Hypersensitivity pneumonitis and metalworking fluids contaminated by Mycobacteria.

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Running title: Metalworking fluids, hypersensitivity pneumonitis and Mycobacteria

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Summary: (198 words)

Question: Metalworking fluids are responsible for hypersensitivity pneumonitis (MWF-HP). The aim is to identify the antigen (Ag) responsible for MWF-HP and to optimize serological diagnosis by definition of a threshold allowing discrimination between HP patients from asymptomatic exposed workers (H-MWF)

Patients and methods: Thirteen patients who were workers at a car-engine manufacturing plant, were suspected of MWF-HP. Microbial analysis of 83 used MWF was carried out. Sera from 13 MWF-HP, 12 H-MWF and 18 UC (healthy unexposed controls) were tested to determine their immunological responses to three Ag including *Mycobacterium immunogenenum* (Mi).

Results: *Mi* was identified in 40% of used fluids by culture and confirmed by DNA sequence. The threshold to differentiate MWF-HP from H-MWF was five arcs of precipitation (Sensitivity (Se) 77%, specificity (Sp) 92%) as determined by electrosyneresis. Using ELISA methods with protein extract from *Mi*, a threshold leading to (Se) 92% and (Sp) 100% was established.

Answer to the question: The detection of specific antibodies against *Mi* Ag in high level in case sera, suggests that *Mi* contaminated-MWF is responsible for MWF-HP. To discriminate MWF-HP from H-MWF, we also put a five arcs threshold forward by electrosyneresis and a 1.6 arbitrary unit for ELISA methods threshold.

Key-words: hypersensitivity pneumonitis, metalworking fluid, *Mycobacterium immunogenenum*, DNA sequence, precipitins, electrosyneresis, ELISA
Introduction

Hypersensitivity pneumonitis (HP) or extrinsic allergic alveolitis is caused by a wide range of antigens, including bacteria. Variability in its clinical presentation and the similarity of HP to other respiratory diseases can lead to under-diagnosis. Exposure to a relevant antigen is the most important predictor of HP (1). Identification of the etiological agent and definition of a threshold for serology between HP patients, asymptomatic exposed patients and healthy unexposed controls are essential for the immunological diagnosis of HP. Exposure to metalworking fluids (MWF) is described in the literature as being responsible for MWF-HP. In the United States, 1.2 million persons have been exposed to MWF. More than one hundred cases of MWF-HP have been described in North American (2,3). Rapidly Growing Mycobacteria (RGM) were considered to be the most likely etiologic agent of MWF-HP (4). The French automobile industry has become concerned about the emergence of pathologies related to exposure to MWF.

Thirteen workers from a car engine-manufacturing plant in Lille, France, were referred to the local University Hospital as consecutive patients between 2004 and 2007. They suffered from respiratory symptoms, consistent with HP, and, after other causes had been ruled out, MWF-HP was suspected. The aim of the study was to identify the main antigen that may be associated with MWF-HP and to define a threshold to discriminate HP patients from asymptomatic exposed workers (H-MWF) and healthy unexposed controls (UC). The following strategy was used:

1/ Cases of MWF-HP were confirmed by assessment of clinical, functional, radiological, and BAL data,

2/ Microbiological analysis of the metalworking fluid from the plant was performed,

3/ Antigen extracts from the most frequently isolated micro-organisms
\textit{(Mycobacterium immunogenenum (Mi), Bacillus simplex (Bs) and Fusarium solani (Fs)}

were done,

4/ The immunological response of the patients (MWF-HP), asymptomatic controls (H-MWF) and unexposed healthy controls (UC) to these antigens was assessed using electrosyneresis and ELISA methods. A Receiver Operating Characteristic (ROC) curve method was used to define a threshold between groups.
Methods:

Subjects:

This study compared exposure and immunological response for MWF-HP cases (n=13), asymptomatic H-MWF (exposed controls) (n=12) and unexposed healthy controls (UC) (n=18). Informed written consent was obtained from all subjects. The protocol was approved by the local review board for research involving human subjects.

- Case subjects were diagnosed with MWF-HP in our department according to clinical, biological and radiological criteria (1,5,6). (Medical investigations are given in results section)

- H-MWF were volunteers exposed to MWF at the same plant, the same workshop and the same task as the MWF-HP workers. They were selected and examined by the occupational medical doctor when they were included in the study and one year later.

- The 18 unexposed healthy controls (UC), were volunteers recruited health insurance company employees, living in urban areas, (towns of up to 5,000 residents). They were examined by the occupational medical doctor on an annual visit and gave responses to a medical questionnaire. H-MWF and UC groups were similar to the MWF-HP patients group with respect to age (+/-5 years), sex and all were non smokers.

Microbiological analyses

Eight series of microbiological analyses were performed (one per month) between November 2006 and July 2007 on metalworking fluids (MWF) from the machine shops where the 13 MWF-HP patients had been working. (There were more than 50 workers per shop).

Sampling
Samples of 50 ml were taken from each vat of used MWF (n= 83), new MWF (n = 4), industrial water (n= 2), osmosed water (n= 5), ultra filtrated water (n=2) and additives (n=1) included in the preparation of MWF.

**Microbiological techniques**

MWF and other samples were analyzed for bacteria, (RGM) rapidly growing mycobacteria, actinomycetes and fungi on three different solid media : Actinomycetes Difco™ (Becton Dickinson®, Cockeysville, USA), Mueller Hinton (Becton Dickinson®, Cockeysville, USA), and Sabouraud chloramphenicol agar (Bio-Rad®, Marnes la Coquette, France). Petri dishes plated with 0.1 mL of one of the samples were incubated at 30°C for three weeks. Colonies of bacteria and fungi were identified and numerated. The limit of detection was 10 CFU/mL (colony forming unit per millilitre).

Screening of *Mycobacterium immunogenenum (Mi)* was done by PRA (PCR-Restriction Analysis) of *hsp* genes, using a combination of patterns obtained after digestion with three different enzymes, as described previously (Hha I, HinF I and Msp I) (4), and compared to 5 referenced RGM strains, including Mi.

Identification of suspected Mi isolates, was confirmed by PCR amplification then sequencing of *hsp* (Heat Shock Protein) and *rpo* (RNA polymerase, β subunit) genes, using previously described PCR methods (7,8). Mycobacteria were identified by using BLAST searches of their sequences compared with sequences in the GenBank data base (www.ncbi.nlm.nih.gov/BLAST). Identification of species was determined by a similarity of both hsp and rpo genes of over 97% (9).

**Immunological methods:**

**Strains, culture and Antigens production**

One strain of Mi, isolated during the first survey of MWF samples from the car-engine manufacturing plant was cultured on Mueller-Hinton plates at 30°C for one week to obtain a
microbial mass. The structures of $Mi$ were recovered by scraping and filtration. Two kinds of antigens were produced: one total aqueous lyophilised extract of $Mi$ (somatic antigens) used for electrosyneresis on cellulose acetate (10-11) and one protein extract of $Mi$ used for ELISA tests. (12).

Somatic antigen from $Mi$, $Fs$ and $Bs$ were produced as follows: strains were cultured on Mueller-Hinton (for $Mi$ and $Bs$) or Dichloran-glycerol 18 agar (Oxoid, Unipath, Basingstoke, England) with 0.5% chloramphenicol (Merck®, Darmstat, Germany) (for $Fs$) culture media for 2 weeks, crushed with an Ultraturax (IKA Labortechnik, Staufen, Germany), sonicated, extracted overnight in NH$_4$CO$_3$ (6 g/L) at 4°C, centrifuged at 13,000 rpm, ultrafiltered with a Centricon 10 (Amicon Millipore®, Saint Quentin en Yvelines, France), and lyophilised (100mg/ml).

Protein extract from $Mi$ was produced as follows: mycobacteria from 20 densely grown Mueller-Hinton culture media were first suspended in 60mL of 1M Tris-HCl buffer pH7.5 supplemented with protease inhibitors (1mM Phenyl-Methane-Sulfonyl-Fluorure, 1mM Phenantroline, 1µM Pepstatin (Sigma-Aldrich®, St Louis, USA)). The mixture was lysed by grinding with an Ultraturrax® device then by sonication using 5 cycles of alternate 1 min pulsing and 1 min chilling on ice. Cell fragments were eliminated by two subsequent centrifugations for 3 min at 10,000 rpm at 4°C. The supernatant was kept, then 100µl of 0.1% deoxycholic acid solution (Sigma-Aldrich®) per millilitre was added and the preparation was incubated for 5 min at room temperature. Proteins were pelleted on ice for 15 minutes with 70µl of 0.6 M trichloroacetic acid (Sigma-Aldrich®) per millilitre of supernatant. Precipitated proteins were subsequently pelleted by centrifugation (Beckman Coulter®, JA 20.1 rotor, 4°C, 15 min, 30,000×g). Protein extracts were purified with an SDS-Page Clean-Up Kit (Roche Diagnostics®, Basel, Switzerland) as recommended by the manufacturer. Protein
concentrations of the purified extracts were determined using the DC Protein Assay (Bio-
Rad®, Marnes-la-Coquette, France) according to manufacturer's instructions.

Electrosyneresis on celluloseacetate (ES) Samples of 15 µL of each serum were placed on
three spots on the anode side, and a 15µL line of somatic antigen was placed on the cathode
side 3.5 cm from the first deposit (figure 1). The samples were washed and stained after 150
min of migration in a 90mV current. The number of arcs was determined for each serum and
results were analysed by ROC curve to determine a threshold between groups. (10)

Enzyme Linked Immunosorbutent Assay (ELISA) As previously described (10,12), the 96
well plates (PolySorp Immunomodule, Nalge Nunc®, Rochester, NY) were coated with
200µL of 1µg/mL protein antigen solution in 50 mmol/L K₂HPO₄ buffer (Sigma-Aldrich®),
pH 8.5 at 4°C for 48 hours. Excess binding sites were blocked at 37°C for 1 hour with 250µL
of 50 mmol/L NaH₂PO₄ (Sigma-Aldrich®), containing 0.5% bovine serum albumin (Sigma-
Aldrich®) and 60 g/L sorbitol (Sigma-Aldrich®). One-hundred microliter serum samples
diluted 1:200 were added in triplicate to all wells. The plates were incubated at 37°C for 1
hour under constant agitation. Plates were washed four times with washing buffer
(100mmol/L Tris-HCl, pH 7.5, 0.25% tween). Next, 100µL of peroxidase conjugated goat
anti-human IgG diluted 1:4000 (Sigma-Aldrich®) were added to all wells and the plates were
incubated at 37°C for 1 hour. The washing procedure was repeated once and 100µL of
3.3’5.5’ tetramethylbenzidine solution (TMB One-Step Substrate System, RD Biotech®,
Besançon, France) at room temperature were added to the wells for 10 minutes. To stop the
color reaction, 100 µL of an acid solution (0.5N H₂SO₄) were added. Wells were read
spectrophotometrically at 450 nm (Titertek Multiskan®, Huntsville, Alabama) and the results
were expressed in arbitrary units (AU).
**Statistical analysis:**

Concentrations of serum and antigens used were preliminarily determined by dilution assays. Sera were deposited on plates in triplicate and variation coefficients corresponding to standard deviation divided by mean of the triplicate were calculated. If a variation coefficient was above 15%, the serum was tested again. This was done only for OD above 0.050. A reference serum which was a pool of two sera of non exposed subjects, was deposited in triplicate on each plate. The ratio “mean of the triplicate for tested serum” under “mean of triplicate for reference pool” was used for analysis. This ratio was expressed in arbitrary units (AU). ROC curve analysis was chosen to compare diagnostic performance of immunological tests. The optimum cut-off level was determined with sensitivity and specificity tables provided by STATA Software Release 9 (StataCorp LP, College Station, TX). The value selected corresponded to the maximum sensitivity and specificity. The area under the curve indicated the discriminating capacity of the antigens. A high value for the area under the ROC curve indicated good sensitivity and specificity. Values under 0.5 indicated that the test could not discriminate patients (MWF-HP) from exposed controls (H-MWF).

A bilateral non-paired Student test was done to compare the ELISA and ES results (in AU and number of precipitins arcs) of patient and control groups. STATA Software Release 9 was used for this test too.
Results:

Subjects:

- MWF-HP was diagnosed in 13 male patients referred to the University Hospital of Lille between January 2004 and September 2007. Their mean age was 46.3 years (range from 34 to 56 years). All had been chronically exposed to MWF with a mean duration of exposure of 9 years (range from 3 to 20 years). Twelve patients were workers on a car-engine assembly line coating metal parts with semi-synthetic MWF. The other had been intermittently exposed to MWF once or twice a day when supervising the car-engine machine shops. All 13 MWF-HP patients showed significant improvement of clinical symptoms after stopping work for 1 month. Subjects with MWF-HP had clinical symptoms compatible with HP (crackles, cough, and/or fever, loss of weight, asthenia), low CO-diffusing capacity, lymphocytisis on bronchoalveolar lavage (BAL) (>20%). Radiographic imaging was done [high-resolution computed tomography (HRCT) lung scans] in order to assess nodules, ground-glass opacities, septal thickening, consolidation, honeycombing, traction bronchiectasis and bronchiolectasis, reticular pattern, pleural effusion and adenopathy (1,5,6). HRCT was performed as soon as possible after working periods. In cases 2, 3, 4 and 5, HRCT was performed from 6 to 9 weeks after exposure. HRCT was normal in 3 cases (cases 2,4,5). For these three patients, other criteria for HP diagnosis were present. Cases that had suggestive HRCT were classified as confirmed patients (n=10), and those who hadn’t were classified as probable patients (n=3). CO diffusion capacity was measured and bronchoalveolar lavages carried out (BAL). Serological analysis of sera were performed routinely to look for Aspergillus fumigatus and Pseudomonas antibodies, by electrosyneresis and ELISA. All were negative. Consequently serology research was extended to other antigens effectively present in MWF. The clinical characteristics of the MWF-HP patients are reported Table I.
- H-MWF were only non-smoking workers, with no underlying respiratory disease. All were asymptomatic, they had no crackles, lung function and transferred CO were normal. (HRCTscan and BAL were not performed).
- UC were also only non-smoking workers. They had no crackles at auscultation and gave negative responses to question about respiratory symptoms.

**MWF microbiological results:**

*M* was identified in 33 used MWF samples from the car-engine machine shops. No *M* was found in any samples of osmotic- ultrafiltered or industrial water or additives. All samples from new oil were sterile. Microbial contamination was found in 74/83 (89%) samples of used MWF.

*M* was isolated in 33/83 (40%) used MWF. Concentration of *M* varied from 2 to >4000 CFU/mL (mean 847 CFU/mL). *Bacillus* spp. (including *B. simplex* *(Bs)* was isolated in 35/83 (42%). Gram-negative bacteria (excluding *Pseudomonas* spp.) were present in less than 12% of used MWF. Fungi were isolated in 9/83 (11%) samples of used MWF, mainly *Fusarium solani* *(Fs)* (3/83, 4%) and *Aspergillus* spp. (5/83, 6%; *A. fumigatus* & *A. flavus*). In one case *A. fumigatus* was isolated with *Curvularia lunata* in the same sample

A polymorphic bacterial flora (including RGM in one case) was found in samples of osmosed water but no *M* (*Mycobacterium Llatzerense*) was isolated from these or from the washing effluents. Microbiological results by culture method are presented in Table 2.

**Precipitins and IgG research against Mi, Fs and Bs antigens:**
ES was performed to detect precipitin arcs against *M. immunogenum, F. solani* and *B. simplex* antigens, corresponding to micro-organisms present in fluids samples (table 3). For *M.i* antigen, the number of precipitin arcs was significantly higher for MWF-HP than for the H-MWF controls (p=0.002, T test). A five precipitin arcs threshold (superior or equal) showed area under ROC curve to 0.853 (95% confidence interval : 0.68-1), 77% for sensitivity and 92% for specificity. For *Fs* and *Bs* antigens, the number of precipitin arcs was not significantly higher for MWF-HP than for H-MWF controls (p=0.76 and p=0.65 respectively, T test). Areas under ROC curve were 0.468 (95% CI : 0.28-0.65) for *Fs* antigen and 0.571 (95%CI : 0.35-0.79) for *Bs* antigen. ROC curves were presented in figure 2.

The mean of AU results by specific *Mi* ELISA IgG was significantly higher in patients (MWF-HP) [2.6 (SD = 1.2)] than in H-MWF controls [1.1 (SD=0.3)] (p<0.001, T test) or unexposed controls (UC) [1 (0.4)] (p<0.001, T test). The difference between mean AUs for H-MWF and unexposed controls was not significant (p=0.29, T test).

Results in arcs for ES are reported table 3 and comparison of performances for three antigens by ROC curves were given in figure 1. Performances for ES and ELISA using *Mi* antigens were reported in table 4 with two different comparisons: results for H-MWF controls were compared first with results for all patients with MWF-HP (probable and confirmed patients, n=13), and second with results for patients with abnormal CT scan (confirmed patients, n=10). Determinate thresholds with ES and ELISA were equal for both comparisons. Performances of ELISA (sensitivity and specificity) were similar if we considered probable or confirmed patients. Sensibility and likelihood ratio of positive test for ES were slightly decreased when we considered only confirmed patients instead of all patients. Area under ROC curves were 0.853 (95%CI: 0.681-1) for ES and 0.936 (0.810-1) for ELISA in considering all patients and were 0.833 (0.615-0.955) for ES and 0.917 (0.718-988) for ELISA when considering confirmed patients only (figure 3 and 4).
Discussion:

Our study reports an outbreak of 13 cases of MWF HP arising at a single automobile factory between 2004 and 2007. To our knowledge, this is the second such episode of grouped cases arising in an automobile factory in Europe. The study demonstrates that *Mi* were identified in MWF even though *Mi* is weakly susceptible to common antiseptics. The detection of specific precipitins for *Mi* in sera, enabled a strong link to be established between MWF-HP patients and *Mi* contaminated-MWF. A threshold to differentiate MWF-HP cases from H-MWF controls is suggested.

Working procedures to limit exposure to MWF are particularly developed in the USA (1,13) where most MWF-HP have been described. Large volumes of metalworking fluids are used in manufacturing industries for cooling and lubrication of metal pieces and tools during machining, especially in the manufacture of automotive parts. Respiratory manifestations have been described, such as an increase in bronchial hyperresponsiveness, asthma (14), and HP (2, 15-17). MWF may be toxic in different ways, for example by inhalation of toxic particles, endotoxins (18,19), or a complex population of microbial flora (20). Bernstein and Coll. reported HP with the use of semisynthetic MWF, called “machine operator’s lung” (15). Aerobic gram-negative rods such as *Pseudomonas aeruginosa*, *Acinetobacter* (13), fungi, but also members of the *Mycobacterium chelonae* complex, including *M. abscessus*, *M. chelonae* and *M. immunogenum* have been identified in MWF (21-24). Mycobacteria and *Pseudomonas* may also be identified in MWF by real-time PCR assays (25). Role of microorganisms in the induction of manifestation of HP is commonly established by precipitins or specific immunoglobulin (IgG) response. Precipitins against *Pseudomonas fluorescens* were observed in serum from six workers from a plant in 1992 (15). Dawkins and Coll. (26) described 12 cases (2003-2004) of MWF-HP who had precipitins against *Acinetobacter* and
*Ochrobactrum*, but precipitins were also present in a similar proportion of exposed control workers without disease at the same plant. Many of MWF-HP were linked to MWF containing nontuberculous rapidly growing mycobacteria (RGM) (21,24,27-29). Wallace and Coll. showed that 95% of mycobacteria isolates recovered from industrial MWF in plants with cases of machinist HP belong to *Mi* (4, 24). MWF containing *Mi* induced granulomatous lung lesions and peribronchiolar lymphocytosis consistent with HP in laboratory mice (30). The present study brings additional evidence for the role of *Mi* in HP related to MWF exposure in humans. Gram-negative bacteria, such as *Pseudomonas*, are often predominant organisms in MWF (31-33). In our study, predominant bacteria were *Mi* and *Bacillus* spp. whereas gram-negative bacteria were present in less than 12% of used MWF.

Identification of pathogen in MWF and/or specific antibodies directed against the pathogen is essential for HP diagnosis. In the present work, we also suggested a threshold for *Mi* precipitins to differentiate MWF-HP cases from H-MWF controls. The ELISA method seems to be the stronger test to differentiate MWF-HP from H-MWF in this series. This difference may be related to the antigen extract which are different comparing ELISA and Electrosyneresis methods, (purified protein for ELISA). Previous studies have also suggested serological scores or thresholds to differentiate HP from exposed asymptomatic persons in farmer’s lung and bird breeder’s lung (34-36).

Without specific procedures to prevent MWF-HP and to identify cases by occupational MDs, there is a risk that only severe forms of the disease will be diagnosed. At the time of writing, 12 clustered cases have been described in Europe in UK (26), about 100 cases in USA (2,3). NIOSH (National Institute of Occupational Safety and Health) published a recommended exposure limit (REL) for MWF in 1998 to prevent respiratory disorders associated with
industrial lubricants (37). The REL is 0.4 mg/m$^3$ (37). Cohen and Coll. recently suggested that manufacturing companies failed to control the level of exposure to below 1 mg/m$^3$ and that protective equipment or medical surveillance programs are insufficient (37). Gupta & Rosenman indicate that HP arose from an environment with exposures well below the Occupational Safety and Health administration (OSHA) permissible exposure limit (PEL) for MWF, and in one case from exposures well below the NIOSH recommended exposure limit (REL) (38). We deduce that particular levels are insufficient to assess whether or not a risk of HP exists. In our opinion, microbiological surveys, especially with PCR-rt, are needed to assess presence or absence of $M_i$ to evaluated HP risk in a plant. Implementation of specific medical surveillance programs is essential in manufacturing plants. Factors associated with reduced aerosol exposure included machining aluminium, milling, the height of the machine shop roof and the presence of mechanical machine shop ventilation (39).

**Conclusion:**

The presence of large amounts of $M_i$ in more than 40% of used MWF samples taken from several machine shops at the same manufacturing plant and the detection of specific precipitins against $M_i$ Ag in sera, suggests that $M_i$ contaminated-MWF is responsible for MWF-HP. The threshold of five arcs by ES differentiates MWF-HP cases from H-MFW controls with a sensitivity of 77% and a specificity of 92%. An ELISA method using protein antigen improves this discrimination (Se 92%, Sp 100%). Clearly any investigation of contamination of MWF in a manufacturing plant must include identification and quantification of RGM with special focus on $M_i$. The introduction of a prevention program of regular sampling and analysis the MWF, adequate protective action and medical surveillance of exposed workers may prevent or minimize HP disease.
Future studies involving several plants with new cases of MWF-HP are needed to confirm that the thresholds are able to discriminate between patients and exposed controls.
References:


Table 1: Characteristics of MWF-HP patients (n=13)

<table>
<thead>
<tr>
<th>Case</th>
<th>Smoking</th>
<th>Clinical presentation</th>
<th>Crackles</th>
<th>DL₅₀ (%) predicted</th>
<th>PaO₂ (mmHg)</th>
<th>HRCT scan</th>
<th>BAL Lympho (%)</th>
<th>BAL Neutro (%)</th>
<th>BAL Cells/ml (x10⁴)</th>
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<td>1</td>
<td>NS</td>
<td>Chronic progressive</td>
<td>Yes</td>
<td>47</td>
<td>66</td>
<td>Ground glass, nodules, honey, combing and reticular patterns</td>
<td>61</td>
<td>10</td>
<td>41</td>
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<td>2</td>
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<td>Acute intermittent</td>
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<td>3</td>
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<td>4</td>
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DL\textsubscript{CO} = diffusing capacity of the lung for CO/ alveolar volume ; PaO\textsubscript{2} = oxygen partial pressure in mmHg ; HRCT scan = high-resolution computed tomography ; # HRCT scan was performed 6 to 8 weeks after exposure in case 2,3,4 and 5. BAL = bronchoalveolar lavage ; Lympho = lymphocytes; Neutro= neutrophiles ; NS = non smoker ; cig/d = cigarettes /day ; p/y = tobacco packet / year ; ND = not determinated
Table 2: Isolation frequencies for the microorganisms in fluids samples, water and additive (total=97)

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Number of positive samples</th>
<th>Number of sterile samples</th>
<th>Mi</th>
<th>Ml</th>
<th>A. f</th>
<th>Fus</th>
<th>Oth fun</th>
<th>Bac</th>
<th>Other bacteria</th>
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<td>New MWF</td>
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<td>0</td>
<td>0</td>
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<td>0</td>
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<td>0</td>
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<tr>
<td>Used MWF</td>
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<td>33</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>35</td>
<td>9</td>
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<td>0</td>
<td>0</td>
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<td>Osmosed water</td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Ultrafiltrated water</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

MWF = metalworking fluids; Mi = Mycobacterium Immunogenum; Ml = Mycobacterium latzerense; A. f = A. fumigatus; Oth. fun = Other fungi (Aspergillus flavus n=1, Curvularia lunata n= 1, Acremonium sp. n=1, Penicillium sp.n=1); Bac= Bacillus spp.; Other bacteria (non mycobacteria, non actinomycetes)
Table 3: Number of precipitin arcs for three antigens by electrosyneresis in metal-working fluid associated hypersensitivity pneumonitis (MWF-HP) and exposed controls (H-MWF).

<table>
<thead>
<tr>
<th>Group</th>
<th>Case number</th>
<th>Mycobacterium immunogenum</th>
<th>Fusarium solani</th>
<th>Bacillus simplex</th>
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<tbody>
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<td>9</td>
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<td>7</td>
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<td>0</td>
</tr>
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<td>5</td>
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<td>2</td>
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<td>0</td>
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<tr>
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<td>12</td>
<td>0</td>
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</tr>
</tbody>
</table>
Table 4: Parameters for immunological tests using antigens of *Mi* and comparing

(1) Total of patients (n=13) to exposed controls (H-MWF) (n=12) and

(2) Confirmed patients (n=10) to H-MWF (n=12).

<table>
<thead>
<tr>
<th>Method</th>
<th>threshold</th>
<th>(1) total of patients <em>versus</em> exposed controls</th>
<th>(2) Confirmed patients <em>versus</em> exposed controls</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(1 total of patients)</td>
<td>(2 Confirmed patients)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(versus exposed controls)</td>
<td>(versus exposed controls)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Method</td>
<td>Se % (CI 95%)</td>
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<tr>
<td>ES (in arcs)</td>
<td>5</td>
<td>77</td>
<td>(46-94)</td>
</tr>
<tr>
<td>ELISA (in AU)</td>
<td>1.6</td>
<td>92</td>
<td>(62-99)</td>
</tr>
</tbody>
</table>

ES = electrosyneresis; ELISA = Enzyme Linked Immunosorbent Assay; AU = arbitrary unit; AuC = area under the curve; CI95%: 95% confidence interval; Se % = percent of sensitivity; Sp % = percent of specificity; LR+ = likelihood ratio of positive test; LR- = likelihood ratio of negative test.
Fig. 1 Electrosyneresis with Mycobacterium immunogenum antigen against 3 sera of MWF-HP cases

Serum 1: case n°8 (3 arcs)
Serum 2: case n° 11 (7 arcs) from May 2007
Serum 3: case n° 11 (7 arcs) from September 2007
Figure 2: ROC curve comparing three antigens in Electrosyneresis.
Figure 3: ROC curves comparing Electrosyneresis and ELISA results with Mi Ag for total of patients (n=13) and exposed controls
Figure 4: ROC curves comparing Electrosynthesosis and ELISA results with Mi Ag for confirmed patients (n=10) and exposed controls