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Work in progress: Design and evaluation of a microarray that differentiates *Brucella sp* from organisms that may cause abortion in animals, elicit serological cross-reactivity or are phylogenetically related

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Abstract

An oligonucleotide microarray prototype was designed and is being evaluated permitting rapid identification of some pathogenic bacteria that may cause abortion in animals such as *Brucella sp*, *Campylobacter fetus subsp fetus*, *Campylobacter jejuni subsp jejuni*, *Campylobacter jejuni subsp doyle*, *Campylobacter coli*, *Listeria monocytogenes*, *Listeria ivanovii*, *Salmonella enterica ssp enterica serovar Abortusovis*, *Salmonella enterica ssp enterica serovar Dublin*, bacteria that may elicit serological cross-reactivity to *Brucella sp* in an infectious process as *Vibrio cholerae*, *Yersinia enterocolitica O9*, *Escherichia coli O157:H7* and phylogenetically related bacteria of the order *Rhizobiales* of the *Alphaproteobacteria*; *Ochrobactrum anthropi*, *Mycoplana dimorpha*, *Ensifer melliloti*, *Phyllobacterium myrsinacearum*, *Rhizobium radiobacter* and *Rhizobium leguminosarum* were also included in the study. The microarray uses oligonucleotide sequences that are species specific to the organisms being investigated, it also contains sequences for virulence genes found in *Brucella sp* and reported in the literature. The microarray was designed and is being evaluated with ATCC/NCTC/DSMZ reference strains.
Preliminary hybridization results were analyzed with clustering software, each organism tested was identified by distinct patterns able to reveal the presence or absence of the selected sequences. All of the zoonotic agents of abortion were correctly classified according to the presence of their species specific genes. Previously identified virulence factors of *Brucella sp* were present to some degree in all of the different *Brucella sp* tested and most were absent in all other zoonotic agents of abortion. Preliminary results confirmed microarray hybridization as a powerful tool, which allows rapid identification of the organisms being tested.

Introduction

Brucellosis is an infectious disease affecting both humans and animals all around the world, organisms of the genus *Brucella* are intracellular Gram - bacteria. There eight recognized host- specific *Brucella* species. *B. abortus* preferentially infects cattle, *B. melitensis* infects sheep and goats, and *B. suis* infects pigs, *B. canis* is isolated from dogs all four of these species can infect man. 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. It is also of importance that other pathogenic bacteria that may cause abortion in animals such as; *C. fetus subsp fetus, C. jejuni subsp jejuni, C. jejuni subsp doyle, C. coli, L. monocytogenes, L. ivanovii, S. enterica ssp enterica serovar Abortusovis, S. enterica ssp enterica serovar Dublin* be present or be the cause of the abortion. 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).
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), antimicrobial and virulence genes (1,2). In this study we chose microarray because microarray procedures might enable harmonization of methods, easily standardizeable and their increased use will enable pattern-recognition processes to be automated making it a simple robust method (11) applicable in any laboratory.
An oligonucleotide microarray was designed containing species specific sequences to pathogenic bacteria that cause abortion, bacteria that elicit serological cross-reactivity to *Brucella sp* in an infectious process such as *V. cholerae, Y. enterocolitica O9, E. coli O157:H7* and phylogenetically related bacteria of the order *Rhizobiales* of the *Alphaproteobacteria*; *O. anthropi, M. dimorpha, E. melliloti, P. myrsinacearum, R. radiobacter* and *R. leguminosarum*. The microarray is being evaluated to rapid identification of the organism, it also contains various genes pertinent to the virulence of the *Brucella sp* under study.

Materials and Methods

The oligonucleotides were designed by the use of OligoPicker software (13) and extended published PCR primers. In the absence of dissimilar sequences a comparison of published genomes (14) was performed. Previously published sequences were used for positive and negative controls sequences (15). Oligonucleotides were then checked for their selectivity with BLAST searches in GenBank (16) . 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 there are no palindromic hairpin sequences(19, 20).

DNA extraction and labeling, and microarray experiments.

DNA was extracted from *Brucella sp* strains with the Wizard Genomic DNA Purification Kit (Promega, Milano, Italy) according to the manufacturer's protocole. Extracted DNAs were then quantified using a Nanodrop Spectrophotometer (Nanodrop Technologies, Celbio Srl., Milan, Italy). An amount of DNA corresponding to 300 ng to 3 µg was brought to a total volume of 21 µl by dessication (Savant SpeedVac®, ArrayIt, USA) and resuspension in water. DNA was then labeled with the Invitrogen's Bioprime DNA labelling system kit (Invitrogen Life Technologies, Milano, Italy) as described previously (2). Labeling efficiency and the percentage of dye incorporation were then determined by measuring the absorbance at OD_{260} for the nucleic acids and OD_{650} or OD_{555} for the Dye, using the Nanodrop Spectrophotometer, and then by incorporating the results in the following link: http://www.pangloss.com/seidel/Protocols/percent_inc.html.

Table 1: Bacteria tested by the microarray.

Organism	ATCC*/NCTC°/DSM§
Brucella abortus biovar 1	Strain 544
Brucella abortus S19	Weybridge
Brucella canis	NCTC 10854
Brucella maris	NCTC 12890
Brucella melitensis biovar 1	16M
Brucella neotomae	NCTC 10084
Brucella ovis	Weybridge
Brucella suis biovar 1	Weybridge
Campylobacter coli	NCTC 11353
Campylobacter fetus subsp. fetus	ATCC 27379
Campylobacter jejuni subsp. doylei	NCTC 11159
Campylobacter jejuni subsp. jejuni	NCTC 12560
Ensifer meliloti	University of Edinburgh
Listeria ivanovii ssp ivanovii	ATCC 19119
Listeria monocytogenes	ATCC 19111
Listeria monocytogenes	ATCC 9525
Yersinia enterocolitica O:9	NCTC 10964
Yersinia enterocolitica O:8	NCTC 23715
Rhizobium leguminosarum	University of Edinburgh
Rhizobium radiobacter	DSM 30205
Phyllobacterium myrsinacearum	DSM 5892
Ochrobactrum anthropi	DSM 6882
Mycoplana dimorpha	DSM 7138
Salmonella enterica subsp. enterica serovar Abortusovis	NCTC
Salmonella enterica subsp. enterica serovar Dublin	NCTC
Vibrio cholerae O1 biovar eltor	Sieroterapico

*ATCC: American Type Culture Collection (http://www.lgcpromochem-atcc.com/)

°NCTC: The National Collection of Type Cultures (http://www.hpacultures.org.uk/)

§DSM: German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de/index.htm)

Figure 1: Clustogram of strains in Table 1 tested on microarray.

Hybridizations were performed following a protocol derived from Hamelin et al. (2). Briefly, for each hybridization, 500 ng of labeled DNA were dried under vacuum in a rotary dessicator (Savant SpeedVac®, ArrayIt, USA). Dessication was not complete and heat was not applied. Dried labeled DNA was resuspended in hybridisation buffer (Dig Ease Buffer, Roche Diagnostics spa, Milano, Italy). Before hybridization, microarrays were pre-hybridized during at least one hour at 42°C with a pre-heated pre-hybridization buffer containing 5X SSC, 0.1% SDS and 1.0% BSA. After pre-hybridization, the microarrays were hybridized with a solution that consisted of 25 µl of hybridisation buffer, 20 µl of Bakers tRNA (10 mg/ml) (Sigma Aldrich spa, Milan, Italy) and 20 µl of Sonicated Salmon Sperm DNA (10 mg/ml) (Sigma Aldrich spa, Milan, Italy), together mixed with the labeled DNA which has previously been denatured. Microarrays were hybridized overnight at 42°C in a SlideBooster (Advaltyix, ABI, Milan, Italy). After hybridization, stringency washes were performed with Advawash (Advaltyix, ABI, Milan, Italy) using 1X SSC, 0.02% SDS preheated to 42°C. Microarrays were then scanned on ScanArray® with ScanArray Gx software (Perkin Elmer, Milan, Italy).

Statistical Analysis

Microarray data were first normalized as described previously (1).The median value for each set of quadruplicate spotted probes was compared to the median value for the buffer or negative control spots after subtraction of local background intensity. Normalized microarray data were then analyzed with the ScanAlyze and Cluster software according to the Cluster and TreeView Manual (19).

Results and Conclusions

Table 1 lists the organisms reported in this paper, the microarray has been tested with all the *Brucella* species and biovars. Initial cluster analysis gave positive results, as may be observed in Figure 1 the *Brucella sp* strains are clustered together, the other species form separate clusters as may be seen in tree of Figure 1. Replicate hybridizations per gave similar results. The microarray gave excellent resolution for the strains tested.

"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 not requiring any PCR amplification. As the efficiency of fluorescent incorporation will increase this method could be used directly on field specimens of *Brucella* sp. The microarray prototype is an effective and rapid diagnostic tool for the correct identification of *Brucella* sp, other pathogenic bacteria that may cause abortion in animals such as; *C. fetus subsp fetus, C. jejuni subsp jejuni, C. jejuni subsp doyle, C. coli, L. monocytogenes, L. ivanovii, S. enterica ssp enterica serovar Abortusovis, S. enterica ssp enterica serovar Dublin*, bacteria that elicit serological cross-reactivity to *Brucella sp* in an infectious process such as *V. cholerae, Y. enterocolitica O9, E. coli O157:H7* and phylogenetically related bacteria of the order *Rhizobiales* of the *Alphaproteobacteria*; *O. anthropi, M. dimorpha, E. melliloti, P. myrsinacearum, R. radiobacter* and *R. leguminosarum*. This microarray is presently very useful for interspecies and intraspecies differentiation.

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Keywords: Brucella, microarray

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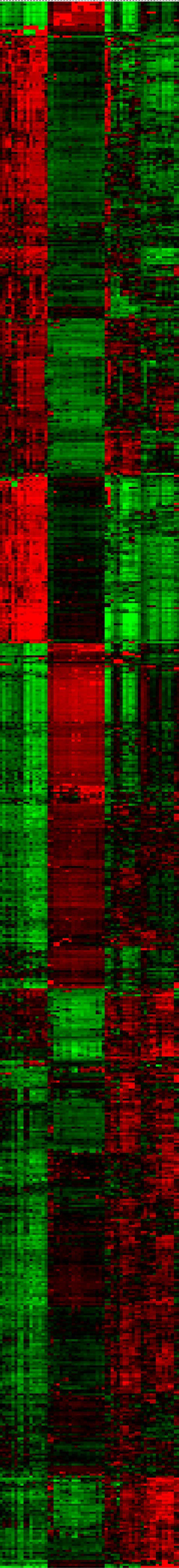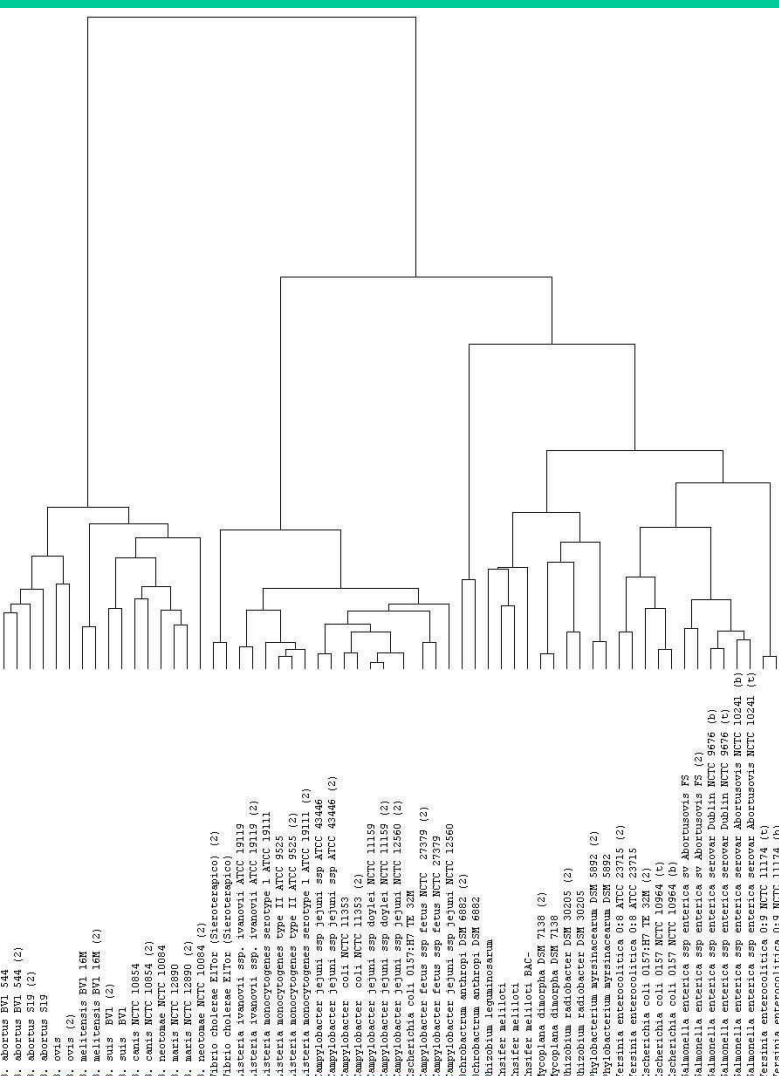(19) Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc.Natl.Acad.Sci.U.S.A. 1998 Dec 8;95(25):14863-14868.