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Molecular detection and identification of fatal infectious agent, isolated from patients in an outbreak occurred in Sistan & Balochistan province of Iran

Abstract

An outbreak of tularemia or plague like syndrome occurred in the province of Sistan and Balochistan of Iran, from May to June 2007. Tularemia and plague had not been reported in this region for last 100 years and before. Thirteen eight cases were identified with ulcer glandular syndrome dominant to all cases with age from children to elderly ages. With fatality rate of 26% and death of 8 patient that have been reported by villagers very late, all other patients and new cases recovered after antibiotic therapy and other heath measures to prevent the spread of diseases. Targeted chemoprophylaxis, sanitation, and vector control played a crucial role in controlling the outbreak. Coco bacillus like agents was isolated from the blood samples of the patients. Epidemiologic, microbiological and molecular analysis of samples findings suggested the possible existence of a local animal reservoir, food or water contamination during this period, but its origin could not be determined. This sudden and unexpected reemergence of tularemia or plague like disease in this province with no background history of rodents or other animal death from the disease or any human cases is important for molecular epidemiology and root finding.

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Introduction

Tularemia is a highly infectious disease which can be caused by a small amount of bacteria; it is naturally occurring. Typically found in rural areas, it is common in animal populations, and functions as a zoonosis if transmitted via the bite of an infected animal (in particular rodents, rabbits, and hares), or if transmitted via a tick or deer fly bite.[1] The disease can be contracted through the handling inhaling the bacteria which causes the illness. If inhaled and left untreated, it can lead to severe respiratory illness, pneumonia, and systemic infection. Tularemia, a zoonotic disease caused by the highly infective, virulent, non-sporulating Gram-negative coccobacillus *Francisella tularensis* It is a very small bacterium, 0.7-1.0 microns in length, that is non-motile and does not form spores... the organism can be isolated from contaminated environmental sources such as water and mud.[2] Humans can become infected with tularemia through many different routes, and age and sex factors do not seem to affect susceptibility. Common routes of infection are: through insect bites including ticks and mosquitoes, handling of contaminated animal tissue or fluid, recurrent contact act with contaminated water, food or soil, halation of infective aerosols.

Tularemia has a 3-5 day incubation period and the initial symptoms are flu-like in nature. These symptoms include: sudden fever, chills, headache, diarrhea muscle aches, dry cough, and progressive weakness. Other symptoms are more specific and depend on the route of infection. Skin ulcers and swollen glands may occur through coetaneous exposure to tularemia. Pneumonic tularemia occurs when the bacteria is inhaled. Symptoms for this form of tularemia include chest pains, bloody sputum and difficulty breathing.[2] Francisella tularensis infection occurs in Iran is supported by a study published in 1973, that serological evidence of infection was found in animals scattered throughout.[3] There is no data on epidemiologic or presence of tularemia cases in sistan and baluchestan provinces.

Plague is primarily a bacterial zoonosis affecting rodents. It is caused by *Yersinia pestis* and is transmitted from animal to animal by fleas. Humans usually become infected through the bite of an infected rodent flea, inhalation of infected aerosols or ingestion of contaminated food and water. Bubonic plague, a severe infectious disease which, in the absence of appropriate antimicrobial drug therapy, can evolve to a rapidly fatal septicemia or pneumonia, can develop. A pneumonia form, which enables direct transmission to contacts, can be responsible for highly lethal outbreaks.[4] Currently, plague natural foci persist in Asia, the Americas, and Africa (where most human cases occur).[5] Plague foci have previously existed in the Kurdistan province of Iran.[6]

The surveillance of territories surrounding the plague focus of Kurdistan province in Iran, by inspection of wild rodent burrows, permit to reveal the existence of an epizootic in a new focus located in the Eastern Azerbaijan province, where plague was never reported. In one study 14 strain of *Y. pestis* were isolated from Different rodents and from the fleas. The eventual relationship between these two areas separated by about 200km was investigated: neither attempts to isolate *Y. pestis* nor serological surveys permit to reveal any sign of plague enzootic in the zone between the Kurdistan focus nor the new described focus of the Eastern Azerbaijan.[7-10] There is no report of plague in Sistan and baluchestan of Iran.

Methods

Sistan-Balochistan province is located in extreme southeastern Iran. The combined Sistan and Baluchestan province today accounts for one of the driest regions of Iran. During May and Jun 2007, several patients with signs of severe infection and painful inflammatory adenopathy were admitted to the health branch of. Khash, saravan and other local health care centers. After eliminating all other possible differential diagnoses, clinicians suspected tularemia. Blood samples collected from patients were sent to the Microbiology Department of Zahedan tropical disease laboratory and Institute Pasteur of Iran. All samples were examined with standard bacteriologic methods.

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Direct examination of smears was performed after Wayson and Gram staining. Blood samples were cultured in Castaneda medium and examined daily. Further subcultures and biochemical tests were performed according to standard microbiological protocols.

PCR analysis

PCR was performed on DNA extracted from isolated bacteria from blood cultures. We have uses primers specific for F1 gene of the yersinia pestis as bellow protocols:

DNA template	1ml
Primer F	1ml
Primer R	1ml
DNTPs	2ml
MgCl2	0.75
PCR buffer 10X	2.5
Taq DNA pol	0.5
D.D.W	upto 25

PCR program

Stage	temp.°C	time(min)	cycle no.
Hot start	94	5	
Denat.	94	1	
Annea.	62	1	35
Exten.	72	1	
Final Ext	72	7	

Nested PCR

We have used inner primers specific for the F1 gene sequence to confirm the PCR product. The protocol is the same as above (Table 1).

Table1 Biochemical characteristic of different biovars of *Yersinia pestis*, Iranian type is identified as Mediavalis

Biovar	Glycerol	Arabinose	Nitrate	PCR and nested PCR
Antique	+	+	+	+
Mediavalis	+	+	-	+
Orientalis	-	+	+	+
Microtus	+	-	-	+
Zahedan-khash		+	-	+

Sequencing

For final confirmation, PCR product was sequences and nucleotide sequence were analyzed by Bioinformatics programs like BLAST to compare the sequence with databases (Table 2).

Results

Samples taken from members of one family in the same village, reveled several Gram negative coocobacill shape bacteria similar to *Francisella tularensis* and *Yersinia pestis*. Further bacteriological analysis In Institute Pasteur in Teheran demonstrated that bacteria are more similar to *Yersinia pestis* with Negative in Nitrate reduction and positive in Arabinose. Diagnosis of plague was suspected and confirmed by isolation of a bacterial DNA and molecular analysis. Figure 1 demonstrates the PCR product of 530 bp from F1 gene of the *Yersinia pestis*. Figure 2 is result of Nested PCR with inner primers to f1 gene and as its shown 2 fragment of 530 bp original gene and 280 bp is shown. The sequencing and PCR data demonstrate that Bacteria had F1 gene sequence (Figure 3-5).

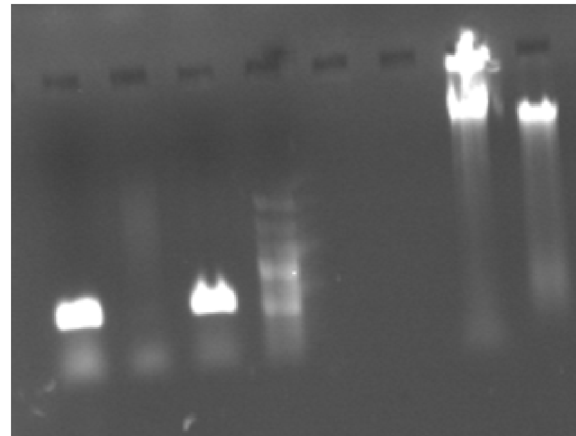

Figure 1 PCR of DNA extracted from bacteria isolated from Blood culture of patients . Primers F1 and R1 from gene specific to *Yersinia pestis*.

1. PCR product of patient no 1,
2. Negative control,
3. Positive control.
4. Molecular marker.

5 and 6. Total DNA extracted from bacterial samples.

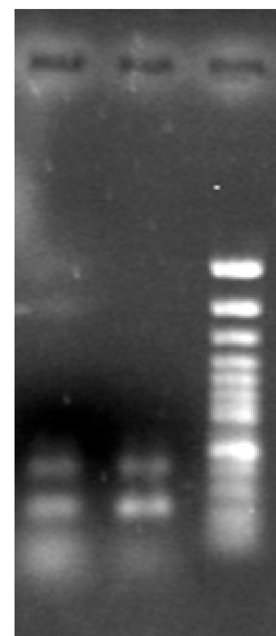

Figure 2 Nested PCR of the product from first PCR with inner primers.

1. Sample from PCR 1, two correct fragments is visible
2. Positive control DNA,
3. MW

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>PCR product Yersinia pestis plasmid pMT size: 540bp

AATTGCGGAATTCTTATTGGTTAGATACGGTTACGGTTACAGCATCAGTGTATTTACCTGCTGCAAGT
TTACCGCCTTTGGAACCAATTGAGCGAACAAAGAAATCCTGGCTGCCCGTAGCCAAGACGACGTCATC
CCCCACAAGGTTCTCACCGTTTACCTTAGGAGAGATATCAAAATCTCTAGAATCCTTGCCAATCACTT
TTGTAGTGAATTGGTGGTTATTTCCATCCTGAGAAGTAAATGTTAAGTACATGGGATCACCCGCGGCA
TCTGTAAAGTTAACAGATGTGCTAGTGGTTCCTGTTTTATAGCCGCCAAGAGTAAGCGTACCAACAAG
TAATTCTGTATCGATGTTTCCATTGTCCATAATTGTAATTGGAGCGCCTTCCTTATATGTAAGAGTGA
TGCGGGCTGGTTCAACAAGAGTTGCCGTTGCAGTGGTGCTTGCAGTTAAATCTGCCGCATTAGCAGTT
GCAATAGTTCCAAATAATGCAATGGCGATAACGG AACTGATTTTTTTCATATGTTACCTCAAAG

Figure 3 Nucleotide sequence of PCR fragment.

```
DEFINITION  Yersinia pestis envelope antigen F1 (caf1) gene, partial cds.
ACCESSION   AF542378
VERSION     AF542378.1  GI:27261280
KEYWORDS    .
SOURCE      Yersinia pestis
  ORGANISM  Yersinia pestis
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Yersinia.
REFERENCE   1  (bases 1 to 510)
  AUTHORS   Karami,A. and Kamali,M.
  TITLE     Nucleotide sequence of the Yersinia pestis envelope antigen F1
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 510)
  AUTHORS   Karami,A. and Kamali,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (31-AUG-2002) Molecular Biology, Institute of
            Immunology, P.O. Box 19615-471, Tehran, Iran
FEATURES             Location/Qualifiers
     source          1..510
                     /organism="Yersinia pestis"
                     /mol_type="genomic DNA"
                     /strain="IR"
                     /db_xref="taxon:632"
     gene            1..>510
                     /gene="caf1"
     CDS             1..>510
                     /gene="caf1"
                     /note="AK-F1"
                     /codon_start=1
                     /transl_table=11
                     /product="envelope antigen F1"
                     /protein_id="AAN87623.1"
                     /db_xref="GI:27261281"
                     /translation="MKKISSVIAIALFGTIATANAADLTASTTATATLVEPARITLTY
                     KEGAPITIMDNGNIDTELLVGTLTLGGYKTGTTSTSVNFTDAAGDPMYLTFTSQDGNN
                     HQFTTKVIGKDSRDFDISPKVNGENLVGDDVVLATGSQDFFVRSIGSKGGKLAAGKYT
                     DAVTVTVSNQ"
ORIGIN
        1 atgaaaaaaa tcagttccgt tatcgccatt gcattatttg gaactattgc aactgctaat
       61 gcggcagatt taactgcaag caccactgca acggcaactc ttgtcgaacc agcccgcatc
      121 actcttacat ataaggaagg cgctccaatt acaattatgg acaatggaaa catcgataca
      181 gaattacttg ttggtacgct tactcttggc ggctataaaa caggaaccac tagcacatct
      241 gttaacttta cagatgccgc gggtgatccc atgtacttaa catttacttc tcaggatgga
      301 aataaccacc aattcactac aaaagtgatt ggcaaggatt ctagagattt tgatatctct
      361 cctaaggtaa acggtgagaa ccttgtgggg gatgacgtcg tcttggctac gggcagccag
      421 gatttctttg ttcgctcaat tggttccaaa ggcggtaaac ttgcagcagg taaatacact
      481 gatgctgtaa ccgtaaccgt atctaaccaa
//

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Figure 4 Blast Comparision of PCR Sequence with Gen bank, reveals the 100% similarity of tested DNA with *Yersinia pestis* F1 gene.

```
0.SEQ          51  ACTATTGCAA CTGCTAATGC GGCAGATTTA ACTGCAAGCA CCACTGCAAC   100
1.SEQ          51  ACCACAGCAA CTGCTA---- ---------- ---------- ----------   100
2.SEQ          51  ACTATTGCAA CTGCTAATGC GGCAGATTTA ACTGCAAGCA CCACTGCAAC   100
3.SEQ          51  ACTATTGCAA CTGCTAATGC GGCAGATTTA ACTGCAAGCA CCACTGCAAC   100
42B90~1.SEQ    51  ACTATTGCAA CTGCTAATGC GGCAGATTTA ACTGCAAGCA CCACTGCAAC   100
5.SEQ          51  ACTATTGCAA CTGCTAATGC GGCAGATTTA ACTGCAAGCA CCACTGCAAC   100
7.SEQ          51  ACTATTGCAA CTGCTAATGC GGCAGATTTA ACTGCAAGCA CCACTGCAAC   100
8.SEQ          51  ACTATTGCAA CTGCTAATGC GGCAGATTTA ACTGCAAGCA CCACTGCAAC   100
9.SEQ          51  ACTATTGCAA CTGCTAATGC GGCAGATTTA ACTGCAAGCA CCACTGCAAC   100
GORGIA.SEQ     51  ACTATTGCAA CTGCTAATGC GGCAGATTTA ACTGCAAGCA CCACTGCAAC   100
KARAMI.SEQ     51  ACTATTGCAA CTGCTAATGC GGCAGATTTA ACTGCAAGCA CCACTGCAAC   100
PMT1.SEQ       51  AGTA------ -----AATG- -------TTA AGTACATG-- ----------   100
R.SEQ          51  AGTA------ -----AATG- -------TTA AGTACATG-- ----------   100
                          110        120        130        140        150
0.SEQ         101  GGCAACTCTT GTCGAACCAG CCCGCATCAC TCTTACATAT AAGGAAGGCG   150
1.SEQ         101  -----GCTTA GTTGAACCTG CCAGAATCAC ACTTACTTAC AAAGAGGGTG   150
2.SEQ         101  GGCAACTCTT GTTGAACCAG CCCGCATCAC TCTTACATAT AAGGAAGGCT   150
3.SEQ         101  GGCAACTCTT GTTGAACCAG CCCGCATCAC TCTTACATAT AAGGAAGGCG   150
42B90~1.SEQ   101  GGCAACTCTT GTTGAACCAG CCCGCATCAC TCTTACATAT AAGGAAGGCG   150
5.SEQ         101  GGCAACTCTT GTTGAACCAG CCCGCATCAC TCTTACATAT AAGGAAGGCG   150
7.SEQ         101  GGCAACTCTT GTTGAACCAG CCCGCATCAC TCTTACATAT AAGGAAGGCG   150
8.SEQ         101  GGCAACTCTT GTTGAACCAG CCCGCATCAC TCTTACATAT AAGGAAGGCG   150
9.SEQ         101  GGCAACTCTT GTCGAACCAG CCCGCATCAC TCTTACATAT AAGGAAGGCG   150
GORGIA.SEQ    101  GGCAACTCTT GTTGAACCAG CCCGCATCAC TCTTACATAT AAGGAAGGCT   150
KARAMI.SEQ    101  GGCAACTCTT GTCGAACCAG CCCGCATCAC TCTTACATAT AAGGAAGGCG   150
PMT1.SEQ      101  ---------- ---GGATCA- CCCGCGGCAT C------TGT AAAG------   150
R.SEQ         101  ---------- ---GGATCA- CCCGCGGCAT C------TGT AAAG------   150
                          160        170        180        190        200
0.SEQ         151  CTCCAATTAC AATTATGGAC AATGGAAACA TCGATACAGA ATTACTTGTT   200
1.SEQ         151  CTCCAATAAC CATTATGGAT AATGGCAATA TCGACACAGA GCTTCTAGTT   200
2.SEQ         151  CTCCAATTAC AATTATGGAC AATGGAAACA TCGATACAGA ATTACTTGTT   200
3.SEQ         151  CTCCAATTAC AATTATGGAC AATGGAAACA TCGATACAGA ATTACTTGTT   200
42B90~1.SEQ   151  CTCCAATTAC AATTATGGAC AATGGAAACA TCGATACAGA ATTACTTGTT   200
```

Figure 5 Multiple sequence similarity analysis of sequence of PCR product with several F1 gene sequence for different biovars of *Yersinia pestis*.

Table 2 Analysis of primer used in this study

Sequences producing significant alignments	(bits)	Value
gi ׀ 39754771 ׀ gb ׀ AY450847.1 ׀ Yersinia pestis strain 482 plasm_ _ _	48	4e-04
gi ׀ 39754755 ׀ gb ׀ AY450846 .1 ׀ Yersinia pestis strain 1351 piss_ _ _	48	4e-04
gi ׀ 39754751 ׀ gb ׀ AY450845 .1 ׀ Yersinia pestis strain EV plasmi_ _ _	48	4e-04
gi ׀ 3883003 ׀ gb ׀ AF 074611 _ 1 ׀ Yersinia pestis is KM plasmid pliT -1 _ _ _	48	4e-04
gi ׀ 2996286 ׀ gb ׀ AF 053947 _ 1 ׀ Yersinia pestis is KM plasmid OTT 1, _ _ _	48	4e-04
gi ׀ 45357241 ׀ gb ׀ AE017045 _1 ׀ Yersinia pestis biovar Mediaeval _ _ _	48	4e-04
gi ׀ 5834685 ׀ am:do ׀ AL117211 _1 ׀ YP PliT1 Yersinia pestis C092 plasm_ _ _	48	4e-04
gi ׀ 52537981 ׀ emb ׀ AJ 698720_ 1 ׀ Yersinia pestis pG8786 plasmid	48	4e-04
gi ׀ 48620 ׀ emb ׀ X61996.1 ׀ YPCAFY. pe se is genes call, caf1M, caf _ _ _	48	4e-04

Discussion

Epidemiologic investigation did not identify any other tularemia or plague patients before this outbreak. It is unlikely that other cases occurred and remained undetected during this period. It seems that bacterial agent gives severe infection with high fatality rates. In this investigation we used molecular methods to diagnose identify the infectious agent of unknown origin for the first time. This coordinated experience to rapidly diagnose and contain the severe fatal infection by using molecular methods can be uses for any other similar situations. The bacteriologic diagnosis is a long procedure (at least 4 days) and, in this epidemic molecular method contributed to the effectiveness of the response. The outbreak occurred in a poor rural area of Khash and Saravan villages, with inadequate sanitation. The resident's interview revealed increase in the population of commensal rodents but no unusual rodent mortality was noted during the weeks preceding the outbreak.

A crisis management program was developed based on standardized case management, prophylactic treatment and follow-up of contacts sharing the patients, and vector control. No natural focus of tularemia or plague had ever been described in these provinces. Assumption for the original sources of infection have been claimed as contaminated meat, possible source of rabbit or other hunted animals or transported goat from border so the reappearance of human cases in this area can be explained in 3 ways: a recent importation of infected animals, contaminated meat from hunting or a sudden manifestation of a natural focus that had remained silent for decades and finally possible bioterrorist act. These has been observation and claims by villagers and officials of the region of unknown disease and death of goats in the villages central to the epidemic and other rural areas. Preliminary examination of few carcasses fund in the region by Veterinary member of the group did not ravel any evidence but there is no scientific report of tests or laboratory analysis of samples from these carcasses. So, it is not possible to exclude any of this assumption without further molecular epidemiology of the region and exact examination of samples from animals, rodents, vectors, water and environmental samples and follow up of rural population by serology tests. The outbreak occurred at the region with no cultivation or agricultural activity. The current challenge in terms of public health is to determine the source, genotype and the type exposure of people to the infectious agent.

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Conflicts of interest

Author declares that there is no conflict of interest.

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