of *Salmonella spp.* in difficult samples in relation to the Norm ISO 6340 o ISO 6579. Until 26% of the samples with this type of pathogen present, couldn't be detected with the current official method.

11-The use of Broth Salmonella-Shigella could enhance in a 67% the sensibility in the detection of *Shigella* in difficult samples in regards to the Norm 21567 or adaptations to the ISO for Salmonella. A 67% of the specimens with this pathogen couldn't be detected with the current official method. The results are still not concluded, but they are showing this direction.

12- Potency, accuracy and precision are deficient with some methods. There are an excessive numbers of samples showing out of range results with the Agar GVPC, Agar TSC and even more with the Agar m-CP, this one was invalidated because its sensibility, accuracy and precision were almost cero. The TSC is also detecting under the real counts, less than 10% of the number and therefore we do no recommend it for control counts in water. The agar Baird Parker is detecting in the real counts, less than 29% and should not be used for water analysis. The Agar GVPC counts are in many occasions less than 2 log of the inoculated value, consequently it has a very deficient sensibility for detection. The Agar YEA Nutriente obtained also many samples out of range (>20 %) when compared with their homologous YEA-Cromogenic and Compact-Dry-Plates®-TC, probably due to confusion between real colonies and another ones that are not, which is inacceptable in the two cromogenics tests compared.

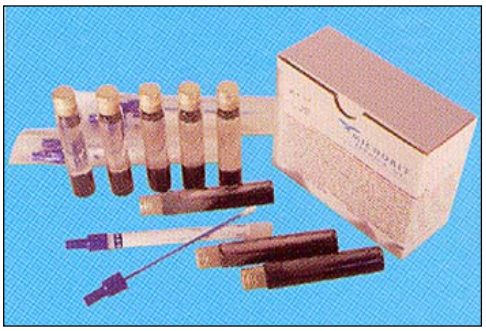
13- It is not strange to the laboratories to obtain counts systematically above of the certified, in the quantitative strains used, when they used media and kits of extraordinary quality, since the certificates are referred to media from other companies with different characteristics (no matter how good the are).

14- We hope that all this work, done with great efforts during the last 7 years, with the participation of 70 laboratories of Spain, which are using some methods, media and /or kits of MICROKIT, will be well accepted for all the institutions of accreditation, normalization and inspection. The goal is to improve with these methods the analytical control with less cost and waste for laboratories and natural resources. This will be an incentive for our company, so we can continue designing in Spain most efficient methods for other microbiological parameters which avoid that the laboratories accredited with ISO 17025 and the ones authorized by the Health system will not be in stagnation with classical methods. We demonstrated here how to optimize then, using a concept that should be remembered: every laboratory should work for improvement without bureaucracy.

❖ PICTURES ANEX

Compact-Dry-Plates ® TC

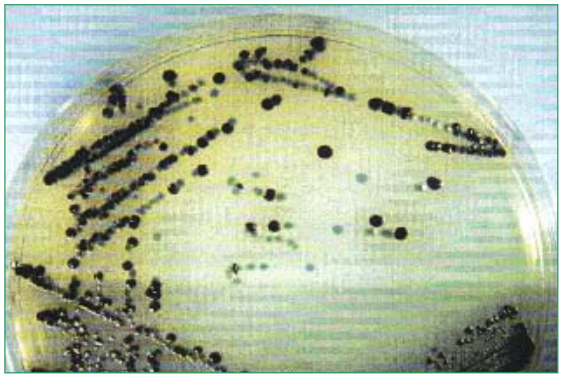
Pseudomonas aeruginosa P/A Broth MICROKIT®



Legionella pneumophila GVPC Broth MICROKIT®

Staphylococcus aureus P/A Broth MICROKIT®

SS Broth MICROKIT®



Chromosalm Agar MICROKIT®

Enterocult P/A Broth MICROKIT®

MCC Colicult P/A Broth MICROKIT® Compact-Dry-Plates ® EC

Clostricult P/A Broth MICROKIT

❖ BIBLIOGRAPHY

- REAL DECRETO 1074/2002 de 18 de Octubre sobre aguas de bebida envasadas. BOE 29.10.2002
- REAL DECRETO 140/2003 de 7 de Febrero sobre Calidad de Aguas de Consumo Humano. BOE 21.02.2003
- REAL DECRETO 865/2003 de 4 de Julio sobre prevención y control de la Legionelosis. BOE 18.07.2003
- Norma ISO 6222. Calidad del agua. Enumeración de microorganismos cultivables. Recuento de colonias por siembra en medio de cultivo de agar nutritivo (YEA).
- Norma ISO 9308. Calidad del agua. Detección y recuento de *E. coli* y de bacterias Coliformes.
- Norma ISO 7899. Water quality-Detection and enumeration of intestinal enterococci.
- Norma UNE 12780. Calidad del agua. Detección y recuento de *Pseudomonas aeruginosa* por filtración de membrana.
- Norma ISO 11731. Calidad del agua: detección y recuento de Legionella
- Norma ISO/CD 26461. Calidad del agua. Detección y Recuento de los esporos de microorganismos anaerobios sulfito-reductores (Clostridia).
- Norma ISO/CD 6461-2:2002 Water quality-Detection and Enumeration of *Cl.perfringens*
- Norma ISO 6340. Water quality-Detection of Salmonella species.
- Validación del medio cromogénico de MICROKIT ® para Salmonella (Chromosalm) mediante un estudio intercolaborativo. Jorge Sanchis Solera, Laboratorios MICROKIT y otros 14 autores. TECNICAS DE LABORATORIO 281, Mayo 2003.
- Validación del método P/A (presencia/ausencia) para la detección de patógenos e indicadores en aguas, mediante un estudio intercolaborativo y otro intercomparativo. Jorge Sanchis Solera, Laboratorios MICROKIT y otros 62 autores. TECNICAS DE LABORATORIO 282, Junio 2003.
- Validación microbiológica de los kits presencia/ausencia (P/A) MICROKIT, frente a la filtración de membrana (MF), en los servicios intercomparativos SEILAGUA®, mediante una novedosa hoja de cálculo. Jorge Sanchis Solera, María Morales, Sylvia Ajates, Laboratorios MICROKIT, S.L. TECNICAS DE LABORATORIO 312, Junio 2006.
- Informes SEILAGUA® 1 a 30 (Total: 1.110 páginas), Laboratorios MICROKIT, Abril-2002 a Julio-2009
- PRT-SEILA-002, Protocolo GLOBAL **VALIDADO** para la ejecución correcta de análisis de aguas (e intercomparativos SEILAGUA®) (39 páginas)
- PRT-AL/AG-020, Análisis microbiológico del ambiente y operarios en industria agroalimentaria y en potabilizadoras de agua (61 páginas)
- PNT-AG-001, Aguas: Recuento de Anaerobios (15 páginas)
- PNT-AG-002, Aguas: Recuento de Anaerobios Sulfito-Reductores (17 páginas)
- PNT-AG-003, Aguas: Determinación y Recuento de Sulfato-Reductores (16 páginas)
- PNT-AG-005, Aguas: Detección y recuento de Enterococos fecales (46 páginas)
- PNT-AG-006, Aguas: Detección y recuento de Coliformes y *E.coli* (48 páginas)
- PNT-AG-007, Aguas: Detección y Recuento de *Legionella pneumophila* (52 páginas)
- PNT-AG-008, Aguas: Detección y recuento de *Pseudomonas aeruginosa* (54 páginas)
- PRT-COSM/AG-009, Investigación de *Burkholderia cepacia* en cosméticos y aguas (33 páginas)
- PNT-AG-011, Aguas: Detección y Recuento de *Clostridium perfringens* y sus esporas (46 págs)
- PNT-AG-012, Aguas: Recuento de Cianobacterias toxigénicas a niveles peligrosos (52 páginas)
- PRT-VAL-001 Protocolo para VALIDACIÓN en microbiología (69 páginas).
- PRT-VAL-1+2, Idem, incluido CD con hojas de cálculo en Excel.
- Orden SCO/778/2009 de 17 de Marzo sobre métodos alternativos para el análisis microbiológico del agua de consumo humano, BOE 78 de 31-Marzo-2009
- Certificado de Validación y estudio de equivalencia de Laboratorios MICROKIT sobre MUGPLUS Agar y MCC Colicult Broth (incl. kits P/A) para coliformes y *E.coli*. Marzo de 2009.