REVIEW

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Bacillus anthracis, *Francisella tularensis* and *Yersinia pestis*. The Most Important Bacterial Warfare Agents — *review*

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ABSTRACT. There are three most important bacterial causative agents of serious infections that could be misused for warfare purposes: *Bacillus anthracis* (the causative agent of anthrax) is the most frequently mentioned one; however, *Fracisella tularensis* (causing tularemia) and *Yersinia pestis* (the causative agent of plague) are further bacterial agents enlisted by *Centers for Disease Control and Prevention* into the category A of potential biological weapons. This review intends to summarize basic information about these bacterial agents. Military aspects of their pathogenesis and the detection techniques suitable for field use are discussed.

Abbreviations

B.a. *Bacillus anthracis* *F.t.* *Francisella tularensis* *Y.p.* *Yersinia pestis*

BWA	biological warfare agent
CDC	*Centers for Disease Control and Prevention*
LAPS	light addressable potentiometric sensor
NBC	nuclear, biological and chemical (weapon)
PCR	polymerase chain reaction
RAPID	ruggedized advanced pathogen identification device
SPR	surface plasmon resonance

CONTENTS

1 Introduction 263
2 *Bacillus anthracis* 264
3 *Francisella tularensis* 266
4 *Yersinia pestis* 267
5 Conclusions 269
References 269

1 INTRODUCTION

BWA belong to mass destruction weapons destroying or damaging selectively living organisms including human beings, livestock and even plants. In biological warfare, one can consider a system consisting of BWA and supporting equipment for delivering and spreading in the target area, and in some cases also chemical additives stabilizing the formed bioaerosol. The threat of mass destruction weapons has changed after termination of the Cold War. One can distinguish nuclear, biological and/or chemical weapons (NBC) as means of mass destruction. The nuclear weapons are typical of superpowers, the latter two types become bogey of the so-called asymmetric war and nowadays the term bioterrorism has appeared (Kortepeter *et al.* 2001). Due to the low cost and rather simple production of chemical and biological weapons, these are frequently called as "weapons of the poor".

For construction of BWA, many pathogens and toxins can be used. It has been reported that at least 1400 infectious organisms including >200 viral and 500 bacterial species are pathogenic to humans (Taylor *et al.* 2001). On the other hand, only few of them were found to be really effective. The frequently used classification of bioagents comes from the CDC (**www.cdc.gov**). BWA are divided into categories A, B and C. In category A, there are enlisted agents that can be easily disseminated or transmitted from person to person, resulting in high mortality rates. The list consists of the following items: *B.a.*, *Clostridium botulinum* toxin, *Y.p.*, *Variola major*, *F.t.* and viral hemorrhagic fevers (such as Ebola, Marburg and Machupo). Category B includes low-mortality agents moderately easily disseminating, and category C consists of pathogens (toxins) that could be engineered for mass dissemination in the future.

This review aims to summarize the most important knowledge about the bacterial BWA from the category A: *B.a.*, *F.t.*, and *Y.p.* Basic information about each microorganism, its pathology, detection techniques and the currently available as well as novel analytical procedures are mentioned.

2 *Bacillus anthracis*

B.a. is a G^+ endospore forming bacterial rod. Robert Koch used this microbe to demonstrate pathogenesis as a pathogen–host relation in 1877. *B.a.* causes the well known disease anthrax predominantly infecting domesticated and wild animals, especially herbivores (cows, sheep, horses, mules, *etc.*). This disease was historically called also the "wool-sorter's disease". Humans usually become infected through contact with various body parts of diseased animals. The spores are very resistant to harsh physical conditions and even long-term stability up to 200 years was observed; for this reason, virulent cadavers should be burned (Titbal *et al.* 1991).

Three forms of anthrax are known. The most common one is the cutaneous form where spores of *B.a.* pass through minor skin breaks, resulting in the formation of dermal ulcers. The second form is gastrointestinal and it occurs most commonly after ingestion of poorly cooked meat contaminated with spores. The intestinal ulcers are formed initially and later spread into the lymphatic system, finally causing septicemia. The last form is inhalation (pulmonary) anthrax following after breathing in some 8000–40 000 spores. The first symptoms are similar to influenza. After 2–3 d, high fever with hemorrhage continues, resulting from systematic infection. Gastrointestinal and inhalation anthrax are fatal without treatment and high mortality is reported even if anthrax was not diagnosed early and antibiotics were not dosed. *B.a.* is susceptible to penicillin; on the other hand, β-lactamase-positive strains have been isolated. Ciprofloxacin, erythromycin, tetracycline, doxycycline and chloramphenicol are alternative drugs (LaForce 1994).

Though *B.a.* was described as BWA, attempts were also made to misuse it for terrorist attacks. The unsuccessful attempts to use *B.a.*, *Vibrio cholerae* and botulinum toxin for their own biological weapons construction were made by the Aum Shinrikyo cult in Japan. They sent their members to Africa to collect samples of the Ebola virus (Olson 1999). Aum Shinrikyo launched nine biological attacks. In 1993, they spread aerosolized cloud of *B.a.* from the roof of the headquarters building of their sect in Kameido, near Tokyo. Fortunately, they were unsuccessful due to using the non-virulent strain Sterne 34F2, which is normally used for prophylaxis (Keim *et al.* 2001). After these failures, they used nerve gas sarin in the Tokyo subway system in 1995, resulting in 12 deaths and ≈3800 injured.

In 2001 (shortly after the World Trade Center tragedy), terrorists used the United States postal system to distribute *B.a.* lethal spores (Canter *et al.* 2005). The panic which broke out afterwards, was surprising. The FBI perpetrator has not been captured so far (**`http://www.fbi.gov/anthrax/amerithrax.htm`**). Five letters were sent (Josefson 2001). The first two letters were sent to the NBC television in New York and to the New York Post on 18th September 2001. Nobody died but some people became ill. These letters were not very high-leveled considering purity of the agent; they contained ≈10 % spores. The one of the most virulent strains of natural *B.a.* (the Ames strain) was used (Higgins *et al.* 2002). The following three letters, one sent to the Florida's tabloid newspaper The Sun and two to Washington DC to the office of Senator Leahy and Daschle on 9th October 2001. The most shocking information was about the included particles. The obtained *B.a.* samples were pure spores (meaning microbial purity as ratio of spores to the vegetative cells) prepared as homogeneous 10 μm particles chemically stabilized. The particle size is one of the most important characteristic for biological weapons and 10 μm is considered to be optimal for penetration into lungs. Their preparation by milling was discussed but more probable was preparation by a more sophisticated method – spray-drying. Five people died after manipulation with the letters and about two dozens were infected. Letters were very well closed and paper served as filter for aerosol. Connection between the perpetrator and Al Qaida or any totalitarian regime has not been proved.

A reliable identification of *B.a.* is quite difficult due to the close relationships in the *Bacillus cereus* group where *B.a.*, *B. cereus*, *B. thuringiensis* and *B. mycides* are classified (Keim *et al.* 1997; Leonard *et al.* 1998). Several papers indicated that these bacteria may be considered as subspecies of *B. cereus* according to comparative analysis of the 16S-rRNA sequences (Ash *et al.* 1991). A false-positive signal would occur when *B.a.* is assayed in the presence of *B. cereus* contamination; especially, when soil particles are present in the samples. The confusion is serious also due to fact that *B. cereus* is a common bacterium widely occurring in soil and environment; moreover, it is completely harmless to humans. *B. subtilis* is another bacterium with surface markers typical of the *Bacillus* genus (Seydlová and Svobodová 2008). Pathogenicity of *B.a.* depends upon the presence of the glutamyl-polypeptide capsule and detection of its presence is a crucial diagnostic step in identification. The cultivation medium for *B.a.* should contain thiamine in contrast to

B. cereus and *B. thuringiensis*. Sensitivity to the Gamma phage is a possibility for detection purposes, too. *B.a.* lack of β-hemolysis on sheep and horse blood agar plates and/or microscopic evaluation of lack of motility are other options for identification.

At present, instrumental detection methods are in focus. A *B.a.*-specific PCR for chromosomal and pXO1/pXO2 plasmid sequences has been described (Ramisse *et al.* 1996; Beyer *et al.* 1999). The PCR for *B.a.* is a very specific method, making possible the species identification (when the method is performed precisely; Kiel *et al*. 2008). The above principle is, *e.g.*, utilized in commercially available real-time PCR devices, such as RAPID (*Idaho Technology*, Salt Lake City, UT, USA; **www.idahotech.com**), are approachable for routine assay of several microorganisms including *B.a*., *F.t.* and *Y.p.* This RAPID device (Fig. 1) is used in NATO armies including the Czech Army. Another portable PCR is the hand-held device RAZOR (*Idaho Technology*; Fig. 2), being suitable for simple field tests due to low size and mass (4.1 kg).

Fig. 1. Mobile real-time PCR device RAPID.

Fig. 2. Mobile real-time PCR device RAZOR.

Several studies reported the performance of biosensors, quite good results being found when LAPS was applied. It is the type of biosensor including a semiconductive potentiometric sensor part. Uithoven *et al.* (2000) obtained some promising results: their device included biotin-coated nitrocellulose tape able to detect safe alternatives, such as *B. subtilis* through streptavidin and purified polyclonal antibody. The secondary antibody complex including urease as label was able to provide pH change as a primary signal. The whole device had eight channels and provided limit of detection at 3×10^3 CFU/mL of *B. subtilis* and measuring range 10^4–10^6 CFU/mL. This principle was employed in the BioDetector instrument, developed by the US Army; the filtration capture immunoassay is evaluated using the LAPS originally marketed as the Threshold system by *Molecular Devices* (**www.moleculardevices.com**).

B.a. spores were detected in powder form by the fluorescence-based fiber-optic biosensor (Tims and Lim 2004). Primary antibody was immobilized on the waveguide surface and cross-linked through the biotin–avidin (bound on antibody Fc part) connection. The secondary antibody was labeled by Cy-5. The limit of detection was evaluated as 3.2×10^5 spores in 1 mg of powder matrix.

3 *Francisella tularensis*

F.t. is the causative agent of tularemia; it is a small and nonmotile G^- coccobacillus requiring aerobic conditions. Tularemia was first notified in Californian's Tulare Country in 1911 and the subsequently isolated G^+ microorganism was named *Bacterium tularensis* (McCoy *et al.* 1912). The human disease was recognized and described by Edward Francis in 1922. Lately, the taxonomic position was changed to *Pasteurella* and even *Brucella*; the final taxonomic position was proposed in 1947 and the genus was called *Francisella* (Dorofe'ev 1947). *F.t.* was the only species in the genus *Francisella.* Hollis *et al.* (1989) studying fatty acids and DNA from *Yersinia philomiragia* stated that *Y. philomiragia* should be renamed to *Francisella philomiragia*.

There are several subspecies of *F.t.*: formerly, it was divided into subtypes A and B; at present, four subspecies are described. The most virulent is the subspecies *tularensis* (subtype A; also named as *nearctica* by investigators in the former Soviet Union) occurring in North America. Recently, *F.t.* subsp. *tularensis* was reported in Europe (Gurycova 1998). This subspecies is able to ferment glycerol as well as L-citrulline (Olsufjev *et al.* 1959). Analysis of 16S rRNA is more important for distinguishing this subspecies (Forsman *et al.* 1990; Sandtröm *et al.* 1992) rather than metabolic activity studies. The case-fatality rate for this subspecies reached 1.4 % in the United States during 1985–1992 (Dennis *et al.* 2001). Subspecies *holartica* (subtype B; can be referred to as *palaeartica*) is found in North America as well as in Eurasia. Zhang *et al.* (2006) proved that this subspecies was present in nearly 5 % of rodents in China. The *holarctica* subspecies can be divided into three biovars (Olsufjev and Meshcheryakova 1983): the erythromycin-sensitive biovar I, erythromycin-resistant biovar II and biovar japonica. The subspecies *holartica* exerts a 10 % lower case-fatality rate in comparison with the *tularensis* subspecies (Olsufjev and Meshcheryakova 1983). The subspecies *mediaasiatica* is spread mainly in central Asia, being less virulent than *tularensis*; on the other hand, it metabolizes L-citrulline and glycerol and exhibits high genomic similarity to the *tularensis* subtype. Broekhuijsen *et al.* (2003) using the microarray technique confirmed genetic similarity between the *F.t.* subspecies *mediaasiatica* and *tularensis* and a close genetic connection between *mediaasiatica* and the *tularensis* strain Schu S4.

The last subtype of *F.t.* is *novicida*, being isolated from water supplies in Utah and first described as a separate species. After genetic distinction, this species was proposed to be a subspecies of *F.t.* (Hollis *et al.* 1989). A better distinguishing of *F.t.* subspecies will be simpler after characterization of diagnostic markers obtained by proteome analysis. Tens of specific proteins were described for every subspecies. An important factor about virulence should be obtained after complete genome analysis, which is now starting (Prior *et al.* 2001).

F.t. causes zoonotic disease tularemia predominantly localized in the northern hemisphere. Naturally, this disease spreads among mammals, mainly rodents, rabbits and hares. The most frequent vectors are small arthropods such as ticks, flies and mosquitoes. For example, 2.1–2.8 % of *Deracentor reticulatus* in the Czech Republic and Austria are natural vectors of *F.t.* (Hubálek *et al.* 1998). The infection can also be obtained from contaminated food, water supply and soil (Hopla 1974). The natural foci of tularemia were well described (Pikula *et al.* 2004). One documented case exemplifies the possibility of tularemia dissemination: 15 people were infected by tularemia from the contaminated dog's fur disseminated when the dog shook itself among guests during a common dinner (Siret *et al.* 2006). The most frequent disease presentations are ulceroglandular, glanular, oculoglanular, oropharyngeal, pneumonic, typhoid and septic (Pullen and Stuart 1945). The onset of tularemia is quite fast. Symptoms such as high fever of 38–40 °C (Plourde *et al.* 1992), body pain and dry cough can be observed (Dennis *et al.* 2001; Shapiro *et al.* 2002; Haristoy *et al.* 2003). The infection process was also intensively investigated in multiple vectors (Banďouchová *et al.* 2009; Pohanka 2007). For disease treatment, several antibiotics are convenient. Streptomycin and gentamicin are widely recommended but tetracycline and chloramphenicol are acceptable alternatives (Enderlin *et al.* 1994). In history, vaccination against tularemia was realized using the live attenuated vaccine in the former Soviet Union in the tularemia endemic areas and in the US Army research facility (Burke 1977). Lymphocyte blast transformation reactivity to *F.t.* remained at least for 1.5 years (Koskela and Herva 1982). Due to the low infection dose – 10–50 organisms in aerosol (Dennis *et al.* 2001) – and simple cultivation *F.t.* was enrolled by CDC into category A.

Several methods are suitable for the assay of *F.t.* When cultivation tests are used, the best growth is observed in cysteine-enriched broths and blood or chocolate-supplemented agars. The characteristically opalescent colonies are formed after 1–2 d of incubation at 37 °C in wet atmosphere. A wide range of immunoassays were described. Serological diagnosis of patient sera was possible by microagglutination (Gaultney *et al.* 1971; Özcürümez *et al.* 2004) and ELISA (Carlsson *et al.* 1979; Bevanger *et al.* 1989; Özcürümez *et al.* 2004; Schmitt *et al.* 2005). The PCR is typically targeted to the *tul4* and *fopA* genes encoding 17 and 43 kDa outer membrane proteins, respectively. This technique was employed, *e.g.*, for tissue samples from infected

mice (Emanuel *et al.* 2003); for tissue specimens from brown hares (*Lepus europaeus*) infected during the epizootic period of tularemia in Austria in 1997 (Grunow *et al.* 2000) and for confirmation after micro-agglutination tests when the infection broke out in Turkey in February 2004 (Celebi *et al.* 2006). DNA extraction from soil spiked with *F.t.* SHU-4 was optimized for PCR assay purposes; the lowest limit of detection reached only 20 CFU/g of soil (Whitehouse and Hottel 2006). Sellek *et al.* (2008) performed real-time PCR based on SYBR Green I and *tul4* gene for *F.t.* LVS assay. They achieved the limit of detection of 0.69 fg of genomic DNA. The PCR is strongly specific for identification of *F.t.* in comparison with immunoassays with typical cross-reactivity with *Brucella* spp., *Escherichia coli*, *Burkholderia* spp. and *Pseudomaonas* spp. (Fonseca *et al.* 2008; Grunow *et al*. 2000; Quinn *et al.* 1984).

Some applications of piezoelectric and amperometric immunosensors were extensively studied for the detection of whole cells in a liquid medium and serological diagnosis (Pohanka and Skládal 2005, 2007*a*,*b*; Pohanka *et al*. 2007*a*–*c*). Piezoelectric biosensors are mass-sensitive devices advancing in the possibility to intercept an antibody as recognition element and consequent assay of microorganism in the medium without any labeling. For example, *F.t.* was identified (limit of detection approximately millions of CFU) without any cross-reaction to *E. coli* and *B. subtilis* (Pohanka and Skládal 2007*b*). The physical principle of the assay consists in the piezoelectric effect and decreased frequency of oscillation by measured by a frequency counter. It seems that biosensors could act as low-cost and reliable devices suitable for routine assay. The potentiostat with amperometric detector (Fig. 3) constructed in the *Military Institute of Defense* (Brno, Czech Republic) was used for immunosensor-based assays (Pohanka and Skládal 2007*a*).

Fig. 3. Electrochemical device with four peristaltic pumps (*top*); flow-through cell with integrated reference electrode and screen-printed strip with four working electrodes (*bottom*).

The bioanalytical devices usually need to be supplied with a liquid sample for assay. For monitoring purposes at public buildings, airports and underground systems, the presence of potentially dangerous bioaerosols should be detected. Therefore, the sampled air is first passed through a collecting solution using a suitable cyclone system, and then the obtained sample is further analyzed. Small compact cyclone systems certified also for the homeland security systems are available from the *Research International* (Monroe, WA, USA; **www.resrchintl.com**). The system SASS 2300 (Fig. 4) can operate as a stand-alone device, but it can also be linked to various detection systems for nearly real-time monitoring of air.

4 *Yersinia pestis*

Y.p. is a nonmotile, slowly growing G^{-} coccobacillus from the family *Enterobacteriaceae*; it is the causative agent of the well known disease plague. The relationship between humans and plague has been known from ancient history. The Justinian plaque epidemic spread from Egypt into Mediterranean Europe after 541 A.D. (Russel 1968). Plague (Black Death epidemic 1347–1351) influenced the European population resulting in up to 40 % casualties. This plaque pandemy was spread in consequence of tragic events. Probably at the beginning, Tatar forces in 1346 during the battle for the seaport city Kaffa placed in today's Ukraine catapulted plague victims into the city in order to spread this disease (Christopher *et al.* 1997;

Mayor 1997). The Genoese merchants who escaped from Kaffa and docked in Genoa in October 1347 probably transferred to Mediterranean ports infected rats and bubonic plague was consequently spread in Europe (Derbes 1966). In the 19th century, plague was spread into Hong Kong from China and consequently to the remaining parts of Asia, America and Africa (Perry 1997).

Fig. 4. The portable cyclone SASS 2300 (*Research International*) is suitable for efficient collection of bioaerosols from air into liquid phase; the obtained samples can be used for further off-line analysis or directly transferred to various (bio)analytical devices providing continuous monitoring systems.

Other species of *Yersinia* cause gastroenteritis, *Y.p.* being the most virulent species. The bubonic plague is characterized by sepsis and local lymphadenopathy. It starts within a week of being bitten by a vector – an infected flea (such as the oriental flea *Xenopsylla cheopis*). After an incubation period of 2–6 d, sudden illness follows with fever, malaise, nausea, vomiting and diarrhea (Reyn *et al.* 1977); the lymphadenopathy occurs consequently. The septicemic plague is diagnosed with positive blood cultures but with no palpable lymphadenopathy. When the contaminated respiratory droplets from patients are inhaled by other person(s), the most serious form – pneumonic plague – follows. Fewer than 2 % of the plague cases are contributed to this form (Craven *et al.* 1993). It progresses rapidly from flu-like illness to bloody sputum. The short incubation period of 1–3 d is typical of this form. Treatment as well as prophylactics of plague employs many antibiotics. Streptomycin was one of the choices but tetracyclines are commonly considered as more potent, now. *Y.p.* is sensitive to penicillin *in vitro* but it was found to be ineffective against extended human disease (Crook and Tempest 1992). Though *Y.p.* is unstable in aerosol for longer times, which makes its use as BWA difficult, CDC enlisted it into category A due to the high mortality and high virulence.

A laboratory diagnosis of plague is similar to other infections. Blood is recommended as specimen for analysis; bubo aspirates and sputum are other options. *Ad hoc* of cultivation, *Y.p.* would be cultivated; however, it belongs between the less viable organisms *in vivo* (Byvalov *et al*. 2008). *Y.p.* can be cultivated on routine laboratory culture media, such as sheep blood agar. MacConkey and eosin–methylene-blue agars are chosen as selective media. Cultivation at 37 °C for 2 d is optimal to produce visible colonies. Specific lysis by bacteriophage can be used for very selective identification (Doll *et al.* 1994). Some of the cultivation techniques are convenient for challenging specific marker synthesis; the recent experience of Fedorova *et al*. (2005)should be mentioned: they were able to induce biosynthesis of YopE (plasmid coding effector protein qualifying pathogenicity of *Y.p.*). Biosynthesis was proved at cultivation in the presence of substances from phagolysosome and/or typical conditions in them.

The presence of the F1 antigen (capsular) excreted by *Y.p.* in human body at 37 °C (Williams *et al.* 1984; Chanteau *et al.* 1998) and in zoonotic vectors (Kilonzo *et al.* 2006) is detectable by ELISA. F1 is specific for *Y.p.*; moreover, it is not only a good marker approachable for identification but also for immunization of protecting against wild variants of plague (Wang *et al*. 2008). Another promising antigen are ATP-binding cassette transporter proteins consisting of OppA, PstS, YrbD, and PiuA (Tanabe *et al.* 2006). The recent effort pointed at a plausible application of antigens being used for vaccine construction as good markers for identification of *Y.p.* during an immunoassay (Smither *et al*. 2007). Protein–protein interactions relating to specific antigens of *Y.p.* were characterized by SPR representing a tool able to evaluate interactions and associations as well as dissociation rate constants (Swietnicki *et al*. 2004). Well described is also the fluorescent antibody assay assessing specific markers. On the other hand, ≈60 % cases exhibited cross reactivity with *Y. pseudotuberculosis* (Devdariani *et al*. 1993).

McDonough *et al.* (1988) have introduced DNA hybridization using a structural gene for the plasminogen activator *pla*. PCR assays frequently employ *pla* and a structural gene for the F1 antigen *caf1* (Norkina 1994; Rahalison 2000). This gene was also found to be long-term stable and in this way suitable for an interesting study of plague victims from the 16th–18th century (Bianucci *et al.* 2008). In contemporary trends, PCR techniques are based on multiple primers. For example, Stewart *et al*. (2008) used a quadruplex real-time PCR assay: they proposed an assay suitable for differentiating *Y.p.* strains. They would be simply distinguished from *Y. pseudotuberculosis* by real-time PCR due to genotyping of the O-antigen gene clusters (Bogdanovich *et al.* 2003; Matero *et al*. 2009). Another promising way to accurately identify *Y.p.* is based on an assay for the 16S rRNA gene target (Tomaso *et al.* 2003). Electrochemical microarray would be simply connected with multiplex PCR for better characterization of the reaction products (Elsholz *et al*. 2009).

Assays based on biosensors could also be suitable for *Y.p.* (Pohanka *et al.* 2007*d*); Cao *et al.* (1995) were able to detect <5 ng/mL of the surface antigen with a fiber optics biosensor employing fluorescence-labeled antibodies. Electrochemical biosensors able to capture the F1 antigen on intercepted antibodies were described by Meyer *et al.* (2007) who achieved good limits of detection. Though they did not work with the whole cells, the antigen was detected from 2.5 ng/mL of buffered solution.

5 CONCLUSIONS

Within the military area, concern with BWA exists for many decades. The main interest was directed to the development and testing issues from the beginning and during the 2nd World War period. Recently, the focus moved towards detection technologies employing bioanalytical principles. This turn was initiated by the *United Nations Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological and Toxin Weapons and Their Destruction*, and especially by the political changes after attacks from the September 11th, 2001. Afterwards, the public concern about BW appeared as a fear of their bioterroristic misuse. Even though the real importance of this issue might be questionable, one can face many attempts and plans for protection of large governmental buildings and public facilities as airports and metro systems. The fear of microbes as "invisible enemies" resulted also in individual protection and detection means within the homeland security system.

The purpose of this review was to characterize the three most dangerous bacterial agents and summarize both the classical microbial as well as the novel bioanalytical and biosensor-based detection technologies.

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REFERENCES

ASH C., FARROW J.A.E., DORSCH M., STACKEBRANDT E., COLLINS M.D.: Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. *Internat.J.Syst.Bacteriol.* **41**, 343–346 (1991).

BANĎOUCHOVÁ H., SEDLÁČKOVÁ J., HUBÁLEK M., POHANKA M., PECKOVÁ L., TREML F., VITULA F., PIKULA J.: Susceptibility of selected murine and microtine species to infection by a wild strain of *Francisella tularensis* subsp. *holoarctica*. *Vet.Med*. **54**, 64–74 (2009).

BEVANGER L., MAELAND J.A., NAESS A.I.: Competitive enzyme immunoassay for antibodies to a 43,000-molecular-weight *Francisella tularensis* outer membrane protein for the diagnosis of tularemia. *J.Clin.Microbiol.* **27**, 922–926 (1989).

BEYER W., POCIVALSEK S., BOHM R.: Polymerase chain reaction–ELISA to detect *Bacillus anthracis* from soil samples – limitations of present published primers. *J.Appl.Microbiol.* **87**, 229–236 (1999).

BOGDANOVICH T., CARNIEL E., FUKUSHIMA H., SKURNIK M.: Use of O-antigen gene cluster-specific PCRs for the identification and O genotyping of *Yersinia pseudotuberculosis* and *Yersinia pestis*. *J.Clin.Microbiol.* **41**, 5103–5112 (2003).

BIANUCCI R., RAHALISON L., MASSA E.R., PELUSO A., FERROGLIO E., SIGNOLI M.: Technical note: a rapid diagnostic test detects plague in ancient human remains: an example of the interaction between archeological and biological approaches (southeastern France, 16th–18th centuries). *Am.J.Phys. Anthropol.*, in press (2008).

BROEKHUIJSEN M., LARSSON P., JOHANSSON A., BYSTRÖM M., ERIKSSON U., LARSSON E., PRIOR R.G., SJÖSTEDT A., TITBALL R.W.: Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent *F. tularensis* subsp. *tularensis*. *J.Clin.Microbiol.* **41**, 2924–2931 (2003).

BYVALOV A.A., GAVRILOV K.E., KRUPIN V.V., CHEBOTAREV E.V., ZHELUDKOVA E.V., DRUBKOV V.I., SMIRNOV A.E., MALKOV V.N., DUPIASHEVA T.I., PECHENKIN D.V., BONDAREV V.P.: Biological and physicochemical properties of *Yersinia pseudotuberculosis* cultures carrying the *Yersinia pestis* fra operon. *Mol.Gen.Mikrobiol.Virusol*. **1**, 14–18 (2008).

CANTER D.A., GUNNING D., RODGERS P., O'CONNOR L., TRAUNERO C., KEMPTER C.J.: Remediation of *Bacillus anthracis* contamination in the *U.S. Department of Justice* mail facility. *Biosecur.Bioterror*. **3**, 119–127 (2005).

CAO K.L., ANDERSON G.P., LIGLER F.S., EZZEL J.: Detection of *Yersinia pestis* fraction 1 antigen with a fieber optic biosensor. *J.Clin. Microbiol.* **33**, 336–341 (1995).

CARLSSON H.E., LINDBERG A.A., LINDBERG G., HEDERSTEDT B., KARLSSON K.A., AGELL B.O.: Enzyme-linked immunosorbent assay for immunological diagnosis of human tularemia. *J.Clin.Microbiol.* **10**, 615–621 (1979).

CELEBI G., BARUONU F., AYOGLU F., CINAR F., KARADENIZLI A., UGUR M.B., GEDIKOGLU S.: Tularemia, a reemerging disease in northwest Turkey: epidemiological investigation and evaluation of treatment responses. *Japan J.Infect.Dis.* **59**, 229–234 (2006).

CHANTEAU S., RABARIJAONA L., O'BRIEN T., RAHALISON L., HAGER J., BOISIER P., BURANS J., RASOLOMAHARO M.: F1 antigenaemia in bubonic plague patients, a marker of gravity and efficacy of therapy. *Trans.Roy.Soc.Trop.Med.Hyg.* **92**, 572–573 (1998).

CHRISTOPHER G.W., CIESLAK T.J., PAVLIN J.A., EITZEN E.M.: Biological warfare: a historical perspective. *J.Am.Med.Assoc.* **278**, 412–417 (1997).

CRAVEN R.B., MAUPIN G.O., BEARD M.L., QUAN T.J., BARNES A.M.: Reported cases of human plague infections in the United States, 1970–1991. *J.Med.Entomol.* **30**, 758–761 (1993).

CROOK L.D., TEMPEST B.: Plague: a clinical review of 27 cases. *Arch.Intern.Med.* **152**, 1253–1256 (1992).

DENNIS D.T., IGLESBY T.V., HENDERSON D.A., BARTLETT J.G., ASCHER M.S., EITZEN E., FINE A.D., FRIEDLANDER A.M., HAUER J., LAYTON M., LILLIBRIDGE S.R., MCDADE J.E., OSTERHOLM M.T., O'TOOLE T., PARKER G., PERL T.M., RUSSELL P.K., TONAT K.: Tularemia as a biological weapon – medical and public health management. *J.Am.Med.Assoc.* **285**, 2763–2773 (2001).

DERBES V.J.: De mussis and the great plaque of 1348: a forgotten episode in bacteriological war. *J.Am.Med.Assoc.* **196**, 59–62 (1966).

DEVDARIANI Z.L., VERENKOV M.S., FEODOROVA V.A., SOLODOVNICOV N.S., BELOV L.G.: Identification of *Yersinia pestis* with varied plasmid composition using monoclonal and polyclonal fluorescent immunoglobulins. *FEMS Immunol.Med.Microbiol*. **6**, 31–35 (1993).

DOLL J.M., ZEITZ P.S., ETTESTAD P., BUCHOLTZ A.L., DAVIS T., GAGE K.: Cat-transmitted fatal pneumonic plague in a person who traveled from Colorado to Arizona. *Am.J.Trop.Med.Hyg.* **51**, 109–114 (1994).

DOROFE'EV K.A.: Classification of the causative agent of tularemia. *Symp.Res.Works Inst.Epidemiol.Microbiol.Chita* **1**, 170–180 (1947).

ELSHOLZ B., NITSCHE A., ACHENBACH J., ELLERBROK H., BLOHM L., ALBERS J., PAULI G., HINTSCHE R., WORL R.: Electrical microarrays for highly sensitive detection of multiplex PCR products from biological agents. *Biosens.Bioelectron.* **24**, 1737–1743 (2009).

EMANUEL P.A., DANG J., GEBHARDT J.S., ALDRICH J., GARBER E.A.E., HENRIETA K., STOPA P., VALDES J.J., SCHULTZ A.D.: Recombinant antibodies: a new reagent for biological agent detection. *Biosens.Bioelectron.* **14**, 761–770 (2000).

FEDOROVA V.A., PETROVA A.V., DEVDARIANI Z.L.: Influence of cultivation conditions on the expression of *Yersinia pestis* YopE. *Zh.Mikrobiol.Epidemiol.Imunobiol*. **4**, 3–7 (2005).

FONSECA A.P., CORREIA P., EXTREMINA C.I., SOUSA J.C., TENREIRO R., BARROS H.: Molecular epidemiology of *Pseudomonas aeruginosa* clinical isolates from Portuguese central hospital. *Folia Microbiol*. **53**, 540–546 (2008).

FORSMAN M., SANDSTRÖM G., JAURIN B.: Identification of *Francisella* species and discrimination of type A and type B strains of *F. tularensis* by 16S rRNA analysis. *Appl.Environ.Microbiol.* **56**, 949–955 (1990).

GAULTNEY J.B., WENDE R.D., WILLIAMS R.P.: Microagglutination procedures for febrile agglutination tests. *Appl.Microbiol.* **22**, 635–640 (1971).

GRUNOW R., SPLETTSTOESSER W., MCDONALD S., OTTERBEIN C., O'BRIEN T., MORGAN C., ALDRICH J., HOFER E., FINKE E.J., MEYER H.: Detection of *Francisella tularensis* in biological specimens using a capture enzyme-linked immunosorbent assay, an immunochromatographic handheld assay, and a PCR. *Clin.Diagn.Lab.Immunol.* **7**, 86–90 (2000).

GURYCOVÁ D.: First isolation of *Francisella tularensis* subsp. *tularensis* in Europe. *Eur.J.Epidemiol.* **46**, 797–802 (1998).

HIGGINS J.A., COOPER M., SCHROEDER-TRUCKER L., BLACK S., MILLER D., KARNS J.S., MANTHEY E., BREEZE R., PERDUE M.L.: A field investigation of *Bacillus anthracis* contamination of *U.S. Department of Agriculture* and other Washington, D.C., buildings during the anthrax attack of October 2001. *Appl.Environ.Microbiol.* **69**, 593–599 (2002).

HOLLIS D.G., WEAVER R.E., STEIGERWALT A.G., WENGER J.D., MOSS C.W., BRENNER D.J.: *Francisella philomiragia* comb.nov. (formerly *Yersinia philomiragia*) and *Francisella tularensis* biogroup novicida (formerly *Francisella novicida*) associated with human disease. *J.Clin.Microbiol.* **27**, 1601–1608 (1989).

HOPLA C.E.: The ecology of tularemia. *Adv.Vet.Sci.Comp.Med.* **18**, 25–53 (1974).

HUBÁLEK Z., SIXL W., HALOUZKA J.: *Francisella tularensis* in *Dermacentor reticulatus* ticks from the Czech Republic and Austria. *Wien.Klin.Wochenschr.* **110**, 909–910 (1998).

JOSEFSON D.: US fear of bioterrorism spreads as anthrax cases increase. *Brit.Med.J.* **323**, 877–878 (2001).

KEIM P., KALIF A., SCHUPP J., HILL K., TRAVIS S.E., RICHMOND K., ADAIR D.M., HUGH-JONES M., KUSKE C.R., JACKSON P.: Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. *J.Bacteriol.* **1979**, 818–824 (1997).

KEIM P., SMITH K.L., KEYS C., TAKAHASHI H., KURATA T., KAUFMANN A.: Molecular investigation of the Aum Shinrikyo anthrax release in Kameido, Japan. *J.Clin.Microbiol.* **39**, 4566–4567 (2001).

KIEL J.L., PARKER J.E., HOLWITT E.A., MCCREARY R.P., ANDREWS C.J., DE LOS SANTOS A., WADE M., KALNS J., WALKER W.: Geographical distribution of genotypic and phenotypic markers among *Bacillus anthracis* isolates and related species by historical movement and horizontal transfer. *Folia Microbiol*. **53**, 472–478 (2008).

KILONZO B.S., MBISE T.J., MWALIMU D.C., KINDAMBA L.: Observations on the endemicity of plague in Karatu and Ngorongoro, northern Tanzania. *Tanzan.Health Res.Bull.* **8**, 1–6 (2006).

KORTEPETER M.G., CIESLAK T.J., EITZEN E.M.: Bioterrorism. *J.Environ.Health* **63**, 21–24 (2001).

LAFORCE F.M.: Anthrax. *Clin.Infect.Dis*. **19**, 1009–1014 (1994).

LEONARD C., ZEKRI O., MAHILLON J.: Integrated physical and genetic mapping of *Bacillus cereus* and other Gram-positive bacteria based on the IS231A transposition vectors. *Infect.Immun.* **66**, 2163–2169 (1998).

MATERO P., PASANEN T., LAUKKANEN R., TISSARI P., TARKKA E., VAARA M., SKURNIK M.: Real-time multiplex PCR assay for detection of *Yersinia pestis* and *Yersinia pseudotuberculosis*. *APMIS* **117**, 34–44 (2009).

MAYOR A.: Dirty tricks in ancient warfare. *Mil.Hist.Quart.* **10**, 1–37 (1997).

MCCOY G.W., CHAPIN C.W.: Further observations on a plaque-like disease of rodents with a preliminary note on the causative agent, *Bacterium tularense. J.Infect.Dis.* **10**, 61–72 (1912).

MCDONOUGH K.A., SCHWAM T.G., THOMAS R.E., FALKOW S.: Identification of a *Yersinia pestis*-specific DNA probe with potential for use in plague surveillance. *J.Clin.Microbiol.* **26**, 2525–2519 (1988).

MEYER M.H., STEHR M., BHUJU S., KRAUSE H.J., HARTMANN M., MIETHE P., SINGH M., KEUSGEN M.: Magnetic biosensor for the detection of *Yersinia pestis*. *J.Microbiol.Meth*. **68**, 218–224 (2007).

NORKINA O.V., KULICHENKO A.N., GINTSBURG A.L., TUCHKOV I.V., POPOV Y.A., AKSENOV M.U., DROSDOV I.G.: Development of a diagnostic test for *Yersinia pestis* by the polymerase chain reaction. *J.Appl.Bacteriol.* **76**, 240–245 (1994).

OLSON K.B.: Aum Shinrikyo: once and future threat? *Emerg.Infect.Dis.* **5**, 513–516 (1999).

OLSUFJEV N.G., EMELYANOVA O.S., DUNAEVA T.N.: Comparative study of strains of *B. tularense* in the Old and New World and their taxonomy. *J.Hyg.Epidemiol.Microbiol.Immunol.* **3**, 138–149 (1959).

OLSUFJEV N.G., MESHCHERYAKOVA I.S.: Subspecific taxonomy of *Francisella tularensis* MCCOY and CHAPIN 1912. *Internat.J.Syst. Bacteriol.* **33**, 872–874 (1983).

ÖZCÜRÜMEZ M.P., KISCHEL N., PRIEBE H., SPLETTSTÖSTER W., FINKE E.J., GRUNOW R.: Comparison of enzyme-linked immunosorbent assay, Western blotting, microagglutination, indirect immunofluorescence assay, and flow cytometry for serological diagnosis of tularemia. *Clin.Diagn.Lab.Immunol.* **11**, 1008–1015 (2004).

PERRY R.D., FETHERSTON J.D.: *Yersinia pestis*-etiologic agent of plague. *Clin.Microbiol.Rev.* **10**, 35–66 (1997).

PIKULA J., BEKLOVÁ M., HOLEŠOVSKÁ Z., TREML F.: Ecology of European brown hare and distribution of natural foci of tularaemia in the Czech Republic. *Acta Vet.Brno* **73**, 267–273 (2004).

PLOURDE P.J., EMBREE J., FRIESEN F., LINDSAY G.: Glandular tularemia with typhoidal features in a Manitoba child. *Can.Med.Assoc.J.* **146**, 1953–1955 (1992).

POHANKA M.: Evaluation of immunoglobulin production during tularaemia infection in BALB/c mouse model. *Acta Vet.Brno* **76**, 579–584 (2007).

POHANKA M., SKLÁDAL P.: Piezoelectric Immunosensor for *Francisella tularensis* detection using immunoglobulin M in a limiting dilution. *Analyt.Lett.* **38**, 411–422 (2005).

POHANKA M., SKLÁDAL P.: Serological diagnosis of tularemia in mice using the amperometric immunosensor. *Electroanalysis* **19**, 2507–2512 (2007*a*).

POHANKA M., SKLÁDAL P.: Piezoelectric immunosensor for the direct and rapid detection of *Francisella tularensis*. *Folia Microbiol.* **52**, 325–330 (2007*b*).

POHANKA M., PAVLIŠ O., SKLÁDAL P.: Diagnosis of tularemia using piezoelectric biosensor technology. *Talanta* **71**, 981–985 (2007*a*).

POHANKA M., PAVLIŠ O., SKLÁDAL P.: Rapid characterization of monoclonal antibodies using the piezoelectric immunosensor. *Sensors* **7**, 341–353 (2007*b*).

POHANKA M., TREML F., HUBÁLEK M., BANĎOUCHOVÁ H., BEKLOVÁ M., PIKULA J.: Piezoelectric biosensor for a simple serological diagnosis of tularemia in infected european brown hares (*Lepus europaeus*). *Sensors* **7**, 2825–2834 (2007*c*).

POHANKA M., SKLÁDAL P., KROČA M.: Biosensors for biological warfare agent detection. *Def.Sci.J.* **57**, 185–193 (2007*d*).

PRIOR R.G., KLASSON L., LARSSON P., WILLIAMS K., LINDLER L., SJOSTEDT A., SVENSSON T., TAMAS I., WREN B.W., OYSTON P.C.F., ANDERSSON S.G.E., TITBALL R.W.: Preliminary analysis and annotation of the partial genome sequence of *Francisella tularensis* strain Schu 4. *J.Appl.Microbiol.* **91**, 1–7 (2001).

PULLEN R.L., STUART B.M.: Tularemia: analysis of 225 cases. *J.Am.Med.Assoc.* **129**, 495–500 (1945).

QIOMM R., CAMPBELL A.M., PHILLIPS A.P.: A monoclonal antibody specific for the A antigen of *Brucella* spp. *J.Gen.Microbiol*. **139**, 2285–2289 (1984).

RAHALISON L., VOLOLONIRINA E., RATSITORAHINA M., CHANTEAU S.: Diagnosis of bubonic plague by PCR in Madagascar under field conditions. *J.Clin.Microbiol.* **38**, 260–263 (2000).

RAMISSE V., PATRA G., GARRIQUE J.L, GUESDON J.L., MOCK M.: Identification and characterization of *Bacillus anthracis* by multiplex PCR analysis of sequences on plasmids pXO1 and pXO2 and chromosomal DNA. *FEMS Microbiol.Lett.* **145**, 9–16 (1996).

REYN C.F., WEBER N.S., TEMPEST B., BARNES A.M., POLAND J.D., BOYCE J.M., ZALMA V.: Epidemiologic and clinical features of an outbreak of bubonic plague in New Mexico. *J.Infect.Dis.* **136**, 489–494 (1977).

RUSSELL J.C.: That earlier plague. *Demography* **5**, 174–184 (1968).

Sandström G., Sjöstedt A., Forsman M., Pavlovich N.V., Mishankin B.N.: Characterization and classification of strains of *Francisella tularensis* isolated in the central asian focus of the Soviet Union and in Japan. *J.Clin.Microbiol.* **30**, 172–175 (1992).

SCHMITT P., SPLETTSTÖSSER W., ÖZCÜRÜMEZ M.P., FINKE E.J., GRUNOW R.: A novel screening ELISA and a confirmatory Western blot useful for diagnosis and epidemiological studies of tularemia. *Epidemiol.Infect.* **133**, 759–766 (2005).

SELLEK R., JIMENEZ O., AIZPURUA C., FERNANDEZ-FRUTOS B., DE LEON B., CAMANACHO M., FERNANDEZ-MOREIRA D., YBARRA C., CARLOS CABRIA J.: Recovery of *Francisella tularensis* from soil samples by filtration and detection by real-time PCR and cELISA. *J.Environ.Monit.* **10**, 362–369 (2008).

SEYDLOVÁ G., SVOBODOVÁ J.: Development of membrane lipids in the surfactin producer *Bacillus subtilis*. *Folia Microbiol*. **53**, 303–307 (2008).

SIRET V., BARATAUD D., PRAT M., VAILLANT V., ANSART S., LECOUSTUMIER A., VAISSSAIRE J., RAFFI F., GARRE M., CAPEK I.: An outbreak of airborne tularemia in France, August 2004. *Eur.Surveill.* **11**, 58–60 (2006).

SMITHER S.J., HILL J., VAN BAAR B.L., HULST A.G., DE JONG A.L., TITBALL R.W.: Identification of outer membrane proteins of *Yersinia pestis* through biotinylation. *J.Microbiol.Meth.* **68**, 26–31 (2007).

STEWART A., SATTERFIELD B., COHEN M., O'NEILL K., ROBISON R.: A quadruplex real-time PCR assay for the detection of *Yersinia pestis* and its plasmids. *J.Med.Microbiol.* **57**, 324–331 (2008); mass spectrometry: *J.Biol.Chem.* **279**, 38693–38700 (2008).

SWIETNICKI W., O'BRIEN S., HOLMAN K., CHERRY S., BRUEGGMANN E., TROPEA J.E., HINES H.B., WAUGH D.S., ULRICH R.G.: Novel protein–protein interactions of the *Yersinia pestis* type III secretion system elucidated with a matrix analysis by surface plasmon resonance. *J.Biol.Chem.* **279**, 38693–38700 (2004).

TANABE M., ATKINS H.S., HARLAND D.N., ELVIN S.J., STAGG A.J., MIRZA O., TITBALL R.W., BYRNE B., BROWN K.A.: The ABC transporter protein OppA provides protection against experimental *Yersinia pestis* infection. *Infect.Immun*. **74**, 3687–3691 (2006).

TAYLOR L.H., LATHAM S.M., WOOLHOUSE M.E.J.: Risk factors for human disease emergence. *Phil.Trans.Roy.Soc.Lond.* B **356**, 983–989 (2001).

TIMS T.B., LIM D.V.: Rapid detection of *Bacillus anthracis* spores directly from powders with an evanescent wave fiber-optic biosensor. *J.Microbiol.Meth.* **59**, 127–130 (2004).

TITBALL R.W., TURNBULL P.C.B., HUSTON R.A.: The monitoring and detection of *Bacillus anthracis* in the environment. *J.Appl.Bacteriol.Symp.* **70**, 9–18 (1991).

TOMASO H., REISINGER E.C., AL DAHOUK S., FRANGOULIDIS D., RAKIN A., LANDT O., NEUBAUER H.: Rapid detection of *Yersinia pestis* with multiplex real-time PCR assays using fluorescent hybridisation probes. *FEMS Immunol.Med.Microbiol*. **38**, 117–126 (2003).

UITHOVEN K.A., SCHMIDT J.C., BALLMAN M.E.: Rapid identification of biological warfare agents using an instrument employing a light addressable potentiometric sensor and a flow-through immunofiltration-enzyme assay system. *Biosens.Bioelectron.* **14**, 761–770 (2000).

WANG T., QI Z., WU B., ZHU Z., YANG Y., CUI B., DAI R., ZHANG Q., QIU Y., WANG Z., WANG H., GUO Z., WANG X., YANG R.: A new purification strategy for fraction 1 capsular antigen and its efficacy against *Yersinia pestis* virulent strain challenge. *Protein Expr.Purif.* **61**, 7–12 (2008).

WHITEHOUSE C.A., HOTTEL H.E.: Comparison of five commercial DNA extraction kits for the recovery of *Francisella tularensis* DNA from spiked soil samples. *Mol.Cell Probes* **21**, 92–96 (2006).

WILLIAMS J.E., GENTRY M.K., BRADEN C.A., LEISTER F., YOLKEN R.H.: Use of enzyme-linked immunosorbent assay to measure antigenaemia during acute plague. *Bull.WHO* **62**, 463–466 (1984).

ZHANG F., LIU W., CHU M.C., HE J., DUAN Q., WU X.M., ZHANG P.H., ZHAO Q.M., YANG H., XIN Z.T., CAO W.C.: *Francisella tularensis* in rodents, China. *Emerg.Infect.Dis.* **12**, 994–996 (2006).