Detection of *Klebsiella pneumoniae* from foods, animals and environmental samples

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ERFAN Laboratory training course 17-21 October 2022

*"*Detection and characterization of *Listeria monocytogenes*, *Klebsiella pneumoniae* and *Salmonella* spp.*"*

One Health EJP MedVetKlebs

MedVetKlebs: *Klebsiella pneumoniae*: from ecology to source attribution and transmission control.

Start:	1 January 2018
Duration:	2 Years
Domain:	Foodborne Zoonoses
Keywords:	*K. pneumoniae*; Harmonization of detection and isolation, environmental niches, population biology, transmission modelling.
Contact:	Sylvain Brisse (IP)

https://onehealthejp.eu/jrp-medvetklebs/

One Health EJP MedVetKlebs

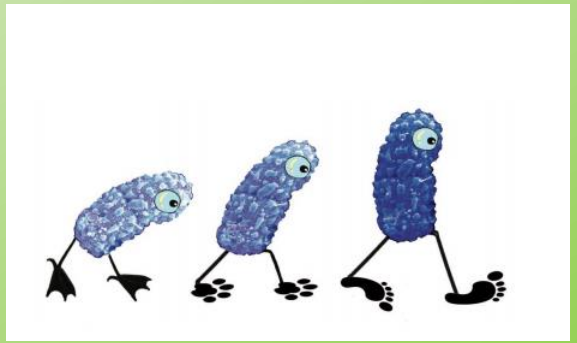
- 2018-2020
- 6 European countries: France, Italy, Ireland, Austria, Denmark, Netherlands
- IP, INRA, ANSES, AGES, SSI, DTU, UCD, IZSAM, NCOH, NUIG

MedVetKlebs is a multidisciplinary international project that aims to define the ecology of Klebsiella pneumoniae and the sources of infections of humans and animals in order to investigate transmission routes and to find an optimal way to control them.

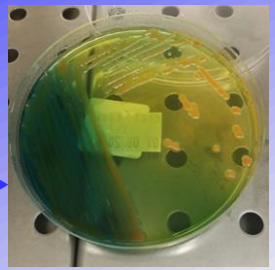
One Health EJP MedVetKlebs

MedVetKlebs aims to improve of public and animal health through a better control of Kp infections by:

1. Developing and harmonizing detection and isolation methods;
2. Ensuring a broad sampling of ecological niches and deep sampling of potential sources;
3. Performing genomics analysis and transmission modeling, including development of new methods for source attribution and risk assessment.



MedVetKlebs: workflow



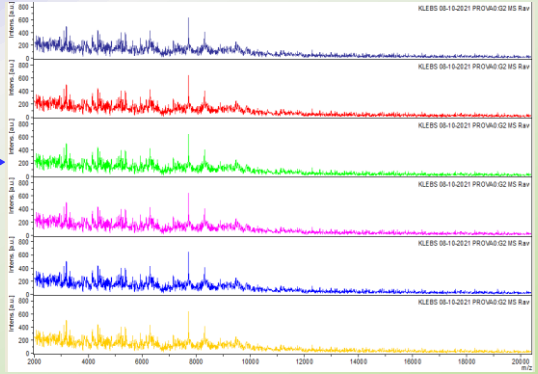


Plate-culture method optimization

Broad sampling >2000 samples

zkir Real Time PCR

MALDI-TOF MS identification

Phenotipic chacterization of AMR

Genomic chacterization of AMR

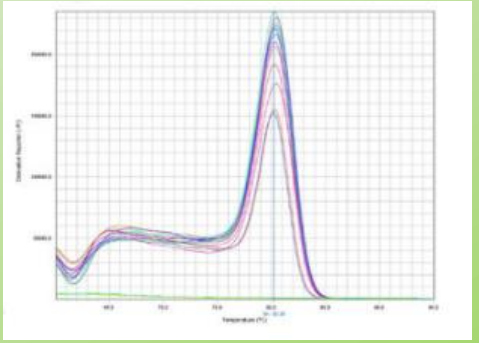


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Plate Code: **GN3F**

	1	2	3	4	5	6	7	8	9	10	11	12
A	AMI	A/S2	FAZ	CEP	ETP	GEN	P/T4	SXT	TAZ	TGC	AXO	TET
	64	32/16	32	16	16	16	128/4	4/76	32	8	64	16
B	AMI	A/S2	FAZ	CEP	ETP	GEN	P/T4	SXT	TAZ	TGC	AXO	TET
	32	16/8	16	8	8	8	64/4	2/38	16	4	32	8
C	AMI	A/S2	FAZ	CEP	ETP	GEN	P/T4	SXT	TAZ	TGC	AXO	TET
	16	8/4	8	4	4	4	32/4	1/19	8	2	16	4
D	AMI	A/S2	FAZ	CEP	ETP	GEN	P/T4	SXT	TAZ	TGC	AXO	TET
	8	4/2	4	2	2	2	16/4	0.5/9.5	4	1	8	2
E	AMP	AZT	FEP	MERO	FUR	CIP	FOX	POD	TAZ	TIM2	AXO	TET
	32	32	32	8	32	4	32	16	2	64/2	4	1
F	AMP	AZT	FEP	MERO	FUR	CIP	FOX	POD	TAZ	TIM2	AXO	TET
	16	16	16	4	16	2	16	8	1	32/2	2	0.5
G	AMP	AZT	FEP	MERO	FUR	CIP	FOX	POD	TOB	TIM2	AXO	NEG
	8	8	8	2	8	1	8	4	8	16/2	1	CON
H	AMP	AZT	FEP	MERO	FUR	CIP	FOX	POD	TOB	POS	POS	POS
	4	4	4	1	4	0.5	4	2	4	CON	CON	CON

ANTIMICROBICS

AMI	Amikacin
AMP	Ampicillin
A/S2	Ampicillin/sulbactam 2:1 ratio
AZT	Aztreonam
FAZ	Cefazolin
FEP	Cefepime
CEP	Cephalothin
MERO	Meropenem
ETP	Ertapenem
FUR	Cefuroxime
GEN	Gentamicin
CIP	Ciprofloxacin
P/T4	Piperacillin / tazobactam constant 4
FOX	Cefoxitin
SXT	Trimethoprim / sulfamethoxazole
POD	Cefpodoxime
TAZ	Ceftazidime
TOB	Tobramycin
TGC	Tigecycline
TIM2	Ticarcillin / clavulanic acid constant 2
AXO	Ceftriaxone
TET	Tetracycline

IZS TERAMO

WOAH Collaborating Centre for animal production food safety

Reference Centre — World Organisation for Animal Health, Founded as OIE

Plate-culture method optimization → Broad sampling >2000 samples → zkir Real Time PCR → MALDI-TOF MS identification → Phenotipic chacterization of AMR → Genomic chacterization of AMR

Institut Pasteur

Klebsiella Sequence Typing

Sequences and profiles database

Isolates database

KLEBORATE

KLEBWIN VIZ

Pathogenwatch

MedVetKlebs: WP1

Evaluation and Optimization of culture based method

Productivity, Selectivity and Specificity calculated for 3 different selective media according to ISO 11133:2014

- Simmons Citrate Agar + Inositol (SCAI) (IZSAM, Italy)
- Klebsiella ChromoSelect Selective Agar base (Sigma-Aldrich, Missouri, USA)
- Chromatic Detection chromogenic agar plate (Liofilchem, Italy)
- 50 *Klebsiella* spp. Reference Strains from Institut Pasteur
- 8 strains of closely related species
- A non – selective agar for comparison e.g. Nutritive agar (IZSAM, Italy)

MedVetKlebs: WP1

Scai agar	Ingredients	Quantity
BASIC MEDIA	Sodium citrate	2.0 g
	Sodium chloride (NaCl)	5.0 g
	KH2PO4	1.0 g
	Ammonium dihydrogen phosphate ($NH_4H_2PO_4$)	1.0 g
	Magnesium sulphate	0.2 g
	Bromothymol blue	0.08 g
	Agar	12 g
	Sterile deionized water	1.000 ml
SUPPLEMENTS	Myo-Inositol	1.0 g
	Sterile deionized water	100 ml

MedVetKlebs: WP1

90925 Klebsiella *ChromoSelect* Selective Agar Base

Klebsiella *ChromoSelect* Selective Agar for selective isolation and easy detection of *Klebsiella* species from water and other sources. This medium can also be used in membrane filtration procedure.

Composition:

Ingredients	Grams/Litre
Peptone, special	12.0
Yeast extract	7.0
Sodium chloride	5.0
Bile salts mixture	1.5
Chromogenic mixture	0.2
Sodium lauryl sulfate	0.1
Agar	15.0

Final pH 7.1 +/- 0.2 at 25°C

Store prepared media below 8°C, protected from direct light. Store dehydrated powder, in a dry place, in tightly-sealed containers at 2-25°C.

Appearance:	Faintly beige to yellow coloured, homogeneous, free flowing powder.
Gelling:	Firm
Color and Clarity:	Light amber coloured, clear to slightly opalescent gel forms in petri plates.

MedVetKlebs: WP1

Instructions For Use
ENGLISH

Chromatic™ Detection

Chromogenic medium for the enumeration and identification of microorganisms directly from clinical and nonclinical specimens.

DESCRIPTION

Chromatic™ Detection is a chromogenic medium used for the enumeration and identification of microorganisms directly from clinical and nonclinical specimens.

The medium allows to carry out indole test for confirmation of *Escherichia coli*.

TYPICAL FORMULA	(g/l)
Peptone	14.0
Tryptone	6.0
Yeast Extract	3.0
Sodium Chloride	5.0
Chromogenic Mix	13.1
Agar	15.0

Final pH 7.2 ± 0.2 at 25°C

METHOD PRINCIPLE

Peptone and tryptone provide amino acids, nitrogen, carbon, vitamins and minerals for organisms growth. Yeast extract is a source of vitamins, particularly of B-group. Sodium chloride maintains the osmotic balance of the medium. Chromogenic mix allows to identify microorganisms on the basis of the color and morphology of the colonies. Agar is the solidifying agent.

ID Table.

Microorganism	Typical colony color
E. coli	Pink-reddish-mauve
Klebsiella spp, *Enterobacter* spp, *Serratia* spp	Green-blue
Proteus spp	Brown
Pseudomonas spp	Yellowish-green
S. aureus	Cream
E. faecalis	Green-turquoise
S. saprophyticus	Light pink

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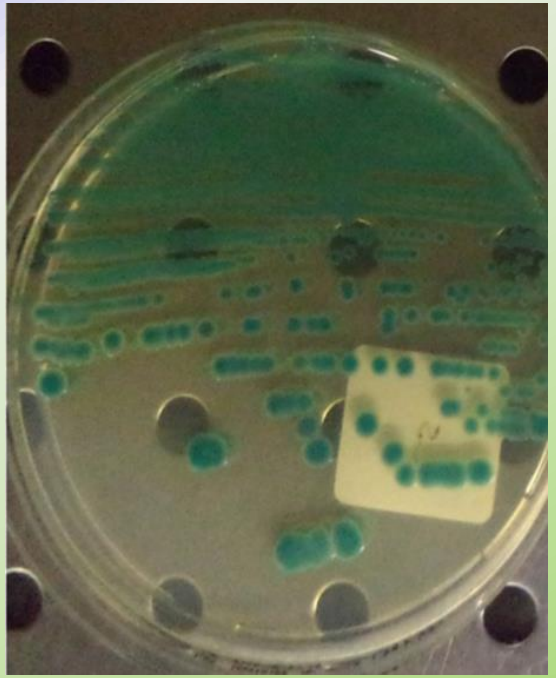
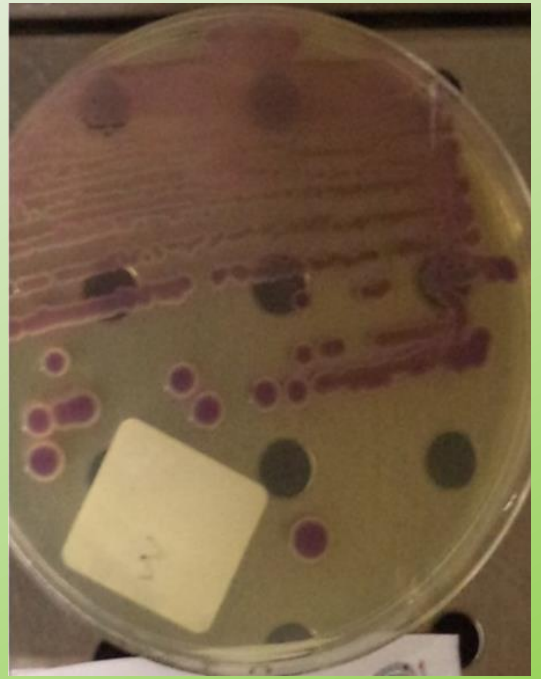
Reference Centre
World Organisation for Animal Health
Founded as OIE

SCAI

LIOFILCHEM CHROMATIC DETECTION

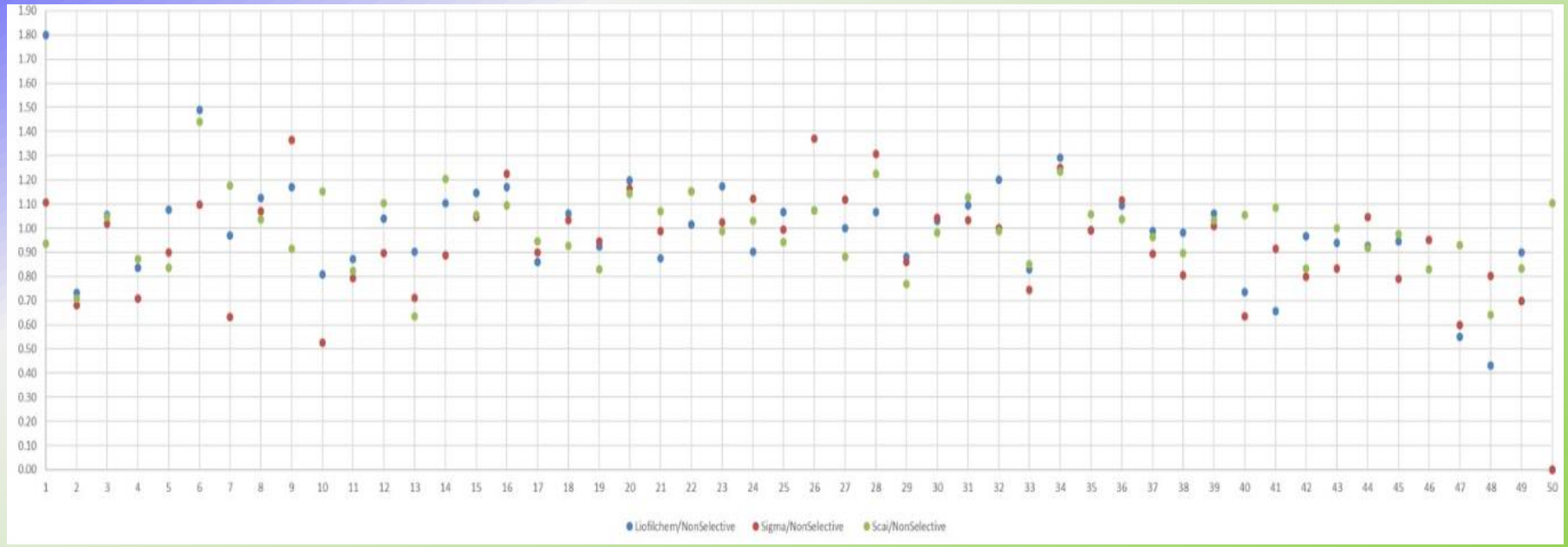
CHROMO SELECT SIGMA-ALDRICH





MedVetKlebs: WP1

The 3 media were compliant for productivity, Pr value was >0.5 for almost all target strains and showed similar productivity.



MedVetKlebs: WP1

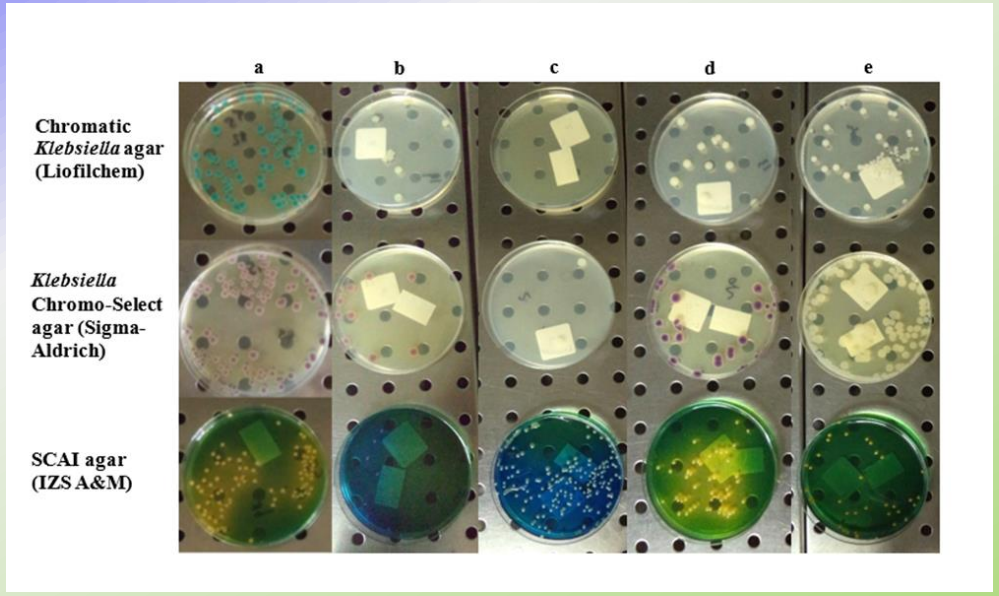
For selectivity (S_F), 7 non target strains (*Cronobacter* spp., *Citrobacter koseri*, *Citrobacter freundii*, *Serratia marcescens*, *Serratia liquefaciens*, *Serratia rubidaea*, and *Pantoea agglomerans*) were tested on the SCAI and *Klebsiella* ChromoSelect selective agar.

Media did not comply with the selectivity criteria (SF >2) for most of the non target strains considered;
Klebsiella ChromoSelect agar was selective for *Cronobacter* spp. and *Citrobacter freundii*.

Id strain	species	Scai	Sigma
SB539	*Enterobacter aerogenes*	-0,11	-0,10
SB538	*Enterobacter aerogenes*	0,00	0,11
SB3629	*Enterobacter aerogenes*	0,49	0,15
3428LN18	*Cronobacter* spp.	9,58	0,05
3556LN18-3	*Citrobacter koseri*	0,50	-0,54
3656LN18-10	*Serratia marcescens*	-0,29	0,09
4726LN18-5	*Serratia liquefaciens*	0,33	0,89
4726LN18-9	*Serratia rubidaea*	0,20	0,26
5136LN18-3	*Pantoea agglomerans*	0,11	0,33
5136LN18-5	*Citrobacter freundii*	3,96	0,31

MedVetKlebs: WP1

Regarding specificity, the selective media tested showed variable results regarding the morphological features of observed colonies, based on the nontarget strains considered. The selective media, indeed, allowed the growth of some non-Klebsiella species strains, and in few cases, colonies were morphologically similar to the target strains.

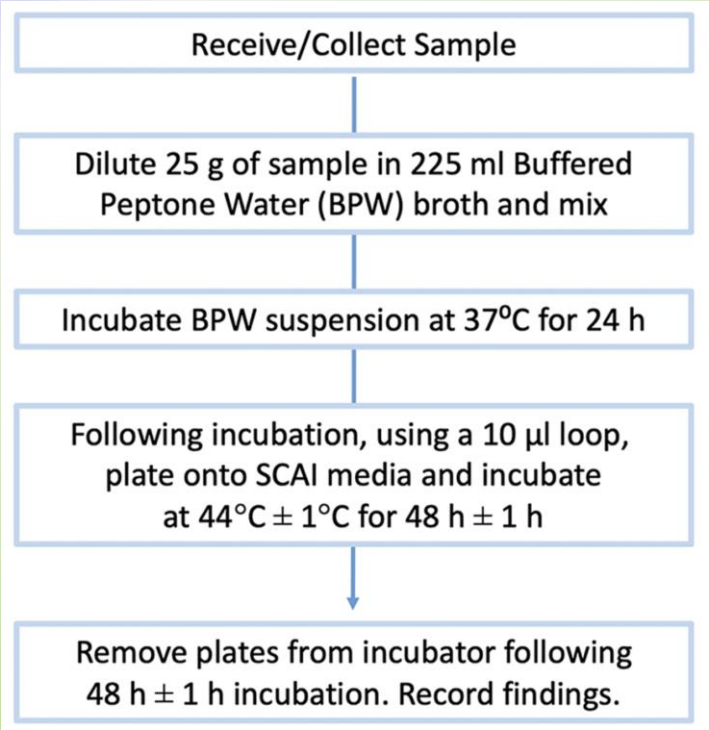


Growth of 4 non-target microorganism on different media compared to the target *Klebsiella pneumoniae* (a); *Citrobacter freundii* (b); *Cronobacter spp.* (c); *Serratia rubidaea* (d); *Serratia marcescens* (e).

Selection of SCAI medium for the detection of Kp

MedVetKlebs: WP1

- We compared two temperatures (37°C and 44°C) for the incubation of SCAI plates
- A slightly higher percentage of positive samples was recorded following incubation of SCAI plates at 44°C



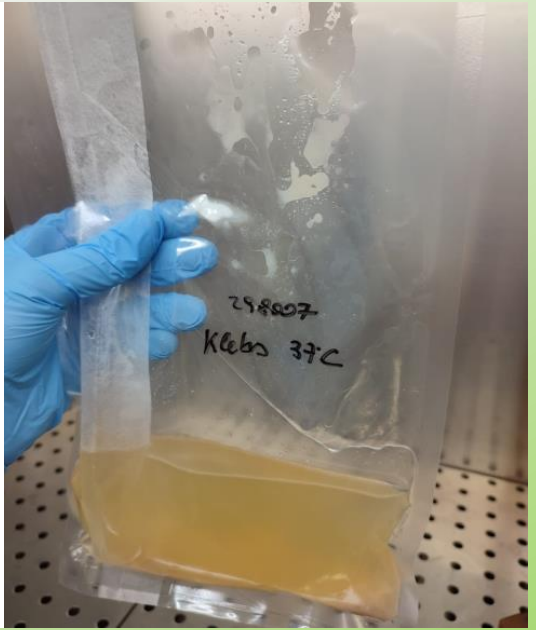
dx.doi.org/10.17504/protocols.io.baxtifnn)

Kp: Detection from foods

- 25 g or 25 ml of analytical portion+ 225 ml of Buffered Peptone Water (BPW).
- For analytical portions < 25 g/ml, add to the analytical portion a volume of BPW to maintain a weight/volume ratio of 1:10.

Kp: Detection from foods

Mix the sample for 60 seconds
using a stomacher or manually



Incubate
Enrichment
Broth at **37° C**
For 18-24 h

Kp: Detection from water samples

- Samples of seawater or wastewater (250 ml)
- Laboratory Filtration system with Vacuum Pump and 0.45 µm nitrocellulose membrane.
- Filter + 225 ml BPW.
- Incubation of enrichment broth at **37 °C** for 18-24 h.

Kp: Detection from animals

- Samples of faeces, brain, intestine, tissues and other diagnostic samples.
- 25 g or 25 ml of analytical portion+ 225 ml of Buffered Peptone Water (BPW).
- For analytical portions < 25 g/ml, add to the analytical portion a volume of BPW to maintain a weight/volume ratio of 1:10.
- Incubation of enrichment broth at **37 °C** for 18-24 h

Kp: Detection from environmental samples

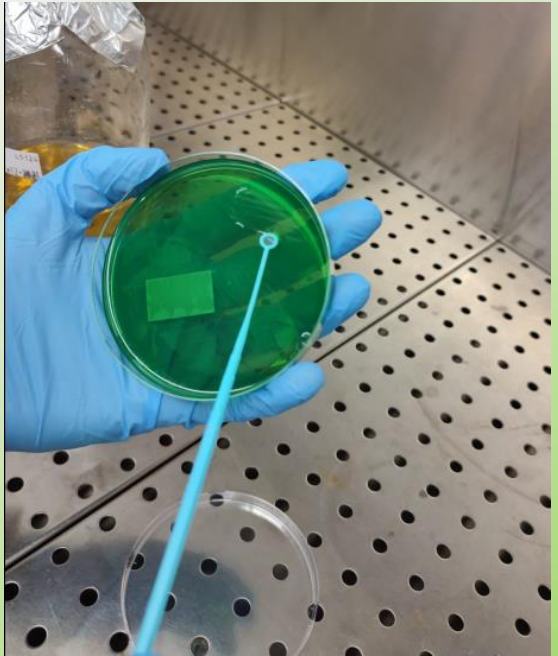
- The sponge is ready to use and is hydrated with 10 ml of diluent or Dey Engley neutralizing broth for environmental surfaces sampling.

- After surface sampling add **90 ml** of BPW to the sponge and incubate at **37 °C** for 18-24 h

Kp: Plate-culture

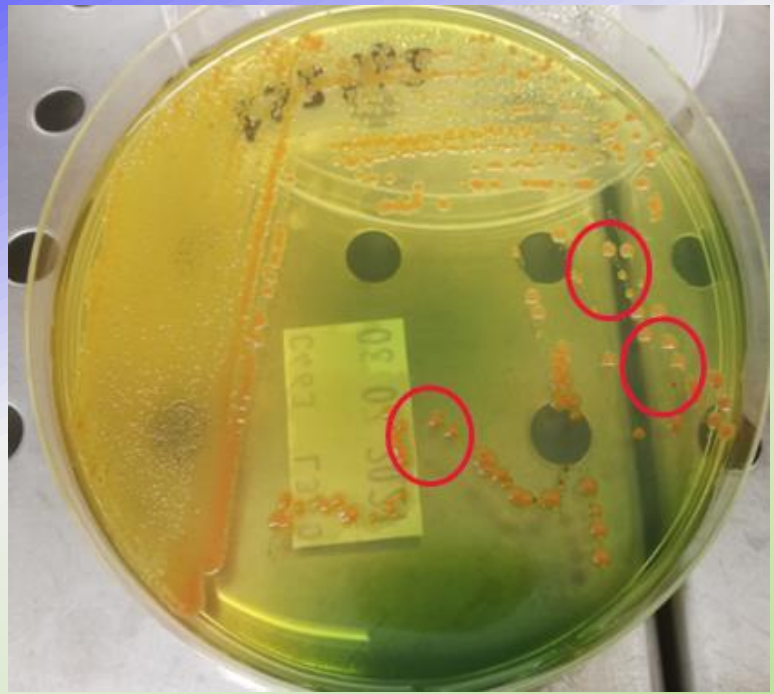
After Incubation of BPW at 37° C

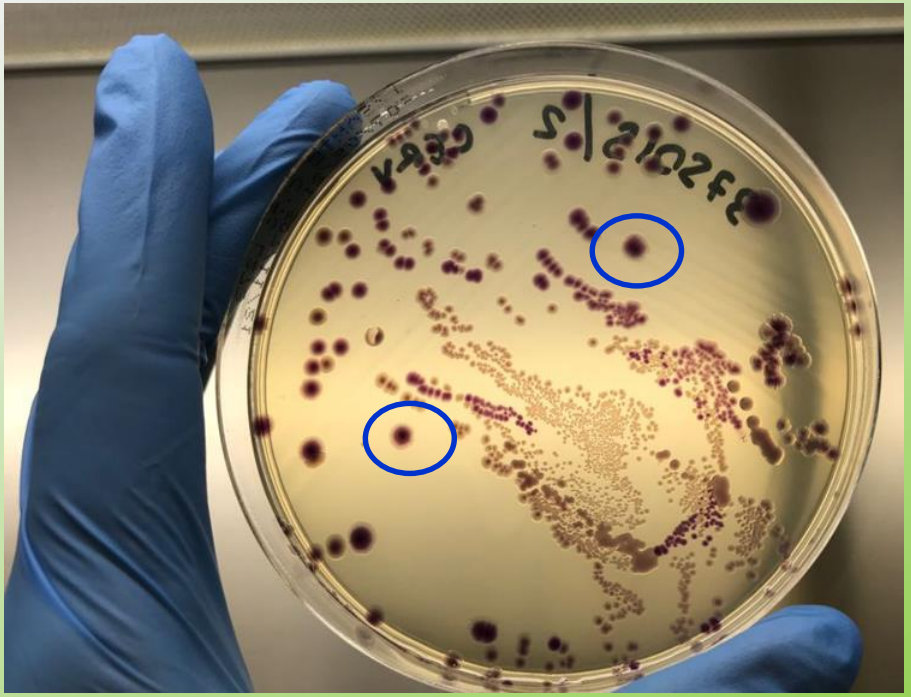
Streak 10 µl loop on SCAI plates or other selective media



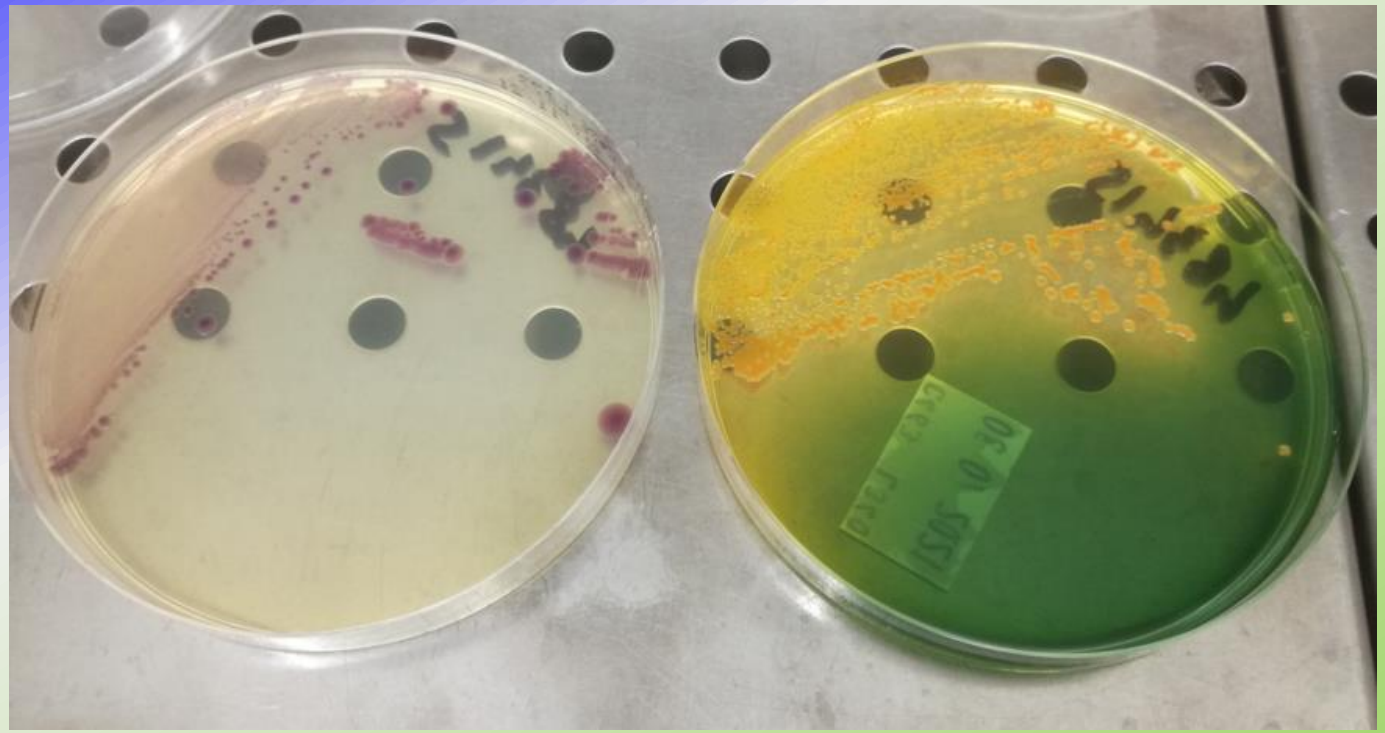
Incubation of plates at **44 °C** for **48 h**

Kp: Selection of suspected colonies





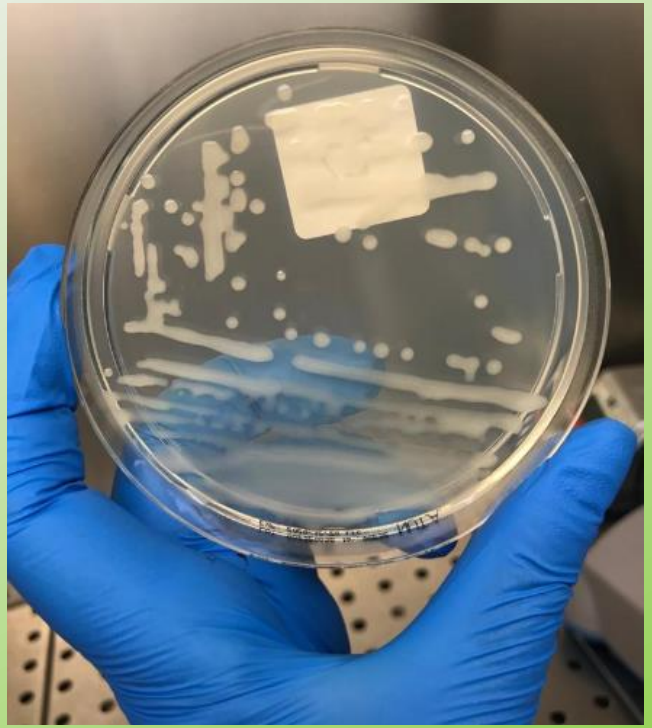
Kp: Selection of suspected and typical colonies



At 44 °C less interferences caused by other bacterial species

Kp: Selection of suspected and typical colonies

- Selection of 5 presumptive colonies (if present) or all the colonies if <5
- Sub-culture onto Nutrient agar
- Plates incubated at 37 °C for 24h to obtain single pure colonies



Kp: Identification

Enterotube

VITEK2 Biomeriuex

API20E

oxidase -ve
indol –ve
H2S –ve
citrate +ve

Kp: Identification

VITEK2 Lab. Report

Istituto Zooprofilattico G.Caporale - TE

Cliente bioMerieux:
N. sistema:

Lab report

Stampato 21-mar-2019 08:55 CET
Stampato da: batteriologia1

Gruppo di isolati: 1278LN19/3-1

Tipo di card: GN Test dello strumento: 00000EBB56E9 (2249)

Bionumero: 4607734777565252
Quantità organismo:

Commenti:	

Informazioni sull'identificazione	**Card:**	GN	**Numero di lotto:** 2410732103	**Scade:**	28-nov-2019 12:00 CET
	Completato:	20-mar-2019 18:32 CET	**Stato:** Finale	**Tempo di analisi:**	5,75 ore
Microrganismo selezionato	Probabilità 96%		**Klebsiella pneumoniae ssp pneumoniae**		
	Bionumero:	4607734777565252		**Affidabilità:**	Identificazione eccellente

Dettagli biochimici

2	APPA	-	3	ADO	-	4	PyrA	+	5	IARL	-	7	dCEL	+	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	+	29	TyrA	+	31	URE	+	32	dSOR	+
33	SAC	+	34	dTAG	+	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	+
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	+	47	ODC	-	48	LDC	+	53	IHISa	-	56	CMT	+	57	BGUR	(-)
58	O129R	+	59	GGAA	-	61	IMLTa	+	62	ELLM	-	64	ILATa	+			

Kp: Identification

VITEK2 Lab. Report

Istituto Zooprofilattico G.Caporale - TE

Cliente bioMerieux:
N. sistema:

Lab report

Stampato 21-mar-2019 08:54 CET
Stampato da: batteriologia1

Gruppo di isolati: 1277LN19/5-1

Tipo di card: GN Test dello strumento: 00000EBB56E9 (2249)

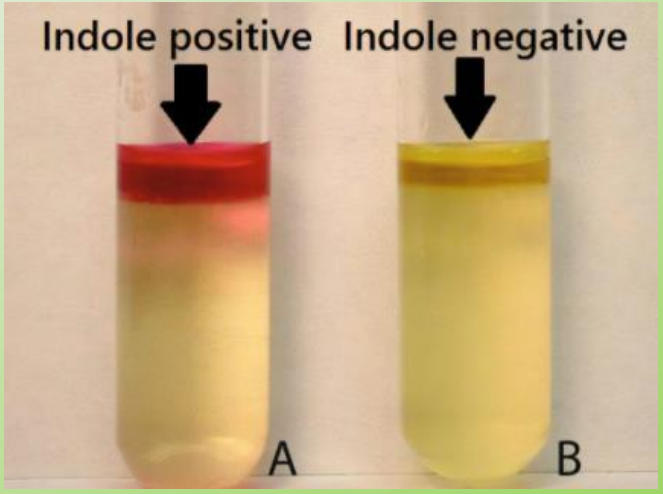
Bionumero: 6607735577565352
Quantità organismo:

Commenti:	

Informazioni sull'identificazione	**Card:**	GN	**Numero di lotto:**	2410732103	**Scade:** 28-nov-2019 12:00 CET
	Completato:	20-mar-2019 22:17 CET	**Stato:**	Finale	**Tempo di analisi:** 9,50 ore
Microrganismo selezionato	**Low Discrimination**				
	Bionumero:	6607735577565352			**Affidabilità:** Discriminazione insufficiente
Microrganismo SRF					

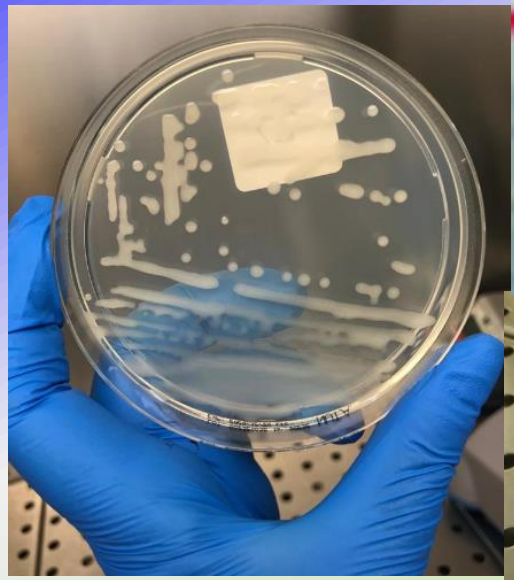
Microrganismi di analisi e test da separare:

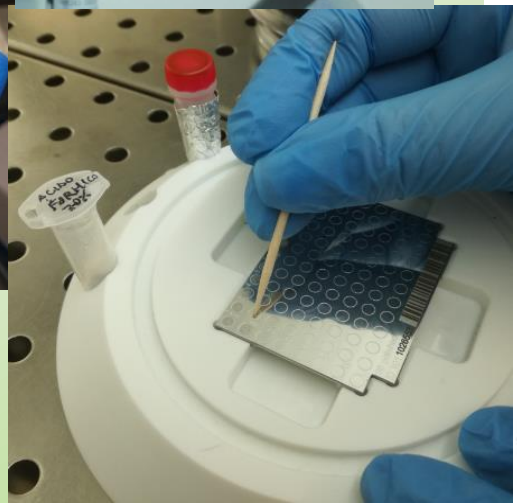
Low Discrimination Organism	
Klebsiella pneumoniae ssp pneumoniae	IND(1),
Klebsiella oxytoca	IND(99),



K. oxytoca ***K.pneumoniae***

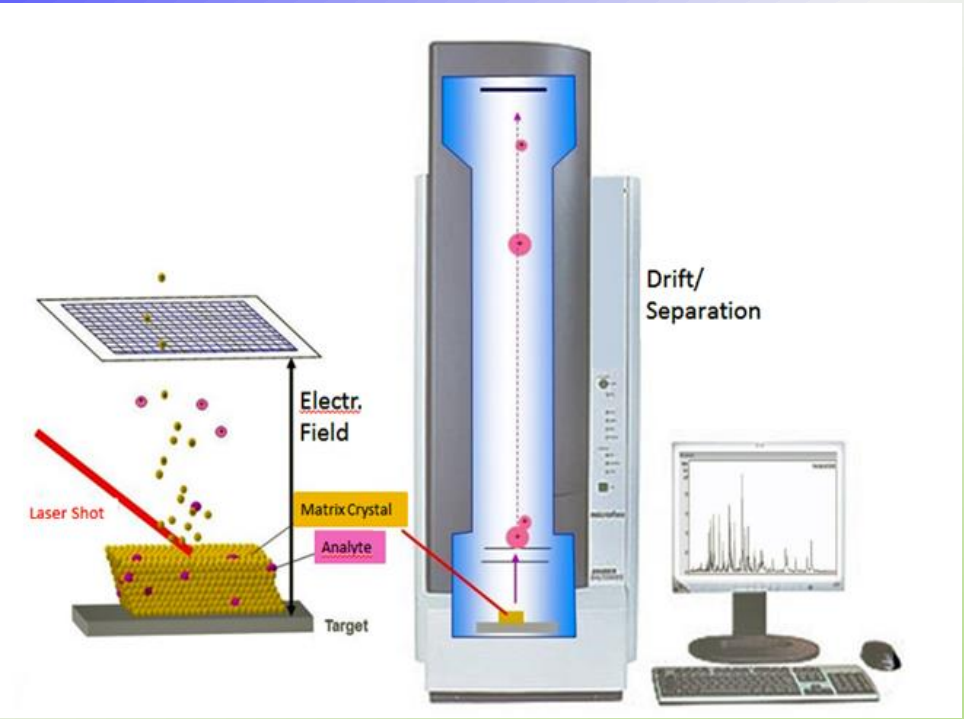
Kp: Identification





MALDI-TOF MASS SPECTROMETRY

Kp: Identification



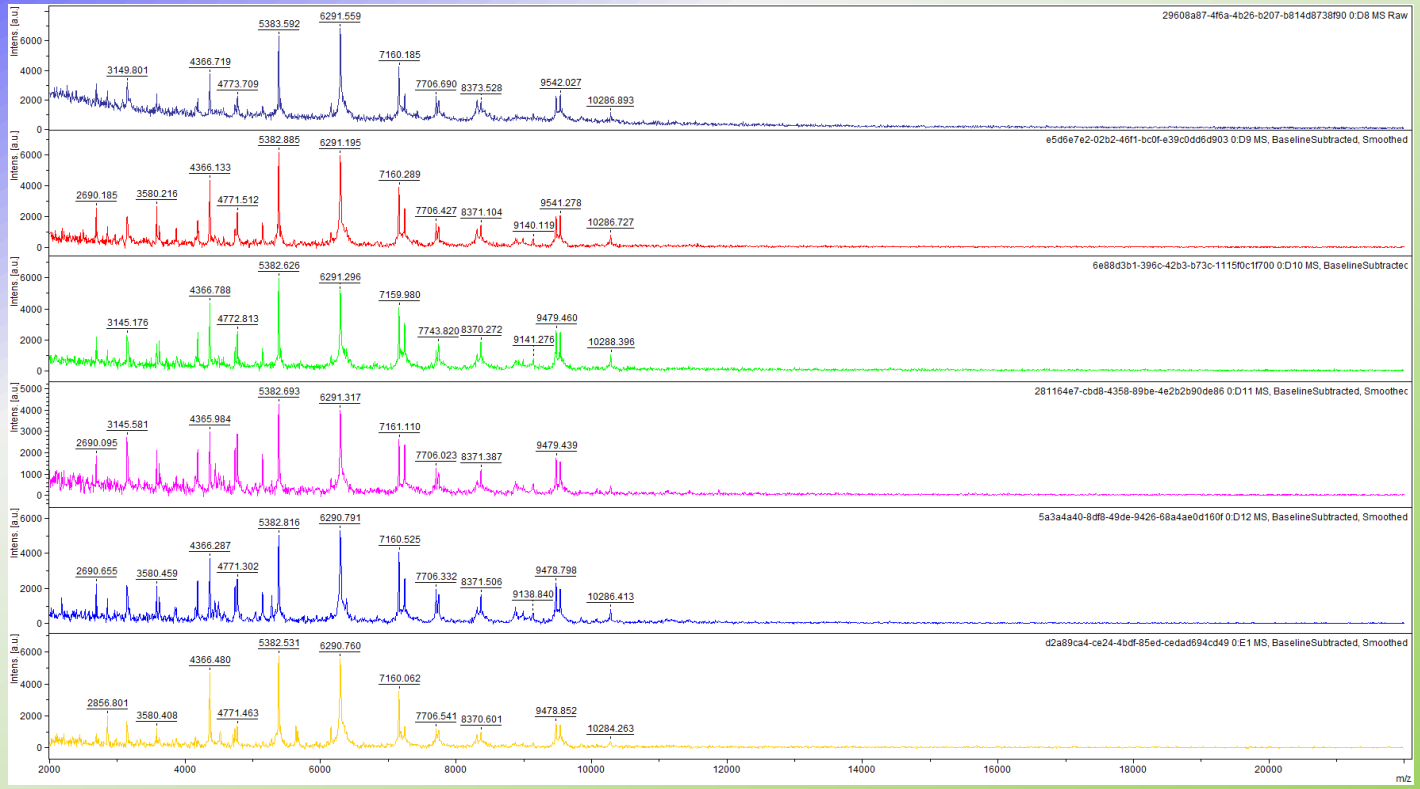
Result Overview

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
A1 (+++) (A)	294259 col. A (Standard)	Klebsiella pneumoniae	2.35	Klebsiella pneumoniae	2.18
A2 (+++) (A)	294259 col. B (Standard)	Klebsiella pneumoniae	2.18	Klebsiella pneumoniae	2.09
A3 (+++) (A)	294259 col. D (Standard)	Escherichia coli	2.30	Escherichia coli	2.12
A4 (+++) (A)	4834IS col. A (Standard)	Klebsiella variicola	2.13	Klebsiella variicola	2.10
A5 (+) (B)	4834IS col. B (Standard)	Klebsiella variicola	1.82	Klebsiella variicola	1.80

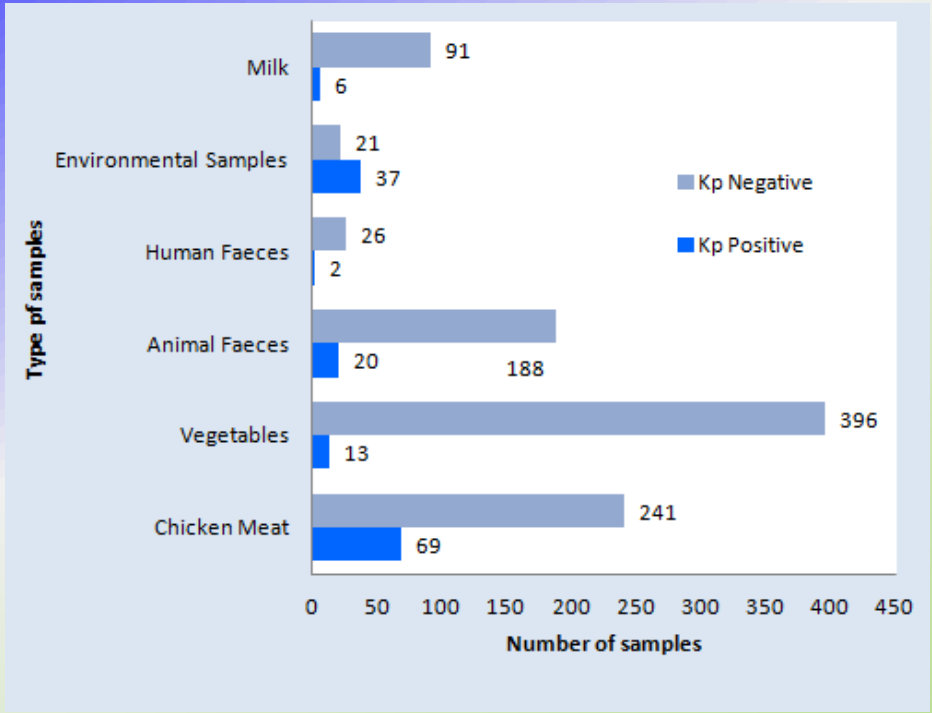
Meaning of Score Values

Range	Interpretation	Symbols	Color
2.00 - 3.00	High-confidence identification	(+++)	green
1.70 - 1.99	Low-confidence identification	(+)	yellow
0.00 - 1.69	No Organism Identification Possible	(-)	red

Kp: MALDI-TOF Spectra



MedVetKlebs: some results



Raw milk: 6,2 %
Environment (food production): 63 %
Human faeces: 7,1 %
Animal faeces: 9,6 %
Vegetables: 3,2 %
Chicken meat: 22,3 %

MedVetKlebs: some results

AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology Spectrum

Microbiol Spectr. 2022 Jan-Feb; 10(1): e02376-21.
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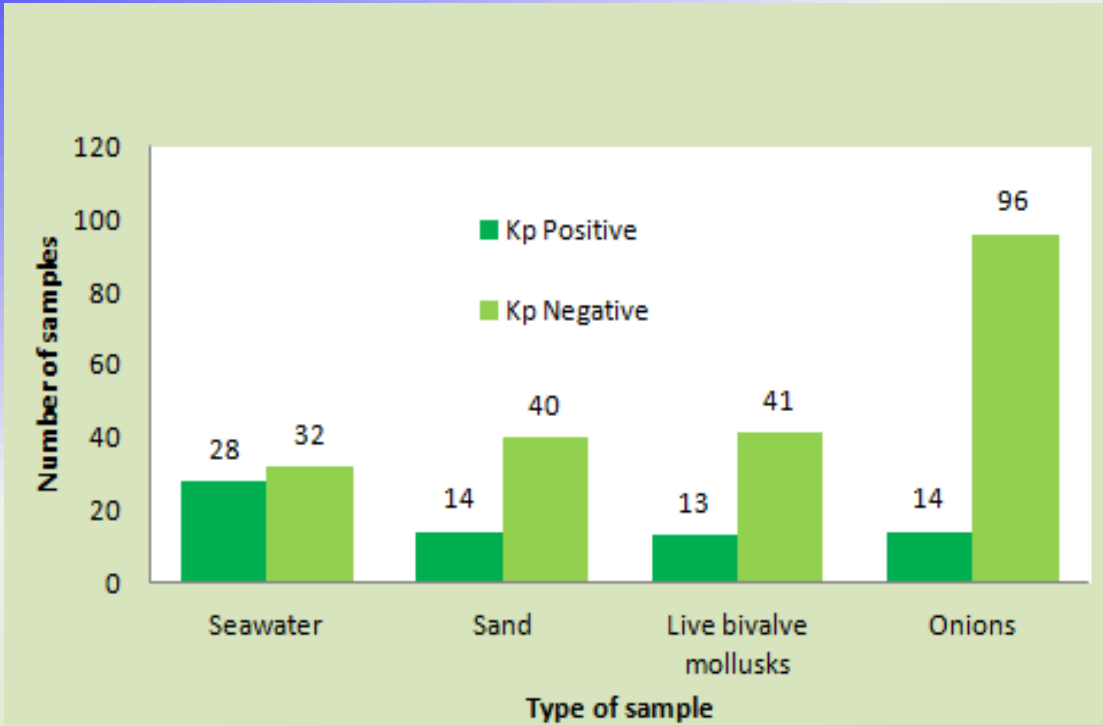
PMCID: PMC8865463
PMID: 35196810

High Prevalence of *Klebsiella pneumoniae* in European Food Products: a Multicentric Study Comparing Culture and Molecular Detection Methods

Carla Rodrigues,[a] Kathrin Hauser,[b] Niamh Cahill,[c] Małgorzata Ligowska-Marzęta,[d] Gabriella Centorotola,[e] Alessandra Cornacchia,[e] Raquel Garcia Fierro,[f] Marisa Haenni,[f] Eva Møller Nielsen,[d] Pascal Piveteau,[g] Elodie Barbier,[h] Dearbháile Morris,[c] Francesco Pomilio,[e] and Sylvain Brisse[a]

Nilton Lincopan, Editor

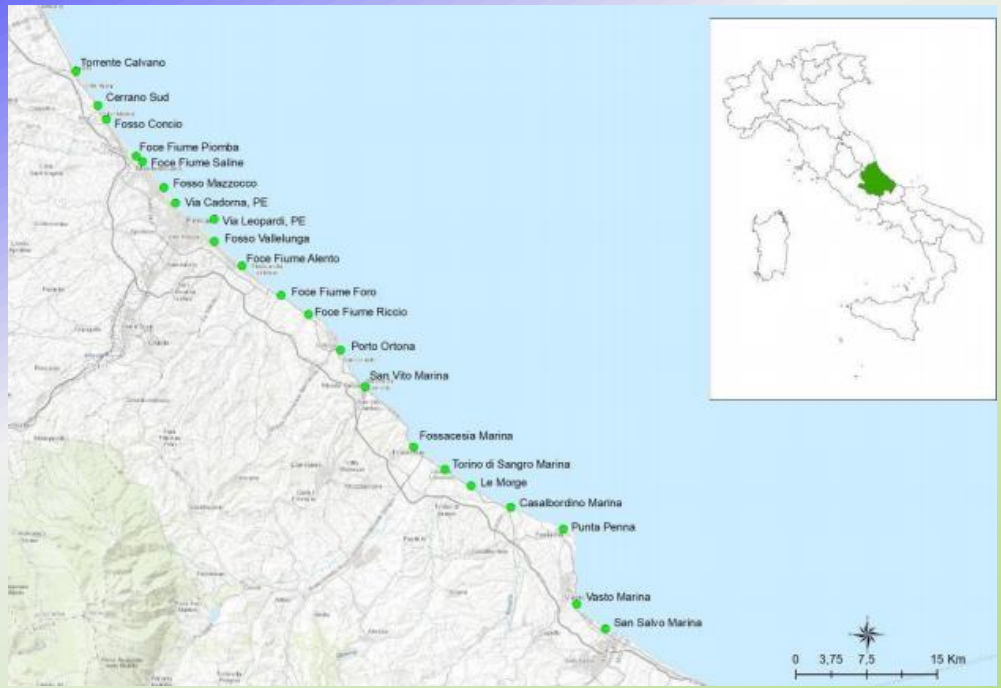
MedVetKlebs: some results

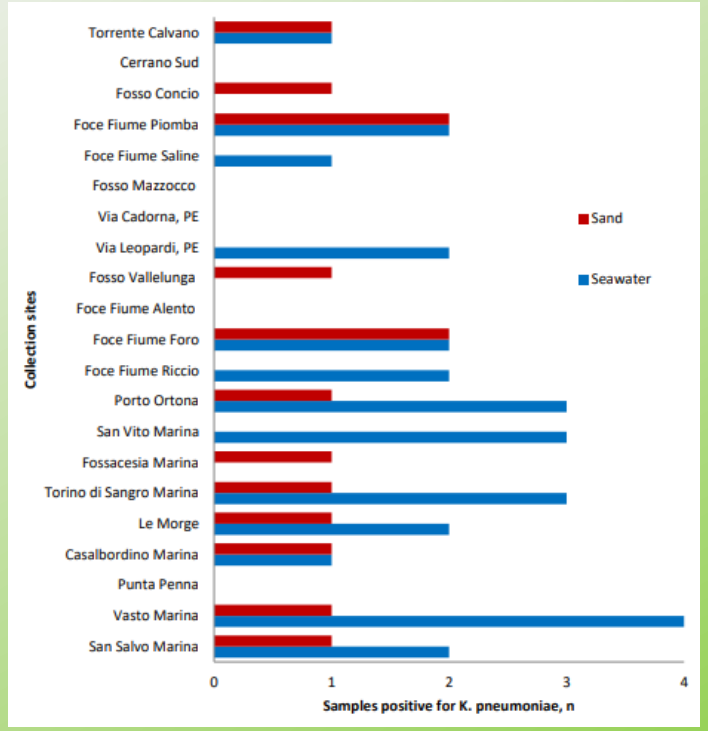


Seawater: 46,6 %
Sand: 25,9 %
Live bivalve mollusks: 24 %
Onions: 12,7 %

MedVetKlebs: some results

Presence of *Klebsiella pneumoniae* in sand and seawater for bathing along the Abruzzo Region shoreline





MedVetKlebs: some results

- National and Regional Plan for surveillance of disease in Wild Fauna
- **420 wild animals** (18 mammals' and 15 birds' species)
- **457** diagnostic samples including faeces (n = 316), intestine (n = 95) and brain (n = 46)
- **106** *Klebsiella* spp. isolates collected (85 were *Kp*)

MedVetKlebs: some results

› Microbiol Resour Announc. 2022 Jun 16;11(6):e0014022. doi: 10.1128/mra.00140-22. Epub 2022 May 17.

Whole-Genome Sequences of Two Klebsiella pneumoniae Strains (Sequence Types 23 and 35) from Wildlife

A Cornacchia [# 1], A Chiaverini [# 1], G Centorotola [1], M Di Domenico [1], A Cocco [1], M Ancora [1], C Cammà [1], N D'Alterio [1], C E Di Francesco [2], F Pomilio [1]

Affiliations + expand

PMID: 35579460 PMCID: PMC9202408 DOI: 10.1128/mra.00140-22

Free PMC article

› Animals (Basel). 2022 May 25;12(11):1347. doi: 10.3390/ani12111347.

Phenotypic and Genetic Characterization of *Klebsiella pneumoniae* Isolates from Wild Animals in Central Italy

Alexandra Chiaverini [1], Alessandra Cornacchia [1], Gabriella Centorotola [1], Elga Ersilia Tieri [1], Nadia Sulli [1], Ilaria Del Matto [1], Giorgio Iannitto [1], Domenico Petrone [1], Antonio Petrini [1], Francesco Pomilio [1]

Affiliations + expand

PMID: 35681810 PMCID: PMC9179660 DOI: 10.3390/ani12111347

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Reference Centre
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Founded as OIE

THANK YOU FOR YOUR ATTENTION!

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