

Pakistan's experience of a bioterrorism-related anthrax scare

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خبرة باكستان في الخوف من الجمرة الخبيثة المرتبطة بالإرهاب البيولوجي
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الخلاصة: تلقى المعهد الوطني للصحة في إسلام آباد، باكستان خلال الفترة بين تشرين الثاني/نوفمبر 2001 وأذار/مارس 2002، 230 عينة من 194 مصدراً مختلفاً لتحليلها بحثاً عن أبواغ الجمرة الخبيثة. وقد أخذت هذه العينات من رسائل وطرود اشتبه بأنها تحتوي على الجمرة الخبيثة، ومن الأشخاص الذين تعرضوا لتلك الرسائل والطرود البريدية. ولدى زراعة العينات على الأغار بدم الخراف، أنتجت 141 عينة نموات توحى بأنواع من العصويات. وانطلاقاً من خصائص تلك النموات مثل غياب الإختلال الدموي الليبتائي وغياب الحركة أو الشك بوجودها إلى جانب الخصائص الشكلية للمستفردات الملونة مملون غرام، فقد اعتبرت 62 من المستفردات مشتبهاً وتم تلقيح القبيعات به. وقد بقيت الحيوانات الملقحة بالمستفردات المشبوهة بصحة جيدة مدةً أطول من المدة المطلوبة للسرقة والتي تقدر بخمسة أيام، مما أدى إلى القول بسلبية جميع نماذج وعدم احتوائها على العصويات الجمرية. وقد نوقش نظام التعامل مع المواد التي يشتبه بتلوثها بجراثيم الجمرة وتحليلها.

ABSTRACT From November 2001 to March 2002, the National Institute of Health, Islamabad, Pakistan, received 230 samples from 194 different sources for analysis for anthrax spores. These samples were taken from letters/packages suspected of containing anthrax and from individuals exposed to them. When cultured on sheep blood agar, 141 samples yielded growth suggestive of *Bacillus* species. On the basis of growth characteristics, absence of beta-haemolysis, absent or doubtful motility and morphological characters of the isolates on Gram stain, 62 isolates were considered suspicious and were inoculated into guinea-pigs. Inoculated animals remained healthy well beyond the required observation period of 5 days. All the samples were therefore reported as negative for *B. anthracis*. Systems for handling and analysing suspected anthrax-contaminated materials are discussed.

Expérience d'une alerte au charbon liée au bioterrorisme au Pakistan

RESUME De novembre 2001 à mars 2002, l'Institut national de la Santé d'Islamabad (Pakistan) a reçu 230 échantillons de 194 sources différentes à analyser pour détecter la présence de spores du charbon. Ces échantillons provenaient de lettres/paquets suspectés de contenir le bacille du charbon, et d'individus exposés. Lorsqu'ils ont été mis en culture sur gélose au sang de mouton, 141 échantillons ont produit une croissance faisant penser à des espèces du bacille. Sur la base des caractéristiques de la croissance, de l'absence de bêta-hémolyse, de l'absence de motilité ou de son aspect douteux et des caractères morphologiques des isolats à la coloration de Gram, 62 isolats ont été considérés comme suspects et ont été inoculés à des cobayes. Les animaux inoculés sont restés sains bien au-delà de la période d'observation requise de 5 jours. Tous les échantillons ont donc été déclarés négatifs pour *B. anthracis*. Des systèmes pour la manipulation et l'analyse des matériels suspectés d'être contaminés par le bacille du charbon sont examinés.

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Introduction

Described as a disease of antiquity, anthrax is primarily a zoonosis predominantly affecting sheep, goats, cattle and other herbivores from which man is secondarily infected [1]. Caused by a Gram-positive, spore-forming bacillus called *Bacillus anthracis*, the human disease occurs in 3 main clinical forms: cutaneous anthrax, inhalation (or pneumonic) anthrax and gastrointestinal anthrax. The cutaneous form is the most common and carries the least mortality while inhalation anthrax is much less common but is almost 100% fatal if untreated [2].

Because of the long life of its spores and the fact that the spores can be prepared in bulk in powder form and can be used as a dispersible aerosol, anthrax has long been considered an ideal weapon for biological warfare [3]. Although no major deployment of anthrax as a biological weapon has been recorded, the Japanese used it in the Manchurian War in the early 1940s [2] and there is evidence that during the Second World War, the Allies came close to using anthrax 'spore bombs' against the Germans [4]. Recently, there have been widely publicized reports of letters containing anthrax spores being mailed to individuals and organizations in an apparent spate of terrorist attacks that are analogous to explosive 'letter bombs' that are used to target individuals [1].

Since the September 11 2001 terrorist attacks in the United States of America (USA), there have been a number of incidents of anthrax-related bioterrorism in the USA and other countries of the world. In Pakistan, the first package suspected of containing anthrax was found in Islamabad in late October 2001, and was followed by similar incidents throughout the country during subsequent months. Soon after the

first incident, the National Institute of Health in Islamabad was designated as the National Reference Centre for the diagnosis and management of anthrax. This paper documents the experience of the National Institute of Health in handling and analysing samples of suspected anthrax from letters/packages and from the individuals exposed to them.

Methods

Sample collection and transport

Samples collected were of 2 types: non clinical/environmental samples and clinical samples.

Three types of non-clinical and environmental samples were collected: the suspected powder; the envelope, letter or package in which the powder was received; and wipe swabs taken from inanimate objects or surfaces thought to have been contaminated by the suspected powder. Universal precautions were followed according to the National Institutes of Health and World Health Organization (WHO) guidelines for the collection and transport of contaminated samples [5-7].

Clinical samples collected from exposed individuals consisted of nasal swabs from anterior nares and skin swabs from areas of skin in contact with the suspect powder or the letter, envelope or package. Wherever possible, swabs were collected in duplicate, 1 for culture and 1 for direct staining. Blood samples, rarely indicated, were taken directly into blood culture bottles using standard protocols for blood culture.

Detailed instructions were issued to all relevant people about the proper transport of samples, preferably through a personal courier [5]. The senders were instructed to ship the sample containers sealed in an outer container made of non-porous material.

This was enclosed in yet another container made of strong thick plastic or metal.

Specimen handling in the laboratory

Samples were processed according to the USA Centers for Disease Control (CDC) guidelines [7]. All procedures, from the opening of sample containers to the issuing of the final report, were carried out inside a class II biological safety cabinet (BSL-II), in a separate room specified for this purpose. Only designated members of laboratory staff handled the samples using protective clothing, gloves, masks, etc. All items, including instruments and disposables used in the test, were discarded into a container of 10% sodium hypochlorite. Subsequently, reusable items were autoclaved and disposables were incinerated. After finishing work, all surfaces were wiped with hypochlorite solution.

Non-clinical/environmental samples

For analysis of powders, a small amount was suspended in 1.0 mL sterile distilled water in a sterile universal container, then 0.1 mL of suspension was inoculated onto sheep blood agar plate and incubated at 37 °C for 18–24 hours.

For analysis of envelopes, the envelope was opened and any powder inside was cultured as above. The letter was pulled out and a piece 2.5 × 2.5 cm was cut out, preferably from an area free from writing. The piece was transferred to 1.0 mL of sterile distilled water or saline and shaken for about 2 minutes. Then 0.1 mL of this fluid was inoculated on a sheep blood agar plate and incubated as above.

For analysis of soil or dusts, about 2.0 g of material was suspended in sample processing solution (phosphate buffered saline with 0.3% polysorbate-20 or normal saline), shaken vigorously and left for 2–3 minutes. The supernatant was divided into

2 aliquots, 1 of which was heated at 65–70 °C for 10 minutes and allowed to cool, then 0.1 mL from heated and unheated aliquots was streaked on sheep blood agar and incubated as above.

Environmental swabs were placed in 3 mL of sample processing solution or normal saline and shaken. The fluid was divided into 2 aliquots, 1 of which was heated at 65–70 °C for 10 minutes, then 0.1 mL from both aliquots was streaked on sheep blood agar and incubated.

Clinical samples

Swabs from anterior nares and skin were streaked onto sheep blood agar plates and incubated for 18–24 hours. From the second swab (or from the same swab used for streaking), smears were made on slides and stained with Gram stain for direct microscopic examination.

Blood cultures were incubated for up to 7 days. From those showing growth, subcultures were made on sheep blood agar.

Analysis of cultures

After incubation for 18–24 hours, inoculated plates showing growths were examined for colony characters, haemolysis, morphology, spore formation, motility and catalase test. If these tests indicated growth of *Bacillus* species, the isolates were then subjected to tests for species characterization. A 3-step strategy was adopted for identification of suspected isolates.

Level A

Cultures yielding large (3–5 mm) colonies especially those with ground-glass or medusa head appearance, which were non-beta haemolytic and on Gram stain showing large spore-forming bacilli in chains, were non-motile and catalase positive, were considered as suspicious and subjected to further (level B) tests.

Level B

Representative colonies from the above plates were subcultured on MacConkey agar, bicarbonate agar for capsule formation (trypticase soy agar containing 0.8% NaHCO₃, incubated in a candle jar) and a gelatin stab culture. Mueller–Hinton agar was inoculated in duplicate, 1 for penicillin susceptibility (10 U) and 1 for the pearl-string test.

For the pearl-string test, a heavy single streak was made on Mueller–Hinton agar. A 10 U penicillin disc was applied over the streak, which was overlaid with a coverslip. After incubation for 3–6 hours, growth from beneath the coverslip was examined microscopically for the presence of strings of spherical cellular forms of the organism. Presence of such 'strings of pearls' is considered characteristic of *B. anthracis* [8].

B. anthracis does not grow on MacConkey agar, forms mucoid colonies on bicarbonate agar [9], gives an inverted fir-tree appearance in gelatin stab culture after a 2–3 day incubation, is susceptible to 10 U penicillin and may yield a positive pearl-string test. An india ink preparation was made from any mucoid colonies on bicarbonate agar and examined microscopically for capsule formation. The capsule appears as a well-defined clear zone around the bacilli. Also a smear from mucoid colonies was stained with polychrome methylene blue (McFadyean reaction) and examined microscopically for capsule formation [7,10]. A presumptive identification of *B. anthracis* could be made on the results of these tests.

Level C

For final confirmation, all presumptive positive cultures were inoculated into guinea-pigs. Guinea-pig is highly susceptible to *B. anthracis*. Inoculation of 0.5 mL of a 24-

hour broth subcutaneously will usually kill the animal within 48 hours [11]. Bacilli can be seen in a direct smear from heart blood of the dead animal and can be isolated from blood and other organs [11]. Colonies were emulsified in 1.0 mL of sterile normal saline to give a slightly turbid suspension. Then 0.5 mL of this suspension (or 0.5 mL of an overnight broth culture) was injected subcutaneously over the thigh area of each of 2 guinea-pigs. A third animal was injected with 0.5 mL of sterile saline as control. Animals were observed for a minimum of 5 days. To speed up the results, all primary cultures giving suspicious growth (level A) were also directly inoculated into guinea-pigs along with level B tests.

Smears were made from swabs, powder suspensions and from suspicious growth on blood agar and stained with malachite green (5% aqueous) for 45 minutes and counterstained with safranin for the demonstration of spores. Malachite green stains the spores green while the bacilli are stained red by safranin [7].

Results

Over a 5-month period from November 2001 to March 2002, a total of 230 samples from 194 sources were sent to the National Institute of Health for analysis. Samples were sent from all parts of the country. Table 1 lists the organizations and individuals who were recipients of parcels/letters suspected of containing anthrax.

Details of the types of clinical and non-clinical samples processed are given in Table 2. When cultured on sheep blood agar, 141 samples yielded growth suggestive of *Bacillus* species (Table 3). On the basis of growth characteristics, absence of beta haemolysis, absent or doubtful motility and morphological characters of the isolates on

Table 1 Recipients of parcels/letters suspected of containing anthrax

Type of organization	No. of samples
Foreign missions	18
Foreign media organizations, banks and multinational corporations	17
Institutions/universities/hospitals	61
Government officials	48
Individuals/miscellaneous	50
Total	194

Gram stain, 62 isolates were considered suspicious for *B. anthracis* and were therefore inoculated into guinea-pigs. Inoculated animals remained healthy well beyond the required observation period of 5 days. All

Table 2 Types of samples collected for analysis of suspected anthrax

Source	No. of samples
<i>Site of clinical samples</i>	
Anterior nares	42
Skin	26
Blood	2
Culture for identification	1
Total	71
<i>Source of non-clinical and environmental samples</i>	
Powders ^a	135
Swabs from inanimate objects/surfaces	10
Papers, envelopes, packages, plastics	12
Miscellaneous	2
Total	159
Total	230

^aIncludes a few samples of wood shavings, flour or soil.

the samples were therefore reported as negative for *B. anthracis*.

None of the isolates yielded mucoid colonies on bicarbonate agar, inverted fir-tree appearance in gelatin stab or a positive pearl-string test. However, for unexplained reasons a limited number of isolates were found to be susceptible to penicillin.

Discussion

One of the most reliable criteria for the preliminary screening of *Bacillus* isolates for *B. anthracis* is non-motility of the isolate. Any *Bacillus* isolate exhibiting motility may be safely assumed to be a species other than *B. anthracis*. The reverse, however, is not always true as some strains of *B. mycooides* (*B. cereus* var *mycooides*) are also non-motile [12].

Susceptibility to gamma phage has been widely accepted as a reliable test of identification for *B. anthracis* [7]. However, a number of *B. mycooides* strains are also reported to be susceptible to gamma phage [13,14]. Experience at the Pakistan National Institute of Health during the present study has shown that, in the absence of supplies of gamma phage (which CDC has been unable to provide) or a polymerase chain reaction (PCR) facility, animal inoculation remains the mainstay of a definitive identification of *B. anthracis*. It is not uncommon to isolate saprophytic members of the *Bacillus* group from the environment. In fact, they are the commonest contaminants in a clinical laboratory. But no other member of this group, except *B. anthracis*, will kill a guinea-pig within 48 hours when injected with a pure culture [11]. Our experience also underlines the difficulties inherent in the definitive diagnosis of anthrax by a routine microbiology laboratory. Without facilities for animal inoculation (or PCR or

Table 3 Results of cultures and animal inoculation with suspected samples of anthrax

Type of sample	Type of isolate					
	Suggestive of <i>Bacillus</i> spp. (n = 230)		Suggestive of <i>B. anthracis</i> ^a (n = 230)		Positive on guinea-pig inoculation (n = 62)	
	No.	%	No.	%	No.	%
Clinical samples	14	6	5	2	0	–
Powders	120	52	56	24	0	–
Environmental samples	7	3	1	<1	0	–
Total	141	61	62	27	0	–

^aIsolates with typical morphology on Gram stain, non-motile non-beta haemolytic, catalase-positive.

n = total number of cultures tested.

gamma phage), a presumptive diagnosis of *B. anthracis* based on inconclusive findings could have led to unforeseen complications.

It maybe no coincidence that since 11 September 2001, the USA has experienced a number of anthrax-related attacks with 5 deaths that were directly attributable to these attacks. All died of inhalation anthrax; all but one received or had contact with letters containing anthrax spores [15]. It has been speculated that Pakistan could also be a target for bioterrorism. Fortunately, in spite of a number of suspected incidents, this has proved to be mere speculation. It is well known that production of weapons-grade anthrax is a highly sophisticated technology. Our experience underlines the fact that the capability to produce anthrax parcel/letter bombs is not easily available to a potential terrorist; the only success of the perpetrators of these incidents has been to create an atmosphere of panic among the general public.

Although the incidents described in this report proved to be hoaxes in every case, they nonetheless served to demonstrate the

public health implications of an actual or potential bioterrorism attack as well as the strengths and weaknesses of the systems in place to deal with it. The threats called for a prompt and coordinated response on the part of several government agencies, notably in health, law enforcement and information. In spite of the fact that Pakistan has never faced a bioterrorism attack before, its systems performed surprisingly well. Facilities for laboratory testing and prompt reporting were fully in place right after the first incident. In some cases, the law enforcement agencies not only investigated the incident but also collected and delivered the suspect letter or package to the testing laboratory from distant regions. Communication and coordination with the original recipients of the object and the general public on the one hand and the public health, the law enforcement and mass media on the other, demonstrated the professionalism and confidence with which the episodes were handled by the authorities concerned, particularly in the Ministry of Health and the National Institute of Health. Availability of a first-rate laboratory,

backed by animal testing facilities, made this task considerably easier.

Conclusions

It is concluded on the basis of strong scientific evidence that all the incidents of suspected anthrax parcel/letter bombs in Pakistan were hoaxes. Nevertheless, standard operating procedures for disaster management in a bioterrorism setting should be formulated and their periodic review should be ensured. Isolates which are provisionally labelled as *B. anthracis* on the basis of presumptive tests should not be reported unless confirmed by a reference laboratory. It should be noted that animal inoculation, although no longer generally practised in clinical microbiology laboratories, still has a place in public health laboratory practice in developing countries with scarce resources.

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Public health response to biological and chemical weapons: WHO guidance

This second edition of WHO's 1970 publication *Health aspects of biological and chemical weapons* includes information designed to guide preparedness for and response to the deliberate use of biological and chemical agents that affect health. While noting that the probability of an attack with such weapons may be low, the guide underscores the magnitude of potential impacts on civilian populations and the corresponding need for public health authorities, in close cooperation with other parts of government, to develop contingency plans. For such plans to be effective, collaborative arrangements involving all partners have to be established and tested well before an incident or emergency occurs. Recommendations and advice draw on the expertise of many specialists around the world.

The publication can be obtained from: Marketing and Dissemination, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland (tel: +41 22 791 2476; fax: +41 22 791 4857; email: bookorders@who.int). It is also available on line at: <http://www.who.int/csr/delibepidemics/biochemguide/en/index.html>