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1 Expansion of Microbial Forensics

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12 Running Head: Expansion of Microbial Forensics

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18 Abstract

19

20 Microbial forensics has been defined as the discipline of applying scientific methods to
21 analyzing evidence related to bioterrorism, biocrimes, hoaxes, or the accidental release of a
22 biological agent or toxin for attribution purposes. Over the past 15 years technology, particularly
23 massively parallel sequencing, and bioinformatics advances now allow characterization of
24 microorganisms for a variety of human forensic applications, such as human identification, body
25 fluid characterization, post-mortem interval estimation, and biocrimes involving tracking of
26 infectious agents. Thus, microbial forensics should be more broadly described as the discipline of
27 applying scientific methods to analyzing microbial evidence in criminal and civil cases for
28 investigative purposes.

29

30 Key words: Microbial forensics, bioterrorism, biocrime, human identification, epidemiology,
31 validation

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32 The anthrax letter attacks of 2001 in the United States ushered the world in a new reality and
33 increased awareness of global vulnerability to bioterrorism. In addition, the event demonstrated
34 that as a nation the United States was woefully unprepared to characterize the biological
35 evidence associated with the case. As a consequence, the field of microbial forensics was
36 developed to build a robust forensic capability to help investigate bioterrorism and biocrime.
37 Microbial forensics is the discipline of applying scientific methods for analyzing evidence from a
38 bioterrorism attack, biocrime, hoax, or inadvertent release of a biological agent or toxin with
39 attribution as the ultimate goal (1). Attribution of microbial evidence is to determine an
40 associated source and/or perpetrator or group of individuals to the highest degree possible. The
41 microbial forensics field is built on a network of multiple specialties (e.g., microbiology,
42 genetics, bioinformatics, forensic science, immunology, population genetics, biochemistry,
43 molecular biology, epidemiology, etc.) and the law enforcement, public health, policy, and
44 intelligence communities. While the field was formalized shortly after the anthrax letter attacks,
45 its roots are well-established as they are embedded in the same practices used for decades in
46 epidemiology and public health to investigate disease outbreaks. Epidemiologists focus on the
47 outbreak, the population(s) at risk, spread of disease, possible reservoirs, and characterization of
48 the etiologic agent (2), to serve primarily the health care system. Both epidemiology and
49 microbial forensics are employed together to attempt to determine if an outbreak is natural,
50 accidental, or intentional. Therefore, the two disciplines are integrated and specialists tend to
51 work together with the latter concentrating on individualization of the agent or toxin and/or how
52 it was produced and disseminated. In addition, traditional forensic methods, such as fingerprints,
53 human DNA, trace materials, and handwriting, are exploited in a microbial forensic investigation
54 as ultimate attribution is identification of the perpetrators of the crime.

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55

56 There are over 1400 microbial species or strains that are potential health threats to humans (3)
57 and the number expands by orders of magnitude when considering plant and animal pathogens.
58 While high consequence agents (4) have been targeted for preparedness, they are but a small
59 percentage of possible biothreats, and it is not practical to continue to develop assays directed at
60 single targets. There are simply far too many targets. Only 20 years ago, it took 13 months and
61 cost more than $870,000 for the first bacterial genome to be sequenced and assembled by the
62 Institute of Genome Research (Rockville, MD) (5, 6). Seven years later for about the same
63 amount of time and a lesser cost (approximately $200,000-$300,000 for the first genome)
64 genomic sequences were obtained of the *Bacillus anthracis* Ames strains from the evidence in
65 the letter attacks and purported reference samples (7–9). Technical advancements in recent
66 years, through the advent of massively parallel sequencing (MPS) (which also has been referred
67 to as next-generation sequencing (NGS) and high-throughput sequencing (HTS)), allow analysis
68 of microbes with a throughput and speed that was not thought possible a short time ago. MPS, a
69 disruptive technology and a boon to microbial forensics, may overcome the challenge of
70 identifying unknown pathogens, hoax microorganisms, and low-abundance microorganisms even
71 in complex mixture samples. With its substantially increased throughput and continued
72 development of powerful bioinformatics pipelines, MPS may be used to characterize any
73 microbe, abundant or trace, degraded or intact, and even genetically engineered genomes with
74 one unifying approach. MPS provides an ability to rapidly diagnose and monitor infections
75 using culture-independent methods (thereby reducing cost and turn-around time) and track
76 disease outbreaks in real-time using whole-genome comparisons (10–12). Indeed, Cummings
77 *et al.* (13) showed several years ago the forensic capability of MPS to rapidly and reliably

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78 sequence multiple whole genomes. Since then epidemiologists have applied MPS to several
79 outbreak investigations (10–12, 14–17), and it is anticipated that MPS eventually will become
80 the routine method for genetic analyses. In addition, MPS provides a methodology for human
81 microbiome studies, which provide inference into different health and disease states and impact
82 on conditions such as obesity, inflammatory bowel syndrome, effects from antibiotic use, and
83 cancer (18–20). These same tools have been used to characterize the complex community of the
84 human microbiome and have been demonstrated for use in human forensic applications, such as
85 human identification, body fluid characterization, and time-since-death decomposition analysis.
86
87 Since the field of microbial forensics was developed in response to exigent circumstances, it was
88 narrowly defined concentrating on the immediate concern - bioterrorism. Other examples of
89 microbial forensics investigations included tracing transmission of human immunodeficiency
90 virus (HIV) and hepatitis C virus (HCV) in criminal health related matters (21–28) which fall
91 under the biocrime category (Table 1).Therefore, microbial forensics focused on investigations
92 where the microbe or its products, (e.g., toxins) were used as weapons or biothreats. However,
93 over the past 15 years technological advances and the realization of the vastness and abundance
94 of the microbial world prompt the field to expand in other areas where microbes and their
95 products may help other types of forensic investigations, including human identification (29) and
96 post-mortem interval estimation (30). This expansion of exploiting microbes forensically beyond
97 investigating bioterrorism and biocrime requires a more comprehensive definition for the field of
98 microbial forensics. Microbial forensics now should be broadly defined as the discipline of
99 characterizing microbiological evidence to develop investigative leads in criminal (and civil)
100 cases.

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101

102 The expansion of the microbial forensics field extends into the realm of forensic human identity
103 testing. Human microbiome analysis can be combined with traditional human DNA testing (e.g.,
104 short tandem repeat (STR) and single nucleotide polymorphism (SNP) analyses) to potentially
105 provide additional data for stronger associations and better chances to exclude individuals falsely
106 associated with biological evidence (Figure 1). Human DNA-based identity testing allows for
107 analysis of stable, inherited markers in the human genome related to individualization, kinship,
108 ancestry, and phenotype. In contrast, forensic microbiome testing allows for the analysis of both
109 stable and fluctuating changes in microbial communities on and in the body related to
110 individualization, diet, health, recent geolocation, and post-mortem time intervals. Microbial
111 genetic markers can be used to expand upon current forensic genetic testing capabilities.

112

113 Microbial Forensics and Human Identity Testing

114

115 Humans are born with approximately 20,500 genes and die with over 1,000,000 genes (31–33).
116 This change in gene count is due to the accumulation of microorganisms as part of the normal
117 development and existence of human beings. Indeed, there are ten times more bacterial cells in
118 and on the body than there are human cells, making us reconsider what makes us human. The
119 substantial microflora that humans carry is known collectively as the human microbiome (32,
120 33). The majority of human microbes are autonomous, self-replicating, transmissible,
121 unavoidable, and, in general, ubiquitous, although they may vary to some degree or substantially
122 from human-to-human (34, 35). The microorganisms, and their accompanying nucleic acids, that
123 are carried by humans are shed, deposited, and exchanged routinely, in a similar fashion to

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124 human DNA which is exploited for identifying people involved in sexual assaults, murders and
125 burglaries. These microbial genomes from many different microbial species collectively are
126 complex and variable and may provide forensic signatures that could be as individualizing as are
127 the current set of human short tandem repeat markers (36) used for identity testing purposes. In
128 addition, since microorganisms that reside in different areas of the human body vary, additional
129 investigative value could be obtained for determining the tissue source of forensic biological
130 evidence. Thus, the human microbiome may be another target that could be used to identify (or
131 exclude) humans involved in crimes.
132
133 When people touch items, they often transfer their DNA onto objects (via primary, secondary
134 and tertiary transfer) (37, 38). Therefore, an individualizing signature is left behind that can be
135 exploited to determine the identity of an individual who may have handled an object. Forensic
136 DNA typing characterizes genetic signatures from human biological samples. The current DNA
137 typing methodologies focus on markers in the human genome and are sensitive, highly
138 discriminating and well-validated (for examples see (39–41)). Unfortunately, the amount of
139 human DNA deposited by touching an object often is very low, and most technologies cannot
140 reliably type such low levels of DNA. To attempt to obtain results from such limited samples,
141 modifications of current methods are made to increase the sensitivity of detection of human
142 DNA. Collectively, the suite of methods that increase the sensitivity of human DNA typing
143 protocols are known as low copy number (LCN) typing (42–44). Under LCN typing, limited
144 template analyses suffer from exaggerated stochastic effects and with increased sensitivity there
145 is a greater potential for contamination. This lack of reproducibility with LCN typing results has
146 not deterred the interest in LCN typing and its potential use for developing investigative lead

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147 purposes. One way to partially overcome the uncertainty of LCN typing methodology is to
148 employ an orthogonal approach. In fact, each technology, i.e., LCN typing and the orthogonal
149 methodology, may not be robust, but when performed together, the information may be
150 sufficiently corroborating to decrease uncertainty, enabling better use results from trace
151 biological sample analyses for developing investigative leads.
152
153 Given the much greater number of bacterial cells compared with human cells, it is conceivable
154 that more bacterial cells and thus gene targets are deposited on touched items than are human
155 markers. Indeed, Grice *et al.* (45) showed that 10,000 bacteria/cm^2 and 50,000 bacteria/cm^2
156 could be collected by swabbing and scraping, respectively, the skin. Of course the population of
157 bacterial cells is comprised of many species with varying abundance levels. However, a
158 combination of enrichment methods, such as PCR, and sequencing can be used to detect those
159 species that may be used to individualize their human hosts. Goga (46) sought to analyze
160 bacterial DNA from shoes as the quantity of human DNA often was at LCN typing levels. They
161 demonstrated that there are sufficient bacteria in shoes and the plantar skins of individuals and
162 that the microbiome communities were unique among the individuals tested. A level of
163 "matching" was possible between shoe and wearer profiles. However, the results varied
164 substantially and in a few comparisons the wearer and another individual could not be resolved.
165 Tims *et al.* (47) analyzed the microbiome before and after hand washing and observed similar
166 variation. They opined that constant bacterial contamination by touching may impact the results.
167 These findings were not similar with those of Fierer *et al.* (48, 29) who collected reference
168 bacterial samples without washing and successfully matched reference microbial communities to
169 those deposited on touched objects, such as keyboards and computer mice. Fierer *et al.* (48)

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170 suggested that washing may have changed the composition of the hand skin microbiome. For
171 hand microbiome profiling, an unwashed hand may be a better starting point for studies. More
172 study is needed to determine the suitability for touch sample analyses.
173
174 Gut microbiome profiling also may be an important tool for forensic human identity testing.
175 There is evidence that core gut microflora of an individual are stable (49)(50) but can be
176 affected by environmental changes and antibiotic use (51)(52). Fecal material has been found at
177 some crime scenes and determining the source of such evidence may be an important
178 investigative lead. Typically, human STR profiling is attempted but with mixed success.
179 However, fecal material, the primary material used to study the gut microbiome, carries a
180 microbiome profile that could be exploited for identification purposes. The stable microbiome
181 may provide identity information while fluctuating or transient microbiota could indicate recent
182 diet or geolocation.
183
184 Identification of the tissue source of forensic biological samples can be critical in some
185 investigations to reconstruct crime scenes and events, but current techniques are limited.
186 Presumptive tests for the specific body fluids are used as screening tests and tend to have
187 specificity limitations. Screening, often quick and inexpensive, is used to select the best
188 candidate samples for more in-depth testing. Confirmatory tests identify tissues with high
189 specificity. While gene expression (mRNA typing) (53, 54) and methylation (55) have been
190 described, most presumptive and confirmatory tests are protein-based enzymatic or immunologic
191 assays. The exception is microscopic visualization of sperm for identification of semen. Proteins
192 are less stable than DNA and a negative result may not indicate reliably the tissue source of a

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193 sample or the amount of DNA in a sample. Most tissue assays are non- or semi-quantitative
194 without a defined threshold for a positive reaction. Because of the different assay formats for
195 each tissue specific target, presumptive and confirmatory tests cannot be run in a similar parallel
196 manner, adding to labor demand and difficult to automate in a cost effective way.
197
198 Unique bacterial genetic signatures may aid in biological sample tissue source determination. For
199 example, *Lactobacillus crispatus, L. jensenii* and *Atopobium vaginae* have been associated with
200 vaginal secretions, while *L. iners, L. gasseri* and *Gardnerella vaginalis* have been found in other
201 body fluids as well (56–58). Tissue specific in the vagina could be informative in rape cases.
202 Nakanishi *et al.* (59) showed that *Streptococcus salivarius* and *S. mutans* could be detected in
203 mock forensic saliva samples and were not present in other forensically-relevant tissue sources.
204 Choi *et al.* (60) had similar findings for *S. salivarius*. These species are relatively abundant and
205 thus may be easily detected.
206
207 Nose and throat (respiratory and digestive) commensal bacteria are likely candidates for
208 geographical and recent contact information because they demonstrate high temporal stability
209 (persistence), are contagious organisms (airborne and fomites), exhibit clonal and geographical
210 variation and diversity, colonize a large portion of the population, and are easy to collect (e.g.,
211 see (61, 62)). Scheidegger and Zimmerli (63) have shown that *Staphylococcus aureus* is a high
212 incidence bacterium among drug users. Shared drug paraphernalia can be an avenue for bacterial
213 transmission (64) which can be colonized transiently or permanently (65). A study of
214 inhalational drug users detected similar *S. aureus* strains between users and drug paraphernalia
215 (66). The *S. aureus* strain analysis traced back fourteen social networks (known groups of

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216 individuals connected by social interaction and reported drug use) linked to a crack house, while
217 only two of the biological networks (nasal culture and drug paraphernalia linked by significant
218 similarity among isolates) were identified by social network analysis. These data demonstrate
219 that behavioral, social and environmental networks may be identified by analysis of a targeted
220 species of the human microbiome. Because environment is related to social networks, hygiene,
221 diet, geography, etc., microbiome analyses could be an additional tool for intelligence gathering.
222 Lastly, the profiles could be used to confirm or refute what persons of interest claim about where
223 they have been and with whom they have associated. If microbial profiles of an individual reflect
224 the unique nature of a complex microbial community, microbial profiles may be better than
225 human DNA typing for recent geolocation as the environment (including contact with groups of
226 individuals) may carry location specific microorganisms that will be exchanged through human
227 contact.
228
229 Tracking sexually transmitted diseases in child molestation, rape cases and questioned
230 malpractice cases in healthcare provider outbreaks have been well documented for HIV and
231 HCV cases (21–28) and have been part of the microbial forensics arena. A full scale analysis
232 which demonstrated the potential of such investigations was reported by Gonzáles-Candelas *et*
233 *al.* (28). These authors described in detail a case of a Spanish anesthetist convicted of
234 malpractice by infecting over 270 of his patients with hepatitis C virus (HCV). The case is
235 particularly interesting due the time frame of the investigation some 25 years after the first
236 suspected transmission(s) and by the number and complexity of potential infectious events (>
237 300 candidates from two hospitals). Thus, scientifically challenging, but forensically obvious
238 questions were posed to the experts: 1) Was the suspect the source responsible for the outbreak?;

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239 2) Could it be ascertained whether the patients that had been infected share a common source
240 and thus could be included in the outbreak?; 3) Alternatively, which patients could have been
241 infected from other sources?; 4) Could these alternative sources or the existence of different but
242 simultaneous outbreaks be determined?; 5) Could the duration of the outbreak be determined?;
243 6) Could the time of infection for each patient in the outbreak be estimated?; and 7) Could the
244 date of infection of the anesthetist be estimated? These questions were tackled by sequencing
245 229-nucleotides of a conservative nonstructural NS5B gene using standard Sanger sequencing
246 technology. After systematic and rigorous analyses the authors concluded that the suspected
247 health-care professional was indeed the source of the HCV infection of a portion of infected
248 individuals and provided likelihood ratios to support the findings vs an alternative hypothesis. As
249 in many other forensic DNA investigations the data were not used to prove if the suspect was
250 guilty as the results only provide identity of the virus and strain (or quasi-species). The data
251 combined with other evidence were used to convict the anesthetist.
252
253 The human virome also can be used potentially for characterizing unidentified cadavers, even in
254 cases of skeletonized remains. Toppinen *et al.* (67) are the first to describe the suitability of bone
255 as source for exploration of DNA viruses (as ancient DNA researchers have shown describing
256 bacterial strains of plague victims from past pandemics (68–70)), but instead for forensic identity
257 purposes. They showed parvovirus B19 DNA sequences were found abundantly in 70-year-old
258 long bones of putative casualties from World War II. The reported viral sequences were
259 exclusively of a genotype, which disappeared from circulation in 1970´s, or of a genotype which
260 has never been reported in Northern Europe. By adding the viral information to that from human

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261 mitochondrial and Y-chromosome profiling, the authors concluded a dispute of the origin of two
262 individuals found in the battlefield between Finland and Russia.
263
264 These cases provide insight into the potential of exploiting the microbial world for forensic
265 purposes. It is clear that the human microbiome holds substantial information to assist the
266 forensic science community's analysis of a variety of case scenarios.
267
268
269 MPS Approaches
270
271 Most microbial analyses methods, and in particular metagenomics, have focused on the single
272 target 16S rRNA gene, which lacks species level resolution. Some of the above mentioned
273 studies targeted only a few known species that may be unique to a body fluid or tissue, such as
274 saliva or vaginal secretions. They only require a positive/negative result for tissue sourcing.
275 However, most of the studies were not sufficient to unequivocally identify the targeted species
276 among a complex metagenomics background necessary for forensic investigations, and generally
277 they were not sufficient to provide high-depth characterization at the sub-species or genotype
278 level. Lastly, such tissue sourcing assays often require redesign of primers to increase specificity
279 and continual evaluation of potential false-positives with increased sampling and databases as
280 they are updated with new genomic sequences. If specialized primer design is required for
281 detection of every, or most microorganisms of interest, there will be great demand on technology
282 development and require substantial and costly validation.
283

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284 Current major genetic and health efforts are describing the diversity of the human microbiome
285 (18, 32, 35, 45, 71–73). However, little work has been carried out on how individualizing the
286 profiles may be, what microorganisms are common to all humans, what portion of these
287 common human microbiome species are stable throughout an individual's lifetime (or at least for
288 reasonably long time periods), and what portions vary due to environmental conditions. Such
289 questions cannot be answered until 1) a defined set of resolving markers are developed to assay
290 the human microbiome; and 2) a defined set of bacterial species are determined that are best
291 suited for human identity testing.
292
293 There are technical challenges to identify bacteria of interest within highly complex
294 metagenomic samples, to distinguish those of interest from near-neighbors and from the vast
295 complex background that constitutes a microbial sample taken from body areas, and the degree
296 of confidence that can be assigned to a potential species that is detected in such complex
297 samples. One approach to meet these objectives is to develop a panel of bacterial markers that
298 have the potential of differentiating human hosts and that can be multiplexed in a MPS system. A
299 targeted bacterial marker panel would provide an efficient and cost-effective method that
300 balances sequence coverage and throughput and yet provides species level resolution. MPS could
301 provide a single, unifying methodology so the power of the assay can be realized and resources
302 for testing and validation can be used in a cost-efficient manner. Sensitive and accurate bacterial
303 DNA detection is imperative for forensics analyses. Validation is an essential part of any assay
304 and should be become a routine part of technology development and implementation (see
305 below). The approach to achieve the goals of human identification through the microbiome is
306 essentially targeted genomics to survey the variety of microorganisms present in a specific

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307 sample. Microbes of interest for human identity testing should be common among all healthy
308 individuals and sufficiently abundant to be detected routinely. Human microbiomes represent
309 unique ecosystems comprised of complex mixtures of microorganisms. For the needs of forensic
310 diagnostics analysis of metagenomic samples can be more focused than total community
311 structure and organism abundance studies. Instead molecular analyses can entail identification of
312 specific microorganisms that provide an individualizing signature to the degree possible. Such
313 information requires resolution of selected microorganisms from near neighbors as well as from
314 all other microorganisms of the community that create noise and reduce the sensitivity of
315 detection of an assay.
316
317 Most metagenomic analyses characterize whole microbial communities at the phylum level often
318 reporting different abundance ratios of partially resolved taxa. While interesting and informative
319 for ecosystem analyses, that level of resolution and the variation in abundance from sample-to-
320 sample likely will not achieve human individualization. Instead, for human identification it is
321 imperative to 1) identify key bacteria that are common to all individuals so identity testing can be
322 informative with a routine target set of microorganisms; 2) select those bacteria that are
323 relatively abundant to reduce stochastic sampling effects; and 3) target genes or sequences that
324 contain sufficient variation to generate a profile that would provide a high degree of
325 individualization of the donor(s) of bacteria-containing biological evidence.
326
327 Current metagenomic approaches apply MPS and target a single phylogenetic marker, the 16S
328 rRNA gene, or perform shotgun whole-genome sequencing. The former approach provides
329 deeper coverage but rarely can differentiate at the species level. The latter approach may be

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330 able to differentiate at the species level but lacks depth of coverage and thus stochastic
331 effects reduce the ability to achieve taxonomic resolution. The 16S rRNA gene is the most
332 commonly used bacterial genetic marker in phylogenetic studies and broad bacterial
333 identification. The conserved and variable regions of the gene, a number of databases (e.g.,
334 see (74–76)), and significant volume of 16S rRNA studies add to the appeal of using this
335 marker. However, there are limitations to using solely 16S rRNA which include insufficient
336 species resolution (77), PCR bias (78, 79), copy number variation (80) and sequence variability
337 among a single bacterium (81), inaccurate phylogenetic relationships based on key variability
338 outside of the marker region (82), and horizontal transfer of the entire gene region (83, 84).
339 These limitations of using a single gene target to identify bacteria in a complex community will
340 lead to inaccurate abundance ratios and can confound phylogenetic analyses. Whole-genome
341 shotgun sequencing provides the ability theoretically to sequence all DNA molecules in a given
342 sample, potentially covering any given region(s) of many genomes. Whole-genome shotgun
343 sequencing can obtain species or strain level characterization of a given genome by producing
344 sequence reads of species/strain-specific informative markers. However, the more area of
345 any given genome that is attempted to be sequenced, the less depth of coverage will be
346 obtained for any particular site, potentially reducing the confidence for speciation. Highly
347 complex metagenomic samples can contain thousands of species within a sample thereby
348 limiting the coverage of any one genome, especially those at low abundance, such as may
349 be inherent with trace biological samples.
350
351 A novel metagenomics approach that employs the use of multiple informative phylogenetic
352 markers can allow greater depth of coverage by interrogating far less genome target than would

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353 be achieved by whole genome sequencing. Markers suited for the task of species-level resolution
354 are housekeeping genes, containing appropriate conserved and variable regions, to differentiate
355 among species. The use of housekeeping genes for bacterial species and strain identification is
356 not a new concept. Multi-locus sequence typing (MLST), first described almost 20 years ago
357 (85), is one of the most commonly used bacterial typing methods. MLST typically uses seven
358 housekeeping genes and the loci composite sequence creates a profile for comparison
359 purposes. Online MLST databases are available for identification purposes and data storage
360 (86–88). With MPS, an augmented MLST method has been developed using 20 genes (or
361 21 genes (89)), called MLST-seq (90). In addition, bioinformatics software packages are
362 freely available to analyze MLST profiles from shotgun sequencing data (88, 91, 92). Even with
363 these advances there are limitations with the MLST method, which include non-universal gene
364 panels and insufficient species level resolution. Seven markers simply are not sufficient for
365 speciation. In these cases MLVA (multi-locus variable number tandem repeat analysis) and
366 SNP (single nucleotide polymorphism) analyses have proved beneficial for additional
367 resolution of specific species (93). An expanded MLST approach has been developed, called
368 rMLST (ribosomal multi-locus sequence typing), utilizing 53 housekeeping genes to type
369 bacteria down to the subspecies level (94). Another method using housekeeping genes as
370 genetic markers was described by Baldwin *et al.* (95). Their approach consisted of a PCR-
371 based assay using 16 different primer pairs for amplifying 9 housekeeping genes followed by
372 electrospray ionization mass spectrometry for genus and species-level characterization (95).
373 This mass spectrometry based assay is very appealing and supports the concept of multiple
374 markers for speciation, but is limited to detecting only the most abundant 1-3 species in a sample,
375 i.e., better suited for testing infected individuals with high titer of the target species.

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377 Bioinformatics programs can retrieve and use housekeeping genes as genetic markers from
378 shotgun sequencing data. AMPHORA (AutoMated PHylogenOmic infeRence) has been
379 developed using a panel of 31 housekeeping genes for better taxonomic resolution than solely
380 using 16S rRNA (96). These genes were selected because they are found within all bacteria,
381 mostly present in single copies, and believed to be fairly resistant to horizontal gene transfer
382 (96). This 31 housekeeping gene panel was modified and another bioinformatic program,
383 called MetaPhyler, was developed to analyze the data (97). Phyla-AMPHORA was developed
384 utilizing thousands of phyla-specific phylogenetic markers to improve resolution of
385 phylogenomic analyses over the initial 31 genes (98). Additionally, PhyloSift enables
386 phylogenetic analysis of metagenomes, which expands on AMPHORA, and includes 37
387 bacterial and archaeal genetic markers (PhyEco markers) (99) in the prokaryote core marker
388 set (100). PhyloSift also provides capabilities for extended and custom marker sets and has
389 software packages for metagenomic dataset simulation (to test newly generated custom markers)
390 and statistical analyses (100). Another bioinformatics tool, mOTU, was developed using 40
391 marker genes (101) to profile metagenomes with species-level resolution (102). Additionally,
392 FunGene (the Functional Gene Pipeline and Repository) includes 11 phylogenetic markers in its
393 repository and analysis pipeline(103). Although these programs use different genetic markers
394 (with a subset of overlapping markers), they demonstrate that the technology and software exist
395 to develop a custom genetic panel consisting of informative phylogenetic markers to use for
396 species level identification in human microbiome samples.

397

398 Validation

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399 Validation is essential in the development of diagnostic methods and those used in microbial
400 forensics are no exception. The most important consideration is that microbial forensic methods
401 may develop information that can impact health, life, and freedom of individuals, policy
402 decisions and possibly action by governments on a large scale. Therefore, results generated from
403 the analyses of any microbial forensics analysis (including collection, transfer, analytical
404 method, and interpretation, as well as proper training) must be understood and limitations
405 defined so that a proper degree of confidence can be associated with findings. Accurate and
406 credible results are a requisite. Budowle *et al.* (104) stressed that failure to properly validate a
407 method or misinterpret the results from a microbial forensic analysis or process may have severe
408 consequences.

409 Validation is the process that: "1) assesses the ability of procedures to obtain reliable results
410 under defined conditions; 2) rigorously defines the conditions that are required to obtain the
411 results; 3) determines the limitations of the procedures; 4) identifies aspects of the analysis that
412 must be monitored and controlled, and 5) forms the basis for development of interpretation
413 guidelines to convey the significance of the findings." (104) . The basic features and criteria for
414 validation are listed in Quality Assurance Guidelines for microbial forensics (1) and addressed
415 elsewhere in detail (104, 105). Budowle *et al.* (104) define developmental validation as "the
416 acquisition of test data and the determination of conditions and limitations of a newly developed
417 method to analyze samples" and internal validation "as an accumulation of test data within the
418 operational laboratory to demonstrate that established methods and procedures perform within
419 predetermined limits in the laboratory." Every effort should be made to validate a method
420 thoroughly before implementation. However, cost, resources, and exigent circumstances may
421 require a method to be implemented without extensive validation. Clearly, it is unacceptable to

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422 hold off on any analyses because an available method has not been completely validated
423 especially when an attack is underway. In this context, Schutzer *et al.* (106) described the
424 process of preliminary validation as "an early evaluation of a method that will be used to
425 investigate a biocrime or bioterrorism event." There is still a requirement to acquire some test
426 data to evaluate a potential method so peer review of extant data could be performed quickly by
427 a panel of experts. This panel would recommend limitations and additional studies (if necessary)
428 to use prior to analyzing evidentiary material. However, it is stressed that a preliminary
429 validation should not be used as an excuse for obviating a full validation.

430

431 Budowle *et al.* (105) recently described criteria for validation of MPS procedures. The criteria
432 are not novel as the same approaches are being used for human genome sequencing and clinical
433 diagnostics. Three common areas of MPS validation are: 1) sample preparation, 2) sequencing,
434 and 3) data analysis; but these criteria should be defined in terms of the specific application.
435 There are a number of general topics subsumed within these three areas, which are listed in
436 Table 2.

437

438 Addressing these general areas of the MPS method should provide an analytical tool sufficiently
439 robust for the expanding field of microbial forensics. While the field certainly has matured and
440 no longer can be considered solely the domain of investigating bioterrorism and biocrime, MPS
441 technologies are still quite dynamic. It is anticipated that the speed of obtaining sequencing
442 results will increase and read lengths will be extended with a concomitant decrease in cost.
443 Applications beyond those discussed here will become part of the microbial forensics arena.

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444 Good quality practices will ensure that the tools of microbial forensics and the results obtained
445 will be robust, accurate and reliable.
446
447 As the microbial forensics field expands to incorporate the use of microbiome characterization
448 for human investigative and forensic testing, additional methods can be created to expand the
449 forensic genetic toolbox. Likely, more advanced sequencing methods can be developed to better
450 elucidate the data that can be obtained from complex metagenomic samples which can provide
451 additional investigative value including geolocation, infection source tracking of biocrimes, post-
452 mortem interval, and increased power of discrimination for human identification. As standards
453 and quality practices are developed for MPS and metagenomic microbial forensic testing
454 methods, human microbiome characterization likely will become a routine methodology in the
455 field of microbial forensics.
456
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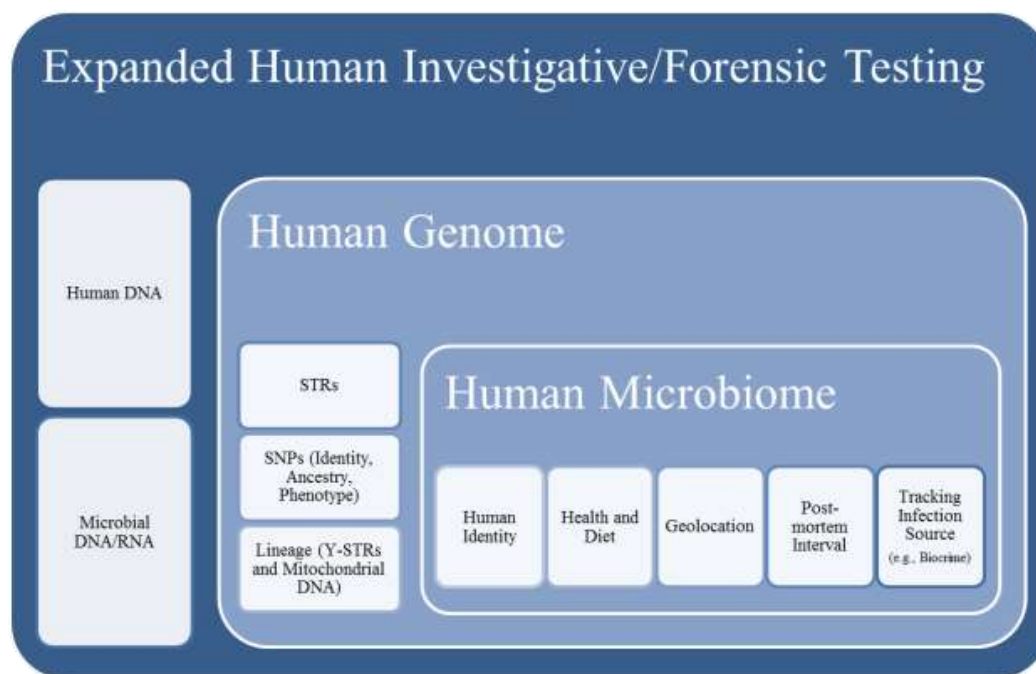

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811 Figure 1. Expanded human investigative and forensic testing to include human genome and
812 human microbiome characterization.

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Table 1 Examples of microbial forensic and epidemiologic (of interest to forensics) investigations

	Year	Case description	Reference
Bacterial	2000-2001	Characterized *Staphylococcus aureus* strains from drug paraphernalia to track drug networks	(66)
	Starting in 2002	Amerithrax investigation-Compared different Ames strain morphotypes to determine the origin of the source material released in the 2001 anthrax attacks	(107, 108)
	2009	Traced the origin of *Bacillus anthracis* from injectional anthrax cases among heroin users in Scotland	(109)
	2010	Traced the origin of the source of the Haitian cholera outbreak after the 2010 earthquake	(10, 110, 111)
	2011	Traced the origin of the 2011 *Escherichia coli* O104:H4 outbreak in Germany	(11)
Viral	1992	First report of molecular tracking of HIV infection from dentist to patients after invasive healthcare procedure	(21)
	1994	Phylogenetic analysis to provide evidence that Dr. Richard Schmidt intentionally injected his girlfriend with HIV/HCV contaminated blood	(25)

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1998	Phylogenetic and molecular clock analysis to provide evidence that a Spanish anesthetist infected 275 patients with hepatitis C virus	(28)
2014	Traced the origin and transmission route of the 2014 Ebola outbreak	(112)
2015	Parvovirus B19 characterization from skeletal remains revealed the likely origin of World War II casualties	(67)

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Table 2 Validation criteria for MPS procedures (from (105))

- Extraction of DNA (or RNA)
  - Quality
  - Purity
- Enrichment
  - PCR
  - Capture
- Library Preparation
- Multiplexing
  - Markers
  - Samples
- Sequencing
- Data Analysis
  - Raw data processing
  - Quality scores
  - Alignment
  - Variant Calls
- Reference materials
  - Test materials
  - Controls
- Databases
  - Test materials
  - Inferences of results

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- Bioinformatics
  - Software tools
  - Data management
  - Data storage
  - Interpretation
  - Taxonomic assignment
  - Abundance
  - Organism classification
  - Community Structure
- Standard Operating Protocols
- Reporting

817

818

819 Biosketch

820 Sarah E. Schmedes, M.S., is a Ph.D. Candidate in the Department of Molecular and Medical
821 Genetics at the University of North Texas Health Science Center (UNTHSC), in Fort Worth, TX.
822 She earned her Bachelor of Science in biochemistry and microbiology from Texas State
823 University in San Marcos, TX and earned her Master of Science in forensic biology from
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825 doctoral program at UNTHSC, she served as a forensic research assistant in the Institute of
826 Applied Genetics at UNTHSC. She has published 8 manuscripts and 1 book chapter during her
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828 recipient of the 2014 Eugene and Millicent Goldschmidt Graduate Student Award, awarded by
829 the Texas Branch-American Society for Microbiology. Her research interests include
830 metagenomics, microbial forensics, and emerging infectious diseases.

831

832 Antti Sajantila, M.D, Ph.D., is a trained forensic pathologist and forensic geneticist from the
833 Department of Forensic Medicine, University of Helsinki, Finland. He received his PhD from the
834 University of Helsinki, Finland and was a post-doctoral fellow in Ludwig Maximilian's
835 University in Munich, Germany. He has published over 200 articles, book chapters and reviews.
836 His research interests are in the fields of human population genetics, human identification,
837 disaster victim identification, and cause of death investigation. He has testified in forensic court
838 cases in Finland, in Australia and in Kenya. He also has served on several national and
839 international committees for application of DNA methods in human identification. He is
840 currently a member of the Interpol's Disaster Victim Identification Standing Committee's
841 Forensic Pathology and Forensic Genetics Working Groups.

842

843 Bruce Budowle received a Ph.D. in Genetics in 1979 from VA Tech. In 1983, he joined the FBI
844 Laboratory Division to carry out research, development, and validation of methods for forensic
845 biological analyses. He has published approximately 550 articles and testified in well over 250
846 criminal cases. He has authored or co-authored books on molecular biology techniques,
847 electrophoresis, protein detection, and microbial forensics. He has been directly involved in
848 developing quality assurance standards for the forensic DNA field. Some of his efforts over the
849 last 20 years have been in microbial forensics and bioterrorism. In 2009 Dr. Budowle became

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850 Executive Director of the Institute of Applied Genetics and Professor in the Department of
851 Molecular and Medical Genetics at the University of North Texas Health Science Center at Fort
852 Worth, Texas. His current efforts focus on human forensic identification, microbial forensics,
853 and emerging infectious disease.

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