Work in progress: validation of an oligonucleotide microarray for the detection of Brucella spp. virulence genes

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Work in progress

"Validation of an oligonucleotide microarray for the detection of *Brucella* sp virulence genes"

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Abstract

The primary aim of this study was to create a diagnostic microarray for the identification of *Brucella sp.* of clinical importance in both the medical and veterinary field. The microarray should be able to identify other organisms that may cause abortions in animals or that may elicit an immunological response similar to that of *Brucella* sp.
Oligonucleotide probes were designed specific to the most common species found to cause zoonotic pathologies; the sequences were designed from the following genes; *16S rRNA*, *16S-23S rRNA* intergenic transcribed spacer region (ITS), β-subunit of the DNA-dependent RNA polymerase (*rpoβ*), heat shock protein (*hsp*), gyrase beta (*gyrβ*) and from other genes usually used as PCR targets. This microarray also contains virulence factor genes specific to the *Brucella* sp.,
Preliminary results confirmed the microarray as an effective one-step method for the identification of *Brucella* sp. from culture even if , the validation is still in progress.

Introduction

Brucellosis is an infectious disease affecting both humans and animals all around the world. The etiological agents are intracellular Gram - microrganisms belonging to the genus *Brucella*. There are eight recognized host- specific *Brucella* species that differ in their preference for certain hosts. *B. abortus* preferentially infects cattle, *B. melitensis* infects sheep and goats, and *B. suis* infects pigs. All three of these species, as well as *B. canis*, can infect humans. Four other species also exist: *B. ceti*, *B. pinnipedialis* ,*B*. ovis and *B. neotomae* (3, 4) Some of these species are subdivided into biovars according to classical laboratory techniques. The correct identification of the different species and biovars is essential for an accurate interpretation of the epidemiological information during the outbreaks of the disease. In this context, molecular biology has made a valuable contribution by greatly reducing diagnosis times and improving accuracy of results (5).
Amongst the molecular techniques, DNA microarrays is a genomic tool presently used to measure the expression of many genes simultaneously (6). They are used to study altered gene expression and cellular protein profiles in human and animal pathologies (7), microarrays have been employed in the study of complex bacterial populations (8), taxonomy (9) and antimicrobial and virulence genes (1,2). Microarray was the chosen method because they have been shown to have higher specificity and higher sensitivity (10) than other molecular techniques, furthermore microarray procedures might enable harmonization of methods because it is easy to standardize, their increased use will enable pattern-recognition processes to be automated making it a simple robust method (11) applicable in any laboratory. Proper and expedient identification of *Brucella* sp. in an infection process will lead to; proper and better management of the disease; informed decisions for the prevention of the disease; proper collection of epidemiological data (12). A microarray containing various genes pertinent to the virulence of the target organism under study might enable the understanding of the mechanism of pathogenesis at the molecular level (13) and their genetic evolution process (14).
"Signature sequences" were used to identify *Brucella* sp. and other bacteria in this study, simultaneous testing of the hybridization efficiency of extracted DNA to multiple sequences on a

unique platform or in a single assay enabled fast and accurate identification of strains with minimal effort. This microarray is a one-step method after growth of organism and does not require any PCR amplification..As the efficiency of fluorescent incorporation will increase this method could be used directly on field specimens.of *Brucella* sp,.

Materials and Methods

The oligonucleotides were designed by the following methods; OligoPicker software (15), extended published PCR primers and comparison of genomes (16) for positive and negative controls previously published sequences were used (17). Oligonucleotides were then checked for their selectivity with BLAST searches in GenBank (18). The resulting unique 'signature sequences' were analyzed with BLAST (18) for sequence homologies. The sequences were accepted when GC content was between 40-60%; less than 75% homology of sequence observed in non-target genes; the calculated ΔT is less than 10-15°C of the Tm's of all the sequences; the non homology between target sequence and non-target genes is less than 14 contiguous base pairs; if do not exist palindromic hairpin sequences (19, 20, 72, 73). Table 1 reports the organisms identified by the microarray..

Slides were designed so that two independent hybridizations may be carried out on each slide using independent cover slips. Each chosen unique sequence was printed four times on Corning Ultra GAPS slides (Corning Canada, Whitby, Ontario). Slides were spotted as reported in Maynard et al. 2005 (97) at the Microarray Laboratory at the NRC – BRI in Montreal, Canada.

Two independent hybridisation were carried out per strain enabling technical replicates (21). DNA was extracted from *Brucella* sp with Wizard Genomic DNA Purification Kit (Promega, Milano, Italy). Extracted DNA concentration was measured using the Nanodrop Spectrophotometer (Nanodrop Technologies, Celbio Srl., Milan, Italy) and an amount of DNA corresponding to 300ng to 3 µg was brought to a total volume of 21µl by essication (Savant SpeedVac®, ArrayIt, USA) and resuspension in water, this DNA was then labelled with Invitrogen's Bioprime DNA labelling system (Invitrogen Life Technologies, Milano, Italy) to the DNA random primers, 20 µl of a 2.5X solution is boiled for 5 min. and then placed on ice for 5 min., from the kit along with 1 µl of high concentration Klenow polymerase (40 U/µl) are added to 5 µl of a deoxynucleoside triphosphate mix (1.2mM dATP, 1.2 mM dGTP, 1,2 1.2 mM dTTP, 0,6 mM dCTP in 10mM Tris [pH 8.0] and 1mM EDTA), to this mix 3 µl of Cy5dCTP or Cy3dCTP (Amersham, Milan, Italy) are added to fluorescently label the DNA. The reaction was then carried out in a water bath in the dark for two hours at 37° C, the reaction is stopped by the addition of 5 µl of 0.5 M EDTA pH8.0. The labelled DNA was then purified by using the Qiagen PCR columns (Qiagen S.p.A., Milan, Italy) following the manufacturer's protocol. The labelling efficiency of the DNA was then measured, the absorbance of the nucleic acid content of the eluted DNA and absorbance maximum of the dye was measured using a Nanodrop Spectrophotometer and by the application of the Beer-Lambert law the following parameters were calculated (13); labelled DNA, flourescent labelled dye and base to dye ratio was calculated at the following link (136); http://www.pangloss.com/seidel/Protocols/percent_inc.html. The percentage of incorporation was between 2% and 8% and the total amount of DNA used per hybridization was about 1.5-2.0 µg.

An appropriate quantity of labelled purified Cy5™ or Cy3™ targets were transferred to an eppendorf tube and vacuum dried Pre-hybridization of the slide was performed, slide was hybridized with a pre-heated pre-hybridization buffer containing 5X SSC, 0.1%SDS and 1%BSA and incubated at 42°C for at least one hour. Slides were prepared for hybridization addinga solution of 20µl of hybridisation buffer, 400µl of Dig Ease Buffer (Roche Diagnostics S.p.A., Milano, Italy), 20 µl Bakers tRNA (10mg/ml)(Sigma Aldrich S.p.A., Milan, Italy) and 20 µl of Sonicated Salmon Sperm DNA (10mg/ml) (Sigma Aldrich S.p.A., Milan, Italy) mixed to the labelled DNA which had been previously denatured and then kept at 42°C. Microarrays were hybridized overnight at 42°C in SlideBooster (Advalytix, ABI, Milan, Italy) stringency washes were performed with Advawash (Advalytix, ABI, Milan, Italy) using 1XSSC, 0.02%SDS preheated to 42°C. Microarrays were then

scanned on ScanArray® with ScanArray Gx software (Perkin Elmer, Milan, Italy). Data was analyzed with ScanAlyze (22), Cluster and TreeView (22).

Data analysis and Software for analysis

Data were analyzed and normalized as follows; the median value of fluorescent spot intensities after subtraction of local background intensity (intensities quantified by ScanAlyze software (22)) for each set of sequences was calculated, the median of quadruplicate spotted probes was compared to of the median of negative control spots. For each slide a cut-off for significant hybridization was established by calculating the mean and median of the signal-to-noise fluorescence ratio for both Brucella sp (PM) and. The cut-off was established as being the difference of the signal-to-noise fluorescence ratio of the greater of the mean or median of MM where the mean or median of PM must be greater than 1.25 that of the MM (23).

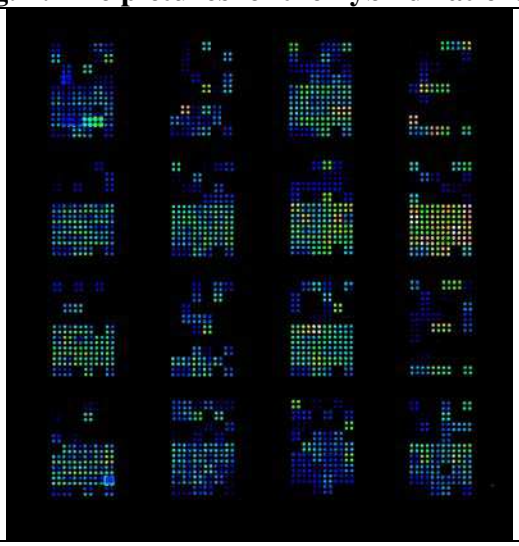
Results

The array pictures of the first hybridizations may be observed in Figure 1, the strains seem to have a picture signature positive and negative control strains give excellent results. Figure 2 describes the layout of the array. This array contains signature sequence oligonucleotides for, ,*Brucella* sp and relative virulence genes.

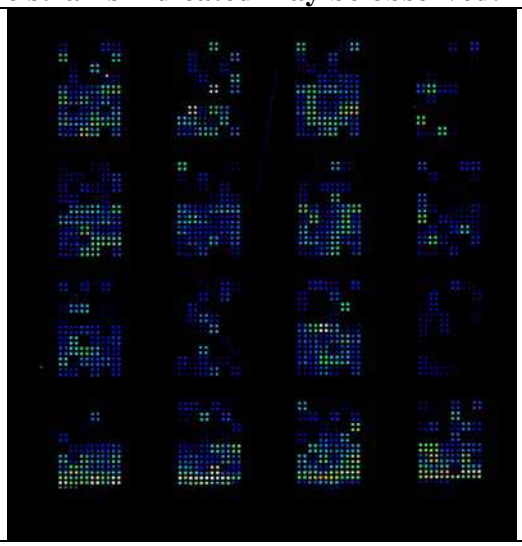
Initial cluster analysis gave positive results, as may be observed in Figure 3 the *Brucella* sp are clustered together in both clusters total [A] or selected [C] genes. Replicate hybridisations gave similar results in most cases, the results were repeatable, signature sequences characterizing both Buck 19 and RB51, *B. abortus* Biovar1, Biovar 3 and Biovar 9 as reported by Ratushna et al. (24) strains gave positive results.

The microarray gave great resolution for the above strains, more work have to be done with *Brucella abortus* by looking at the individual genes as may be seen in Figure 3 F and G , replicates of all biovars and tests on biovar 3, have not yet been performed, it is essential that repeatability, reproduceability and PCR confirm these results, this will be determinant in the production of an excellent prototype.

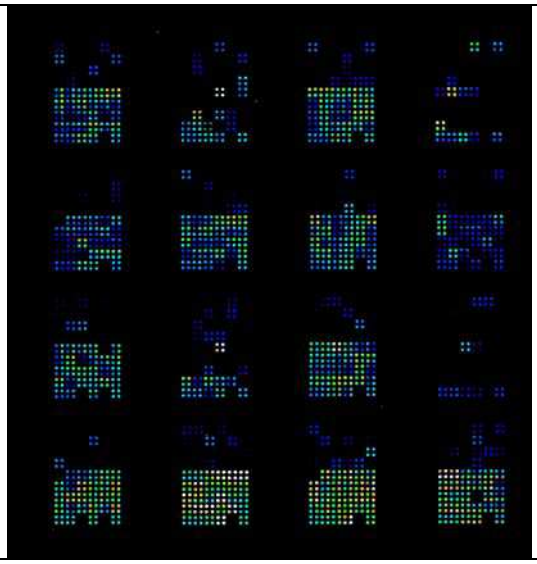
Fig. 1: The pictures of the hybridizations of the strains indicated may be observed.



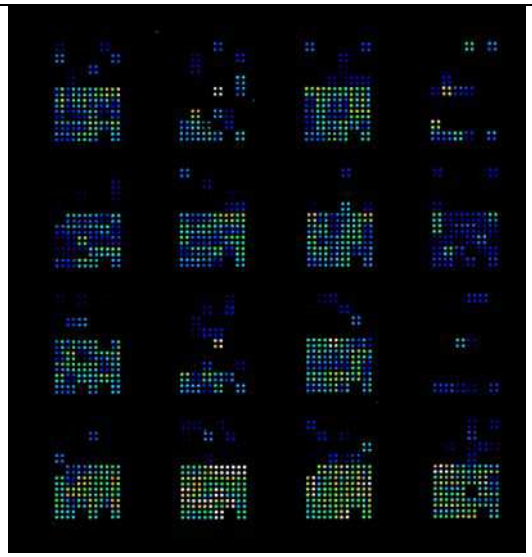
Brucella abortus biovar 1 strain 544



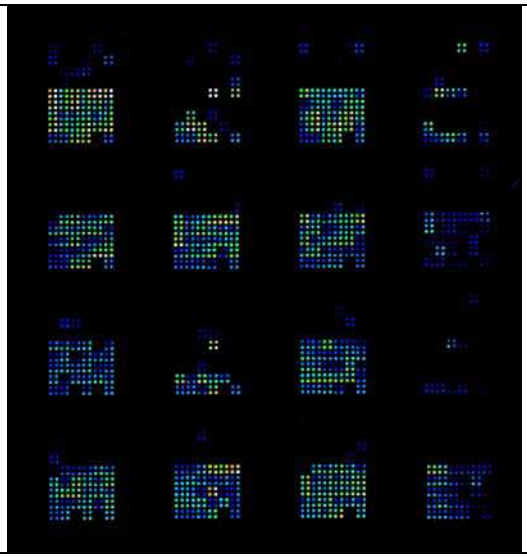
Brucella abortus biovar 2

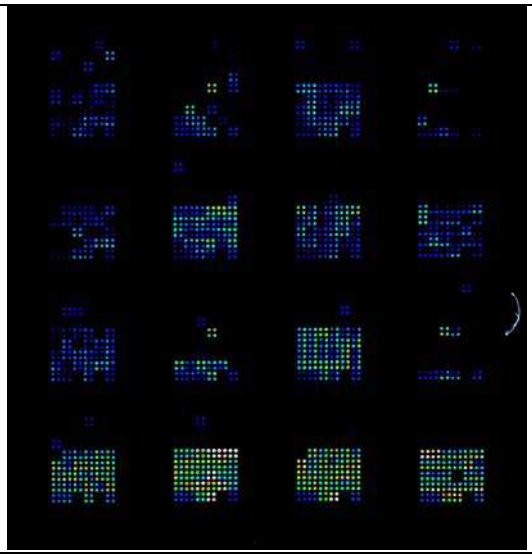


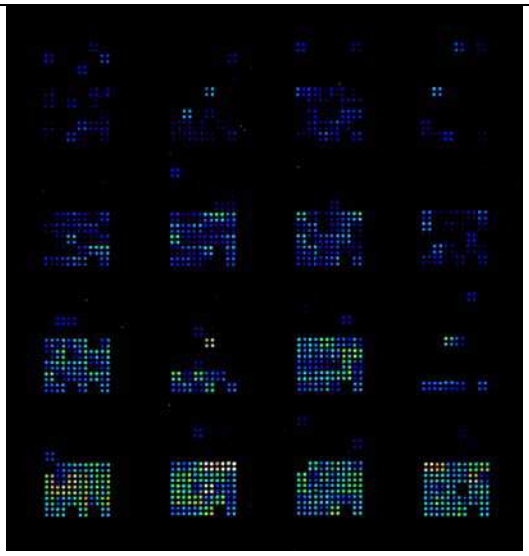
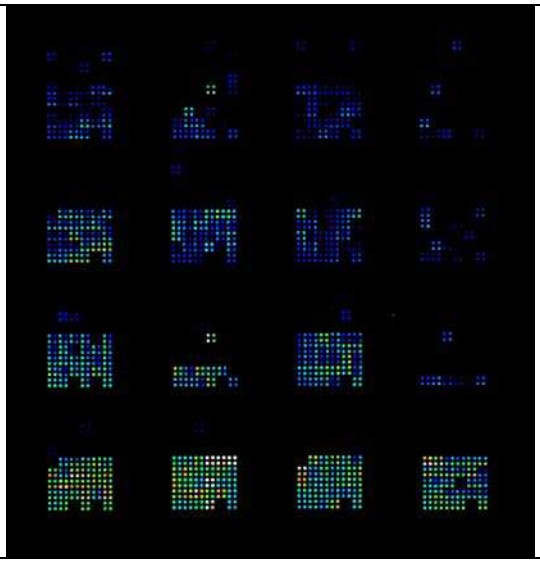
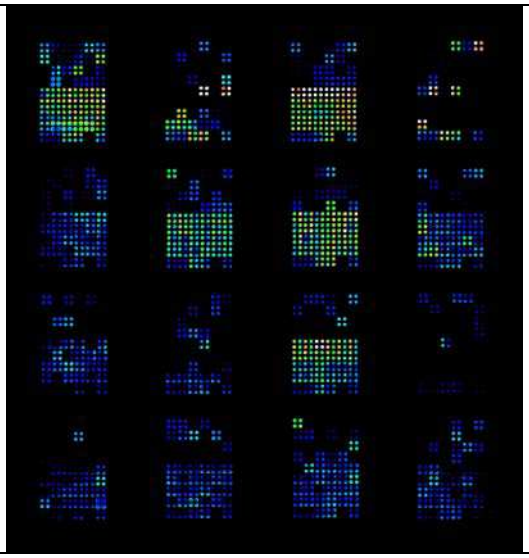
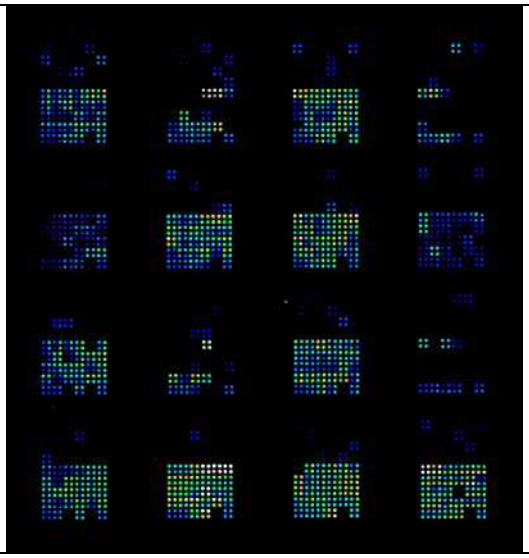
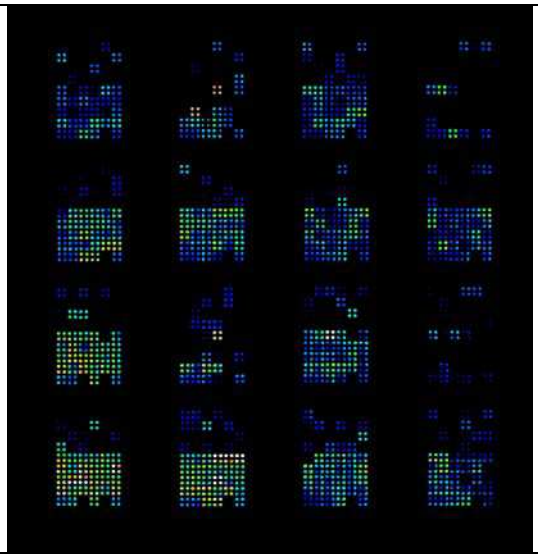
Brucella abortus biovar 4

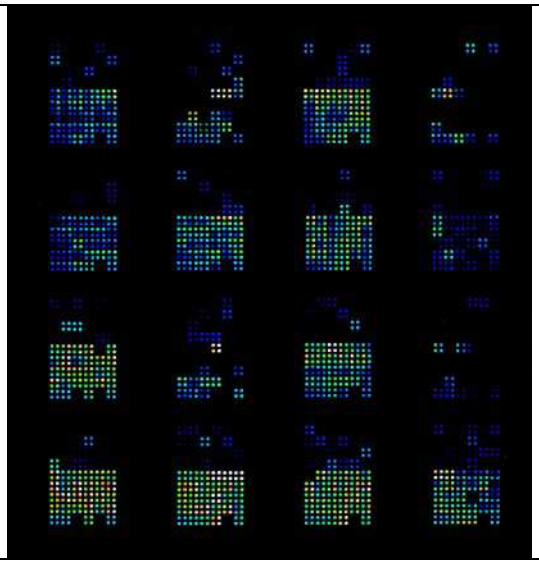
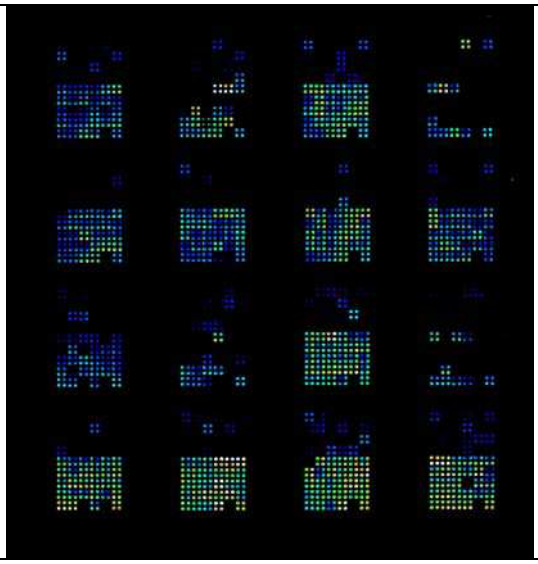
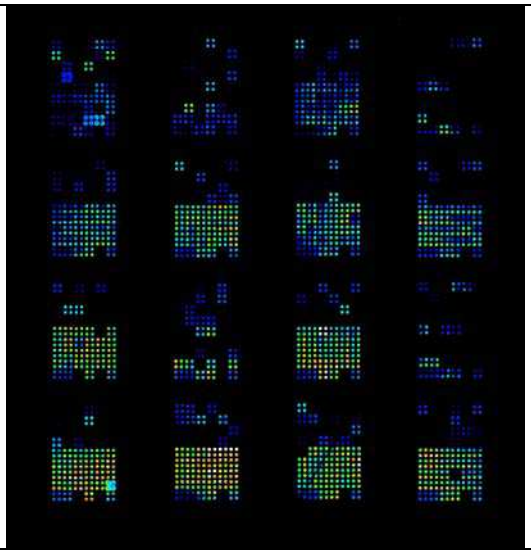
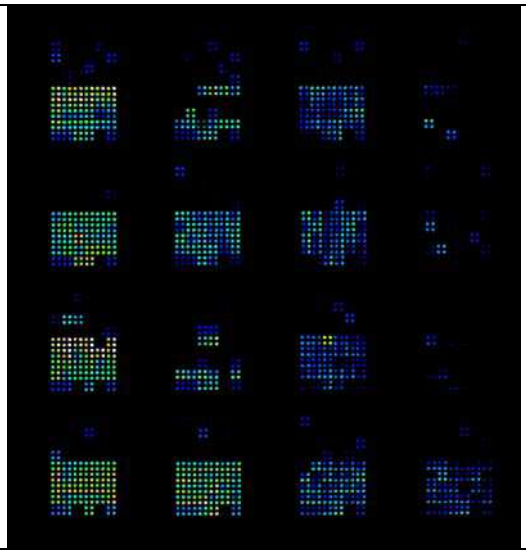
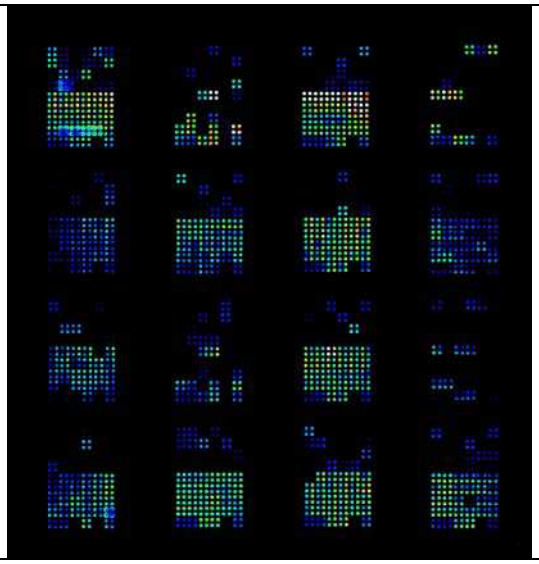
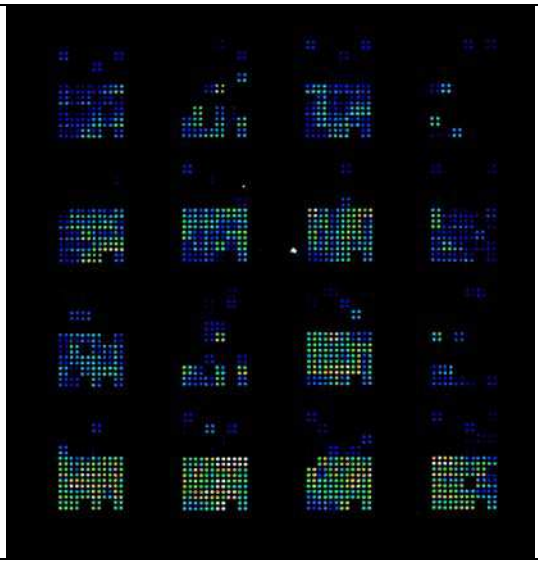


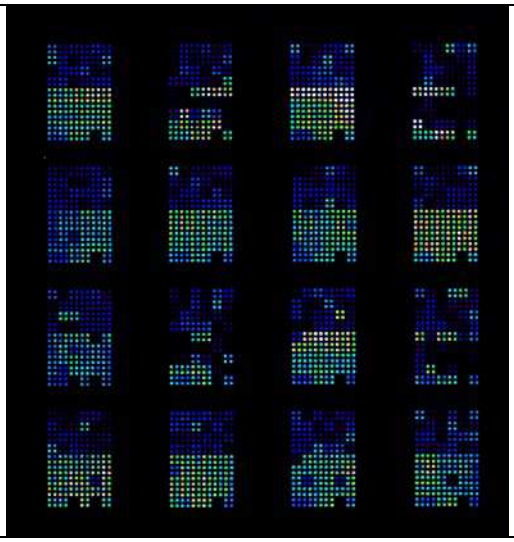
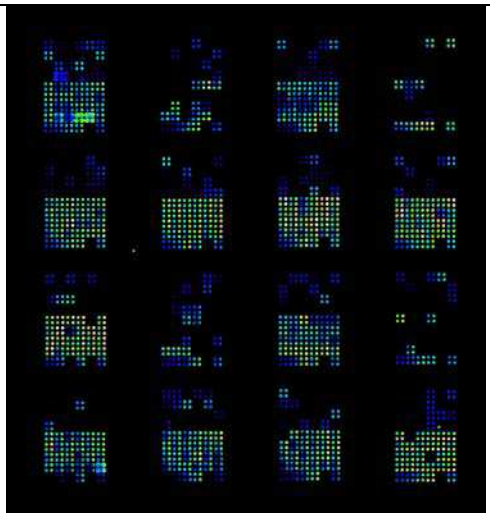
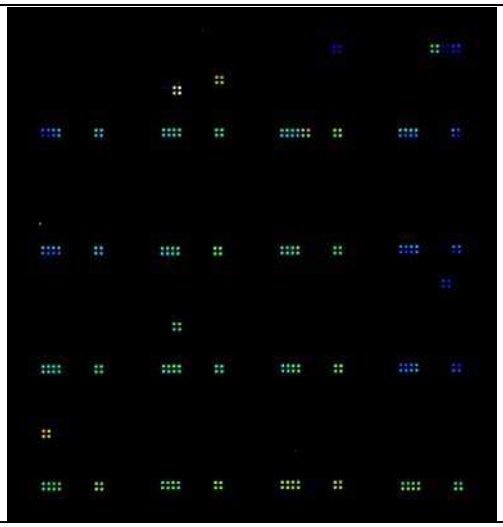
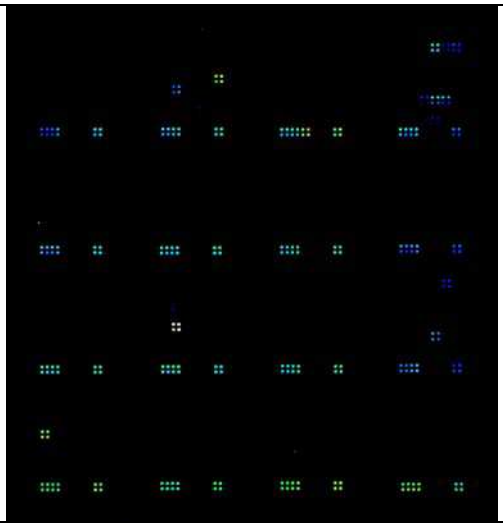
Brucella abortus biovar 5





Brucella abortus biovar 6	*Brucella abortus biovar 7*
	
Brucella abortus biovar 9	*Brucella abortus RB51*
	
BUCK 19	
	

Brucella melitensis biovar 1 strain 16M	*Brucella melitensis biovar 2*
	
Brucella melitensis biovar 3	*Brucella melitensis B115*
	
Brucella suis biovar 1	*Brucella suis biovar 2*
	

Brucella suis biovar 3	*Brucella suis biovar 4*
	
Brucella suis biovar 5	***Brucella ovis***
	

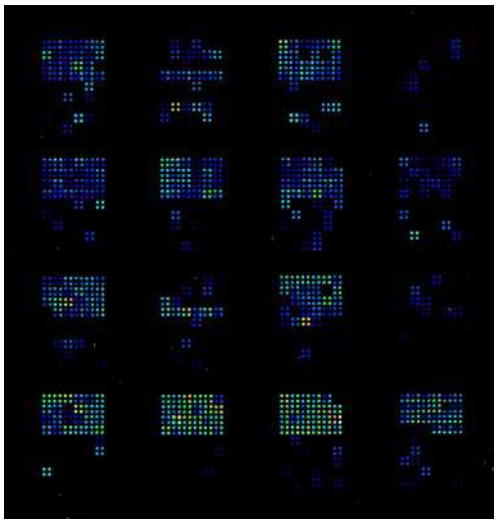
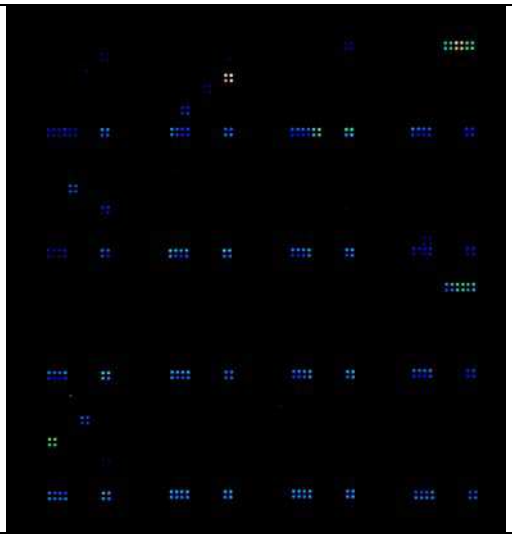
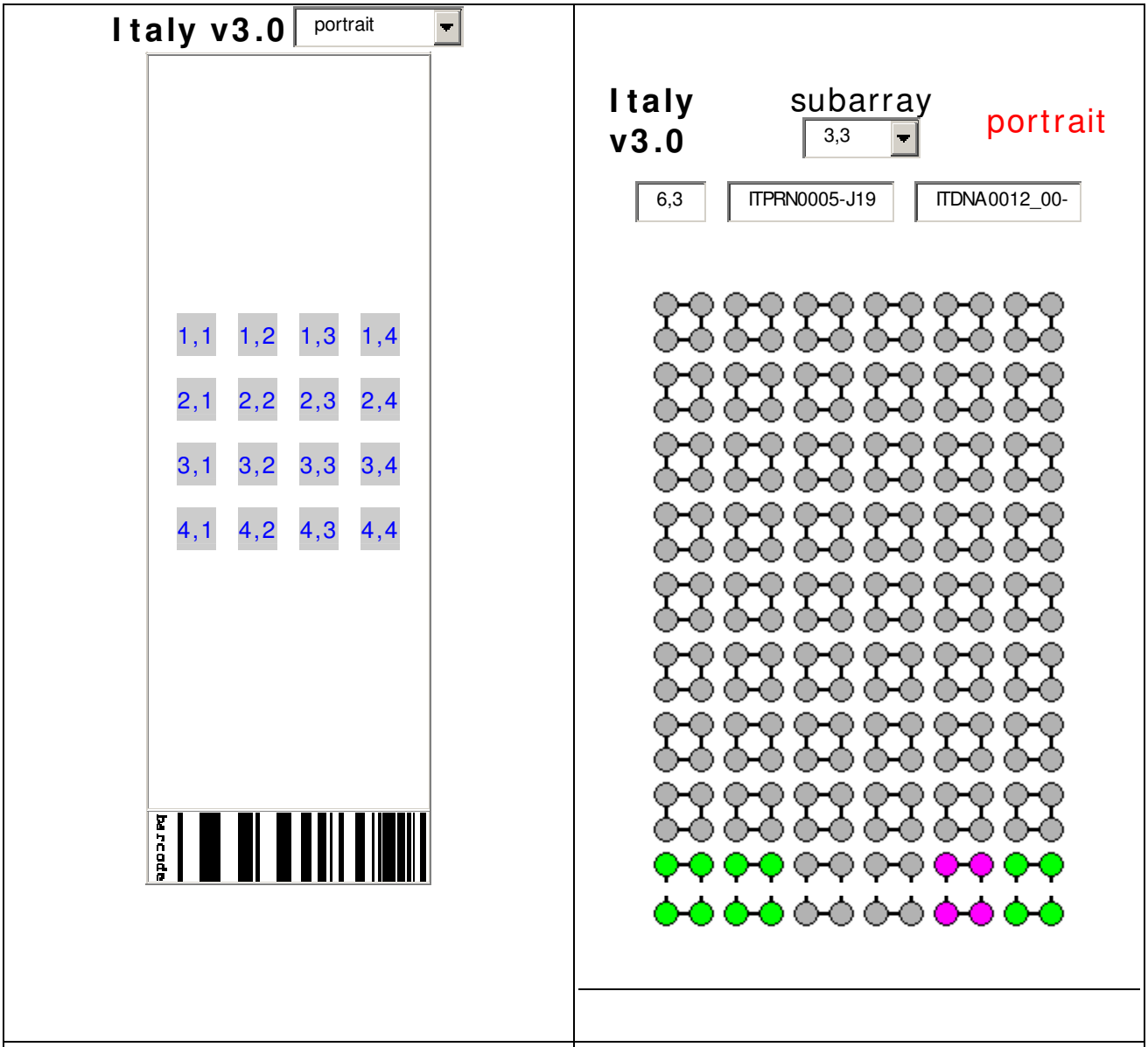
Campylobacter coli	*Campylobacter jejuni ssp jejuni*
	
Mycobacterium bovis BCG	*Corynebacterium pseudotuberculosis*

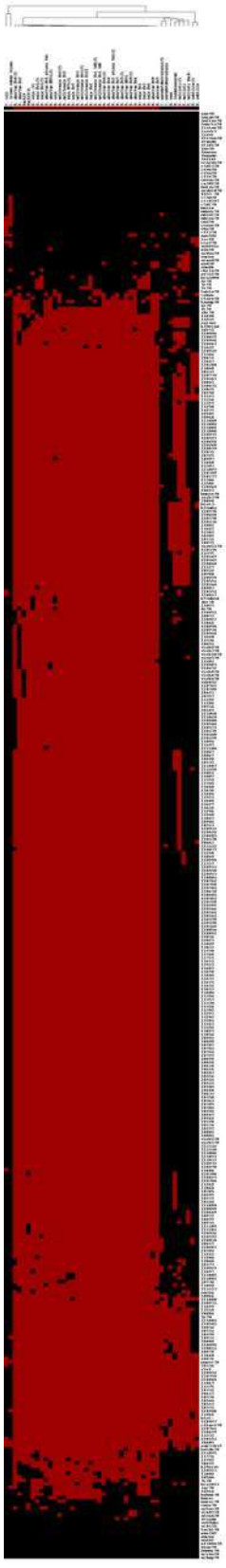
Fig. 2: (A) Layout of array; (B) Layout of typical subarray gray circles are oligonucleotides the square disposition is the number of replicates per oligonucleotide the green circles are positive controls and the fuchsia are negative controls. The positive and negative controls are found on all subarrays. The first four rows of squares are *Mycobacterium sp* genes and the last five rows are *Brucella sp* genes.



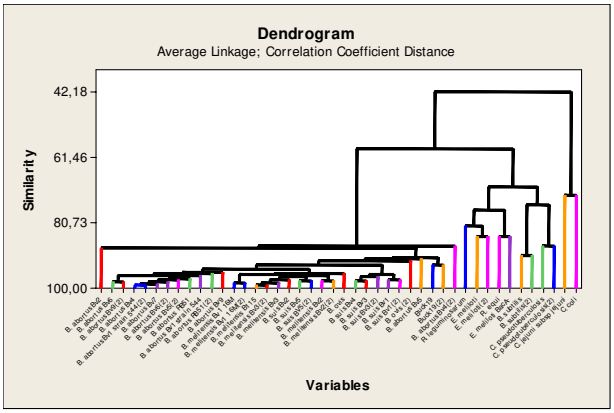
(A) (B)

Fig. 3: [A] Cluster of total organisms and total genes; [B] Dendrogram of cluster with similarity of total organisms with total genes on microarray; [C]Cluster of total organisms with selected genes; [D] Cluster of virulence and only *Brucella* sp; [E] Dendrogram of cluster with similarity of virulence and *Brucella* sp. [F] Selected genes will cluster *Brucella abortus*, [G] Selected genes will cluster *Brucella abortus*.

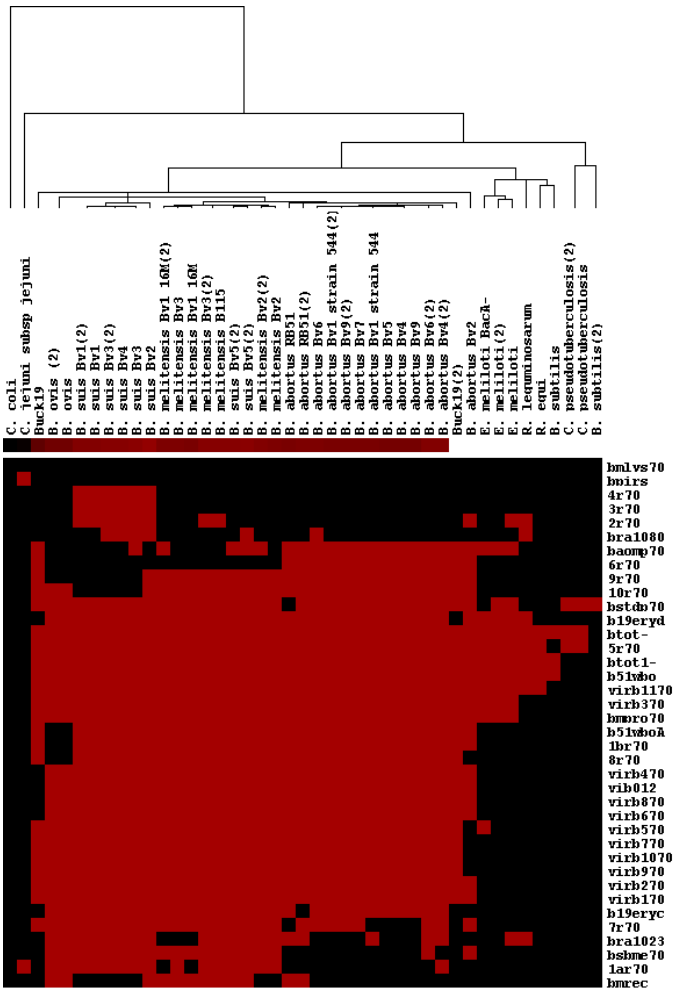
[A]



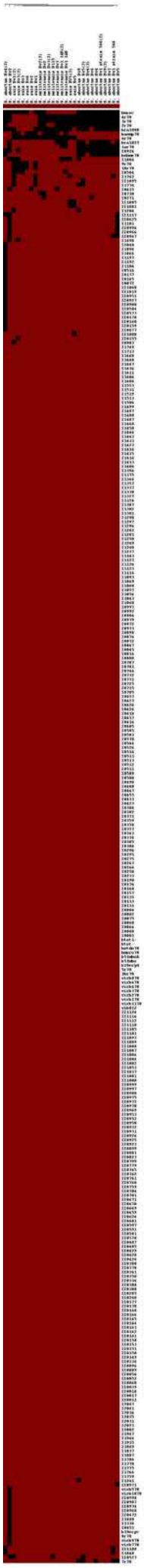
[B]



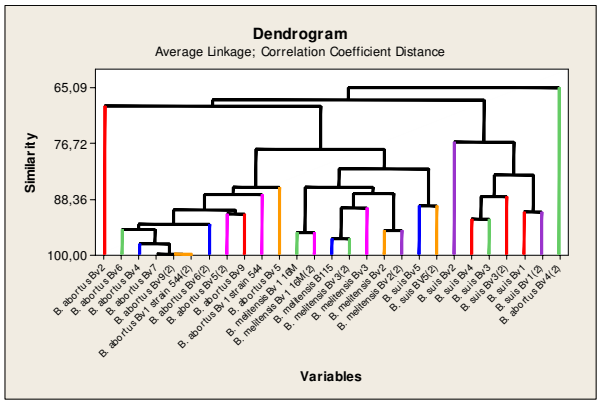
[C]



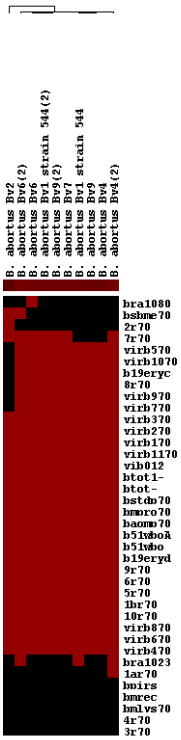
[D]



[E]


Dendrogram
Average Linkage; Correlation Coefficient Distance
Similarity
65,09
76,72
88,36
100,00
B. abortus Bv2
B. abortus Bv6(2)
B. abortus Bv6
B. abortus Bv4
B. abortus Bv7
B. abortus Bv9(2)
B. abortus Bv1 strain 544(2)
B. abortus Bv5(2)
B. abortus Bv5(3)
B. abortus Bv9
B. abortus Bv1 strain 544
B. abortus Bv5
B. melitensis Bv1 16M(2)
B. melitensis Bv1 16M
B. melitensis Bv1(5)
B. melitensis Bv3(3)
B. melitensis Bv3
B. melitensis Bv2(2)
B. melitensis Bv2
B. suis Bv5
B. suis Bv5(2)
B. suis Bv2
B. suis Bv2
B. suis Bv4
B. suis Bv3
B. suis Bv3(2)
B. suis Bv1
B. suis Bv1(2)
B. abortus Bv4(2)
Variables

[F]


B. abortus Bv2
B. abortus Bv6(2)
B. abortus Bv6
B. abortus Bv1 strain 544(2)
B. abortus Bv9(2)
B. abortus Bv7
B. abortus Bv1 strain 544
B. abortus Bv9
B. abortus Bv4
B. abortus Bv4(2)
bra1080
bsbme70
2r70
7r70
virb570
virb1070
b19eryc
8r70
virb970
virb770
virb370
virb270
virb170
virb1170
vib012
btot1-
btot-
bstdb70
bmoro70
baomm70
b51xboA
b51xbo
b19eryd
9r70
6r70
5r70
1br70
10r70
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bra1023
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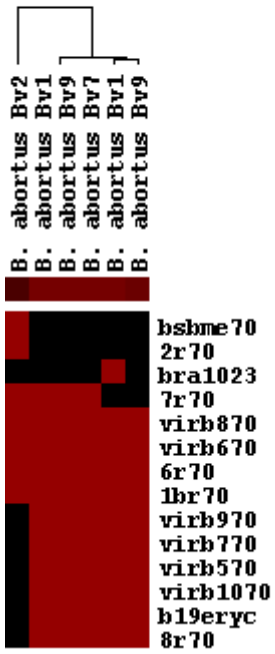
bwirs
bmrec
bmlvs70
4r70
3r70

[G]


B. abortus Bv2
B. abortus Bv1
B. abortus Bv9
B. abortus Bv7
B. abortus Bv1
B. abortus Bv9
bsbme70
2r70
bra1023
7r70
virb870
virb670
6r70
1br70
virb970
virb770
virb570
virb1070
b19eryc
8r70

Conclusions

The microarray prototype is an effective and rapid diagnostic tool for classification of *Brucella* sp. This prototype requires improvement but it is presently very useful for interspecies and intraspecies differentiation even if requires further validation and confirmation of its findings by PCR..
The prototype also contains virulence genes, we have not dealt with this aspect at length in this poster because they were placed in this microarray for future trascriptomic applications for investigations into the process of pathogenesis of the disease but as may be observed they also cluster the organisms.
The cluster analysis tool developed by Eisen et al. (22) facilitates the application of our microarray prototype in the interpretation of the wealth of information generated by it. Eisen's software proved to be indispensable for our purposes, but other informatics tools such as neural networks and wavelet, where profiles of reference strains will be used as training of data and unknowns, will be classified this process will automate, improve further and offer new solution for MDMs (Microbial Diagnostic Microarray) technology.

This tool is an efficient, robust, easily to standardize, one-step method for the analysis of *Brucella* sp and could be usefull when applied directly to DNA extracted from biological specimens received in laboratories. Costs for microarray will most probably decrease in the future when its applications will be implemented in diagnostic laboratories.

References

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Organisms identified by microarray.

Agrobacterium tumefaciens	*Campylobacter jejuni subsp. jejuni*	*Francisella sp.*	*Leptospira interrogans*	*Neospora caninum*	*Salmonella enterica subsp. enterica serovar Dublin*
Agrobacterium rhizogenes	*Campylobacter mucosalis*	*Francisella tularensis*	*L. ivanovii*	*Ochrobactrum anthropi*	*Sarcocystis sp*
Brucella abortus biovar 1 str. 9-941	*Chlamydophila abortus*	*Francisella tularensis subsp. tularensis*	*L. monocytogenes type I, 2, 3*	*Ochrobactrum anthropi*	*Stenotrophomonas maltophilia*
Campylobacter coli	*Coxiella burnetii*	*Francisella tularensis subsp. novicida*	*L. monocytogenes vir. ass. genes*	*Pasteurella multocida subsp multocida*	*Toxoplasma gondii*
Campylobacter fetus s fetus	*E. coli O157:H7*	*Fusobacterium necrophorum ssp funduliforme*	*L. monocytogenes vir. genes*	*Phyllobacterium myrsinacearum*	*Vibrio cholerae O1 biovar eltor*
Campylobacter fetus subsp. venerealis	*E. coli*	*Fusobacterium necrophorum ssp necrophorum*	*Manheimia haemolytica*	*Rhizobium leguminosarum*	*Vibrio cholerae strain non01*
Campylobacter jejuni subsp. doylei	*Ensifer meliloti*	*Leptospira sp*	*Mycoplana dimorpha*	*Salmonella enterica subsp. enterica serovar Abortusovis*	*Yersinia enterocolitica O:9*

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