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Rapid Diagnosis of Highly Pathogenic Clinical Strains of Yersinia Pseudotuberculosis Using PCR and Preservation of 70Kbp Virulence Plasmid

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Abstract

Clinical strains of Yersinia.pseudotuberculosis were screened for 70 Kbp virulence plasmids it was found only 20 % carrying 70Kbp plasmid, when subjected to different concentrations of Naledixin for development of mutant strains , plasmids were lost from one strain Yersinia pseudotuberculosis IP3295, which leaded to the modification of L.B medium into human blood (H.B) L.B medium as culture medium for Yersinia pseudotuberculosis .Yersinia pseudotuberculosis IP3295 showed 70Kbp virulence plasmid with additional small plasmids which proves the incorporation of plasmids with chromosomal DNA and similarity with Yersinia pestis. All strains of Yersinia pseudotuberculosis harboring virulence plasmids were detected for FyuA and Irp2 genes of the High pathogenicity island using PCR. And it was found that all plasmid harboring strains contains high Pathogenicity Island.

Keywords: Yersinia pseudotuberculosis, High pathogenecity island, 70Kbp virulence plasmid

1. Introduction

Yersinia spp. are gram-negative, facultative rods that are motile when isolated from environment but become non-motile in mammalian host. Three species of *Yersinia* cause disease in humans: *Yersinia enterocolitica, Yersinia pseudotuberculosis*, and *Yersinia pestis*. These species differ considerably in invasiveness. *Y. enterocolitica* and *Y. pseudotuberculosis* can cross the gastrointestinal mucosa to infect underlying tissue, but infections usually remain localized in the sub-mucosal area. *Y. pestis* is injected into the body by an insect bite and thus does not have to penetrate a body surface on its own, but once inside the body, it spreads rapidly and causes a systemic infection (Mingrone and Fantasia, 1988; Cornelis, 2002).

Infections due to *Y. enterocolitica* and *Y. pseudotuberculosis* are acquired by ingestion of Contaminated food or water. Both of these species are ubiquitous in the environment and infect a variety of wild and domestic animals as well as humans. *Y. enterocolitica* is a common cause of human disease, whereas *Y. pseudotuberculosis* is primarily an animal pathogen that only occasionally causes disease in humans.

In recent years, *Y. enterocolitica* infections have been particularly common among children, And outbreaks have occurred in day-care centers and schools. Symptoms of *Y. enterocolitica* Infections vary from a mild form of the disease, characterized by diarrhea and abdominal pain, To a more severe form, characterized by fever and abdominal pain so severe that it can be Mistaken for appendicitis. Only a relatively small number of *Y. enterocolitica* strains cause disease. These strains are serogrouped using an O/H type of system similar to the one used to classify strains of *E. coli*. The type of disease caused by *Y. pseudotuberculosis* except that diarrhea is usually not a symptom. *Y. pseudotuberculosis* infections are more likely than *Y. enterocolitica* infections to become systemic symptoms of systemic infection are those generally associated with endotoxemia (fever, chills, weight loss, and shock).

Y. enterocolitica and *Y. pseudotuberculosis* both have an affinity for Payer's patches and probably transit the mucosa through M cells. Once they reach the underlying tissue, they enter the mesenteric lymph nodes (the nodes that drain the tissue around the intestine) and multiply there. The bacteria provoke an inflammatory response that is responsible for the abdominal pain.

Both *Y. enterocolitica* and *Y. pseudotuberculosis* are killed by (PMNs), thus the bacteria can multiply in tissue and blood only as long as they can avoid being ingested by (PMNs).

In some people, *Y. enterocolitica* gastrointestinal infections are followed by arthritis of the peripheral joints, which develops 2 to 6 weeks after the intestinal infection has cleared. This arthritis is called reactive arthritis; because it is thought to be caused by T cells and / or antibodies elicited by antigens that cross-react with host antigen, not by bacterial infection of the joints. Reactive arthritis is seen commonly in people with histocompatibility antigen HLA-B27. Several groups have reported antibodies to bacterial surface antigen that cross-react with human B27, a protein that is exposed on the surface of the host cells. Thus it is possible that fragments of bacteria released during the infection induce antibodies that remain in circulation after the bacteria have cleared. These antibodies bind to B27 triggering a complement-mediated inflammatory response. Alternatively (or additionally) T cells that recognize B27 could attack host cells (Fukushima et al , 2001; Denecker et al., 2002; Hayashidani et al., 2002; Juris et al., 2002).

2. Materials and Methods

2.1 Bacterial Strains

20 clinical Strains of Yersinia pseudotuberculosis were gifted by medical microbiology Institute Munich University, Germany. strains were given on L.B and blood agar media for refreshment, and l.B media with antibiotics such as Nalidexin

2.2 Isolation of Plasmid

Plasmid DNA for large and small Plasmid preparations was extracted by alkaline Lysis method. (Maniatis, 1982)

Nalidexin mutation.

Wild type strains were subjected to different gradients to Nalidexin to reach the consentration 30µgLL Naledixin resistance. Hen clones were grown on Nalidexine containing L.B. medium. (Zaky 2004).

2.3 Preservation of Virulence Plasmids

5% fresh human blood was added to the L.B medium, and poured into plates containing Naledixin. With different concentrations, the strains first grown on the minimal concentration, and incubated in 37ºC, then harvested with spatula after addition of amount of fresh sterile L.B.medium, and then transferred into the next concentration plates and so on until reached 30ºµg/g.

PCR.

Different sets of primers used for PCR amplification were synthesized by Carl Roth (Karlsruhe, Germany).PCR amplifications were performed in an automated thermal cycler (TRIO Thrmoblock; Biometra, Goetingen, Germany) as described by Saiki et al. with TagI polymerase and different pairs of oligonucleotide primers. The initial denaturation step (94º C, 5 min) was followed by 35 cycles of denaturation (94º C, 1 min), annealing (at the annealing temperature Tm, 1min), and extension (72º C, 1 min), with one final extension step (72º C, 8 min). The sequences of the forward (FP) and reverse (RP) primers used for PCR reactions, the size of the amplified fragment (S), and Tm were as follows: (i) irp2 (FP), 5'-AAGGATTCGCTGTTACCGGAC-3' irp2 (RP), 5'-TCGTCGGGCAGCGTTTCTTCT-3'(S, 280bp; Tm, 61 C);(ii)irp-P242(FP),5'-AAGGATTCGCTGTTACCGGAC-3' irp2-505 (RP), 5'-TCGTCGGGCAGCGTTTCTTCT-3'(S, 264 bp; Tm, 58C); (iii) fyuA(FP), 5'- GCGAC GGGAAGCGATTTA-3' fyuA (RP), 5'- CGCAGTAGGCACGATGTTGTA-3' (S,780bp;Tm 60C);(iv) hmsR (FP) 5'- TAAAGAAAGACCCCACCAATC-3' hmsR (RP), 5'-ATCATCGGCATCAAGCAAATC-3'(S, 730 bp; Tm, 56 C; entF (FP), 5'-TATCAGCGTTATCACCATTTG-3' entF (RP), 5'- CCAGTTCCGGCAGCGTTTCTT-3' (S, 511 bp; Tm, 55 C); and Ec-chrom (FP), 5'- TTTATTCCGTTGCGTGAGGTT-3', HPI-5end (RP), 5'- TAGGATACCTTCACGCTGCTGTCGCGC-3' (S, 900 bp; Tm 52 C).

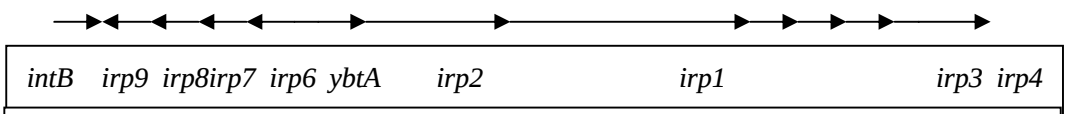

Figure1. Structure of HPI in *Yesinia pseudotuberculosis* and *Yesinia pestis* (Kaper and Hacker, 1999).

3. Results

3.1 Screening of 70 Kbp Virulence Plasmid

20 clinical strains of *Yersinia pseudotuberculosis* were screened for virulence plasmids, and only 5 strains harboring virulence plasmids, and after treatment with naledixin to be naledixin mutants, still harbouring the 70 Kbp virulence plasmids.

Table 1. Clinical strains of *Yersinia pseudotuberculosis* harbouring 70kbp virulence plasmids and high pathogenicity island.

No.	Strain	Type	70kbp virulence plasmid	High pathogenicity island
1	*Yersinia pseudotuberculosis*	I		
2	*Yersinia pseudotuberculosis*	I	+ve	+ve
3	*Yersinia pseudotuberculosis*	H260/91Ib		
4	*Yersinia pseudotuberculosis*	H47/91C Ia		
5	*Yersinia pseudotuberculosis*	H268/91 Ib		
6	*Yersinia pseudotuberculosis*	H191/91 IA	+ve	+ve
7	*Yersinia pseudotuberculosis*	O1A(252)	+ve	+ve
8	*Yersinia pseudotuberculosis*	IA		
9	*Yersinia pseudotuberculosis*	I		
10	*Yersinia pseudotuberculosis*	I		
11	*Yersinia pseudotuberculosis*	IA		
12	*Yersinia pseudotuberculosis*	IH 370-36/88	+ve	+ve
13	*Yersinia pseudotuberculosis*	IH-346-36/88		
14	*Yersinia pseudotuberculosis*	IB		
15	*Yersinia pseudotuberculosis*	IB		
16	*Yersinia pseudotuberculosis*	IP 3295	+ve	+ve
17	*Yersinia pseudotuberculosis*	IA		
18	*Yersinia pseudotuberculosis*	IA		
19	*Yersinia pseudotuberculosis*	O1A 26/8	+ve	+ve
20	*Yersinia pseudotuberculosis*	I		

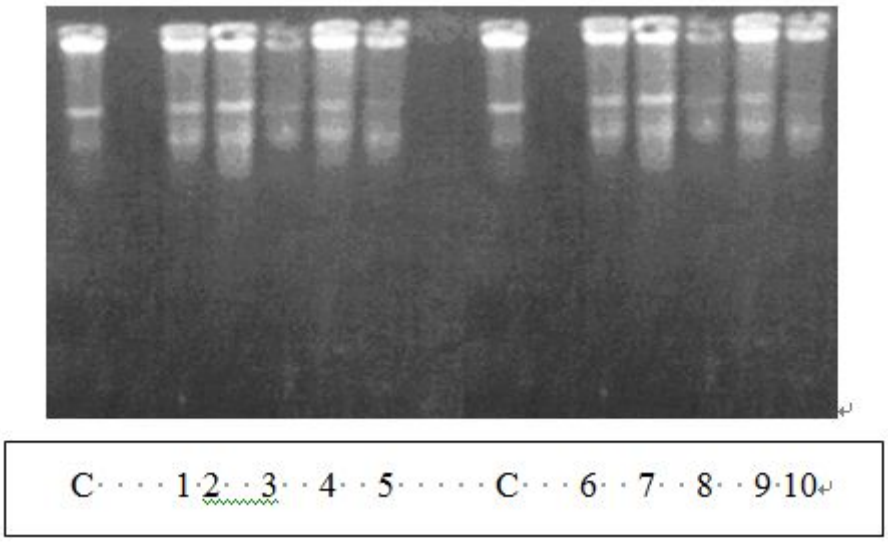

Figure 2. Virulence plasmid 70Kbp screening of clinical strains of *Yersinia pseudotuberculosis. C. Control, Yersinia enterocolitica. From 1 to 5 Wild types. From 6 to 10 Naledixin mutants.*

3.2 Detection of High Pathogenicity Island of Yersinia Pseudotuberculosis

Clinical strains of Yersinia pseudotuberculosis harbouring 70KbP plasmid(wild type and Naledixin mutants) were diagnosed for High pathogenicity island(HPI),with PCR, it was found fragments of FyuA and irp2 Figure.*(3,4).*

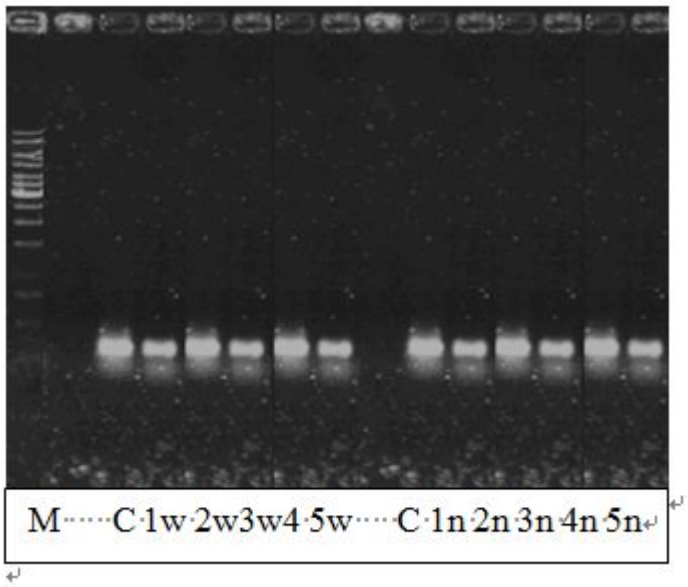

Figure 3. PCR of *fyuA* gene of clinical strains of *Yersinia pseudotuberculosis* Where. C control *Yersinia enterocolitica* 8081-L2, from 1w to 5w, wild types and from 1n to 5n nalidexin mutants.

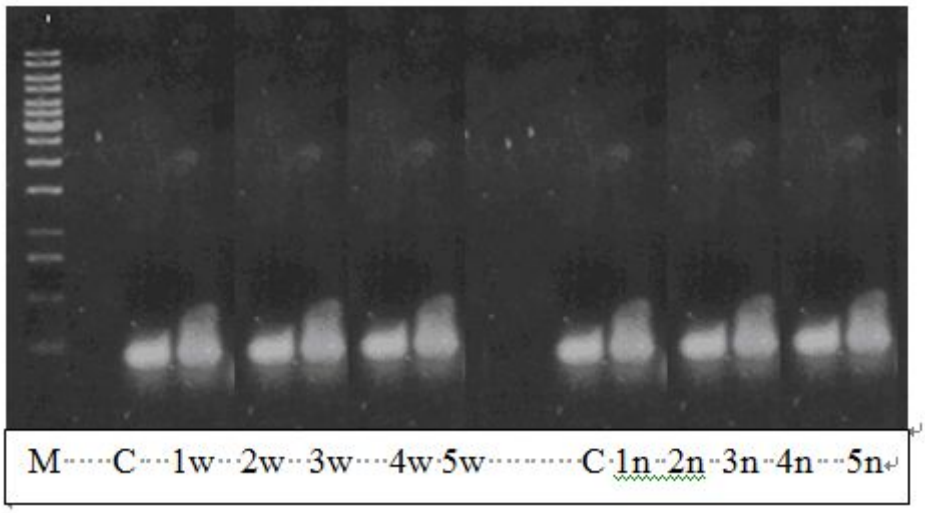

Figure 4. PCR of *irp* gene of HPI of clinical strains of *Yersinia pseudotuberculosis*, where M. 1000bp DNA marker, C, control *Yersinia enterocolitica* 8081-L2.from 1w to 5w , wild types, from 1n to5n nalidexin mutants.

3.3 Preservation of 70kbp Virulence Plasmids

During treatment with naledixin for the plasmid harbouring clinical strains of *Yersinia pseudotuberculosis*, it was found that strain *Yersinia pseudotuberculosis* IP395 lost virulence plasmids, but after growth on modified H.B-L.B medium containing naledixine, the virulence plasmid has recovered with additional small plasmids.(Figure.5).

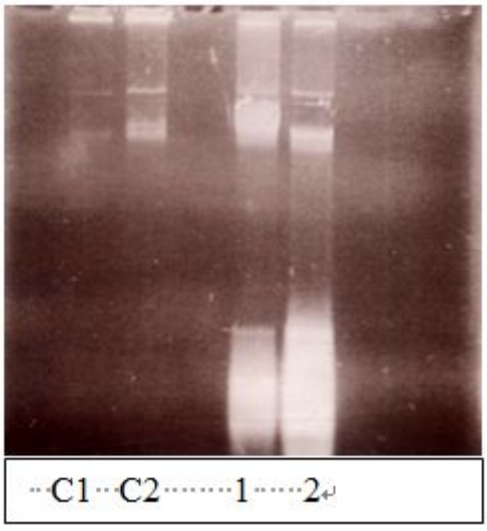

Figure 5. plasmid screening of clinical strain naledixin mutants *Yersinia pseudotuberculosis* IP3295 after treatment with Human blood H.B-LB medium
C1, control1 *Yersinia enterocolitica* 8081-L.2, C2, *Yersinia pseudotuberculosis* IP3295, 1, 2 *Yersinia enterocolitica* IP3295.

4. Discussion

For most bacterial pathogens virulence is a multifactorial process requiring two general classes of determinants. The first encompasses genes that participate in physiological processes necessary for survival in host and non-host environments and these genes are generally found in both pathogenic and non-pathogenic organisms. The second class of virulence genes specifies traits that are unique to pathogens, and not surprisingly, these genes are rarely detected in non-pathogenic organisms, based on the initial characterization of plasmids from *Yersinia* and *Shigella*. Such sequences were originally thought to be confined to extra-chromosomal elements but recently, several virulence cassettes have been mapped to the chromosome of pathogenic organisms. These segments of the chromosome, termed pathogenicity islands (Groisman and Ochman, 1996; Thoerner et al., 2003).

In this study, the detection of virulence plasmid in *Yersinia pseudotuberculosis* proves the virulence of the different strains of *Yersinia pseudotuberculosis*. Pathogenic species of *Yersinia* carry plasmids of about 70 Kb that appear to be necessary for virulence. These plasmids called pYV plasmids, cause requirement for calcium for growth at 37°C pYV-containing Yersiniae from colonies at 28°C but not at 37°C were grown on media deprived of calcium.

In conditions of growth restriction, i.e. 37°C in the absence of calcium, pYV plasmids from *Y. enterocolitica* and *Y. pseudotuberculosis* direct the secretion of at least nine proteins. Some of these proteins are also inserted in the outer membrane, which explain why these proteins are currently referred to as YOPs, for *Yersinia* outer membrane proteins. The structural genes of these proteins (YOP genes) are scattered around the pYV plasimds (Heesemann et al., 1984; Biot and Cornelis, 1988).

Recent studies showed that the virulence of *Y. pseudotuberculosis* depends on the pYV plasmid, which is responsible for invasion into epithelial cells in vitro and facilitation of the translocation of bacteria across the intestinal epithelium. Therefore, *Y. pseudotuberculosis* is similar to *Y. enterocolitica* and *Y. pestis*. This loss of plasmid pYV always correlates with the loss of pathogenicity, however, reported that chromosomal genes also prompt in vivo the replication of *Y. pseudotuberculosis*, although the loss of plasmid pYV is associated with a significant decrease in the level of virulence (Fukushima et al., 1991).

This study dealt with one of the important virulence determinants, which is detected in the *Y. pseudotuberculosis* called high pathogenicity island (HPI).

Survival and proliferation within the host depends on the ability of a pathogen to scavenge essential nutrients such as iron, which is bound by the host molecules ferritin, transferrin, and lactoferrin. Bacteria have developed an efficient strategy to obtain iron from these eukaryotic iron scavengers. They produce and secrete low-molecular-weight siderophores with extremely high affinities for ferric iron. The siderophore was designated Yersiniabactin (Ybt). Ybt displays relatively weak iron binding under in vitro culture conditions. Nevertheless, the siderophore activity was detected only in highly pathogenic Yersiniae that are lethal for mice at low infectious doses, (*Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica).*

The yersiniabactin gene cluster responsible for the manifestation of lethality for mice was

named the high-pathogenicity island (HPI). The complete structure of HPI and the functional core of the island consist of 12 genes. At least six genes encoding Iron-Responsible proteins (Irp1 to Irp5 and Irp9) in *Y. enterocolitica* (Ybt E and Ybt T are *Y. pestis* bacteriocin pesticin. Therefore, loss of pesticin sensitivity correlates with loss of the yersiniabactin binding site and with inability of *Yersinia* to obtain sufficient iron for growth in the mammalian host environment (Heesemann, 1987; Koczura and Kazonwski, 2003; Mokracka et al., 2004).

The fyuA-irp gene cluster identified primarily in yersiniae meets the basic criteria of so called pathogenecity islands, such as (i) a typical G+C content on the 45-kbp chromosomal DNA region, (ii) requiring a cluster of genes for virulence, (iii) the presence of mobility genes (eg, insertion elements) as well as association with a tRNA gene at one boundary, and (iv) instability. Moreover, we recently demonstrated that the fyuA-irp gene cluster comprised two evolutionary lineages, one assigned to *Y. enterocolitica* biotype 1B strains and other *Y. pestis* and *Y. pseudotuberculosis*. Surprisingly, the fyuA gene cluster of the latter lineage could also be detected in four pesticin-sensitive *E.coli* strains indicating that it might be transmissible. Therefore, we investigated the distribution of the fyua-irp gene cluster among some pathogenic species of the family Enterobacteriaceae..

In this study, modification of L.B medium with addition of human blood agar, has returned back the fitness of human pathogenic *Yeresinia pseudotuberculosis*, to express virulence factors, and recovery of large plasmids, is an indication that human blood is a real environment for pathogens, which could be a substitute of mouse passage in the molecular biology of pathogens work. Moreover recovery of plasmids, proves that plasmids have not kicked out of the bacterial cells but incorporated into the chromosomal DNA, with more additional small plasmids, supports the relatedness of *Yersinia pseudotuberculosis* and *Yersinia pestis*.

Genetic relatedness was studied by Kim et al using 16S rDNA for the identification of *Y.pseudotuberculosis*. A phylogenetic tree constructed from 16S proved the relatedness of *Yersinia pestis* and *Yersinia pseudotuberculosis*..(Kim et al, 2003).

Similarity between *Yersinia pestis*, the etiological agent of bubonic plague, and entero-pathogenic *Yersinia pseudotuberculosis* and *Yersinia pestis* possess three plasmids, of which one shared by the enteropathogenic species, mediates a number of virulence factors that directly or promote survival within macrophages and immunosupression. The two remaining plasmids are unique and encode functions that promote acute disease by enhancing bacterial dissemination in tisuues and resistance to phagocytosis by neutrophils. (Brubaker, 1991). It would be recommended more studies should be done using the modified H.B-L.B medium for inhancement of virulence factors of antibiotic-resistant human pathogens, to give a real and clear picture about the reality and phylogeny of the pathogen.

References

Brubaker, R. R. (1991). Factors promoting acute and chronic diseases caused by Yersiniae. *Clinical microbiology reviews*, 309-324.

Cornelis, G. R., Sory, M. P., Iaroche, Y. & Derelaye, I. O.. Genetic analysis of the plasmid region controlling virulence in *Yersinia enterocolitica* O:9 by Mini-Mu insertions and lac gene

fusions. *Microb. Pathog*, *1*, 349-359. http://dx.doi.org/10.1083/jcb.200205077

Cornelis, S. G. R. (2002). *Yersinia* type II secretion: send in the effectors. *J. Cell Biol, 158*(3), 401-8.

Denecker, G., Totemeyer, S., Mota, L. J., Troisfontanies, P., Lambermont, I., Youta, C., Stainier, I., Kermann, M. & Cornelis, G. R. (2002). Effect of low-and high-virulence *Yesinia enterocolitica* strains on the inflammatory response of human umbilical vein endothelial cells. *Infect. Immun*, *70*(7), 3510-20. http://dx.doi.org/10.1128/IAI.70.7.3510-3520.2002

Fukushima, H., Sato, T., Nagasako, R. & Takeda, I. (1991). Acute Mesentric lymphadenitis due to *Yersinia pseudotuberculosis* laking a virulence plasmid. *J. Clinical Microbiology*, 126-1275.

Fukushima, H., Hao, Q., Wu, K., Hu, X., Chen, J., Guo, Z., Dai, H., Quin, C., Lu, S. & Gomyoda, M. (2001). *Yersinia enterocolitica* O:9 as a possible barrier against *Yersinia pestis* in natural plague foci in Ningxia, China. *Curr. Microbial*,42 (1), 1-7. http://dx.doi.org/10.1007/s002840010168

Groisman, E. A. & Ochman, H. (1996). Pathogenecity Islands: Bacterial evolution in Quantum Leaps. *Cell*, 87,791-794. http://dx.doi.org/10.1016/S0092-8674(00)81985-6

Hayashidani, H., Kanzaki, N., Kaneko, Y., Okatani, A. T., Taniguchi, K. K. & Ogawa, M. (2002). Occurrence of Yersiniosis and Listeriosis in wild boars in Japan. *J. Wild. Dis, 38* (1), 202-5.

Heesemann, J., Algermissen, B. & Laufs, R. (1984). Genetically manipulated virulence of *Yersinia enterocolitica*. *Infection and Immunity,* 46, 105-110.

Heesemann, J. (1987). Chromosomal-encoded siderophores are required for mouse virulence of enteropathogenic *Yersinia* species. *FEMS. Microbial Lett,* 48, 229-233. http://dx.doi.org/10.1111/j.1574-6968.1987.tb02547.x

Hassan, A. S. (2000). *Plague: Updated clinical epidemiological and immunological aspects.* M. Sc. Thesis. Faculty of Medicine, Cairo University.

Juris, S. J., Shao, F. & Dixon, J. E. (2002). *Yersinia* effectors target mammalian signaling pathways. *Cell Microbiol*, 4(4), 201-11. http://dx.doi.org/10.1046/j.1462-5822.2002.00182.x

Kim,W., Song, M. O, Song, W, Kim, K. J, Chung, S. I, Choi, C. S & Park, Y. H. (2003). Comparison of 16S rDNA analysis and rep-PCR genomic fingerprinting for molecular identification of *Yersinia pseudotuberculosis*, *83*(2), 125-33.

Kaper, J. B. & Hacker, J. (1999). Pathogenicity Island and other mobile virulence elements. *American society for microbiology*,77-88.

Maniatis, T., Fritsch, E. F. Sambrook, J. (1982). Molecular cloning, a laboratory Manual. *Cold Spring Harbor Laboratory*, 149-171.

Mingrone, M. G. & Fantasia, M. (1988). Characteristics of *Yersinia* spp. isolated from wild and zoo animals. *J. Wild. Dis. 24*(1), 25-9.

Mokracka, J., koczura, R. & Kaznowski, A. (2004). Yersiniabactin and other siderophores produced by clinical isolates of *Enterobacter* spp and *Citrobacter* spp. FEMS. *Immunol Med Microbiol*, *15*, 40.

Thoerner, P., Bin, K. C. I., Boglidtuber., Bissig-Choisat, B., Wassenaar, T. M., Frey, J. & Jemmi, T. (2003). PCR detection of virulence genes in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* and investigation of virulence gene distribution. *Appl. Environ Microbiol*, *69*(3), 1810-6. http://dx.doi.org/10.1128/AEM.69.3.1810-1816.2003