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Of mice and men: Traces of life in the death registries of the 1630 plague in Milano

Alfonsina D'Amato[a], Gleb Zilberstein[b], Svetlana Zilberstein[b], Benedetto Luigi Compagnoni[c], Pier Giorgio Righetti[d,*]

[a] Quadram Institute Bioscience, Norwich Research Park, NR4 7UA, England, United Kingdom
[b] Spectrophon Ltd., Pekeris 4, Rehovot 76702, Israel
[c] Archivio di Stato di Milano, Via Senato 10, Milano 20121, Italy
[d] Department of Chemistry, Materials and Chemical Engineering "Giulio Natta", Politecnico di Milano, Via Mancinelli 7, Milano 20131, Italy

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ABSTRACT

The death registries of the plague epidemic of 1630, stored at the Archivio di Stato of Milano, have been interrogated via the EVA film technology (ethyl vinyl acetate film studded with crushed strong anion and cation exchangers as well as C_8 resins). The EVA diskettes have been left in contact with the lower right margins of 11 different pages pertaining to the peak months of the raging disease (June through end of September) for 60–90 min and then the captured material, after elution and digestion, analysed by mass spectrometry. The main findings: 17 *Yersiniaceae family proteins*, 31 different human keratins, 22 unique mouse keratins, about 400 peptides from different bacterial strains, 58 human tissue proteins and 130 additional mouse and rat tissue proteins. In addition, > 60 plant proteins (notably potato, corn, rice, carrot and chickpeas), likely representing the meagre meals of the scribes, contaminating the pages, were detected. The significance of these unique findings is amply illustrated in the body of the article.

Significance: Archivists, historians, librarians usually explore the texts of ancient and modern manuscript in order to extract the meaning of the writing and understand the mood, feelings, political, philosophical and/or religious ideas therein expressed by the authors. With the present EVA methodology (the only one, at present, able to access our Cultural Heritage without damaging or contaminating it) we interrogate, instead, the support, be it paper, parchment, wood panel, cloth, canvas and the like, in order to extract invisible data, such as the presence of drugs, medicaments, infectious pathogens, human and environmental contaminants. Metabolites, proteins and peptides thus captured are then analysed via mass spectrometry. The unique data mined by this technology should considerably enlarge the (so far) restricted horizon of the writing exploration and add new insight on the environmental conditions in which such documents were produced as well as, importantly, on the health/pathological conditions of the authors. It is believed that the present technology, as here reported, will become the officially accepted one for exploring the world Cultural Heritage.

1. Introduction

Barrages of epidemics, typhus with his brothers and sisters, such as plague, cholera, typhoid and dysentery, have scourged humanity for millennia and have decided more military campaigns than Caesar, Hannibal, Napoleon, to mention just a few. Perhaps, over the centuries, the most devastating one has been plague [1–4]. The first literary citation on this disease can be found in the Iliad of Homer (I, 43–61) and later on in Sophocles, Thucydides, Lucretius, Virgil, Ovid and Seneca, to name just some classic authors.

Bouts of plague kept spreading around Europe at regular intervals, till the end of the XVIII century. In Milano, there were two such episodes that drastically altered the population of the town as well as of the surrounding region of Lombardy. The first one occurred during 1578, at the time of cardinal Carlo Borromeo (later declared a saint). The cardinal daily visited the affected people and organized processions and public masses not in churches but in open squares, so as to avoid strict contact among the believers. Notwithstanding the daily contacts, the cardinal was spared by the disease. A most devastating epidemic occurred in 1630 at the time of cardinal Federico Borromeo, Carlo's cousin. Early episodes were registered around mid-March, the peak being reached in the summer months, June through September, the

* Corresponding author at: Department of Chemistry, Materials and Chemical Engineering "Giulio Natta", Politecnico di Milano, 20131 Milan, Italy.
E-mail address: piergiorgio.righetti@polimi.it (P.G. Righetti).

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epidemic fading away by the end of November 1630. This plague visit claimed a heavy toll as well: about 120,000 died in the entire Lombardy region, eliminating one half of the entire population, during the peak months the victims being estimated at up to 2000 per day. The ravaging of the 1630 plague was amply described in different chapters (XXXI, XXXII and XXXV) of the novel "I Promessi Sposi" (The Betrothed) by Alessandro Manzoni, a book that has been mandatory reading in all junior high schools in Italy for well over 150 years.

We learned that death registries, compiled during the scourging months of plague, were stored at the State Archives in Milano, so we decided to explore these documents in order to extract information usually not accessible to librarians, historians and archivists, who typically try to decode the writing but have no idea of the wealth of information stored on the support (be it paper, cloth or any other material, including, of course, parchment and papyri). We have recently developed a technique, known by the acronym of EVA (ethyl vinyl acetate with embedded resins for capture of positively and negatively as well as hydrophobic analytes) that permits to interrogate our Cultural Heritage while avoiding its contamination or any possible damage. The EVA methodology has given us unique and unexpected results in consulting the margins of the original manuscript of Master y Margarita by Bulgakov, concerning his health state and assumption of medicaments. In a further physico-chemical analysis of the EVA diskettes (or films) their capture ability and absence of insults to the specimens under analysis was also amply proven [5–7]. On these understanding, the director of the State Archives gave us access to these documents. The unique and unexpected results of this exploration are illustrated below.

2. Materials and methods

2.1. Chemicals and reagents

Methanol (LC-MS Ultra CHROMASOLV, > 99.9%), 2-propanol (LC-MS Ultra CHROMASOLV, > 99.9%), acetic acid (eluent additive for LC-MS, > 99.9), ammonium bicarbonate (AMBIC), dithiothreitol (DTT), iodoacetamide (IAA), formic acid (FA, eluent additive for LC-MS, > 99.9), ammonium acetate, trifluoroethanol (TFE) (> 99%), ammonium hydroxide, and water (LC-MS Ultra CHROMASOLV, > 99.9%) were purchased from Sigma-Aldrich (Milwaukee, USA). Microcon YM-5, and C_{18} Zip-tip pipette tips, were from Millipore U.K. Limited. The trypsin was from Promega, USA. Acclaim PepMap C_{18} columns were from Thermo Scientific, USA. The ultrapure water was obtained through a Millipore Milli-Q system (Milford, USA). The mixed-bed cation (SCX)/anion (SAX) exchange resins AG501 and C_8 resins were from Bio-Rad (Hercules, USA).

2.2. Archive material

The following documents pertaining to the death registries of the 1630 bout of plague, stored in the State Archives in Milano, have been explored: Atti di governo, Popolazione parte antica, thread 119; Atti di governo, Sanità parte antica, threads 279, 279bis and 280. A total of 11 pages have been interrogated with the EVA diskettes. In addition, one "grida" (a manifesto read aloud by herald in towns and then pinned to the entrance gates) has been explored, as affixed in Cremona on 17th of June 1630, to warn the population to stop commerce with neighbouring villages on accounts of infection having spread into town (size of the foil: 40 × 30 cm).

2.3. Synthesis and characterization of the EVA film

A special plastic-like film based on ethyl-vinyl acetate (EVA) as binder of ground AG 501 mix-bed cation/anion exchange and C_8 resins (all from Bio Rad) was prepared. A mixture was made comprising 70% 1–10 μm size ground beads and 30% EVA (the melting temperature was 75 °C). This mixture of melted EVA and Bio-Rad resins was poured in a "Brabender" mixer W30 and extruded via a "Brabender" extruder KE19 (both from Brabender GmbH, Duisburg, Germany) in the form of either a thin film or diskettes. The thickness of the diskettes was 200 to 500 μm; they were wetted in doubly distilled water prior to their applications to the surface of the specimens under investigation. The contact time was 60 to 90 min.

2.4. Extraction protocol

The diskettes were gently wetted with ultrapure water and then placed in contact with the surface of the foils of the death registries for 60 to 90 min. The proteins were eluted from the diskettes sequentially with 200 μL of volatile buffers (formate at pH 3, followed by ammonia at pH 10) and finally with volatile solvents (acetonitrile) so as to collect separately positively and negatively charged as well as hydrophobic proteins.

2.5. Mass spectrometry analysis and protein identifications

The dried eluates were suspended in 8 M urea, reduced by 5 mM DTT and alkylated by 15 mM IAA. The buffer was exchanged by filter unit of 5 kDa cut-off, using 50 mM AMBIC. Alternatively, the dried eluates were loaded on precast gradient gels (Invitrogen) and Coomassie stained blue lanes were cut, reduced by 1.5 mg/mL DTT and alkylated by 10 mg/mL IAA. The proteins were digested by 0.5 μg of trypsin, overnight at 37 °C. The peptide mixtures were purified by C_{18} pipette tips and analysed by nLC MSMS, using an Orbitrap Fusion trihybrid mass spectrometer coupled with a nano-flow UHPL system (Thermo Fischer Scientific, USA). The peptides were separated, after trapping on a C_{18} pre-column, using a gradient of 3–40% acetonitrile in 0.1% formic acid, over 50 min at flow rate of 300 nL/min, at 40 °C. The peptides were fragmented in the linear ion trap by a data-dependent acquisition method, by selecting the 40 most intense ions. The raw data were analysed by Mascot (version 2.4.1) by consulting the following protein databases: SwissProt without taxonomy restriction (545536 total sequences, 194023197 total residues), to explore a wide range of potentially organisms and then targeted databases: UniProt_*Yersina pestis* (24291 total sequences, 7342141 total residues), and Swiss Prot, selecting *Viridiplantae* and *Bacteria* taxonomies (38546 and 314940 total sequences). The tolerance on parents was 20 ppm and on fragments was 0.80 Da. The variable modifications allowed were oxidation on methionine. The false discovery rate was below 0.1% and the identified proteins contained at least 2 peptides. The meta-proteomics analyses were performed by consulting the Unipept analysis pipeline (http://unipept.ugent.be) [8,9], using the peptide matches assigned by Mascot to *Bacteria* and *Yersinia pestis* protein families (Supplementary Table 3). The Gene ontology analyses were performed by Panther (http://pantherdb.org/).The identified proteins and their peptides, with all variable modifications, peptide sequences and scores, are shown in Supplementary Tables 1–2. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [10] partner repository with the dataset identifier PXD008103 and https://doi.org/10.6019/PXD008103 (Username: reviewer78323@ebi.ac.uk; Password: Ah0KdyVy.journal).

3. Results

Fig. 1 gives an example of one of the pages analysed. At the top centre is the day in which the deaths were registered. Here, though, it is stated "Die ante dicto" (day specified in the previous pages) since in this day 10 pages listing death people were filled and this is page No. 7. The significance of the various capital letters preceding the name of the person will be illustrated in the Discussion. Each person is listed by name, followed by the age, the reason of death ("*ex peste obiit*" or "morto di peste", i.e. died of plague; in one case "febre acuta maligna", i.e. acute malignant fever). Each entry terminates with the name of the officer who provided the diagnosis, mostly not a physician but a barber (e.g., in the top entry "ind. Jo. Della Porta barbitonsoris", i.e. indicated by Joannes della Porta, barber).