Epidemiology of Anthrax in Domestic Animals of Bangladesh

Farzana Islam Rume

PhD Thesis
June 2016

Department of Microbiology
University of Dhaka
Dhaka-1000, Bangladesh

Dhaka University Institutional Repository

Epidemiology of Anthrax in Domestic Animals of Bangladesh

A dissertation submitted to
the Faculty of Biological Sciences
of The University of Dhaka

in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

Submitted By
Farzana Islam Rume
Registration No.: 155/ 2011-2012
Session: 2011-2012

June 2016

Department of Microbiology
University of Dhaka
Dhaka-1000, Bangladesh

Dhaka University Institutional Repository

CERTIFICATE

This is to certify that the thesis entitled "Epidemiology of Anthrax in Domestic Animals of Bangladesh" was carried out by Farzana Islam Rume, Registration No. 155/ 2011-2012, and Session: 2011-2012 for the partial fulfillment of the degree of Doctor of Philosophy from the Department of Microbiology, University of Dhaka, Bangladesh.

This work was carried out under our supervision and the style and contents of the thesis have been approved and recommended for the award of PhD degree.

Dr. Chowdhury Rafiqul Ahsan
Professor and Supervisor
Department of Microbiology,
University of Dhaka, Bangladesh

Dr. Paritosh Kumar Biswas
Professor and Supervisor
Department of Microbiology and
Veterinary Public Health,
Chittagong Veterinary and Animal
Sciences University, Chittagong,
Bangladesh

Dr. Mahmuda Yasmin
Professor and Supervisor
Department of Microbiology,
University of Dhaka, Bangladesh

Dhaka University Institutional Repository

Dedicated to

My beloved family

ACKNOWLEDGEMENT

At first, I wish to express my gratefulness to the Almighty Allah, who has enabled me to complete the research work and this thesis successfully and to achieve PhD degree in Microbiology from the Department of Microbiology, University of Dhaka, Dhaka, Bangladesh.

I am extremely pleased to express my heartfelt respect, deep sense of gratitude and immense indebtedness to my honorable supervisor Professor Dr. Chowdhury Rafiqul Ahsan, Department of Microbiology, University of Dhaka, Dhaka, Bangladesh, for planning the experiments, scholastic supervision, continuous guidance, constant encouragement, valuable suggestion and constructive criticism during the entire period of the research work and preparation of this thesis. I count myself very fortunate to have been his student and be benefited from his critical guidance on improving my scientific view.

I am deeply indebted to my supervisor, Professor Dr. Paritosh Kumar Biswas, Department of Microbiology and Veterinary Public Health, Chittagong Veterinary and Animal Sciences University, Chittagong, Bangladesh, without whose ample guidance this thesis work would not have been possible. I would like to express my special gratitude to him for sharing his knowledge, experience and important evaluations of the research works, giving me encouragement, offering me opportunities of exposure to international research groups, seminars, continuous guidance throughout the thesis work and training needed to become an independent researcher. I am really thankful to him.

I would like to express my intense gratefulness to my supervisor, Professor Dr. Mahmuda Yasmin, Department of Microbiology, University of Dhaka, Dhaka, Bangladesh, for her sincere and generous co-operation, cordial feelings and affectionate encouragement in completing this research work.

I would like to take the opportunity to acknowledge our international collaborators Dr. Antonio Fasanella, Dr. Alessia Affuso and other members in the research group of Istituto Zooprofilattico Sperimentale of Puglia and Basilicata, Anthrax Reference Institute of Italy, Foggia, Italy; Professor Dr. Martin Hugh-Jones of School of the Coast and Environment, Louisiana State University, Baton Rouge, United States of America; and Dr. Gregor Grass and Dr. Matthias Hanczaruk of Bundeswehr Institute of Microbiology, Munich, Germany for creating the opportunity and providing the laboratory space to conduct the molecular parts of this research work.

I am thankful to the owners of the cattle, officials and personnel of Department of Livestock Services for their permission and great help in sampling. I am immensely grateful to many others from whom I received cordial co-operation, friendly collaboration, fruitful advice and guidance but regret for my inability to mention everyone by name.

I would also like to acknowledge the financial supports obtained from BAS-USDA project.

I am grateful to my beloved parents and parents in law, who inspired me with the best of their prayer, great sacrifice and blessing in carrying out the higher study. Thanks also to my uncle, aunties, brothers and brothers in law, sisters and sister in law and beloved nieces and nephews for putting high ambition about my higher studies.

Finally, I specially thank my beloved husband and soul mate Dr. Md Robiul Karim for his encouragement and support in the fulfillment of this degree. He has been an invaluable partner as a scientist and as a partner in life. Special appreciation goes to my beloved daughter Faiza Karim Umama and son Md Fardin Karim for sacrificing their happiness due to my PhD study.

Dhaka University Institutional Repository

Contents

		Page No.
List of Figures		i
List of Tables		ii
Abbreviations		iii-vi
	Abstract	vii-x
1.0	**Introduction**	1-46
1.1	Overview	1
1.2	Literature review	7
1.2.1	History of anthrax	7
1.2.2	Disease agent of anthrax: *Bacillus anthracis*	9
1.2.2.1	Taxonomy of *Bacillus anthracis*	9
1.2.2.2	Species status in the genus *Bacillus*	9
1.2.2.3	Properties of *Bacillus anthracis*	9
1.2.2.4	The *B. anthracis* genome	11
1.2.2.5	*B. anthracis* toxins	12
1.2.2.6	*B. anthracis* capsule	13
1.2.2.7	Roles of capsule in virulence	13
1.2.2.8	Other virulence factors	14
1.2.2.9	Pathogenesis	14
1.2.3	Epidemiology	15
1.2.3.1	Receptive Host	15
1.2.3.2	Disease distribution	16
1.2.3.2.1	Anthrax worldwide	16
1.2.3.2.2	Anthrax outbreaks in Bangladesh	17

Contents **Page No.**

1.2.3.3	Ecology of anthrax	19
1.2.3.4	Risk factors associated with anthrax in domestic animals	21
1.2.4	Anthrax transmission cycle	22
1.2.5	*B. anthracis* life cycle	23
1.2.6	Clinical signs of anthrax	24
1.2.7	Postmortem examination	24
1.2.8	Treatment and control	24
1.2.9	Diagnostic approaches	25
1.2.9.1	Specimens required for laboratory diagnosis	25
1.2.9.2	Specimen shipment	26
1.2.9.3	Microscopic test	27
1.2.9.4	Blood smear rationale	27
1.2.9.5	Cultural test	27
1.2.9.6	Biological test	28
1.2.9.7	Polymerase Chain Reaction (PCR)	29
1.2.9.8	Molecular characterization	29
1.2.9.9	Differential diagnosis	32
1.2.9.10	Personnel protection	32
1.2.10	Anthrax molecular research in Bangladesh	32
1.2.11	Anthrax vaccine for veterinary use	34
1.2.11.1	Anthrax vaccine: mode of action	35
1.2.11.2	Immune response after vaccination	36
1.2.11.3	Anthrax vaccine and Bangladesh	37
1.2.11.4	Animal models for anthrax vaccine studies	38
1.2.12	Vaccine immunology	39
1.2.12.1	Use of sonication for the preparation of whole cell extract	39

Dhaka University Institutional Repository

Contents **Page No.**

1.2.12.2	Protection of protein from proteases	40
1.2.12.3	Determination of protein concentration	41
1.2.12.4	Detection of protein by Western blot	41
1.2.12.4.1	Gel electrophoresis	42
1.2.12.4.2	Transfer of proteins	43
1.2.12.4.3	Blocking and antibodies	43
1.2.12.4.4	Detection	44
1.3	Objectives of the study	46
2.0	**Materials and Methods**	47-79
2.1	Case-control study	47
2.1.1	Geography and cattle population statistics of Bangladesh	47
2.1.2	Selection of case farm	47
2.1.3	Selection of control farms	49
2.1.4	Data collection	49
2.1.5	Statistical analysis	49
2.2	Molecular characterization of *B. anthracis*	54
2.2.1	Molecular characterization of *B. anthracis* by using 15 VNTR loci	54
2.2.1.1	Sample collection	54
2.2.1.2	Isolation of *B. anthracis*	60
2.2.1.3	DNA preparation and Real Time PCR assay	61
2.2.1.4	PHRANA (Progressive Hierarchical Resolving Assays using Nucleic Acids)	63
2.2.1.5	Canonical Single Nucleotide Polymorphism analysis	63
2.2.1.6	Multiple-Locus Variable Number Tandem Repeat with 15 VNTRs	64
2.2.1.7	Single Nucleotide Repeat analysis	65

Contents **Page No.**

2.2.2	Whole genome sequencing of *B. anthracis*	66
2.2.2.1	Collection of soil samples	66
2.2.2.2	Isolation of *B. anthracis* from soil samples and inactivation	68
2.2.2.3	Extraction of DNA from inactivated culture material	68
2.2.2.4	Diagnostic real-time PCR for chromosomal and plasmid markers of *B. anthracis*	69
2.2.2.5	Analysis of canSNPs by Melt Mismatch Amplification Mutation Assay (Melt-MAMA)	69
2.2.2.6	Multi locus VNTR (variable number of tandem repeats) analysis (MLVA)	69
2.2.2.7	Ion Torrent next generation whole genome sequencing	70
2.2.2.8	Analysis of whole genome sequencing data - SNP calling	70
2.2.2.9	Interrogation for branch-specific SNPs by high resolution melting analysis (HRM-SNP)	71
2.2.3	Genotypes and subgenotypes of *B. anthracis* circulating in Bangladesh	72
2.3	Immunogenicity of anthrax vaccine strain used in Bangladesh	73
2.3.1	Source of anthrax vaccine	73
2.3.2	Serum sample collection	73
2.3.3	Immunological tests on laboratory animal	73
2.3.4	Culture of bacteria by using anthrax vaccine strain	73
2.3.5	Preparation of whole cell extract	74
2.3.6	Bio-Rad Protein assay	74
2.3.7	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)	75
2.3.8	Transfer to Nitrocellulose Membrane	77
2.3.9	Western blotting assay	78

Contents		Page No.

3.0	**Results**	80-104
3.1	Case-control study	80
3.1.1	Spatial distribution of case farms	80
3.1.2	Population statistics of the case and control farms	80
3.1.3	Risk factors	80
3.2	Molecular characterization of *B. anthracis*	85
3.2.1	Molecular characterization of *B. anthracis* by using 15 vntr loci	85
3.2.1.1	Canonical Single Nucleotide Polymorphism (canSNP)	85
3.2.1.2	Multiple- Locus Variable Number Tandem Repeat with 15 VNTRs	86
3.2.1.3	Single Nucleotide Repeats	88
3.2.2	Whole genome sequencing of *B. anthracis*	89
3.2.2.1	*B. anthracis* isolation from environmental samples and initial genotyping	89
3.2.2.2	Whole genome sequencing and assembly	92
3.2.2.3	SNP discovery and analysis suggest two Bangladeshi cluster related to strains from Central Europe	92
3.2.2.4	HRM-SNP analysis verifies the relationships of the genome sequenced strains	95
3.2.3	Genetic identities of *B. anthracis* in Bangladesh: A comparison with the results of this and other two previous studies	95
3.3	Anthrax vaccine immunogenicity	100
3.3.1	Isolation and identification of anthrax bacteria (*B. anthracis*) from anthrax vaccine	100
3.3.2	Protein estimation	100
3.3.3	SDS-PAGE	101
3.3.4	Western blot	102

Contents		**Page No.**

4.0	**Discussion**	105-115
4.1	Case control study	105
4.2	Molecular characterization of *B. anthracis*	108
4.2.1	Molecular characterization of *B. anthracis* by using 15 VNTR loci	108
4.2.2	Whole genome sequencing of *B. anthracis*	110
4.2.3	Circulating genotypes of *B. anthracis*: comparisons with other previous studies	111
4.3	Anthrax vaccine immunogenicity	112
	References	116-143
	Appendix-I	144
	Appendix-II	146
	Appendix-III	152

List of Figures **Page No.**

Figure	Title	Page No.
Figure 1.2.1	The discoverers of *B. anthracis* and fathers of medical microbiology	8
Figure 1.2.2	*Bacillus anthracis.* Gram stain. 1500X	10
Figure 1.2.3	Structure of *B. anthracis*	11
Figure 1.2.4	Global distribution of anthrax	17
Figure 1.2.5	Anthrax zoonotic transmission cycle	23
Figure 1.2.6	The A-B model of the anthrax toxin	36
Figure 1.2.7	Mechanism of detection chemistries	44
Figure 2.1.1	Geographical distributions of anthrax-affected…...…..Brahmaputra	52
Figure 2.1.2	Common practices of carcass disposal in the outbreak areas.	53
Figure 2.2.1	Map of Bangladesh indicating the districts from…………...…..loci	55
Figure 2.2.2	Collection of samples from the field sites	57
Figure 2.2.3	Sample processing and preserving according to the categories	59
Figure 2.2.4	Steps of Isolation of bacteria by GABRI method	60
Figure 2.2.5	Pictorial presentation of DNA extraction and real time PCR assay	62
Figure 2.2.6	Map of Bangladesh with locations of sampling……...........maps.net	67
Figure 3.2.1	*B. anthracis* sub-grouping through canSNP analysis	85
Figure 3.2.2	Map of Bangladesh indicating the genotype…..........… *B. anthracis*	86
Figure 3.2.3	Maximum parsimony tree derived from chromosomal…........2015b	93
Figure 3.3.1	Creamy white colonies showing growth of the vaccine …... at 37°C	100
Figure 3.3.2	Standard curve of protein (BSA)	101
Figure 3.3.3	SDS-PAGE maps showing migration patterns of the .. Sterne F-24	101
Figure 3.3.4a	Western blotting assays for Sterne F-24 whole ….... weight marker	102
Figure 3.3.4b	Western blotting assays for Sterne F-24…....……weight marker	103
Figure 3.3.4c	Western blotting assays for Sterne F-24…..…..........weight marker	104

List of Tables **Page No.**

Table 1.2.1	Taxonomic classification of *B. anthracis*	9
Table 1.2.2	Features of *B. anthracis* genome	12
Table 1.2.3	List of anthrax outbreaks in Bangladesh since 1960	19
Table 1.2.4	MLVA result of Sterne strain by using 8 VNTR loci	35
Table 2.1.1	List of the districts, thana and villages from where ….. collected	51
Table 2.2.1	Data on samples and sampling area	56
Table 2.2.2	Primers used for PCR amplification	62
Table 2.2.3	SNPs and respective primer sequences used for HRM-SNP	72
Table 3.1.1	Population statistics of the case and control ..…….2010-2014	81
Table 3.1.2	Matched-pair analysis of risk factors……………..in Bangladesh	82
Table 3.1.3	Multivariable analysis of risk factor ……………(Initial model)	84
Table 3.1.4	Multivariable analysis of risk factor associated…(Final model)	84
Table 3.2.1	MLVA 15- VNTRs results	87
Table 3.2.2	SNR results	88
Table 3.2.3	Strains of *B. anthracis* from Bangladesh………....A.Br.001/002	90
Table 3.2.4	Results of MLVA-31 typing	91
Table 3.2.5	Results of HRM-SNP typing	94
Table 3.2.6	Comparison among the genotypes of Bangladeshi ……..….loci	97
Table 3.2.7	An overview on the genotypes of *B. anthracis* …………studies	98
Table 3.2.8	Comparisons on the Single Nucleotide.......……previous studies	99
Table 3.3.1	Protein estimation of the sample………… F-24 of *B. anthracis*)	100

Abbreviations

'	Minute
%	Percentage
<	Less than
>	Greater than
≤	Less than or equal
µL	Microliter
µm	Micrometer
^0C	Degree centigrade
1^0 Ab	Primary antibody
2^0 Ab	Secondary antibody
AEBSF	Aminoethyl benzylsulfonyl fluoride
AMP	Adenosine monophosphate
AP	Alkaline phosphatase
APS	Ammonium persulphate
ASV	Anthrax vaccine/Anthrax Spore Vaccine
BARC	Bangladesh Agricultural Research Council
BBS	Bangladesh Bureau of Statistics
BC	Before Christ
Bp	Base pair
BPB	Bromo phenol blue
BSL	Biosafety level
canSNP	Canonical Single Nucleotide Polymorphism
Cap	Capsule
CDC	Centers for Disease Control and Prevention
CDIL	Central Disease Investigation Laboratory
CI	Confidence interval
Cm	Centimeter
CMI	Cell-mediated immunity
DAB	3,3'-diaminobenzidine
dH_2O	Distilled water
DLS	Department of Livestock Services
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOT	Department of Transportation
DU	University of Dhaka
DW	Distilled water
E	East
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EF	Edema factor
ELISA	Enzymelinked immunosorbent assay
ET	Edema toxin
FAO	Food and Agriculture Organization

Abbreviations (Cont.)

FDIL	Field Disease Investigation Laboratory
G	Gram
G	Gravity
GABRI	Ground Anthrax Bacillus Refined Isolation
GPS	Global positioning system
GT	Genotype
H	Hour
H_2O_2	Hydrogen peroxide
HCI	Hydrogen chloride
HEPA	High efficiency particulate air
HHR	History of heavy raining occurred in the last 2 weeks
HRM	High resolution melting
HRP	horseradish peroxidase
IATA	International Air Transporters Association
ICDDR,B	International Centre for Diarrhoeal Disease Research, Bangladesh
IEDCR	Institute of Epidemiology, Disease Control and Research
IgG	Immunoglobulin G
IU	International unit
kDa	Kilo Dalton
L	Liter
LD50	Lethal dose 50
LF	Lethal factor
LRI	Livestock Research Institute
LT	Lethal toxin
mA	Milli ampere
MAPK	Mitogen-activated protein kinase
MAPKK or MEK	MAPK kinase
Max	Maximum
Melt-MAMA	Melt Mismatch Amplification Mutation Assay
Mg	Milligram
Min	Minute
Min	Minimum
Ml	Milliliter
MLVA	Multi-Locus Variable Number of Tandem Repeats Analysis
Mm	Millimeter
mM	Milli mole
MSHA	Mine Safety and Health Administration
N	North
N	Number
NCM	Nitrocellulose membrane
Ng	Nano gram

Abbreviations (Cont.)

NIOSH	National Institute for Occupational Safety and Health
Nm	Nanometer
nM	Nano mole
NMR	Nuclear magnetic resonance
No.	Number
Nt	Nucleotides
^{o}	Degree
OIE	World Organization for Animal Health
OR	Odds ratio
PA	Protective antigen
PAA	Per Acetic Acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHRANA	Progressive Hierarchical Resolving Assays using Nucleic Acids
PLET	Polymyxin-Lysozyme-EDTA-Thallous acetate
pM	Pico mole
PMSF	Phenylmethylsulfonyl fluoride
ProMED	Program for Monitoring Emerging Diseases
Rcf	Relative centrifugal force
RNA	Ribonucleic acid
rPA	Recombinant protective antigen
Rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
S	Second
SAS	Sick animal on farm or a nearby farm slaughtered in the recent past
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SM	Skimmed milk
SNP	Single nucleotide polymorphism
SNR	Single Nucleotide Repeats
sRNA	Small ribonucleic acid
SubGt	Subgenotype
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
TNA	Toxin neutralization assay

Abbreviations (Cont.)

tRNA	Transfer ribonucleic acid
TSMP	Trimethoprime-Sulfamethoxazole-Polymixine
TSPB	Trimethoprime-Sulphomethaxazole Polymyxin B
USA	United States of America
V	Volt
v/v	Volume by volume
VNTR	Variable-Number Tandem-Repeat
Vs	Versus
WCE	Whole cell extract
WHO	World Health Organization
μM	Micro mole

Abstract

Anthrax is an acute, febrile disease of warm blooded animals including humans caused by the bacterium *Bacillus anthracis*. It is an one health disease with global distribution. For sensitive species, anthrax represents a fatal outcome with sudden death and sometimes bleeding from natural orifices. The most common source of infection for ruminants is ingestion of spores during grazing in contaminated pastures or through grass and water contaminated with anthrax bacilli spores. Domestic cattle, sheep and goats can also become infected through contaminated bone meal (used as feed) originating from anthrax-infected carcasses. Humans can get infection through the exposure to infected animals or tissues of infected animals; handling of infected animal products like skin, bone, and flesh; inhalation of anthrax spores from contaminated animal by-products; and also direct exposure to *B. anthracis.* In Bangladesh, anthrax is considered an endemic disease affecting ruminants with sporadic zoonotic occurrences in humans. There have been numerous episodes of anthrax outbreaks in this country since 2009 with having zoonotic consequences in animal owners and villagers involved in handling the infected animals and carcasses or processing and eating the meat of sick animals. However, due to the common practices of incorrect disposal of infected carcasses, the disease is not properly monitored and because of the socio-economic conditions, the situation is under-reported and under-diagnosed.There are many factors thought to be associated with recent outbreaks, only a few of which are reported recently. Furthermore, the molecular genetic characteristics of circulating *B. anthracis* and the immunogenicity of vaccine used against that in the country are poorly understood.Therefore, the present study was aimed:(1) to evaluate the risk factors associated with anthrax infection in cattle by case control study; (2) to isolate and genotypically characterize*B. anthracis* organism from the field sample by hierarchical fingerprinting system (PHRANA) employing canonical single nucleotide polymorphism (canSNP), multilocus variable number tendem repeat (VNTR) analysis (MLVA),single nucleotide repeat (SNR) and also by whole genome sequencing; and (3) to detect the immunogenic properties and to assess the immunogenicity of anthrax vaccine by using immunoblotting assay (Western blot) and by animal model.

A matched case-control study was conducted in Bangladesh by enrolling case smallholdings of cattle affected with anthrax from October 2010 through December 2014 with following mass media and concerned available surveillance reports. In total, 43 case smallholdings were enrolled, and for each case, its control was matched by similarity in herd-size and rearing of animals, selected from a distantly located place but within the same sub-district of the case farm. Data collected by administering a prototype questionnaire were analysed by matched-pair analysis and multivariable conditional logistic regression. Out of the 43 smallholdings, 41 were seen in three adjoining districts: Pabna, Siraganj and Tangail, apparently forming a spatial cluster, could be called “anthrax-hot spot” in Bangladesh. Sick animal on farm or a nearby farm slaughtered in the recent past (Odds ratio (OR) 12.2, 95% Confidence interval (CI) 1.6 – 93.4, p=0.016)), History of heavy raining occurred in the last 2 weeks (OR 13.1, 95% CI 1.2 – 147.1, p=0.037), and Disposing of dead animal into nearby water body (OR 11.9, 95% CI 1.0 – 145.3, p=0.052) were independent risk factors for anthrax in cattle in the country.

Two important international collaborations were used to carry out the research on molecular characterization of *B. anthracis.* During collaboration with the Istituto Zooprofilattico Sperimentale of Puglia and Basilicata (Anthrax Reference Center, Italy), a total of 169 samples (73 soil, 1 tissue, 4 bone and 91 bone meal samples) were collected from 12 different districts of Bangladeshbetween 2012 and 2015, and investigated. Twelve samples (10 soil and 2 bone samples) tested positive for *B. anthracis* by RT-PCR analysis. Biomolecular analyses were conducted starting from the CanSNP to analyze the phylogenetic origin of the strainswhich confirmed that all the strains belonged to lineage A major subgroup A.Br. 001/002. The analysis of genotype, obtained through the MLVA with the analysis of 15 VNTR, demonstrated four different genotypes: two of them were previously identified in the district of Sirajganj. The sub-genotyping, conducted with SNR analysis, revealed the presence of eight subgenotypes. The data concluded that there was no observed correlation between imported cattle feed and anthrax occurrence in Bangladesh and that the remarkable genetic variations of *B. anthracis* were found in the soil of numerous outbreaks.

Research works in collaboration with Bundeswehr Institute of Microbiology, Munich, Germany focused on genomic aspects. Whole genome sequencing and comparative genomic analyses were performed to evaluate the genetic and phylogenetic relationship between Bangladeshi and central Europe strains. We genotyped several strains of *B. anthracis* collected from the districts of Sirajganj and Tangail in 2013. All these strains belonged to canSNP group A.Br.001/002 differing only in a few of 31 tandem-repeat (MLVA)-markers. Whole genome sequences were obtained from these strains and compared with genomic information of *B. anthracis* strains originating from various geographical locations. Characteristic signatures were detected defining two "Bangladesh" clusters potentially useful for rapid molecular epidemiology. From this data, high-resolution PCR assays were developed and subsequently tested on additional isolates from Bangladesh and Central Europe. Remarkably, this comparative genomic analysis focusing on SNP-discovery revealed a close genetic relationship between these strains from Bangladesh and historic strains collected between 1991 and 2008 in The Netherlands and Germany.

In Bangladesh, anthrax vaccine is produced by the Livestock Research Institute (LRI), Mohakhali, Dhaka. The master-seed of this vaccine is living spores of the non-capsulated attenuated Sterne F-24 strain of *B. anthracis,* originated in Australia. An investigation was carried out to characterize the immunogenic properties of this vaccine strain and its immunogenic capacity in cattle and mice. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done for separating proteins. Numerous proteins were detected from 120 to 26 kDa molecular weight after stained with Coomassie Brilliant Blue.Western blotting was performed by probing the blocked membrane with a primary antibody (bovine and mice) that recognized a single or more specific proteins or epitope on a group of proteins (e.g., SH2 domain or phosphorylated tyrosine) of anthrax vaccine. By western blot analysis, no bands were seen in non-vaccinated cattle and control mice. But bands were detected with about 36 kDa proteins from all the vaccinated cattle. In hyperimmunized mice, bands of 83 to 35 kDa proteins were detected. The molecular weight of the protein in the vaccinated cattle and hyperimmunized mice was about 36 kDa which is similar or may be related to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, molecular weight 35974) BA5369. GAPDH (BA5369) protein plays an important role in adhesion to host cells and their colonization. The vaccine strain being used at LRI was proven to be immunogenic in producing anti anthrax IgG

antibody both in the vaccinated cattle and in the hyperimmunized mice. On the other hand, an 83 kDa was identified in the hyperimmune mice sera but not in the vaccinated cattle sera. This 83 kDa protein could be related to the protective antigen (PA), which also has got a molecular weight of 83 kDa. The PA is a very important component of the anthrax toxin in the regard that it plays a major role in anthrax immunity. It seems that the vaccine strain being currently used might not produce sufficient protection against the new genotypes of the organism circulating in Bangladesh due to the difference in the pXO1 molecular structure between the vaccine strain and the field strains. Whether this genetic mismatching between the vaccine strain and the strains circulating in the country is contributing to vaccine failure needs to be studied further. The results of our study on the immunogenic properties of anthrax vaccine in cattle suggest that animal should be boosted for another two times at 2-3 week intervals for the development of antibody against protective antigen.

Dhaka University Institutional Repository

Introduction

Dhaka University Institutional Repository

1.0 Introduction

1.1 Overview

Anthrax is a non-contagious but deadly zoonotic disease caused by *Bacillus anthracis* (*B. anthracis*), a gram-positive, non-motile, spore-forming bacterium (Fasanella et al., 2013a). It is an acute, febrile disease of warm blooded animals including humans. *B. anthracis* has a long history of interest to microbiologists. It was the first organism used to prove Robert Koch's postulate. Since Dr. Louis Pasteur performed these experiments in 1877, *B. anthracis* has remained at the forefront of studies on pathogenic bacteria. Anthrax is blamed for several devastating plagues that killed both humans and livestock. *B. anthracis* is derived from "anthrakis", the Greek word for coal, because anthrax in humans causes black, coal-like lesions on the skin at the site of inoculation (Inglesby et al., 1999). Anthrax is known by many names around the world including charbon, woolsorters disease, ragpickers disease, malignant carbuncle, malignant pustule and Siberian ulcer (OIE, 2008a).

B. anthracis has a wide host range including humans. Anthrax is commonly seen in wild and domestic vertebrate herbivores, including cattle, sheep, and goats worldwide (Fasanella et al., 2013b). The disease was also reported in wild carnivores who consumed the carcasses of anthrax infected animals, whereas the birds are naturally resistant to anthrax (Lindeque and Turnbull, 1994; FAO, 2001). Anthrax, in susceptible animals, has a generally fatal outcome characterized by sudden death and leakage of blood from the natural openings. In bovine animal industry, the disease causes enormous effect due to sudden death of animals as well as the public health implication of the disease. In case of humans, the disease occurs with exposure to infected animals, exposure to tissue from infected animals, or by handling infected animal products like skin, bone, flesh or by inhaling anthrax spores from contaminated animal by-products and also direct exposure to *B. anthracis* (Dixon et al., 1999; CDC, 2009a,b). Anthrax infection in humans can occur in three forms: cutaneous, which affects the skin; inhalation, which affects lungs; and gastrointestinal, which affects the digestive tract (CDC, 2009a,b; Hugh-Jones and Blackburn, 2009). Recently, a fourth fatal form (injectional anthrax) has been reported in drug users as a result of injections of heroin contaminated with anthrax spores (Hicks et al., 2012). Cutaneous anthrax, the most

Dhaka University Institutional Repository

common manifestation of disease, accounts for 95% of cases, whereas pulmonary and gastrointestinal anthrax are much less common and follow inhalation or ingestion of spores, respectively. Inhalational anthrax is rare but particularly deadly, with up to a 90% fatality rate (Mayer et al., 2001).

B. anthracis forms spores when environmental conditions are not conducive to grow. Spore forms are resistant to heat, cold, pH, desiccation, chemicals and irradiation. Because of its hardy spores, *B. anthracis* can survive for several decades in the environment (OIE, 2008a). When a host ingests spores, the spores germinate and subsequently produce vegetative forms that multiply and eventually kill the host. Bacilli then spread into the environment, subsequently sporulate, and are taken up by another animal. Thus spores of *B. anthracis* play an important role for the successful global spreading of this organism (Van Ert et al., 2007a). Due to its strong ability to maintain the vitality and virulence for many decades and to the very low costs of production, *B. anthracis* is considered one of the pathogens of interest such as bacteriological weapon in a hypothetical bioterrorist attack (Freidlander, 1997). *B. anthracis* already received notoriety for its use as an agent of bioterrorism in the 2001 letter attacks in the United States (Hoffmaster et al., 2002), and an unsuccessful aerosol attack in Japan in 1993 (Keim et al., 2001). Now a days, *B. anthracis* is classified by the Centers for Disease Control and Prevention (CDC) as a category A select agent, because of its suitability for use as a biothreat agent in an attack or commission of a biocrime (Inglesby et al., 2002). That's why anthrax must be notified immediately by the authority.

Anthrax outbreaks in animals in nearly 200 countries are recorded by The World Anthrax Data Site, a World Health Organization Collaborating Center for Remote Sensing and Geographic Information Systems for Public Health (Curtis et al., 2005; Hugh-Jones, 2006). The anthrax status of a given country may be classified into one of six categories: hyperendemic/epidemic, endemic, sporadic, probably free, free and unknown. The countries with hyperendemic/epidemic status are more frequent in Africa, although the status of Egypt is "Probably free". Examples of regions with unknown anthrax status are the polar extremes, the Arctic and the Antarctic (http://www.vetmed.lsu.edu/ whocc/). According to official data, the worldwide annual number of human anthrax cases ranges from 2,000 to 20,000 (Hugh-Jones, 1999).

Dhaka University Institutional Repository

Enhanced disease management in animals in developed countries has removed this threat. Nevertheless, anthrax outbreaks have been reported in the USA (Mongoh et al., 2008a), Australia (Durrheim et al. 2009), Italy (Fasanella et al., 2010a), Sweden (Lewerin et al., 2010) and many other European countries within the last 10 years at various frequencies (OIE, 2011). Anthrax is sporadic or endemic in South Asian countries (Vijaikumar et al., 2002; Rao et al., 2005; ICDDR,B, 2009).

The animal anthrax, locally known as 'Torka', is believed to have been enzootic in Bangladesh for a long time, and historically human outbreaks were always preceded by animal outbreaks (Ahmed et al., 2010; Biswas et al., 2012; Ahsan et al., 2013). Over the recent years anthrax has emerged as a potential threat to the veterinary and public health in Bangladesh and seems to be spreading panic across all sections of Bangladeshi society. For poor villagers living on the margins of society, cattle and livestock are valuable commodities to own. For many ownership of a single cow is the only source of livelihood. Needless to say, the anthrax outbreak has sent ripples throughout the rural areas. The Government of Bangladesh declared a red alert due to a sudden explosive outbreak of anthrax in 2010 that hit 12 districts and affected 607 people (Ahmed et al., 2010). The outbreak was investigated and thought to have been caused by the slaughter of infected cattle and selling or eating of contaminated meat. The outbreak was most prevalent in the districts of Pabna, Sirajganj, Rajshahi, Kushtia and Tangail, which have greater cattle populations (Ahmed et al., 2001; Ahmed et al., 2010). Considering the scenario, the most vulnerable segments in Bangladesh are the farmers, butchers and villagers whose profession entails them to be in close proximity with herbivorous animals and who handle the infected animal meat and by-products. The impact of the anthrax outbreak has also economic consequences. That time, trade of red meat came almost to a stand still resulting in economic woes for cattle traders, butchers and others involved in the business. Subsequent anthrax outbreak took a heavy toll on Bangladesh's export-oriented leather industry (Ahmed et al., 2010).

In absence of structured active surveillance for zoonotic diseases in Bangladesh, cases of animal anthrax are hardly come to the surface until mass media report human cases suddenly popped up as consequences of recent butchering and slaughtering of clinically sick animals for meat. Such intermittent reports across the country do not reveal the real

Dhaka University Institutional Repository

load of the disease in the animal populations. Contrary to a very few yearly mass media reports disclosing outbreaks of anthrax in humans attributed to transmission of the organism from animals, unprecedented high numbers of human and animal anthrax cases were reported in the years 2009 and 2010, recorded mostly from two districts namely, Pabna and Sirajganj (Ahmed et al., 2010; Biswas et al., 2012; Chakraborty et al., 2012). In such high epidemic peak of occurrence, a case-control study was conducted previously by enrolling 15 cases (occurred in July-September, 2010) and 15 control cattle smallholdings in that spatial zone (Biswas et al., 2012). Although assessing risks by including anthrax cases only from a narrower time and space might have had merits in a particular geographical area for the epidemic time, such study could have limited impact in that its findings must not be generalized for the country as a whole. To recommend non-vaccine other preventive measures applicable for the entire country, risks for anthrax have to be investigated by enrolling the cases reported across the country for a longer period of time. Because anthrax is a One Health disease, management of risks identified in cattle from broader time and space would eventually reduce human cases in the country.

The clinical course of anthrax ranges from peracute to chronic (Samad, 2008). The peracute, a common form in cattle, is characterized by sudden onset and a rapidly fatal course with only a brief evidence of illness with staggering, dyspnea, trembling, collapse and a few convulsive movements. In acute form, there is an abrupt fever and a period of excitement followed by depression, stupor, respiratory or cardiac distress, staggering, convulsions and death. Because of insufficient time for treatment between clinical onset and death, vaccination against the disease is the first line of consideration for any control plan of anthrax affecting bovine animals. However, in developing countries, such as Bangladesh, vaccine supply cannot be made available for most susceptible populations because of its limited production. Thus, adopting measures targeting the amelioration of identified risks/practices associated with anthrax can be very helpful in such situation for reducing anthrax incidences.

The current development of genetic markers for *B. anthracis* has opened the way to a highly informative approach for monitoring the potential spread and distribution the strains causing the of outbreaks. Various sets of repeat sequences with high mutability

Dhaka University Institutional Repository

became available for fingerprinting protocols after the groundbreaking study of Keim et al. (2000). In application to the epidemiology of anthrax in animals, the first version of a multi-locus variable number of tandem repeats analysis (MLVA) using eight markers was used to analyze outbreak strains in the Kruger National Park, South Africa (Smith et al., 2000). The discriminatory power of the method was later increased by the addition of further markers by Lista et al. (2006) and Van Ert et al. (2007a). Recently after explosive anthrax outbreaks in Bangladesh, Chakraborty et al. (2012) and Fasanella et al. (2013b) conducted molecular studies employing MLVA with using 8 and 15 variable number of tandem repeats (VNTR), respectively, along with single nucleotide repeats (SNR) tool. In such molecular investigations, different types of environmental samples from different anthrax prone regions of the country are usually accessed. This research could be defined as an active surveillance due to the fact that analyzing environmental samples means acting on the source where the spores could be more gathered. With spatial variation in occurrence although anthrax is considered to be an enzootic disease in bovine animals in Bangladesh, its individual occurrences are seldom reported in mass media until humans are clinically affected with the disease due to slaughtering or butchering of clinically sick animals for meat. In absence of structural surveillance for anthrax in the country, reports on it from any reliable global electronic-based surveillance program, such as ProMED-mail (http://www.promedmail.org/) might be a valuable resource in verifying the real causation by producing evidence from laboratory investigations, and in identifying the particular genotype of the pathogen. Such verification and knowledge on particular genotype(s) circulating in a country are also needed in viewpoints of identification of source/origin of the pathogen, exportation of livestock products and investigating any future bioterrorism attack anywhere else in the world using *B. anthracis.*

The Sterne strain of *B. anthracis* is used for immunization and should be administered to livestock in a dose containing up to10 million viable spores (Hambleton et al., 1984). Animals vaccinated >6 months ago might not retain sufficient immunity because Sterne strain vaccine induces immunity that typically lasts for just under 1 year (FAO, 2001). Six months apart from each other two doses of vaccine against anthrax might be ideal for anthrax-prone area (FAO, 2001). Anthrax vaccine containing F-24 strain of *B. anthracis* (Roy et al., 2013) is produced in two laboratories in Bangladesh. Although these two laboratories have a target production of 5.1 million vaccine doses annually, their average

Dhaka University Institutional Repository

annual vaccine production for the last 6 years (July 2004 to June 2010) was 3.8 million doses, whereas the country's total ruminant population is approximately 48.7 million (Tareque et al., 2010). As a result, each year a large number of ruminant population remains out of vaccine coverage. Furthermore, the immunogenic power of this long used vaccine strain is poorly known. Thus far, the immunological characteristics of local anthrax vaccine have been assessed in cattle and goats in a couple of studies (Dipti et al., 2013; Roy et al., 2013). However, the immunogenicity of this vaccine should be evaluated by using immunoproteomics study to know our strength to confront anthrax in the livestock population.

Many studies have been carried out on the molecular epidemiology of anthrax in humans and in a variety of animals worldwide. But such studies are very limited in Bangladesh. Considering the subsequent episodes of anthrax outbreaks in both human and animals in recent years, this lacking of molecular study should be substantiated with high resolution genetic study of *B. anthracis* strains circulating in Bangladesh. On the overview given above towards the need for better understanding of anthrax epidemiology in Bangladesh, the present study was undertaken to: evaluate the risk factors associated with anthrax infection in cattle; characterize genotypically the *B. anthracis* strains circulating in the country by applying higher discriminating power tools such as, hierarchical fingerprinting system (PHRANA): canonical single nucleotide polymorphism (canSNP), multilocus variable number tendem repeat (VNTR) analysis (MLVA), single nucleotide repeat (SNR) and also by whole genome sequencing; and investigate the immunogenic potency of the anthrax vaccine being used in the country. The results obtained would contribute to strategize more effective prevention and control measures against the disease in Bangladesh, safeguarding both animal and public health.

Dhaka University Institutional Repository

1.2 Literature review

1.2.1 History of anthrax

Anthrax made its first recorded appearance in 1491 BC in the early writings of Mesopotamia and the Book of Genesis. The Old Testament description of the 5th and 6th Egyptian plagues showed typical symptoms of anthrax. Before Virgil (70 BC-19 BC) composed the Aeneid, he wrote a selection of poems on agriculture called Georgics. Aside from topics of animal husbandry and veterinary medicine in his third Georgic, he dedicated a section to a disease that manifested itself in sheep, cattle, horses, and wild animals. Sparse comments on anthrax were also described in early Hindi and Greek literature. The infamous “Black Bane” occurred in 1613 and became the first European pandemic. It caused over 60,000 deaths in humans and cattle. The disease derives its name from the Greek word for coal, anthrakis, due to the coal-like black lesions found on the skin in cutaneous anthrax (Blanc, 1890; Inglesby et al., 1999).

Casimir Davaine and Pierre Rayer first observed rod-like organisms present in the blood of anthrax-infected animals and humans in 1850. By 1863, Davaine showed that those rods were most likely the cause of anthrax since unexposed sheep did not develop the disease (Compton, 1987). Robert Koch developed a method for culturing pure *B. anthracis* in 1876. This method allowed him to be the first to elucidate the complete life cycle of anthrax from spore to vegetative bacterium and back to spore again (Compton, 1987). Koch also used *B. anthracis* to develop and prove his postulates regarding the germ theory of disease (Compton, 1987). Figure 1.2.1 shows the photographs of Robert Koch and Louis Pasteur and drawing of *B. anthracis* by Robert Koch together with Ferdinand Cohn.

Louis Pasteur created the first major vaccine against anthrax in livestock in 1881 (Compton, 1987). However, despite the existence of anthrax vaccines since around 1870, the disease remains a threat to livestock and even humans particularly in developing continents such as Asia, Africa and South America (Dutz and Kohout-Dutz, 1981). In addition, *B. anthracis* was documented to have been part of the biological arsenals of many nations, including the U.S. at one time. With the Biological Weapons Convention of 1972, production of these weapons was outlawed (Hilleman, 2002). Even so, *B.*

anthracis is still believed to be part of the biological arsenals of a least 17 nations (Inglesby et al., 1999). Taking into consideration the current world environment and the unpredictable nature of terrorism, developing a highly effective vaccine with the ability to fully protect against all forms of the disease would be an important component to add to the national biological defense arsenal of UK (Compton, 1987; Cimons, 1998).

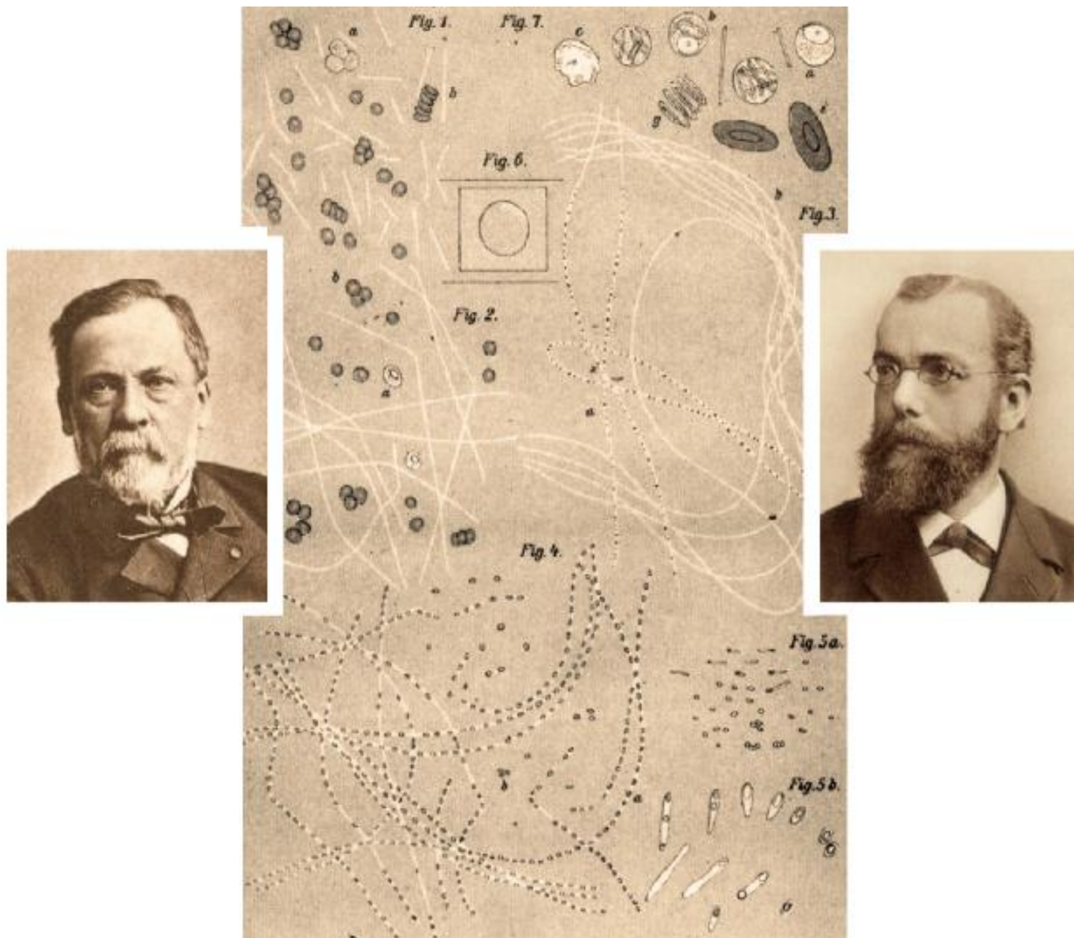

Figure 1.2.1: The discoverers of *B. anthracis* and fathers of medical microbiology: Robert Koch (1843-1910), right, and Louis Pasteur (1822-1895), left, and inset: drawing of *B. anthracis* by Robert Koch together with Ferdinand Cohn (1828-1898) made in 1876 (Hudson et al., 2008).

1.2.2 Disease agent of anthrax: *Bacillus anthracis*

1.2.2.1 Taxonomy of *Bacillus anthracis*

Bacterial taxonomy includes both phenotypic and genotypic characteristics and leads to the biological binomial nomenclature based on the genus (generic name) followed by the species (specific name) (Woese, 1994; Prescott et al., 1999; Moore et al., 2010). The taxonomic classification of *B. anthracis* is shown in Table 1.2.1.

Table 1.2.1 Taxonomic classification of *B. anthracis*

Classification	Name
Empire	Prokaryota
Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Bacillaceae
Genus	*Bacillus*
Species	*Bacillus anthracis*

1.2.2.2 Species status in the genus *Bacillus*

Bacillus anthracis belongs to the large genus of *Bacillus* and it is often placed in the so-called *Bacillus cereus* group (*B. cereus* sensu lato), which is a sub-group of closely related species (Daffonchio et al., 2000). The group comprises *B. anthracis, B. cereus, B. mycoides, B. pseudomycoides, B. thuringiensis* and *B. weihenstephanensis*. These species have been traditionally separated into different species based on phenotypic characteristics, pathogenicity, clinical symptoms, host preference and ecological niche (Rasko et al., 2005).

1.2.2.3 Properties of *Bacillus anthracis*

B. anthracis is an aerobic, gram-positive, non-motile rod (Koehler et al., 1994). The bacterium measures 1-1.5µm by 3-10µm (Ibrahim et al., 1999). Spore formation occurs centrally or paracentrally and causes no bacterial swelling (Dixon et al., 1999; Inglesby et al., 1999). Spore formation occurs when nutrients are depleted as happens after host death and exposure to air (Bartlett et al., 2002). *B. anthracis* spores are highly resistant to various environmental changes and can survive indefinitely in soil, air, water and vegetation despite extreme heat or cold, dessication, chemical treatment or ultraviolet

Dhaka University Institutional Repository

exposure (Dutz and Kohout-Dutz, 1981; Dragon and Rennie, 1995; Ibrahim et al., 1999). The highly resistant nature of the spore aids in the persistence of the disease in an area (Dragon and Rennie, 1995). The properties of *B. anthracis* is presented in Figure 1.2.2.

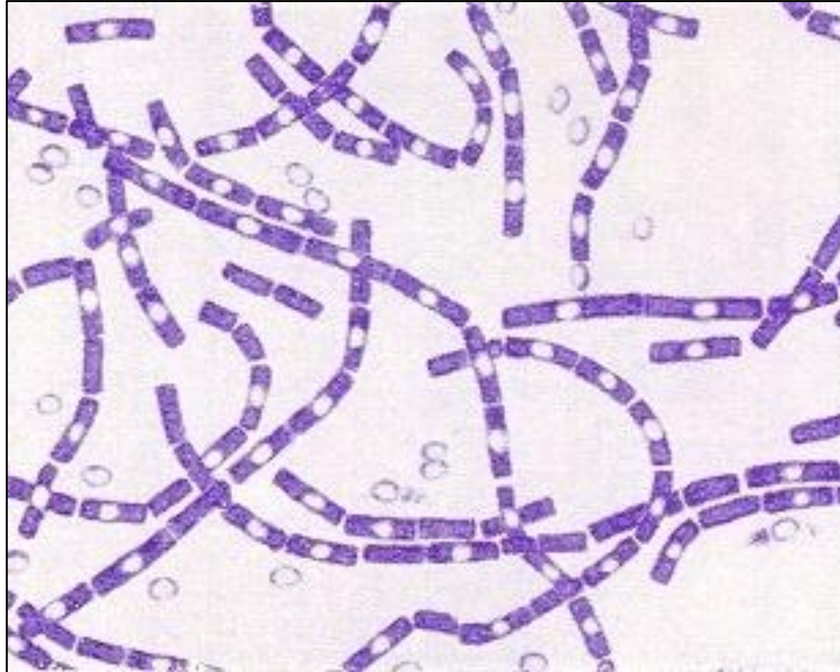

Figure 1.2.2: ***Bacillus anthracis.*** **Gram stain. 1500X.** (The cells have characteristic squared ends. The endospores are ellipsoidal shaped and located centrally in the sporangium. The spores are highly refractile to light and resistant to staining. Source: http://textbookofbacteriology.net/Anthrax.html.)

The bacteria grow readily on all conventional microbiology media at 37^{0}C including sheep blood agar and produce non-hemolytic colonies (Inglesby et al., 1999). Colony appearance on agar is typically 4-5mm rough, white colonies with a characteristic comma shape or tail often referred to as "curly-hair" or “medusa head” colonies (Ibrahim et al., 1999; Inglesby et al., 1999). *B. anthracis* occurs singly or in pairs in tissue and forms long chains in culture giving a classic "boxcar" appearance (Dutz and Kohout-Dutz, 1981).

Dhaka University Institutional Repository

1.2.2.4 The *B. anthracis* genome

The genome of *B. anthracis* includes a single 5.2-megabase chromosome and two large virulence plasmids, pXO1 and pXO2 (182 and 95 kilobases, respectively). Altogether, the genome has 5,838 predicted protein-coding genes (Brossier et al., 2000). The chromosomal sequence and gene organization is quite similar to that of the closely related bacteria *B. cereus* and *B. thuringiensis* (Brossier and Mock, 2001). Figure 1.2.3 shows the structure of *B. anthracis*.

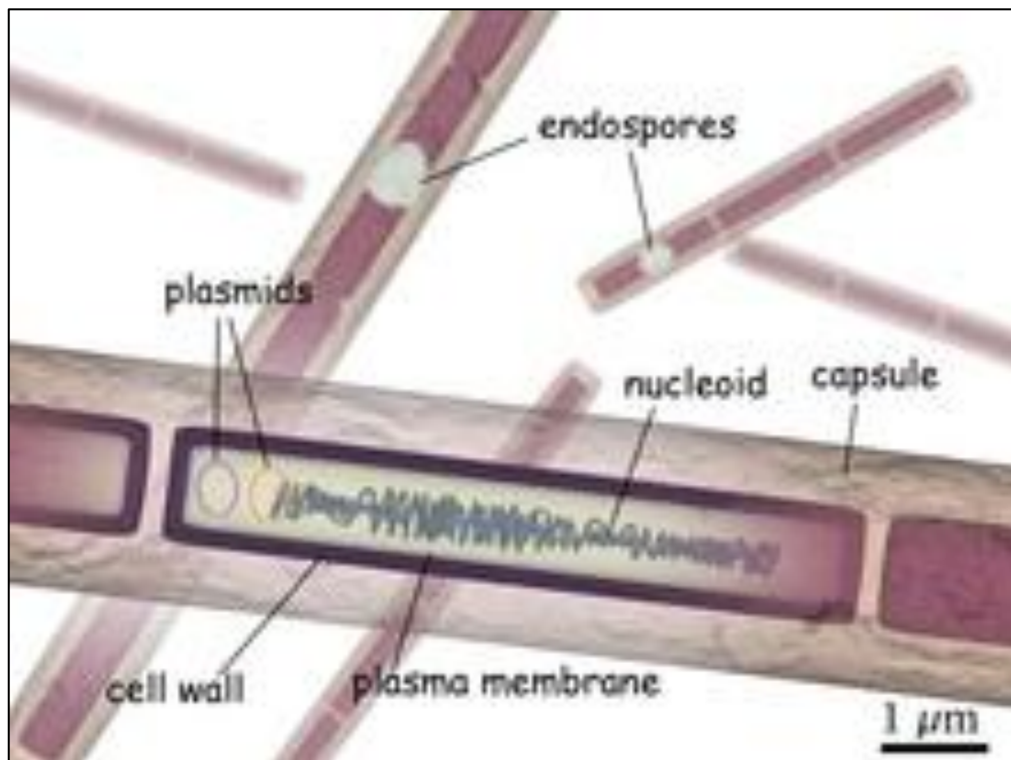

Figure 1.2.3: Structure of *B. anthracis*.

(Source: https://en.wikipedia.org/wiki/Bacillus_anthracis)

B. anthracis likely evolved from a single clone of *B. cereus* that acquired pXO1 and pXO2 from the environment by lateral genetic transfer. Genes required for virulence factor expression and regulation are located on the plasmids (Celli et al., 2003). pXO1 contains a large pathogenicity island which encodes lethal and edema toxins (LT and ET) (Cheville et al., 1993; Celli et al., 2003), while the biosynthetic enzymes of the poly-D-gamma glutamic acid capsule are encoded on a pathogenicity island on pXO2 (Cherwonogrodzky, 1993; Cimons, 1998). Loss of either plasmid significantly reduces

Dhaka University Institutional Repository

virulence in most animal models (Compton, 1987; Corbel, 1997; Cloeckaert et al., 1998, 2003). Features of the *B. anthracis* genome are represented in Table 1.2.2.

Table 1.2.2 Features of *B. anthracis* genome

Feature	Chromosome	pX01	pX02
Size (bp)	5,227,293	181,677	94,829
Number of genes	5,508	217	133
Replicon coding (%)	84.3	77.1	76.2
Average gene length (nt)	800	645	639
G+C content (%)	35.4	32.5	33.0
rRNA operons	11	0	0
tRNAs	95	0	0
sRNAs	3	2	0
Phage genes	62	0	0
Transposon genes	18	15	6
Disrupted reading frame	37	5	7
Genes with assigned function	2,762	65	38
Conserved hypothetical genes	1,212	22	19
Genes of unknown function	657	8	5
Hypothetical genes	877	122	51

(Source: https://en.wikipedia.org/wiki/*Bacillus_anthracis*)

1.2.2.5 *B. anthracis* toxins

The structure and mechanisms of action of the toxins have been intensely studied. The enzymatic effector proteins of the two toxins are called lethal factor (LF) and edema factor (EF). Both LF and EF can bind a third protein, protective antigen (PA). PA is cleaved by mammalian serum and/or cell surface proteases and can bind to at least two specific receptors (TEM8 and CMG2) located on host cell membranes (Coulson et al., 1994; Cosivi and Corbel, 1998). PA forms ring-shaped heptamers, and interacts with LF and EF (Dai and Koehler, 1997), which then enter the host cell by endocytosis (Davis et al., 1994). Upon acidification of the endocytic vacuole, the PA heptamer apparently forms a pore through which the EF and LF moieties are translocated. Molecular targets within mammalian cells have been clearly identified for both toxins. LF is a zinc metalloprotease capable of inhibiting signal transduction through the mitogen-activated protein kinase (MAPK) cascade by cleaving most MAPK kinases (MAPKKs or MEKs), preventing the phosphorylation of MAPKs such as p38, ERK and JNK (Dragon and Rennie, 1995; Doyle, 1996; Dixon et al., 1999). EF is a calcium/calmodulin dependent adenylate cyclase that increases intracellular levels of cyclic AMP (Duesbery et al.,

1998), leading to massive edema. LT and ET appear to impair both the innate and adaptive immune systems, having effects on multiple cell types, including macrophages, dendritic cells and neutrophils.

1.2.2.6 *B. anthracis* capsule

The poly-glutamate capsule appears to be a fibrous structure in electron micrographs of the bacillus surface (Dutz and Kohout-Dutz, 1981). Early data show that capsule consists entirely of poly-D-γ-glutamate (Fraser and Mays, 1986; Ezzell and Abshire, 1988; Friedlander, 2001; Flick-Smith et al., 2002). One-dimensional and two-dimensional nuclear magnetic resonance (NMR) data recently confirmed that capsule has γ-carboxyl peptide linkages, and gas chromatography data recently confirmed that capsule appears to contain glutamic acid only of the D configuration (Gaur et al., 2002). Capsule purified from autoclaved Ames bacilli appears to have an extended random coil structure, as determined by light scatter and circular dichroism data, and a heterologous size range averaging about 700 kDa, as determined by light scatter analysis (Gaur et al., 2002). Capsule synthesis is dependent on four proteins (CapA, B, C and E) encoded by an operon in pXO2 (Cherwonogrodzky, 1993; Guidi-Rontani et al., 1997; Cimons, M. 1998; Gupta et al., 1999; Greenfield and Bronze, 2003). CapD (or DepA), another protein encoded by the *cap* operon, can degrade capsule (Gupta and Ghosh, 2001; Halperin and Marcus, 2001), and the subsequent release of capsule fragments (low molecular weight capsule) has been linked to virulence (Hilleman, 2002). A recent report not only confirms that CapD is required for full virulence in mice and that CapD can degrade capsule, but it also shows that CapD apparently covalently links capsule to cell wall peptidoglycan (Iacono-Connors et al., 1991).

1.2.2.7 Roles of capsule in virulence

Remarkably, crude capsule preparations were shown to be anti-phagocytic in 1907 (Ibrahim et al., 1999), and *B. anthracis* capsule was associated with virulence in 1915 (Inglesby et al., 1999). A 1963 report showed reduction of phagocytosis of strains NP and Sterne by guinea pig polymorpho nuclear cells upon addition of exogenous capsule (Ivins and Welkos, 1986). Addition of purified capsule also enhanced virulence of a Pasteur II strain in guinea pigs. Others have described capsule-mediated inhibition of phagocytosis in neutrophils and macrophages (Ivins and Welkos, 1986, 1988; Gupta et

al., 1999). Conversely, antibody to capsule enhances phagocytosis (Ivins, 1988; Ivins and Welkos, 1988; Ivins et al., 1992). Capsule may also camouflage bacilli from the immune system by binding host proteins. As far back as the 1930s, investigators showed that capsule binds to basic serum proteins, such as lysozyme (Ivins et al., 1994; Ivins et al., 1995). Recent evidence shows that it binds and deactivates antibacterial cationic peptides (Ivins et al., 1998). It has been suggested that capsule fragments might bind to mediators of innate immunity, acting as a sink that drains immune modulators (Gupta and Ghosh, 2001). Complement binding by capsule, perhaps in conjunction with S-layer proteins (Dutz and Kohout-Dutz, 1981), and capsule-mediated inhibition of anthracidal activity of normal horse serum and guinea pig leukocyte extracts have also been reported (Fraser and Mays, 1986).

1.2.2.8 Other virulence factors

In addition to anthrax toxins, capsule and their regulators, a number of genes/proteins that have a measurable contribution to virulence and survival in mice or guinea pig models of infection have been identified. A select few of these, such as specific proteases (Kanaya et al., 1999), may contribute directly to inflicting damage on the animal host; others, such as cell wall-modifying enzymes, may promote evasion of the innate immune system (Karlin et al., 2003). Most of the other genes/proteins known to affect virulence are not virulence factors per se, but appear to promote spore germination (Khanna and Singh, 2001), acquisition of key nutrients (Koehler et al., 1994; Kim et al., 1997; Koehler, 2002; Krishnanchettiar et al., 2002), resistance to oxidative stress or coordination of an overall stress response during replication in the host environment (Laemmli, 1970; Koehler et al., 1994).

1.2.2.9 Pathogenesis

Natural infection in animals usually occurs by ingestion of spores that can germinate in tissues such as the pharynx, mouth or intestines, or by absorption through the skin to cause cutaneous infection. Animals vary in their susceptibility to anthrax according to the route of infection. For most animals, the oral route is the least successful and infection is most easily established by intramuscular injection. However, cattle are easily infected orally and more difficult to infect subcutaneously (Beveridge, 1983). The infection of herbivores (and occasionally humans) via the inhalational route normally

proceeds as follows: Once the spores are inhaled, they are transported through the air passages into the tiny air particles sacs (alveoli) in the lungs. The spores are then picked up by scavenger cells (macrophages) in the lungs and are transported through small vessels (lymphatics) to the lymph nodes in the central chest cavity (mediastinum). Damage caused by the anthrax spores and bacilli to the central chest cavity can cause chest pain and difficulty breathing.

Once in the lymph nodes, the spores germinate into active bacilli that multiply and eventually burst the macrophages, releasing many more bacilli into the bloodstream to be transferred to the entire body. Once in the blood stream, these bacilli release three proteins named lethal factor, edema factor, and protective antigen (Vos et al., 2002; Pimental et al., 2004). Septicemia in anthrax is a terminal event. *B. anthracis* produces a toxic complex of three components: factor I (edema factor), factor II (protective antigen) and factor III (lethal factor). These factors operate in combination and their total effect is to kill phagocytes, damage capillary walls and interfere with the clotting mechanism. The net effect is edema, shock and death. Protective antigen provides the mechanism for lethal factor to enter cells and it is an antibody against this protective antigen that protects animals against infection (Valli et al., 2003).

1.2.3. Epidemiology

1.2.3.1 Receptive Host

Anthrax has a wide host range of animal species, including humans. Under natural conditions the animals that are more susceptible to anthrax are ruminants, both domestic such as cattle, buffalo, sheep, goats, camels and wild such as deer, roe deer, elephant (Hanna and Ireland, 1999; FAO, 2001; Bales et al., 2002; OIE, 2008a; Aikembayev et al., 2010; Biswas et al., 2012; Fasanella et al., 2013a,b). Horses are also receptive and pigs to a lesser extent. Horses in natural conditions are less receptive to anthrax than cattle when the infection is transmitted via food, probably because they are monogastric and the spores ingested with food are quickly neutralised by the acid chloride present in the stomach. In cattle, however, before arriving in the stomach the spores make a long trip and this favours their implantation. On the contrary in anthrax infection transmitted through the skin, horses seem to be more sensitive because in the past, when the Pasteur

vaccines were used, vaccination accidents were more frequent in the horse compared to ruminants. In anthrax outbreaks, because of the activity of biting flies, the value of horses affected/horse population is higher than the value of ruminants affected/ruminant population. Carnivores are sick only exceptionally while birds are refractory (Lindeque and Turnbull, 1994; FAO, 2001). For sensitive species, anthrax represents a fatal outcome with sudden death and sometimes bleeding from natural orifices. The disease usually transmits to humans through contact with infected animals or contaminated animal products. The exposure pattern of *B. anthracis* spores to humans results in three different forms of the disease: cutaneous (penetration of the spores through micro-abrasions or cuts) (Lucey, 2005), gastrointestinal (through the consumption of infected meat) (Ndybahinduka et al., 1984; Sirisanthana and Brown, 2002; Beatty et al., 2003; Kanafani et al., 2003), or inhalational anthrax (via inhalation of the spores) (Lucey, 2005), depending on the site of infection. Cutaneous anthrax is the most common and the most treatable form; inhalational anthrax is rare but poses a much greater risk of death (CDC, 2009a,b; Hugh-Jones and Blackburn, 2009).

1.2.3.2 Disease distribution

1.2.3.2.1 Anthrax worldwide

In Europe and in parts of North America, sporadic outbreaks can follow the disturbance of old anthrax epizootic graves, such as at the creation of a ditch or a road (WHO, 2008). In some Mediterranean countries like Italy (Garofolo et al., 2010, 2011) and Turkey (Durmaz et al., 2012) there are endemic areas in which cattle, humans and wild animals have been infected. In Canada, there have been several reports on anthrax in wild Bison (Nishi et al., 2007; Shury et al., 2009). In Texas, there is an area with almost enzootic anthrax in white-tailed deer (Blackburn and Goodin, 2013). In several Asian countries, the disease is still a large problem and in Cambodia and Vietnam it is even considered enzootic. In Africa, outbreaks occur in national parks e.g., in South Africa, Botswana, Namibia and Uganda. The situation is considered hyperendemic in Zambia and Zimbabwe (WHO, 2008). Figure 1.2.4 shows the global distribution of anthrax.

Dhaka University Institutional Repository

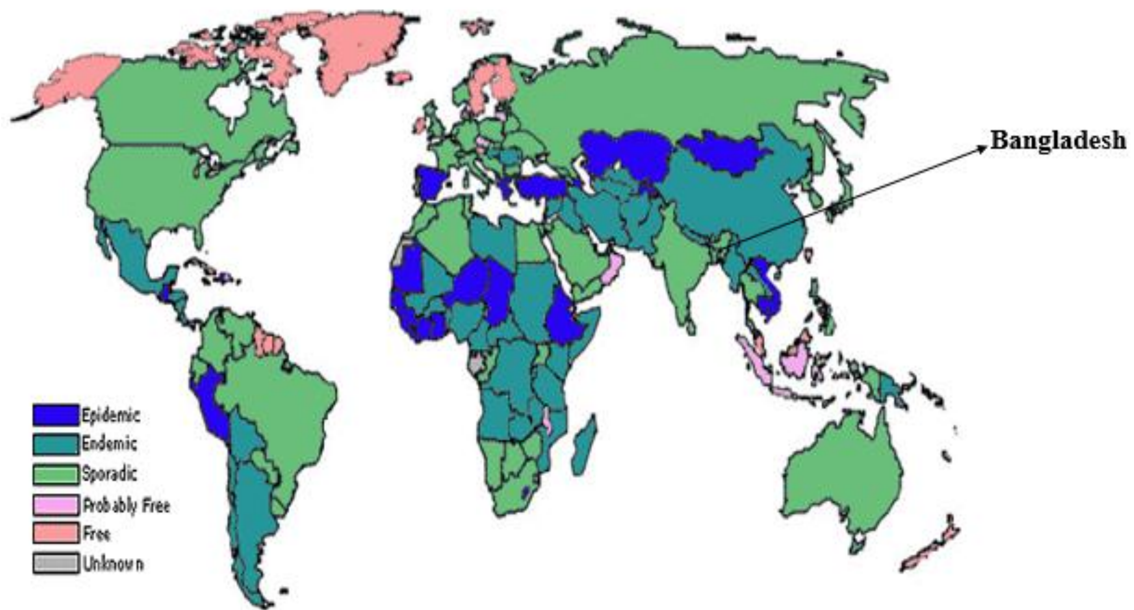

Figure 1.2.4: Global distribution of anthrax (Source: http://www.vetmed.lsu.edu/whocc/).

1.2.3.2.2 Anthrax outbreaks in Bangladesh

The major zoonotic bacterial diseases recorded in Bangladesh are Anthrax, Tuberculosis, Brucellosis, Salmonellosis, Campylobacteriosis and Leptospirosis, of which only Anthrax has been reported as clinical outbreaks form in both humans and cattle (Samad, 2011). The animal anthrax, locally known as 'Torka', is believed to have been enzootic in Bangladesh for a long time, and historically human outbreaks were always preceded by animal outbreaks. Published reports of anthrax in animals in Bangladesh date back to 1948 (Mohan and Ali, 1948). Some aspects of anthrax in Bengal were described and certain relevant features discussed in their report. Anthrax was recognized to occur in Bengal at the inception of the Civil Veterinary Department in 1892. Since then it has caused mortality chiefly in cattle, but to some extent also in other animals. On several occasions the infection has spread to human beings. Generally speaking, the extent of infection and mortality has been low and the outbreaks have been small in size. However, sporadic anthrax outbreaks in cattle, humans (Samad and Haque, 1986) and elephant (Mustafa, 1984) have been reported in Bangladesh. Sixty two animal infections were recorded with 69% deaths from Pabna milk shed areas during 1980-84. Samad and Haque (1986) reported 27 human cases of anthrax during 1980-84 and a research team of

Dhaka University Institutional Repository

Institute of Epidemiology, Disease Control and Research (IEDCR) detected 19 cases out of 624 tannery workers of Dhaka city in 1997.

In 2009-2010, anthrax has dramatically reemerged with greater involvement in this country. The Government of Bangladesh declared a red alert due to a sudden explosive outbreak of anthrax in 2010 that hit 12 districts and affected 607 people. The outbreak was investigated and thought to have been caused by the slaughter of infected cattle and selling or eating of contaminated meat. The outbreak was most prevalent in the districts of Pabna, Sirajganj, Rajshahi, Kushtia and Tangail, which have greater cattle populations (Ahmed et al., 2010; Chakraborty et al., 2012; Islam et al., 2013). Health and livestock officials in Bangladesh expressed great concern over a fresh outbreak of human anthrax prevailed from April to September 2011, mostly affecting two North-Western districts, Sirajganj (61 cases) and Pabna (32 cases). Additionally, districts of Bogra, Meherpur and Tangail had 28, 39 and 14 cases of anthrax, respectively. Due to vaccine coverage in the preceding years, the number of livestock deaths was minimal and only a few infected animals slaughtered were held responsible for human transmission during the 2011 outbreak (ICDDR,B, 2011). Fortunately, there were no anthrax-related human deaths but meat sales drastically declined due to a lack of consumer confidence, and anthrax created mass havoc with significant economic losses related to cattle farming. Although there was no known case fatality, people panicked and mass immunization of livestock was demanded by concerned sections.

In Bangladesh, reports for both animal and human anthrax do not represent the true picture of the disease since it is under diagnosed and/or reported due to many reasons. Because the disease is thought to be enzootic, in agricultural setting humans might always get infection from infected animal. Therefore, anthrax is likely to be prevailing in the country in endemic form (Ahmed et al., 2010; Biswas et al., 2012). The anthrax outbreaks in Bangladesh are listed in Table 1.2.3.

Table 1.2.3 List of anthrax outbreaks in Bangladesh since 1960

SL No.	Year	Animal case no.	Human case no.	Source
1.	1960	1 (Elephant)		Mustafa (1984)
2.	1980	6	00	Samad and Hoque (1984); Ahmed et al. (2010)
3.	1981	7	00	
4.	1982	16	17	
5.	1983	21	10	
6.	1984	12	-	
7.	1984	1 (Elephant)		Mustafa (1984)
8.	1989-1996	333	-	Ahmed et al. (2010)
9.	1997	0	19	
10	2004	1 (Elephant)		Infectious Diseases and Epidemiology Network, www.giedononline.com; International society for infectious diseases, ProMed<promedmail.org> archive 20040618.1629
11.	October 2009-June 2010	55	99	Ahmed et al. (2010)
12.	August 2010-October 2010	140	607	
13.	2011	178	278	DLS (Personal Communication) and IEDCR report (http://www.iedcr.org/pdf/files/anthrax/Anthrax_Cases_Bangladesh_2010-15.pdf access: 17/03/2016)
14.	2012	195	176	
15.	2013	156	327	
Total		1122	1533	

1.2.3.3 Ecology of anthrax

Anthrax spores survive best in soils rich in organic matter and calcium. In the Kruger National Park (Africa) *B. anthracis* spores have been isolated from animal bones estimated to be about 200 years old (Smith et al., 2000). Saile and Koehler (2006) have demonstrated that spores would germinate and establish stable populations of vegetative cells in the rhizosphere of fescue (*Festuca arundinacea*) grass in the laboratory in an otherwise sterile environment. In natural circumstances, the vegetative cells are fragile and die even in simple environments, such as water or milk (Turnbull et al., 1989). It seems that soil encourages sporulation, not germination, and this would explain why vegetative bacilli are not found in nature. Van Ness (1971) defined the “incubator areas” as depressions which collect water, dead vegetation, calcium and other salts washed in from the surrounding slightly higher ground and thus provide a medium suitable for germination and multiplication. However, this hypothesis was never confirmed by

Dhaka University Institutional Repository

scientific study. It has been proposed that rainy water may collect and concentrate spores in 'storage areas' (Dragon and Rennie, 1995). Spores have a high surface hydrophobicity and so could be carried during a rain runoff in clumps of humus and organic matter to collect and concentrate in standing pools or puddles. As they have a high buoyant density, this would result in them and their organic matter clumps remaining suspended in the standing water to be further concentrated as the water evaporated. Thus theoretically 'storage areas' may collect more spores from extended areas to reach increasing spore concentrations over time and be lethally available to potential incidental grazing hosts. Most *B. anthracis* is held in the ground as spores until the ideal conditions are created for its reproductive cycle that occurs in a different habitat, primarily domestic and wild ruminants. Nature provides few opportunities to the bacterium for its replicative cycle and the development of an exceptional pathogenicity is the effective strategy aimed to significantly increase the probability of success against the host's immune mechanisms. Rapid intense multiplication by the vegetative cells quickly takes the host to death. Although many of the new generations of bacteria will be neutralized by putrefactive processes, a good part survives and spreads into the surrounding soil as spores, ensuring the standard of environmental density of the bacteria that is an essential condition for the continuation of the species. Spores would survive passage through the scavenger's intestinal tract who consumed the carcasses of anthrax infected animals, but not vegetative cells. Anthrax spores were recovered from approximately half of the faeces from jackals (*Canis mesomelas*), vultures (*Gyps africanus, Torgos tracheliotus, Trigonoceps occipitalis*) and hyaenas (*Crocuta crocuta*) collected in the vicinity of carcasses in the Etosha National Park, but not at a distance; the faecal spore density was extremely variable (Lindeque and Turnbull, 1994). Insects, primarily necrophilic and haemophagic flies, have been associated in the spreading of anthrax spores. Fasanella et al. (2010b) demonstrated that, under experimental condition, *Musca domestica* can spread the bacterium and additionally that *B. anthracis* is able to germinate within their intestines.

Dhaka University Institutional Repository

1.2.3.4 Risk factors associated with anthrax in domestic animals

In Bangladesh, a community bad practice for moribund cattle is slaughtering for meat. In relation to religious concept, Islam forbids slaughter of pregnant and very young animals and also eating of animals that die from natural causes. The farmers prefer slaughtering moribund cattle and goats for consumption of the meat as a halal food and also for minimizing their financial cost to some extent by selling the meat. The farmers slaughter cattle at the yard of their household premises due to the lack of manpower to remove the moribund cow from the shed. Some farmers also slaughter sick cattle on the open on grazing field because they think that the animals may die before returning back to the farms. The farmers rinse the slaughtering places with water to remove the blood after slaughtering the cattle and throw the butchering waste in nearby ditches, bodies of water, or open fields. Some scavenging animals like dog, and foxes and also some birds enhance the distribution of the butchering waste and thus promote environmental contamination with the pathogen. These types of bad practices by the owners and other unmeasured factors might have causal roles in the occurrences of anthrax outbreaks in the country (Islam et al., 2013).

History of heavy raining occurred in the last 2 weeks of an outbreak was found to be a potential risk factor for the occurrence of anthrax (Islam et al., 2013). Environmental factors including high ambient temperature and relative humidity that provide a milieu for germination of anthrax bacilli spores from infected carcasses thrown into flood waters or in open fields (Chakraborty et al., 2012) might favor the occurrence of anthrax in Bangladesh (OIE, 2010). During the rainy season, grass harvested along with roots could also harbor anthrax spores and when fed, livestock can become infected (Biswas et al., 2012). Throwing the dead carcass on near by water body, a common practice in Bangladesh during monsoon season and flood condition, was also reported as a risk factor (ICDDR,B, 2009). Farmers prefer to discard the carcasses either in the flood waters or in the rivers due to the lack of dry land away from the household premises to bury the carcasses during the monsoon season. Dumping carcasses in the water may lead to contamination of the water body and spread the organism to the remote places (ICDDR,B, 2009). Locations of farms in a river basin and flooding were suspected of having contributory roles in anthrax outbreaks in North Dakota, USA and in Sweden (Mongoh et al., 2008a; Durrheim et al., 2009; Lewerin et al., 2010).

Among factors that are causally associated with bovine anthrax in Bangladesh the catastrophic decline of vultures (*Gyps bengalensis*, *Gyps indicus* and *Gyps tenuirostris*) could be an important one (Prakash, 1999; Pain et al., 2003). The consumption of diclofenac (a non-steroidal anti-inflammatory drug) residues in animal carcasses is a cause of this decline (Oaks et al., 2004). Due to the disappearance of vultures, animal carcasses disposed of in fields or water remain exposed for longer, contaminating the soil and water (Islam et al., 2013). Biswas et al. (2012) reported that feeding animals with up rooted and unwashed grass and feeding water hyacinth (*Eichhornia crassipes*) could be risk factors for anthrax in cattle in the anthrax-prone areas in Bangladesh.

1.2.4 Anthrax transmission cycle

Anthrax spores can live in the soil for many years. Wild or domestic animals (such as cattle, sheep, or antelopes) can contract anthrax through this soil and become infected. Transmission in wildlife and in humans occurs in one of three ways: cuts in the skin (cutaneous), inhalation and ingestion. Infected animals as well as carriers of the disease can shed spores in urine and feces. In wildlife, anthrax outbreaks typically occur in dry summers following periods of rain. Dry weather usually forces grazing animals to feed closer to the ground where spores are often concentrated. Exposure typically occurs through inhalation or ingestion of spore-laden dust. Transmission among susceptible animals may also occur through flies and mosquitoes. Humans can contract anthrax from contact with infected animals through cuts or scratches in the skin, the eyes, nose or mouth; inhaling spores from contaminated objects such as hides of infected animals; and eating under cooked meat from an infected animal (http://wildlifedisease.unbc.ca/anthrax.htm; Gates et al., 2001). Figure 1.2.5 represents the anthrax transmission cycle.

Dhaka University Institutional Repository

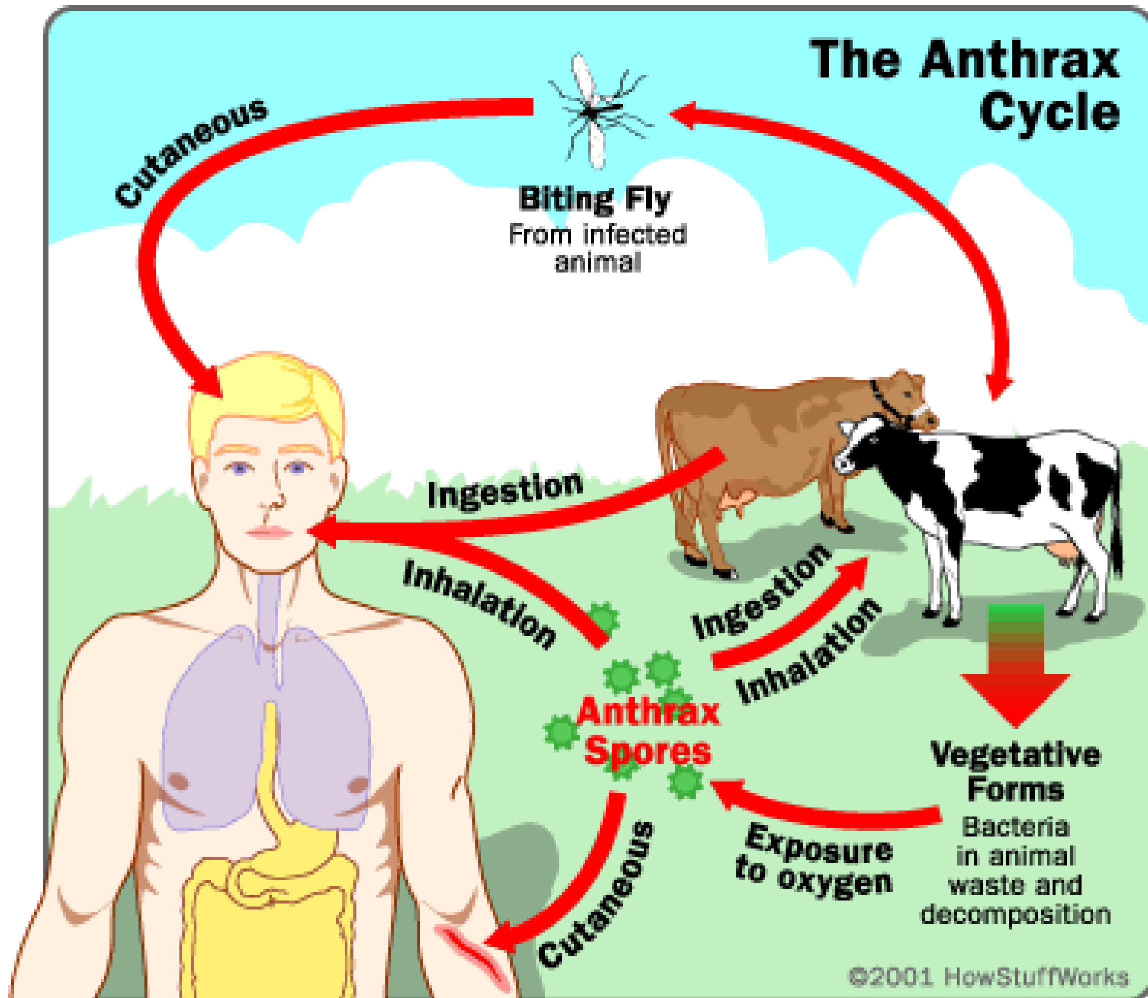

Figure 1.2.5: Anthrax zoonotic transmission cycle
(Source: http://science.howstuffworks.com/anthrax1.htm).

1.2.5 *B. anthracis* life cycle

The oxygen and nutrient-rich blood of warm-blooded hosts represent ideal conditions for anthrax bacteria, and other types of bacteria within the host are outcompeted by anthrax bacteria for resources. When a host dies and oxygen is no longer transported in the blood, *B. anthracis* bacteria become dormant. Other types of bacteria from the gastrointestinal tract of the host animal begin the process of decomposition and can destroy remaining *B. anthracis* bacteria if the carcass remains intact. Scavenging birds and mammals may open the carcass, dispersing anthrax bacteria which, given the correct environmental conditions, form spores that are infectious to other animals or humans (http://wildlifedisease.unbc.ca/anthrax.htm).

Dhaka University Institutional Repository

1.2.6 Clinical signs of anthrax

The signs of anthrax in cattle are variable and may be overlooked in cases of short duration. A number of pathological and environmental factors cause blood stained discharges from the mouth, nose or anus of a carcass. It is normally considered that oozing of unclotted tarry blood from the external natural orifices is suggestive of anthrax. The acute form of the disease is characterized by sudden death in cattle without any prior sign of illness (Jones et al., 1997). The clinical forms of anthrax in animals are traditionally described as (i) peracute or very acute: in which death occurs suddenly (within a few hours at most) of the onset of clinical signs; (ii) acute: in which death occurs from 24 hours to a few days after onset; and (iii) subacute or localized: which lasts for several days and may end in recovery. Anthrax can affect humans and classically causes three types of infection. These are: (i) pulmonary form affecting the lungs; (ii) intestinal form affecting the digestive tract; and (iii) cutaneous form affecting the skin (most dominant form) (Rose, 1940; Samad, 2008).

1.2.7 Postmortem examination

If a carcass of an animal that has died from anthrax is opened, dark unclotted blood and an enlarged, haemorrhagic spleen are immediate indicators of anthrax. However, an enlarged spleen (splenomegaly), cited as a characteristic feature of anthrax and regularly seen in cattle, is uncommon in sheep, pigs and horses. In animals that die, bloody discharges from the body openings may be seen. Decomposition is more rapid than in other conditions and the carcasses become bloated with gases. Rigor mortis or stiffening is not complete. The mesentery may be thickened and oedematous with excess peritoneal, pleural and pericardial fluid. Petechial haemorrhages may be visible in many organs and the intestinal mucosa may be dark red and oedematous, with some areas of necrosis (Vos et al., 2002).

1.2.8 Treatment and control

Minett (1950) mentioned that anthrax is highly fatal and it is difficult to treat affected animals. Long acting penicillin is the antibiotic of choice. Response to treatment may vary; best results are obtained when drugs are administered at earlier phase of infection. If antibiotics are used, vaccination with an anthrax vaccine should be delayed for one to two weeks. The vaccine is a modified-live bacteria and antibiotics will kill or neutralize

Dhaka University Institutional Repository

the vaccine. An effective vaccine is available (non-encapsulated, Sterne strain $34F_2$). The vaccine is relatively safe and provides effective protection on most species of livestock. However, vaccine should be cautiously administered to goats and llamas because clinical cases have been reported in some vaccinated animals belonging to these species.

1.2.9 Diagnostic approaches

Suspicion of anthrax arises from the observation of clinical symptoms, the anatomic pathological findings and epidemiological data. The ecology of the bacterium limits the distribution of the disease that is almost always confined to well-defined territories, in which one can observe, with systematic cyclicity, sporadic outbreaks usually involving a few animals (one or two on average), but the frequency of which tends to increase during dry summers that follow very wet springs (Hugh-Jones, 1999). Less frequent and certainly more dangerous are introductive events that affect animals living in fixed stables which contract anthrax by eating contaminated food (usually forages) coming from high risk areas. This can and does happen in areas normally deemed free of anthrax and commonly in winter when livestock need extra feed which will have been purchased and may be contaminated. Thus, despite a careful epidemiological analysis, this can lead health professionals to misdiagnose suspect cases and, consequently, the subsequent inappropriate management of infected carcasses that leads to an inevitable increase in the risk of infection in humans and other livestock (Kreidl et al., 2006).

1.2.9.1 Specimen required for laboratory diagnosis

When taking a sample from a dead animal suspected of anthrax one need to take precautions to prevent human infection, bacterial sporulation and a resulting environmental contamination. From live animals blood can be collected from the main superficial veins; while from dead animals it can be taken from the peripheral veins, such as in the ear after removal of the auricle with a hot knife; in this way the wound is cauterized and the spilling of blood and contamination of the floor by spores prevented. The blood can be either on a cotton swab or in a vacutainer; the former is better. When using a cotton swab the blood should be allowed to dry, killing the contaminants and encouraging any *B. anthracis* to sporulate. Putrefaction quickly destroys vegetative *B. anthracis*, which can therefore be difficult to isolate from the carcasses just 48 hours after death, especially in hot weather (Stein, 1947b). Traditionally ears are collected as

Dhaka University Institutional Repository

they are convenient and far from the intestinal tract but a better sample are nasal turbinates which are well vasculated and therefore should have plenty of spores but with minimal tissue that is only little affected by putrefaction. One can also take serous liquids from oedematous areas and soft organs but these should be placed in leak-proof containers. At low temperatures (5°C-10°C) it is possible to isolate the bacillus up to 4 weeks after death (Whitford, 1979). However, in reality the sample should be taken as soon as possible since decomposition leads to the rapid disintegration of the bacilli.

In the case of sheep or goats, one can send the entire carcass to the diagnostic laboratory in a leak proof container with a label indicating the suspected disease. This precaution must be taken even with single organs, such as the spleen or a collection of lymph nodes (in particular the prescapular node). When the carcass is too dehydrated, which can present diagnostic problems, one can collect soil from the ground under the animal that may have been contaminated by the leakage of blood and other body fluids from the natural openings and seepage. It should be noted that the longer an animal has been dead the smaller is the probability of getting a positive diagnosis, even with an experienced diagnostic laboratory. Samples should be placed in a plastic bag, double bagged and labeled appropriately and transported to the laboratory for examination (Fox et al., 1973).

1.2.9.2 Specimen shipment

Anthrax specimens must be transported in containers to contain any fluid and material from breakage of slides, syringes or blood tubes. Ice should be used if there is to be any likely delay in transportation. Specimens should be packed and labeled for transport in compliance with the carrier's conditions, government and postal regulations and International Air Transport Association regulations, as appropriate. The laboratory should be advised that suspect anthrax samples are being sent to them. The specimen advice form should be marked in large, clear letters that it is for suspect anthrax specimens. Sharps needles or blades must not under any circumstances be forwarded with the samples but must be disposed of in an appropriate container (Laforce, 1978).

Dhaka University Institutional Repository

1.2.9.3 Microscopic test

A preliminary examination with an unstained fresh blood smear will highlight the presence of rod like forms or typical "*bamboo canes*". The organisms are immobile and well capsulated. The slide may be fixed and stained with Gram stain when *B. anthracis* is coloured in violet. Preferably one can use Giemsa stain which stains the bacilli purple and the capsule a characteristic red mauve or with MacFadyean stain, which is blue methyl polychromatic and stains the capsule pink. Löffler uses methylene blue to which K2CO3 to 1% has been added (Turnbull, 1998). For *B. anthracis* this leads to the metachromatic phenomenon with the bacterial bodies stained blue while the capsule takes on a reddish colour. In the preparation of the slide one must take care to pass the slide several times over the flame because the usual methods of fixing colours do not inactivate the spores, which can represent a significant danger to the staff who will handle these microscopic preparations. Anecdotally are stories of students getting cutaneous lesions from handling sharp-edged broken blood smear slides that were decades old.

1.2.9.4 Blood smear rationale

In most species, at death from anthrax (the pig being a notable exception) the blood is usually teeming with the capsulated anthrax bacilli, provided the animal has not been treated. Numbers may also be lower in immunized animals that succumb to the disease (Turnbull et al., 1990). Published figures for terminal *B. anthracis* blood counts are (approximate cfu/ ml) mice 10^7, guinea-pigs 10^8–10^9, rats 10^4–10^7, sheep and goats 10^8, rhesus monkeys 10^4–10^9, chimpanzees 10^9, zebras 10^6–10^8, elephants 10^6–10^8, cheetah 10^8 (Lincoln et al., 1967; Turnbull et al., 1990; Friedlander et al., 1993; Lindeque and Turnbull, 1994; Fritz et al., 1995; Lindeque et al., 1996). These figures are relevant to understanding the value of the blood smear for diagnosis. Lincoln et al. (1967) found that it became very difficult to observe *B. anthracis* in blood when numbers were below about 5×10^4 per ml.

1.2.9.5 Cultural test

B. anthracis grows easily on normal agars, whether liquid or solid. Using a sterile loop the plates can be sown with material from samples of blood, exudates, oedematous infiltrations, organs or parts of them taken from infected or suspected animals. When one

suspects the presence of spores in the material used in the sample (wool, hair, leather, environmental samples) it is necessary to first incubate the material at 72°C for 30 minutes to destroy contaminating bacteria, yeasts and molds. It is always better to use a semi-selective medium to isolate the bacterium. Moreover, blood-containing media are preferable in comparison to the often-used PLET or a Knisly agar, such as TSPB agar, which is made highly selective against Gram- negative bacteria by supplementation with trimethoprim (13.1 mg/L), sulfamethoxazole (20 mg/L) and polymyxin B (30,000 IU/L) (Tomaso et al., 2006). The plates are then incubated at 37°C for 24 hours. If the bacterium is present in the material used, white colonies will develop, 2-5 mm in diameter, of a pasty consistency, and non-haemolytic. At a small magnification one can see long filaments folded several times on their own that seem to have the appearance of the foliage of a jellyfish, the so-called Medusa's Head.

The ground anthrax *Bacillus* refined identification (GABRI) method is more sensitive in revealing the presence of *B. anthracis* since it involves the use of a non-ionic detergent, such as Tween 20, which allows the separation of spores from soil particles by disrupting hydrophobic interactions with the solid matrices (Dragon and Rennie, 2001). In addition, the method provides the use of an antibiotic, fosfomycin that strongly reduces other microbial contaminants. Each sample is washed with washing buffer (sterile deionized water solution containing 0.5% of Tween 20) and incubated at 64°C for 20 minutes to eliminate vegetative forms of *B. anthracis*. After this incubation, 1 ml of supernatant is mixed with 9 ml of tryptose phosphate broth containing 125 µL/ml of fosfomycin. From this mix, 500 µL are seeded on trimethoprime-sulfamethoxazole-polymixin agar plates (TSMP) and incubated, aerobically at 37°C for 24 hours. Subsequently, suspected colonies (whitish and without hemolysis) are picked up and spread onto 5% blood agar and incubated at 37°C for 24 hours (Fasanella et al., 2013a)

1.2.9.6 Biological Test

It is usual with this kind of test to use particularly sensitive laboratory animals such as guinea pigs. The inoculation of suspect material subcutaneously or intramuscularly is not recommended, especially when the inoculate is full of secondary putrefactive bacteria. It is better to set up a test infection by coating the material on an area of abdominal skin which had been previously shaved and scarified. This technique takes advantage of the

Dhaka University Institutional Repository

ability of *B. anthracis* to penetrate scarified skin, selecting it from the mixed microbial flora. Rabbits die within 72 to 166 hours (Fasanella et al., 2009) and this depends on the virulence of the different strains of anthrax and the number of organisms. However, after a few hours a gelatinous, haemorrhagic oedema forms at the point of inoculation, which is then followed by all the other characteristics of an anthrax lesion.

1.2.9.7 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is the method of choice as a parallel diagnostic test, whether performed directly on clinical samples after non-selective enrichment of mixed cultures, or as a confirmation test for suspect colonies. Additionally, PCR has become necessary for clarification of negative cultural results of positive controls or when spiked agar plates are uninterpretable due to an overgrowth of *B. anthracis* by haemolytic *Bacillus spp*. To confirm suspicious colonies, the DNA template for PCR can be extracted by boiling resuspended colonies in TE buffer for ten minutes. To prepare DNA from a non-selective enrichment culture, a DNA Preparation Kit generally gives a better result. DNA can be prepared directly from spores by simple heating or autoclaving if very high numbers of spores ($>10^6$) are present. However, if only low numbers of spores are present or expected, as in the case of nose swabs or in secondary contamination scenarios, DNA preparation should be preceded by spore germination in culture. To identify virulent *B. anthracis* strains, and for the differentiation of non-virulent strains, the presence of both plasmid pXO1 (toxins) and pXO2 (capsule formation) must be confirmed. Some chromosomal targets of rpoB, S-layer protein genes and Ba813 very often lead to false-positive results from environmental sample (Papaparaskevas et al., 2004), while plcR is able to differentiate *B. anthracis* from *B. cereus* and *B. thuringiensis* (Easterday et al., 2005).

1.2.9.8 Molecular characterization

The full molecular characterization of *B. anthracis* was first completed with the Anthrax Genome Project, which in 2003 led to the publication of the genome of strain *B. anthracis* Ames type (Read et al., 2003). For the scientific community this was a milestone, so that today, in Genbank there are 14 assembly, 5 complete and 4 incomplete sequence projects of 19 strains of anthrax. The genome of *B. anthracis* is composed of

one chromosome with more than 5,000,000 bases and two plasmids, pXO1 with over 180,000 bases, and pXO2 with over 94,000 bases (Read et al., 2003).

B. anthracis represents one of the most genetically homogeneous bacteria, with more than 99% of homologous nucleotide sequences. Searches made before 2001 showed small molecular variations among strains of different origin. In fact the complete sequence of the gene PagA (Protective Antigen) and the MLST (Multiple Locus Sequencing Typing) analysis of housekeeping genes revealed a few mutations (Okinaka et al, 1999; Price, et al 1999). The revealed genetic crystallization is a direct consequence of its modus vivendi. In fact the vegetative form alternates with the spore form in an unpredictable frequency as a consequence of the regional ecology of the disease such that it may take years or even decades between infectious cycles. The evolutionary pressure is extremely low in the dormant (spore) stages of the cycle, remaining minimal during the active phase (vegetative form) because it is estimated that a maximum of 40 bacterial generations can follow (Keim et al., 2004). Consequently *B. anthracis* evolution is temporarily reduced as the number of generations in a year are few when compared with *Escherichia coli* (Gutmann et al., 1994) The precise molecular typing of strains of *B. anthracis* is crucial both for forensic analyses of biosecurity and bioterrorism events and for epidemiological investigations of natural outbreaks. The traditional methods are not very recommended for anthrax genotyping because they are unable to detect small genomic differences.

The genomic diversity is mainly the result of events during the evolution of the bacterium and genomic analysis must rely on molecular markers as polymorphic as possible with a high rate of mutation. The anthrax genotyping methods currently in use test different types of markers in relation to the utility of the analysis.

The genotyping method, considered to have a low resolution, is the analysis of single nucleotide polymorphisms (SNPs) and identifies point mutations in the genome. These markers have a good stability with a genomic mutation rate of 10^{-10}. While there is a low rate of mutation some 35,000 SNPs comprise the entire genome of anthrax (Read et al., 2002; Pearson et al., 2004). At present the opportunity to test all the identified SNPs in any isolate is technically difficult and financially expensive. However, some studies have

Dhaka University Institutional Repository

helped identify 12 canonical SNPs that are the most stable and homoplastic and can be used for phylogenetic investigations. Polymorphism analyses may be carried out using Snapshot or with real time PCR assays with TaqMan MGB probes (Van Ert et al., 2007a).

The high resolution typing assay par excellence is that of multiple locus VNTR analysis (MLVA) as it 481 seeks to identify specific genomic regions known as variable number tandem repeat (VNTR). These 482 regions of repeated DNA in tandem by their nature have a higher rate of mutation. The frequency of mutation of these markers in *B. anthracis* is comparable to 10^{-5} with a high variability depending on the locus (Keim et al., 2004). This technique initially with 8 VNTR was able to identify 89 genotypes among 400 isolates from around the world (Keim et al., 2000), while the 15 VNTR assay increased this to 221 genotypes among 1,033 isolates. The method has now been increased up to 25 loci (Lista et al., 2006), which allows an excellent discrimination. Technically, the VNTR are searched using capillary sequencers to analyze DNA fragments. Lately high resolution assays were reported that examine markers called SNRs (Single Nucleotide Repeats), a sort of VNTR consisting of repeated sequences of poliA. Stratilo et al. (2006) through a bioinformatics analysis identified specific regions with a mutation rate of 10^{-4}. Utilisation of these regions allows discrimination between organisms with the same MLVA pattern and thus allows sub-genotyping. The instability of these loci doesn’t make them homoplastic because back-mutations often occur. Their use can differentiate strains within the same outbreak or epidemic. A recent study has suggested the use of a panel of 4 SNR markers (Kenefic et al., 2008a). The described genotyping methods can be understood in a hierarchical way. The SNPs being at low-power of discrimination can be used for phylogenetic investigations. On the other hand, VNTRs and SNRs have high discriminatory powers. The first for its high diversity and homoplasia is able to correctly define the genotype, while the latter searching for any signs of redundancy is considered suitable for identifying sub-genotypes. All the methods described are best performed by specialized laboratories experienced in molecular biology (Keim et al., 2004).

1.2.9.9 Differential diagnosis

For differential diagnosis, other causes of sudden death should be considered. Among these are African horse sickness, botulism, blackleg (*Clostridium chauvoei*), peracute babesiosis, chemical poisoning (heavy metals, other poisons), ingestion of toxic plants, snake bite, lightning strike or metabolic disorders such as lactic acidosis, magnesium deficiency, bloat. An outbreak of Rift Valley Fever in Kenya in 1997 was initially thought to be anthrax (WHO, 1997). The differential diagnosis list will inevitably vary by species and geographical area, and the above list is not exhaustive.

1.2.9.10 Personnel protection

If anthrax is suspected, a jugular blood sample should be collected aseptically for culture. Producers should take every precaution to avoid skin contact with the potentially contaminated carcass and soil. Protective, impermeable clothing and equipment such as rubber gloves, rubber or leather apron, and rubber boots with no perforations should be used. No skin, especially which is compromised with wounds or scratches, should be exposed. Disposable personal protective equipment is preferable, but if not available, decontamination can be achieved by washing any exposed equipment in hot water and detergent. Disposable personal protective equipment should be burned and buried with the carcass. The *B. anthraces* organisms range from 0.5-5.0 µm in size. Veterinarians and producers working with anthrax suspects or confirmed cases should wear respiratory equipment capable of filtering this size of particle. A National Institute for Occupational Safety and Health (NIOSH), the United States federal agency and Mine Safety and Health Administration (MSHA), an agency of the United States Department of Labor, approved high efficiency respirator, such as a half-face disposable respirator with a high efficiency particulate air (HEPA) filter. This filter is recommended when conducting soil remediation and burial and when applying quicklime to soil (Lindeque and Turnball, 1994).

1.2.10 Anthrax molecular research in Bangladesh

Chakraborty et al. (2012) investigated 14 reported outbreaks of cutaneous anthrax in Bangladesh by a multidisciplinary collaborative team of epidemiologists, physicians, veterinarians, and anthropologists from the Institute of Epidemiology, Disease Control and Research (IEDCR), the Department of Livestock Services (DLS) of the Government

Dhaka University Institutional Repository

of Bangladesh, and International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B) during August 2009-October 2010. In the study, they identified 140 cases of animals and 273 cases of human cutaneous anthrax. Microbiologists of the Central Disease Investigation Laboratory (CDIL), Bangladesh identified gram-positive, chain forming, spore-containing, capsulated bacilli from smears of bone marrow of five slaughtered cattle by using Grams staining and polychrome methylene blue staining. The Center for Disease Control and Prevention (CDC), Atlanta, USA isolated *B. anthracis* from the ocular fluid of two dead goats and also detected the pathogen from bone marrow smears collected from a slaughtered cow and a disposed dead goat by M'Fadyean staining. Microbiologists of IEDCR identified blue-stained bacilli with squared ends by microscopy of vesicular smears by using Loeffler's polychrome methylene blue stain for 23 of the 28 suspected cutaneous anthrax cases. At CDC, *B. anthracis* was detected in three skin biopsy specimens by immunohistochemical analysis and from 10 vesicular swab smears by M'Fadyean staining. The bacterium was isolated at CDC from vesicular swabs of three cases, two from the first outbreak in 2009 and one from the outbreaks in August 2010. MLVA was performed for *B. anthracis* isolated from human and animal specimens at CDC. The MLVA-8 genotype of 2009 isolates confirmed that animals and humans were infected by isolates of the same genotype (Chakraborty et al., 2012)

Another investigation was conducted by Fasanella et al. (2013b) taking samples from eight farms in Sirajganj district where animal cases occurred in December 2010. Human anthrax cases were also registered there. Soil samples, about 50 g each were collected from suspected contaminated sites within each farm compound, such as where sick animals had been slaughtered and butchered or had been buried. Three turbinate bones from three skulls, available on one farm, were recovered, and individually bagged and sent to Istituto Zooprofilattico Sperimentale of Puglia and Basilicata (Italy) for further analysis. The GABRI method was used to recover *B. anthracis* organisms from the soil samples. To obtain higher genetic differentiation in very closely related isolates, VNTR loci were investigated, paired with SNRs loci that are molecular markers with extreme discriminatory power. 5'-fluorescent-labelled oligonucleotides, deprotected and desalted were utilized, specifically selected for the VNTRs and SNRs used. The 15 specific primer pairs for the MLVA (Van Ert et al., 2007a) and four specific primer pairs for

Dhaka University Institutional Repository

SNR reactions were selected (Kenefic et al., 2008b; Garofolo et al., 2010). Fasanella et al. (2013b) recovered *B. anthracis* from soil samples and turbinate bones on six farms. They isolated *B. anthracis* from 16/20 samples, for a total of 99 isolates. The MLVA on 15 VNTRs on the isolates showed the presence of three different novel genotypes, which they labelled Gt/Kha, Gt/ChU, and Gt/KamBel (Fasanella et al., 2012).

1.2.11 Anthrax vaccine for veterinary use

The veterinary vaccine used by Louis Pasteur in 1881 to immunise livestock was one of the first vaccines developed. It consisted of an attenuated strain of *B. anthracis* that resulted from repeated culture for 8 days at elevated temperatures (42-43^{0}C) (Smith, 2012). The culture used by Pasteur probably contained a mixture of bacilli with only the capsule-encoding plasmid pX02 and bacilli complete with both plasmids. Although the Pasteur vaccine strain showed reduced virulence compared to wild-type *B. anthracis*, variations in degree of protection and virulence between batches resulted with the occasional death of a vaccinated animal. The basis of the attenuation remained a mystery for about 100 years until Mikesell et al. (1983) showed that increasing the culture temperature to 42^{0}C results in the partial loss of the toxin-encoding plasmid pX01. Nowadays, live spores of the attenuated, non-encapsulated Sterne strain ($34F_2$) are most commonly used to immunise animals. This strain, prepared by Max Sterne in South Africa in the late 1930s (Sterne, 1939a), is a stable acapsulate mutant that produces all three toxin components of *B. anthracis* (PA, LF and EF), i.e. possesses the pX01 plasmid, but lacks the pX02 plasmid responsible for capsule formation. This live-spore vaccine has proved to be safe and very effective, inducing a profound antibody response against the PA of *B. anthracis*. A single dose provides immunity for only about 1 year, and repeated vaccinations are required for long-term protection. The Sterne vaccine is completely avirulent for most animal species, including humans, although residual virulence can be observed in goats and some laboratory animals. In addition to the Pasteur and Sterne vaccine strains, there are live-spore vaccines that carry both plasmids, yet are still attenuated, including the Italian Carbosap strain (Fasanella et al., 2001) and the Argentinean A strain (Cataldi et al., 2000). The reason for their attenuation remains to be clarified. Table 1.2.4 shows the MLVA result of Sterne strain.

Table 1.2.4 MLVA result of Sterne strain by using 8 VNTR loci

Strain	Laboratories	VNTRs observed bp								Reference
		Vrra	vrrb1	vrrb2	vrrc1	vrrc2	pXO1	pXO2	cg3	
Sterne strain	KCDC, Korea	314	229	162	583	532	129	-	153	Jung et al., 2012

1.2.11.1 Anthrax vaccine: mode of action

Protective antigen (PA) is a very important component of the anthrax toxin for the reason that this protein plays a major role in anthrax immunity after both immunization and infection (Shlyakhov et al., 1997). A number of antigens of *B. anthracis* have been studied for their ability to induce protective immunity against the disease. Of the known antigens including the capsule, S-layer, surface polysaccharides and other proteins, only those proteins, which together make up the anthrax toxin, cause detectable production of antibodies (Mizrahi, 1990; Uchida et al., 1993; Singh et al., 1998). Of the three proteins, EF, LF and PA, only PA elicits antibodies that are protective against the disease (Singh et al., 1989; Stevens et al., 1995). This immunity is thought to occur as a result of neutralizing the activity of the anthrax toxin (Ezzell and Abshire, 1988). Antibodies to PA will either block the protein from binding to host cell receptors or once bound will block the action of furin cleavage. Either situation renders PA biologically inactive. Without active PA bound to the cell, EF and LF cannot enter the cell. Thus, the anthrax toxin's influence on the host is halted (Singh et al., 1998). Therefore, since PA is the only antigen known to induce protective antibodies against anthrax, the protein has become the main focus of anthrax vaccine research (Ivins and Welkos, 1988; Turnbull, 1991). PA, when produced in the absence of LF and EF, has been shown to be capable of producing effective protection both as a purified protein and when used in a recombinant or attenuated vaccine (Turnbull, 1991). However, protection studies have shown that high antibody titers to PA do not correlate with level of protection (Turnbull et al., 1986; Ezzell and Abshire, 1988). In fact, the veterinary live spore vaccine produced from the Sterne strain of *B. anthracis*, gives better and more prolonged protection against infection by the bacterium than merely adjuvanted PA even though antibody levels induced are much lower (Sterne, 1937; Sterne, 1939b; Turnbull et al., 1986; Ezzell and Abshire, 1988; Turnbull, 1991; Shlyakhov et al., 1997). The knowledge that spore vaccines confer stronger, more reliable immunity to the disease seems to point to a role for cell-mediated immunity (CMI) in protection of the host (Turnbull et al., 1988;

Dhaka University Institutional Repository

Mizrahi 1990; Turnbull, 1991; Coulson et al., 1994, Pezard et al., 1995; Shlyakhov et al., 1997). Figure 1.2.6 represents the A-B model of anthrax toxin.

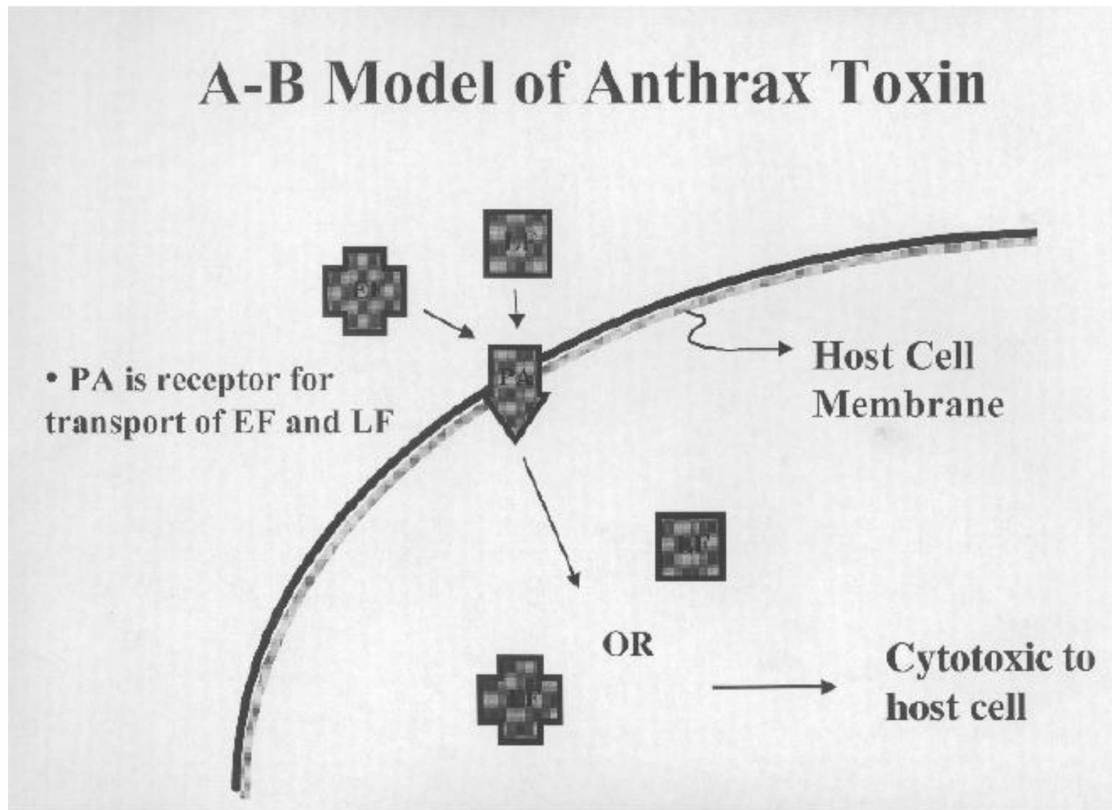

Figure 1.2.6: The A-B model of the anthrax toxin. The A-B model of anthrax consists of a B moiety and 2 A moieties. Seven PA molecules bind to one cell receptor then act as the effector for the binding and internalization of EF and LF. Since there are two A moieties, EF and LF bind competitively to PA (Prince, 2003).

1.2.11.2 Immune response after vaccination

An exposure to a germ after vaccination stimulates the memory B and memory T cells, which recognize the antigen from the germ and respond quickly and effectively to prevent disease. Activated memory B and memory T cells remember the antigen from the vaccine and respond faster and more efficiently against future infection by the same antigen. Plasma B cells produce a molecule called an antibody that recognizes and binds to the antigen on the germ or cells infected with the germ. Antibodies bind to antigens on the germ to prevent infection, as well as to the cells infected with the germ to mark them for killing. Memory T cells encounter and recognize the antigen displayed on the surface of the antigen-presenting cells that initiate the immune response against the germ. This

activates memory T cells. Activated killer T cells bind to and destroy any cells that contain the germ's antigen (Dragon et al., 1999). Vaccines help the body to acquire immunity against disease-causing pathogens and cancer cells. A vaccine contains a killed or weakened form or derivative of an infectious germ. The vaccine has little or no disease-causing ability, but its presence in the body still provokes an immune response. This activates various immune cells that learn from the vaccine to recognize and destroy the germ. A vaccine activates various immune cells because it contains part of the germ, called an antigen that stimulates the body's immune response. An antigen by itself or in a vaccine has little to no disease-causing ability. The first immune cells that encounter the vaccine are called antigen-presenting cells. Each antigen-presenting cell digests an antigen, then displays on its surface a small piece of antigen that can be recognized by T cells. When antigen-specific helper T cells encounter an antigen presenting cell, they become activated and send a chemical messenger to other immune cells e.g., B cells and killer T cells. The chemical messenger helps these immune cells become activated. Once stimulated by the antigen and the chemical message from the helper T cells, the B and killer T cells divide and transform into specialized immune cells that fight back against that specific antigen. Also, a small but important fraction of the B and T cells transform into memory cells that react quicker when they encounter the same antigen again (Dragon et al., 1999).

1.2.11.3 Anthrax vaccine and Bangladesh

In Bangladesh, vaccination is usually practiced to prevent and control anthrax. For this purpose, vaccine prepared from Sterne F-24 strain of *B. anthracis* by Livestock Research Institute (LRI), Mohakhali, Dhaka has long been used in this country. The master seed is F-24 strain of *B. anthracis,* originated from Australia. The dose (1×10^7 spores/mL) is 1 ml and 0.5 ml subcutaneously per cow and goat, respectively, per year and the vaccine can give protection for 6 months (Roy et al., 2013; Hassan et al., 2015). The total yearly anthrax vaccine production was 38.29 million doses (Ahmed et al., 2010). The Sterne strain of *B. anthracis* produces sublethal amounts of the toxin that induces formation of protective antibody. Anthrax vaccine/anthrax Spore Vaccine (ASV) is a glycerinated suspension of live spores of toxinogenic uncapsulated avirluent Sterne strain of *B. anthracis*. ASV can be used to protect all species of animals such as cattle, sheep, goat and elephant but has side effects in some sensitive species due to residual virulence

Dhaka University Institutional Repository

(Rajalakshmi et al., 2010). Immunization of cattle with anthrax vaccine generated high level of anti anthrax IgG antibody response at day 15 (0.396±0.31) of immunization and reached its peak at day 90 (1.551±0.46) and maintained that level up to the end of the study day 180 (1.549±0.78) (Fatema, 2011). This vaccine can provide immunity against anthrax disease for six months according to the instruction of LRI. Laboratory data for anthrax vaccine efficacy are insufficient (Fatema et al., 2011; Murshidul, 2012). Dipti et al. (2013) and Roy et al. (2013) conducted research on immunological response of local anthrax vaccine in livestock. Some factors have negative impact on the successful vaccination program for the prevention and control of anthrax in Bangladesh. An insufficient supply of anthrax vaccine, lack of staffing for vaccination, and an ineffective vaccine strategy that fails to target the highest risk areas, has left livestock susceptible to anthrax infection. Although the official price per dose of anthrax vaccine is low, a post outbreak ring-vaccination strategy is often implemented in high risk areas due to staffing shortages. However, the live-attenuated vaccine can only protect an animal effectively for 6-9 months; therefore, vaccinated cattle are susceptible again during the next year (Turnbull et al., 2004; Mongoh et al., 2008b).

1.2.11.4 Animal models for anthrax vaccine studies

While a vaccine intended for human use would ultimately have to prove safe and efficacious in non-human primates, it is difficult to endorse a single animal model for early evaluation of experimental vaccines. Mice and guinea pigs are generally more difficult to protect from fully virulent *B. anthracis* strains (Fellows et al., 2001). Mice can be used to evaluate toxin-based vaccines if a Sterne-type (pXO2-strain) challenge strain is used (Welkos et al., 1986), and other vaccines if a pXO1- challenge strain is used (Welkos, 1991). Fully virulent challenge strains are useful for evaluating vaccines containing PA in combination with other antigens and novel adjuvants (Chabot et al., 2004). The type of mouse used can be critical, as there is some variability among mouse lines. One study compared 10 types of mice and found all had low LD50s (50% lethal dose) upon subcutaneous inoculation with *B. anthracis* Vollum 1B (5-30 spores), and their mean time of death after challenge with 60 spores varied from 3 days for DBA2J mice to 6.5 days for C57BR/cdJ mice (Welkos et al., 1986). LD50s can vary significantly, however, with attenuated challenge strains. For example, some mice, such as A/J mice, are sensitive to Sterne (LD50 = 1.1×10^3 spores), while other mice, such as

Dhaka University Institutional Repository

C58/J, are resistant (Sterne LD50 > 10^7 spores) (Welkos et al., 1986). In laboratories for which *B. anthracis* is considered too dangerous, vaccines might be evaluated by their ability to protect against purified toxin challenge. Fischer 344 rats are often used, due to their remarkable sensitivity to LT (Beall et al., 1962; Haines et al., 1965). In one study, injecting 12 µg of PA plus 2.4 µg of LF (about 4 × LD50) caused rats to die in less than 2 h, while mice challenged with 4 × LD50 took up to 6 days to die (Welkos et al., 1986). It is intriguing that some animals, such as rats, are very sensitive to toxin but difficult to infect (Klein et al., 1962; Lincoln et al., 1967), while other animals, such as guinea pigs, are more toxin resistant but can be killed with relatively few spores (Keppie et al., 1963). Reasons for these discrepancies are unknown but may speak to differences in host germinants and innate immunity, as well as direct toxin sensitivity (Welkos et al., 1986; Uchida et al., 1993). *In vitro* methods for evaluating vaccines include enzymelinked immunosorbent assays (ELISAs) to measure antibody to toxins, capsule, killed spores and other antigens. Anti-LT response can also be evaluated using toxin neutralization assays (TNAs), in which mouse macrophages are protected from purified LT by antisera (Friedlander, 1986; Pitt et al., 2001; Reuveny et al., 2001). TNAs are generally regarded as the *in vitro* gold standard for evaluating PA-based vaccines, though one study found that TNA titers do not always correlate with protection (Chabot et al., 2004). In that study, immunization with cross linked rPA (recombinant PA) improved protection of mice against Ames challenge but resulted in reduced serum TNA titers. Measurements of cellular immunity are generally not used to evaluate vaccines, as the pathogenesis of anthrax is probably mostly due to extracellular bacilli. However, a thorough evaluation of a new vaccine might include a combination of *in vitro* tests that measure antibody responses, toxin neutralization, killing of bacteria and cellular immunity.

1.2.12 Vaccine immunology

1.2.12.1 Use of sonication for the preparation of whole cell extract

Sonication involves applying sound energy (usually ultrasound) in order to agitate particles in a sample. In the laboratory, it is usually applied using an *ultrasonic bath* or an *ultrasonic probe*. Sonication has various applications including degassing, homogenizing and emulsifying liquids. For biological applications ultrasonic homogenizers, also known as sonificators or sonicators, are mainly used to disrupt the

Dhaka University Institutional Repository

cell membranes and release the cellular contents. Cell disruption via sonication has been the method of choice for many proteomic investigations when working with cells that are difficult to lyse. Sonication has been successfully used to characterize the soluble and membrane fractions of *Mycobacterium leprae* (Coldham et al., 2004; Marques et al., 2004), *B. subtilis* (Antelmann et al., 2000) and *B. anthracis* resulting in the detection of several hundred bacterial proteins. In the case of the latter a full reference map was created containing a total of 534 identified proteins (Wang et al., 2005). In a different study, sonication was used to selectively extract the membrane fraction of *B. anthracis* which allowed identification of more than 80 protein spots including a large number of S-layer proteins (Chitlaru et al., 2004). Sonication has also been applied for the preparation of exosporium samples of the fully virulent *B. anthracis* Ames strain. The sonication was performed for 6-7 min causing partial fragmentation of the exosporium with disturbing the spores (Redmond et al., 2004).The prepared extracts were further characterized by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the protein bands identified using N-terminal sequencing or mass spectrometry (MS) which revealed several spore coat proteins. Sonication has undoubtedly proven very useful for the preparation of protein extracts as it can achieve a high percentage of cell lysis and very good protein yields even from Gram-positive microorganisms (Redmond et al., 2004; Wang et al., 2005)

1.2.12.2 Protection of protein from proteases

The rupture of the cell structures provokes the liberation of various proteases which begin to exert their action on the extracted proteins. This is less of an issue with bacterial cells, in comparison with eukaryotic cells, due to the absence of cellular organelles such as the lysosomes. However, bacteria do contain proteases and the addition of protease inhibitors is recommended in order to keep the integrity of the extracted proteins. If no preventive measures are taken common effects of proteolysis are the presence of artifactual spots and loss of high molecular weight proteins (Gorg et al., 2004).

Some proteases are resistant to denaturation, although solubilising the proteins in a strong denaturing agent may prevent their action. It has been demonstrated that while active in a high concentration of urea the proteases are inhibited by the addition of thiourea to the extraction buffer (Castellanos-Serra and Paz-Lago, 2002). These are also

Dhaka University Institutional Repository

less active at lower temperatures so keeping the samples cool throughout the extraction process is advisable. In addition, proteolysis can often be inhibited by preparing the sample in Tris base, basic carrier ampholytes or by precipitation in trichloroacetic acid (TCA) or TCA/acetone (Canas et al., 2007).

Protease inhibitors are usually added when preparing protein extracts from bacteria. Commonly used inhibitors include phenylmethylsulfonyl fluoride (PMSF), aminoethyl benzylsulfonyl fluoride (AEBSF), EDTA, pepstatin, aprotinin, benzamidine and leupeptin. Microbial proteases are predominantly extracellular and can be classified into four groups based on the catalytic residue of their active site. The four groups are: serine proteases, cysteine proteases, aspartate proteases and metalloproteases. However, aspartate proteases are rare in bacteria and to date none have been reported in pathogenic microorganisms. Metalloproteases, on the other hand, seem to be a common feature in most bacterial pathogens (Firdaus et al., 2005).

1.2.12.3 Determination of protein concentration

The protein concentrations of the samples to be loaded on a gel need to be determined. Quantification for total protein can be achieved by measuring samples at 280 nm on a spectrophotometer, but the buffer must not contain absorbing materials. When the buffer contains absorbing materials, the Bradford assay (Bradford, 1976) can be used where a standard curve is created to determine unknown sample concentrations.

1.2.12.4 Detection of protein by Western blot

The Western (note that in this context ‘‘Western’’ should be spelt with a lower-case ‘‘w’’) blot is commonly used to identify, quantify, and determine the size of specific proteins. Western blotting evolved from Southern blotting, which is used to detect specific DNA sequences among DNA fragments separated by gel electrophoresis, and northern blotting, which is used to detect and quantify RNA and to determine its size, and also involves gel electrophoresis to separate RNA. In the late 1970s, Towbin et al. (1979) enabled proteins to be electrophoretically separated using polyacrylamide-urea gels and transferred onto a nitrocellulose membrane. Burnette (1981) later employed the more widely used sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), which eventually led to this method being termed Western blotting. It is also called protein

blotting or immunoblotting and has rapidly become a powerful tool for studying proteins. Basically, gel electrophoresis is used to separate native or denatured proteins. The proteins are then transferred to a membrane for detection using antibodies specific to the target protein. The technique has continued to evolve, and there are many reports on troubleshooting and improving the technique (Kurien and Scofield, 2009).

1.2.12.4.1 Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) describes a technique widely used in biochemistry, forensics, genetics, molecular biology and biotechnology to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility. Mobility is a function of the length, conformation and charge of the molecule. For proteins, sodium dodecyl sulfate (SDS) is an anionic detergent applied to protein samples to linearize proteins and to impart a negative charge to linearized proteins. This procedure is called SDS-PAGE. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis. Proteins that have a greater hydrophobic content, for instance many membrane proteins, and those that interact with surfactants in their native environment, are intrinsically harder to treat accurately using this method, due to the greater variability in the ratio of bound SDS (Rath et al., 2009).

For optimal separation, it is important to determine the ideal bisacrylamide: acrylamide ratio prior to electrophoresis. Proteins are then separated using gel electrophoresis. Proteins can be separated by isoelectric point, molecular weight, electric charge, or a combination of these. The most common type of electrophoresis uses polyacrylamide gels and buffers loaded with SDS. The procedure of SDS-PAGE is based on the characteristics of SDS, which is a strongly anionic detergent. Because proteins do not all have the same electrical charge, the mixture is treated with SDS, and thus the proteins become denatured and negatively charged. As a result, this allows separation of proteins by molecular weight. Treatment with a reducing agent to remove disulfide bonds and boiling of samples can facilitate denaturing. When voltage is applied to the gel, proteins migrate at different speeds, and these different rates result in separation into bands within each lane. A two dimensional gel can also be used. This type of gel spreads out proteins

Dhaka University Institutional Repository

from a sample in two dimensions (O'Farrell, 1975). Proteins are separated by isoelectric point in the first dimension and by molecular weight in the second dimension.

1.2.12.4.2 Transfer of proteins

Once electrophoresis is complete, the separated proteins can be transferred from within the gel onto a membrane (a Western blot) made of nitrocellulose, polyvinylidene difluoride, activated paper, or activatednylon (Towbin et al., 1979; Kurien and Scofield, 2006). Nitrocellulose is the most commonly used membrane. Electroblotting is the most popular procedure for transferring proteins from a gel to a membrane. Its main advantages are speed and completeness of transfer. This process uses an electric current to pull proteins from the gel onto the membrane. It can be achieved by immersion of a gel-membrane sandwich (wet transfer) or by putting the gel-membrane sandwich between absorbent papers that has been soaked in transfer buffer (semidry transfer). The effectiveness of protein transfer is dependent on the type of gel used, the molecular mass of the protein, and the type of membrane. Some limitations associated with protein transfer include a lower molecular weight limit of 10 kDa, the use of specialized transfer buffers (e.g., 3-(cyclohexylamino)-L-propanesulfonicacid) to facilitate transfer of proteins with a high isolelectric point, and problems associated with using a transfer buffer with a lower pH than the protein's isoelectricpoint (i.e., the protein will run backward).

1.2.12.4.3 Blocking and antibodies

It is important to prevent interactions between the membrane and the antibody chosen to detect the target protein. To block nonspecific binding, the membrane is placed in a dilute solution of protein such as bovine serum albumin and nonfat dry milk. Researchers should ensure that the blocking buffer that is appropriate for the specific antiserum is also appropriate for the type of membrane. Blocking helps mask any potential nonspecific binding sites on the membrane, thus reducing background ‘‘noise’’ in the final product of the Western blot, eliminating false positives and providing a clear result. After blocking, the most popular method is to incubate the membrane with primary antibody, wash, reblock, and then incubate with secondary antibody and wash again. It is important to determine the optimal concentration of antibodies before running all the samples as optimization is a prime determinant of the sensitivity of the assay (Burnette,

1981). The antibody concentration should be optimized to provide the best signal to noise ratio. Both monoclonal and polyclonal antibodies can be used for Western analyses, with advantages and disadvantages in using either type (MacPhee, 2010).

1.2.12.4.4 Detection

The probes that are labeled and bound to the protein of interest need to be detected on the Western blot. For detection methods, colorimetric, radioactive, and fluorescent methods can be used (Hawkes et al., 1982; Blake et al., 1984; Kurien and Scofield, 2003). The most common detection methods use secondary antibodies conjugated to alkaline phosphatase (AP) or horseradish peroxidase (HRP). In these methods, when the enzyme substrate is added, either a colored precipitate is deposited on the blot (colorimetric detection) or a chemiluminescent or fluorescent product is formed and the light signal is captured on film or with a digital imaging system. Secondary antibodies conjugated to fluorophores are gaining popularity and can be directly visualized and captured with a compatible imager, without the need for additional liquid substrate. Mechanisms of detection chemistries of Western blot are shown in Figure 1.2.7.

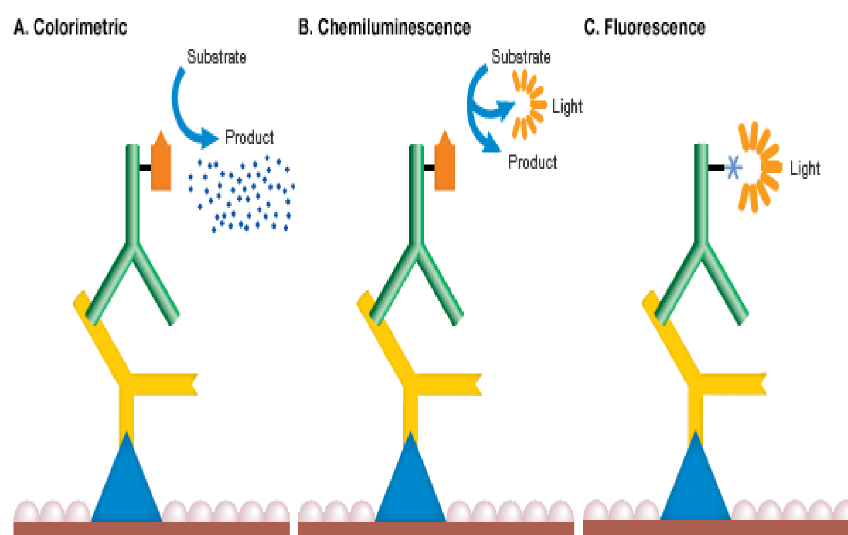

Figure 1.2.7: Mechanism of detection chemistries. In each method of Western blot detection, a detectable signal is generated following binding of an antibody specific for the protein of interest. In colorimetric (A), the signal is a colored precipitate. In chemiluminescence (B), the reaction itself emits light. In fluorescence detection (C), the antibody is labeled with a fluorophore. (Source: http://www.bio-rad.com/en-bd/applications-technologies/detection-methods).

Dhaka University Institutional Repository

However, chemiluminescent detection is used most often. Enhanced chemiluminescence (ECL) is a sensitive method and can be used for relative quantitation of the protein of interest (Kurien and Scofield, 2006; MacPhee, 2010). The primary antibody binds to the protein of interest and the secondary antibody, usually linked to horseradish peroxidase, is used to cleave a chemiluminescent agent. The reaction product produces luminescence, which is related to the amount of protein. Only a single light detector is required, and the light is detected by photographic film or by a charged-couple device camera (more sensitive, greater resolution, and a larger range of exposures than film). It is helpful that many manufacturers produce a variety of ECL-based Western blot detection kits according to the researchers' specific needs. Once exposures have been captured, blots can be washed in a buffer and then ''stripped,'' which involves removing bound antisera to enable reuse of the blot. Blots can then be stored for future reprobing several more times. However, subsequent reprobing can interfere with protein antigens, resulting in a decreased signal (Alegria-Schaffer et al., 2009).

Dhaka University Institutional Repository

1.3 Objectives of the study

The globally distributed deadly zoonotic disease anthrax has a long history in Bangladesh. Until 2009, the disease was reportedly considered as sporadic in bovine animals with few human cases in this country. In 2009-2010, anthrax has dramatically evolved with explosive outbreaks and zoonotic consequences in 12 districts that continued in the subsequent years. Because of economic and public health view points, this sudden outbreak concerned almost all communities in Bangladesh, justifying declaration of the "red alert" then by the government. This unusual urgency also challenged scientific communities to study the disease from different angles. Despite such emergency only a few studies have been conducted so far in relation to epidemiology of the disease and molecular characterizations of the circulating strains of the causative pathogen. Therefore, to illustrate a better picture on the issues mentioned the specific aims of this study were to:

1) - understand the risk factors/practices associated with occurrence of bovine anthrax in Bangladesh.
2) - explore the genotypes of *B. anthracis* circulating in the country along with their probable sources of origin.
3) - assess the genetic relationships of the strains of *B. anthracis* isolated from different outbreak areas.
4) - investigate phylogenetic relationship of *B. anthracis* strains isolated from Bangladesh and some central European countries.
5) - evaluate the immunogenicity of the vaccine strain being used to produce anthrax vaccine in Bangladesh for mass vaccination.

Dhaka University Institutional Repository

Materials and Methods

Dhaka University Institutional Repository

2.0 Materials and Methods

2.1 Case-control study

2.1.1 Geography and cattle population statistics of Bangladesh

Bangladesh lies in the north eastern part of South Asia between 20° 34' and 26° 38' north latitude and 88° 01' and 92° 41' east longitude. The country is divided into eight administrative divisions with 64 districts and 489 sub-districts (upazilas) (an upazila is a lower administrative unit in Bangladesh) (http://www.bangladesh.gov.bd/site/page/812d94a8-0376-4579-a8f1-a1f66fa5df5d). It has the highest density of livestock (cattle, goats, sheep and buffaloes) in the world with an estimated 145 large ruminants per square kilometer of land compared with 90 in India and 20 in Brazil (BARC, 2010). The total cattle and buffalo population of Bangladesh is about 26.22 million (BBS, 2013); most of them are reared by villagers in smallholdings.

2.1.2 Selection of case farm

We verified any reports/rumors on anthrax affecting humans and/or animals intermittently published from the Institute of Epidemiology, Disease Control and Research (IEDCR), the Department of Livestock Services (DLS), Bangladesh, National daily Newspapers and an electronic based surveillance for infectious diseases called "Program for Monitoring Emerging Diseases"-mail (ProMED-mail; http://www.promedmail.org/) from October 2010 through December 2014, and physically visited the reported outbreak sites. Additionally, while visiting an anthrax suspected smallholding in an area following a report mentioned above, any other smallholding(s) having a history of sudden death of ≥1 bovine animals in the recent past with the onset of convulsions or falling down with or without previous reported fever were also visited and collected samples, such as remnants of organs and blood/carcass contaminated soils. Of the farms visited a case farm was enrolled in the study based on any of the following three criteria:

(1) At least one bovine animal from the farm/smallholding died with sudden onset of convulsions or falling down, with or without previous reported fever (seen in the anthrax outbreaks of 2009) (ICDDR,B, 2009) and a large number of

Dhaka University Institutional Repository

characteristic organisms (McFadyean reaction) were revealed in the blood films of the moribund or recently dead animals stained with 1% polychrome methylene blue according to the World Organization for Animal Health (OIE, 2008a). Examination of blood films was performed at the nearby Field Disease Investigation Laboratory (FDIL) or at the Central Disease Investigation Laboratory (CDIL) in Dhaka (in Bangladesh one CDIL and eight FDILs provide livestock disease diagnostic services). Information confirming such examination was verified by interviewing the technical staff working at the FDIL or CDIL and the veterinarian involved with the case management.

(2) A cluster of human anthrax cases that had a common history of being exposed to the slaughtering and butchering associated practice of a sick bovine animal belonging to a smallholding and one or more typical black dermal eschars appeared on the skin of the persons in <3 weeks of such exposures. Such a cluster included all the anthrax cases in the community and the smallholder's family, because the sick animal was slaughtered at the homestead of the smallholder to sell meat to neighbors. Slaughter was performed by the Halal method (Gracey et al., 1999). Practices associated with slaughtering and butchering included skinning a carcass and processing and cooking meat.

(3) Any remnant of organ and/or blood/carcass contaminated soil sample collected from a farm yard was tested positive for *B. anthracis* by GABRI (Ground Anthrax Bacillus Refined Isolation) method (Fasanella et al., 2013a; Rume et al., 2016). And if the isolates resulted from initial screening by GABRI were found positive for capC (pXO2), pagA (pXO1) and Dhp61 (BA_5345) by real-time polymerase chain reactions, as described previously (Turnbull, 2008; Antwerpen et al., 2008). GABRI method is more sensitive in revealing the presence of *B. anthracis* because it involves the use of a non-ionic detergent, such as Tween 20 which allows the separation of spores from soil particles by disrupting hydrophobic interactions with the solid matrices. Real-time PCR assays were conducted using either LightCycler 1.5, LightCycler 480 II (Roche) or the Stratagene MX 3000P (Agilent Technologies).

In total, 43 case farms were finally selected for this study. Here, Table 2.1.1 represents the list of the districts, thanas and villages from where the data were collected. Figure 2.1.1 shows the places of Bangladesh from where the data were collected for case-control study. Figure 2.1.2 indicates the common practices of carcass disposal in the outbreak areas.

2.1.3 Selection of control farms

For each case farm, a control farm was selected within the same sub-district but located quite distantly from the case farm. A control farm was matched with a case farm in terms of similarity in animal rearing and herd size, but a history of freedom for any febrile disease affecting its animals over the last six months from the date on clinical onset of anthrax in the case farm, ensured by interviewing the owner of the control farm.

2.1.4 Data collection

The data from each case and control farm were collected by a structured questionnaire consisting of 46 binomial variables. For that the farm owners of the case and control farms were interviewed face-to-face. Population statistics of the case and control farms were noted, and Global Positioning System (GPS) coordinates of the farms were recorded using personal navigators (eTrex Venture, Garmin, USA). A geographical information system programme (Arc GIS 10.2.2; Environmental System Research Institute, USA) was used.

2.1.5 Statistical analysis

The data were entered into a spreadsheet programme (Excel 2000, Microsoft) (the spreadsheet is available on request). To estimate the strength and statistical significance of association between a risk factor and the disease, we applied the matched-pair (McNemar) test using GraphPad Software Quick Calcs (http:// www.graphpad.com/quickcalcs/McNemar1.cfm). An association was considered significant if a test had $p \leq 0.05$. The data from the spreadsheet were transferred to Stata 11 (StataSE) (Stata Corporation, USA) and in order to examine the independence of effects, multivariable conditional logistic regression was applied, using ‘clogit’ syntax. Any variables with $p < 0.20$ after McNemar test were included in the multivariable

Dhaka University Institutional Repository

conditional logistic regression analysis. The model of risk factors was constructed by backward selection applying the iterative maximum likelihood estimation procedure.

Dhaka University Institutional Repository

Table 2.1.1 List of the districts, thana and villages from where the data were collected

Sl. No.	Case farms				Control Farms			
	District	**Thana**	**Village**	**No. of Case**	**District**	**Thana**	**Village**	**No. of control**
1	Chittagong	Chandgaon	Chandgaon mohor	1	Chittagong	Chandgaon	Chandgaon mohor	1
2	Tangail	Sadar	Katuli	3	Tangail	Sadar	Chowbaria	1
							Rehaiminai	1
							Kharjana	1
		Modhupur	Ambaria	2		Modhupur	Ambaria	2
		Dhonbari-	North Norilla	3		Dhonbari-	North Norilla	2
			Sandalpur	2			Islampur	2
							Jomserpur	1
3	Pabna	Bera	South Bongram	2	Pabna	Bera	Vitapara	2
4	Sirajganj	Kamarkhand	Dhopakandi	6	Sirajgonj	Kamarkhand	Dhopakandi	1
			kazipara	1			Alokdia	2
							Noya chala	1
							Haidarpur	2
							Thakurjipara	1
		Sahajadpur	Ganga Prasad	1		Sahajadpur	Gara doho	6
			Binnadae	1			Tetierkanda	2
			Nukali	1			Daya	1
			Char Koujuri	2			Choto moharajpur	1
			Chithulia	5			Binotia	1
			Jugnidoho	2			Jothpar	1
			Tetierkanda	1			Jamirta	1
			Binotia	1			Nogordala	1
			Jothpar	1			Sarishakoil	1
			Jamirta	1			Chithulia	1
		Solonga	Raghunathpur	1		Solonga	Bhuyagati	1
		Belkuchi	Adachaki	3		Belkuchi	Adachaki,	1
							Somserpur	2
		Ullapara	Samolipara	1		Ullapara	Nobogram	1
			Nandigoti	2			Sonatola	2
Total				43				43

Dhaka University Institutional Repository

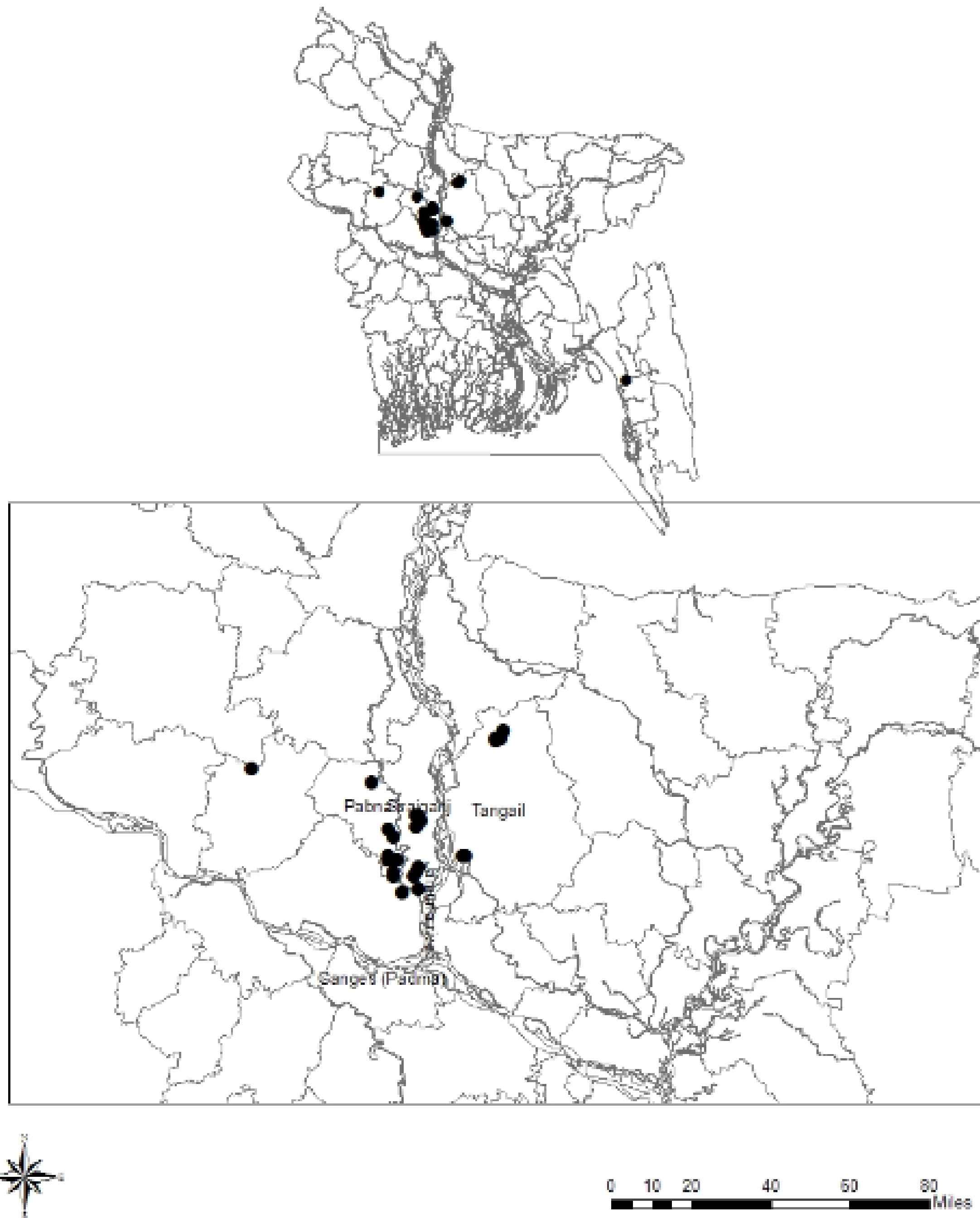

Figure 2.1.1: Geographical distributions of anthrax-affected cattle smallholdings observed in Bangladesh in October 2010 - December 2015 that were enrolled in the case control study as the case farms; A closer view (at bottom) showing 41 of the 43 cases located in three adjoining districts: Pabna, Sirajganj and Tangial lying at the converging area of two rivers, Padma (Ganges) and Jumuna (western (main) branch of Brahmaputra)).

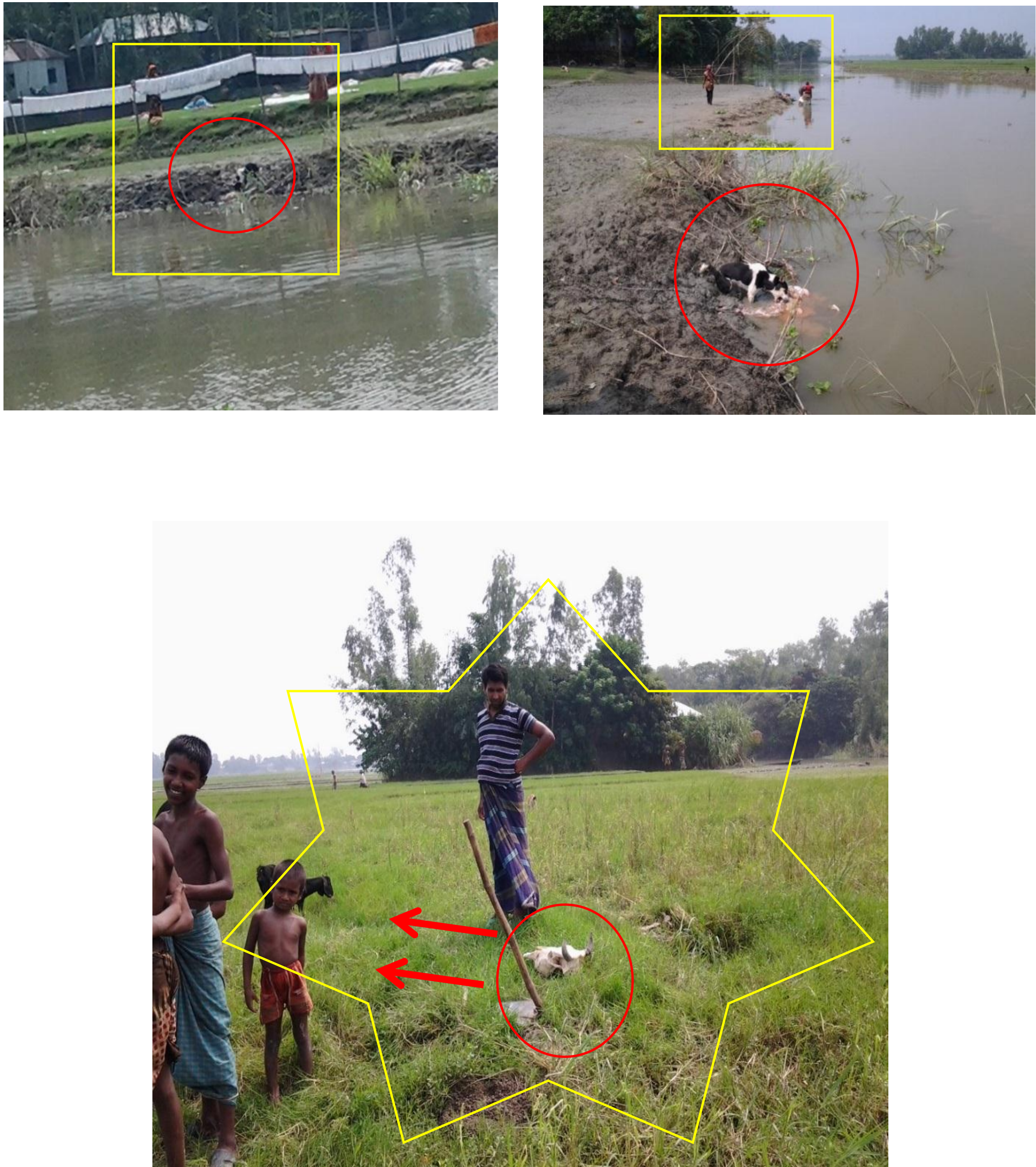

Figure 2.1.2: Common practices of carcass disposal in the outbreak areas. (Red circle indicates the place of disposed dead animal, yellow marking indicates the possible disease distribution ways and the red arrow indicates the possible anthrax transmission cycle).

Dhaka University Institutional Repository

2.2 Molecular characterization of *B. anthracis*

2.2.1 Molecular characterization of *B. anthracis* by using 15 VNTR loci

2.2.1.1 Sample collection

A total of 169 samples collected from 12 different districts of Bangladesh: Pabna, Sirajganj, Rajshahi, Comilla, Thakurgaon, Barisal, Chittagong, Dhaka, Gazipur, Kishoreganj, Mymensingh, andTangail (Figure 2.2.1), were sent to Istituto Zooprofilattico Sperimentale of Puglia and Basilicata, Foggia, Italy. To detect the association between anthrax transmission and imported cattle feed (bone meal), 91 bone meal samples were collected from the cattle feed importer or wholesaler agents from 17 Upazila/Thanas of 10 districts of Bangladesh (Table 2.2.1). On the other hand, several outbreak reports reveal that the disease is most prevalent in Sirajganj and nearby districts like Tangail and Pabna (Ahmed et al., 2010; Biswas et al., 2012). Therefore, a total of 73 soil samples were collected from 9 Upazila/Thanas of three districts (Sirajganj, Tangail and Pabna) and randomly picked soil from home stead or burial sites with or without having the history of anthrax cases (Table 2.2.1). Figure 2.2.2 and Figure 2.2.3 represents the sampling places and storage of samples, respectively.

The sampling was carried out in the period from 2012 to 2015. The study protocol was reviewed and approved by the faculty of Biological Science, University of Dhaka (DU), Bangladesh. This study was carried out in strict accordance with the recommendations found in the guide for the use of tissue samples of the DU. The tissue and bone samples were collected from the open fields where the owner threw their dead animal (Figure 2.2.2). The field team obtained informed verbal consent from respondents. No specific permissions were required for the locations from where the environmental samples (bone meal, soil), tissue samples and bone samples of dead animals were collected. Moreover, none of the specimen collections involved either endangered or protected species. No samples were collected from the live animal. Thus, no permits were required from the farm owner. Only verbal consents from farm owners were taken for the collection of soil from the burial sites. Sampling methods were supported by Anthrax in Humans and Animals- 4th edition, as published by the WHO (2008), OIE (2008a) and FAO (2001).

Dhaka University Institutional Repository

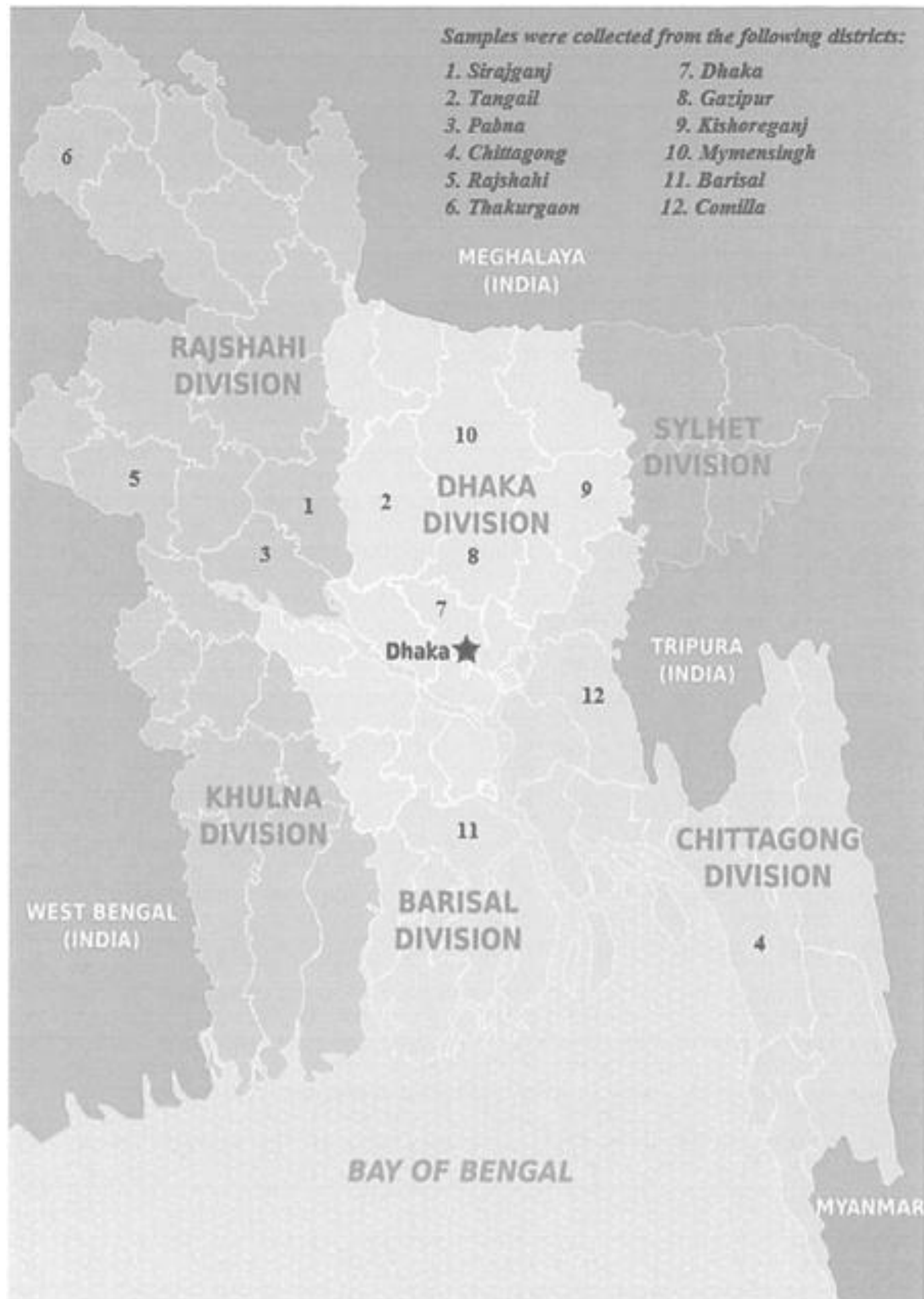

Figure 2.2.1: Map of Bangladesh indicating the districts from where the field samples were collected for molecular study by using 15 VNTR loci.

Table 2.2.1 Data on samples and sampling areas

Sl. No./Districts	Upazilla/Thana (GPS coordinates)	Village	Soil	Bone	Tissue	Bone meal
1. Sirajganj	Kamarkhanda (24.3667° N to 89.7042° E)	Dhopakandi	6	-	-	-
		kazipura	1	-	-	-
		Alokdia	1	-	-	-
		Noya chala	1	-	-	-
		Haidarpur	1	-	-	-
		Thakurjipara	1	-	-	-
	Sahajadpur (24.17° N to 89.5883° E)	Gara doho	2	-	-	-
		Binnadae	2	2	1	-
		Nukali	1	-	-	-
		Daya	1	-	-	-
		Char Koujuri	2	1	-	-
		Chithulia	5	-	-	-
		Binotia	4	-	-	-
		Pathaliapara	3	-	-	-
		Mosipur	1	-	-	-
		Jothpar	4	-	-	-
		Jugnidoho	1	-	-	-
		Jamirta	2	-	-	-
		Nogordala	1	-	-	-
		Sarishakoil	1	-	-	-
	Raigonj Upazila, Salanga Union (24.4133°N to 89.5° E)	Raghunathpur	1	-	-	-
		Bhuyagati	1	-	-	-
	Belkuchi (24.2833° N to 89.7166° E)	Adachaki	4	-	-	-
		Somserpur	2	-	-	-
	Ullapara (24.3166° N to 89.5666° E)	Samolipara	1	-	-	-
		Nandigoti	2	-	-	-
		Nobogram	1	-	-	-
		Sonatola	2	-	-	-
2. Tangail	Tangail Sadar (24.2500° N to 89.9167° E)	Katuli	2	-	-	-
	Madhupur (23.9833° N to 89.91666° E)	Ambaria	4	-	-	-
	Dhanbari (24.6166° N to 90.025° E)	North Norilla	5	1	-	-
		Sandalpur	2	-	-	-
		Islampur	2	-	-	-
		Jomserpur	1	-	-	-
3. Pabna	Bera (24.0667° N to 89.6250° E)	South Bongram	2	-	-	-
4. Chittagong	Kotowali (22.3375° N 91.8389° E)	-	-	-	-	32
	Double Mooring (22.3375° N 91.8083° E)	-	-	-	-	4
	Pahartali (22.3667° N to 91.7750° E)	-	-	-	-	2
	Sitakunda (22.3667° N to 91.7750° E)	-	-	-	-	22
5. Rajshahi	Ghoramara (23.9666° N to 89.05° E)	-	-	-	-	2
	Godagari (24.4666° N to 88.3333° E)	-	-	-	-	1
6. Thakurgaon	Pirganj (25.8666° N to 88.3666° E)	-	-	-	-	1
7. Dhaka	Savar (23.8441° N to 90.2511° E)	-	-	-	-	7
	Mirpur (23.5666° N to 90.6166° E)	-	-	-	-	2
	Tejgaon (23.7666° N to 90.4° E)	-	-	-	-	8
8. Gazipur	Gazipur Sadar, Kashimpur union (23.9833° N to 90.3166° E)	-	-	-	-	2
	Kapasia (24.4166° N to 90.0333° E)	-	-	-	-	2
	Sreepur (24.2000° N 90.4667° E)	-	-	-	-	1
9. Kishoreganj	Bajitpur (24.2166° N to 90.95° E)	-	-	-	-	2
10. Mymensingh	Mymensingh Sadar (24.6333° N to 90.1833° E)	-	-	-	-	1
11. Barisal	Barisal Sadar (22.7000° N 90.3667° E)	-	-	-	-	2
12. Comilla	Comilla Sadar (23.4666° N to 91.1833° E)	-	-	-	-	2
Total			**73**	**4**	**1**	**91**
		169				

Dhaka University Institutional Repository

Figure 2.2.2: Collection of samples from the field sites.

Dhaka University Institutional Repository

This consent procedure was approved by the faculty of Biological Science, University of Dhaka, Bangladesh. The whole shipping-process was in accordance with International Bio-Security rules from NC State University in compliance with the US Department of Transportation (DOT) and the International Air Transporters Association (IATA). The biological materials of this study belonged to "Category B infectious substances" that have the proper shipping name "Biological Substance, Category B" and the identification number UN 3373. The samples were properly tripled packaged and compliant with IATA packing instruction.

Dhaka University Institutional Repository

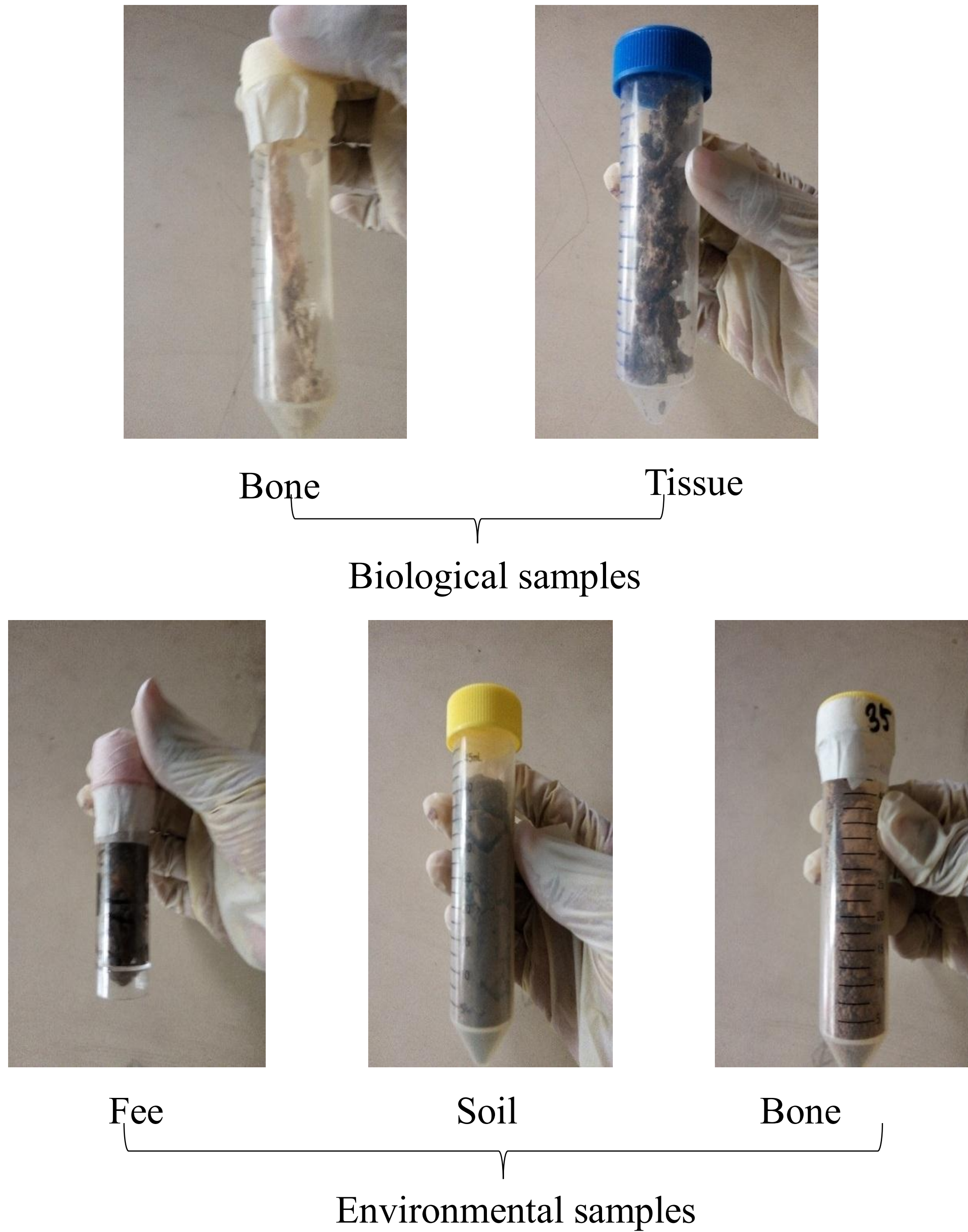

Figure 2.2.3: Sample processing and preserving according to the categories.

2.2.1.2 Isolation of *B. anthracis*

To detect *B. anthracis* from the samples, the Ground Anthrax Bacillus Refined Isolation (GABRI) method was used (Fasanella et al., 2013a), which had been developed in the Istituto Zooprofilattico Sperimentale of Puglia and Basilicata, Italian Reference Centre of anthrax. This test is able to isolate *B. anthracis* in high contaminated soil. The GABRI method is more sensitive in revealing the presence of *B. anthracis* since it involves the use of a non-ionic detergent, such as Tween 20, which allows the separation of spores from soil particles by disrupting hydrophobic interactions with the solid matrices. In addition, the method provides the use of an antibiotic, Fosfomycin that strongly reduces other microbial contaminants. Each sample has been washed with Washing Buffer (sterile deionized water solution containing 0.5% of Tween 20) and incubated at 64°C for 20 minutes to eliminate vegetative forms of *B. anthracis*. Figure 2.2.4 shows the steps of isolation of *B. anthracis* by GABRI method.

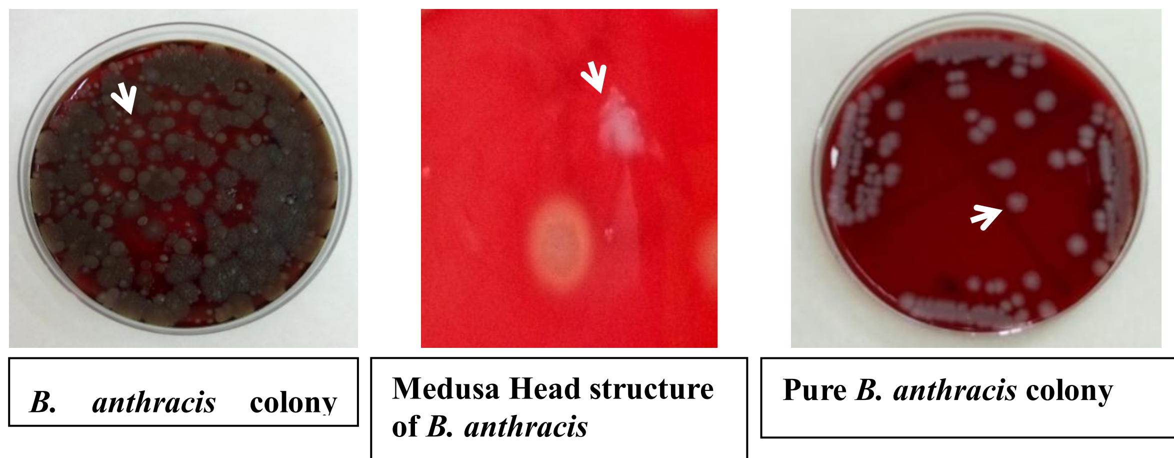

Figure 2.2.4: Steps of isolation of *B. anthracis* by Ground Anthrax Bacillus Refined Isolation (GABRI) method.

After this incubation, 1 ml of supernatant was mixed with 9 ml of Tryptose Phosphate Broth containing 125 µL/ml of Fosfomycin. From this mix, 500 µL was seeded on Trimethoprime-Sulfamethoxazole-Polymixine agar plates (TSMP) and incubated, aerobically, at 37°C for 24 hours. Subsequently, suspected colonies (whitish and without hemolysis) were picked and spread onto 5% blood agar and incubated at 37°C for 24 hours. This protocol was used for the processing of all samples received. All tests were run in the Institute's BL-3 Security Laboratory.

Dhaka University Institutional Repository

2.2.1.3 DNA preparation and Real Time PCR assay

At the same time, a few suspect colonies were scraped off the plate and transferred into 1.5 ml reaction tubes filled with 500 µL of sterile water and subjected to DNA extraction with heat through the use of a thermoblock (98°C for 20 minutes), followed by centrifugation at 12851 relative centrifugal force (rcf) for 10 minutes at 4°C. Specific PCR assays were used to confirm *B. anthracis*. The primers used for PCR amplification are listed in Table 2.2.2. The method is based on the amplification of DNA specific sequences through the use of three pairs of specific primers (Fasanella et al., 2001):

1) The R1/R2 primers are specific for the BA813 gene, located on the chromosome of *B. anthracis*;
2) PAG 23/24 primers are specific for the protective antigen (PA), located on the pXO1 virulence plasmid;
3) CAP 57/58 primers are specific for the capsule, plasmid pXO2.

The amplification was performed using the CFX Connect Real Time PCR Detection System. The Melting curve was determined by increments of 0.5°C starting from a temperature of 65°C to 95°C and was analyzed by CFX Manager^{TM} Software, Version 3.0.

For the following biomolecular analysis, high quality DNA is required. Thus, colonies which had tested positive to the RT-PCR assay and previously seeded on 5% sheep blood agar, were selected for DNA extraction using the DNAeasy Blood and Tissue kits (Qiagen, USA) following the protocol for Gram-positive bacteria. The DNA extraction and real time PCR assay are pictorially presented in Figure 2.2.5.

Dhaka University Institutional Repository

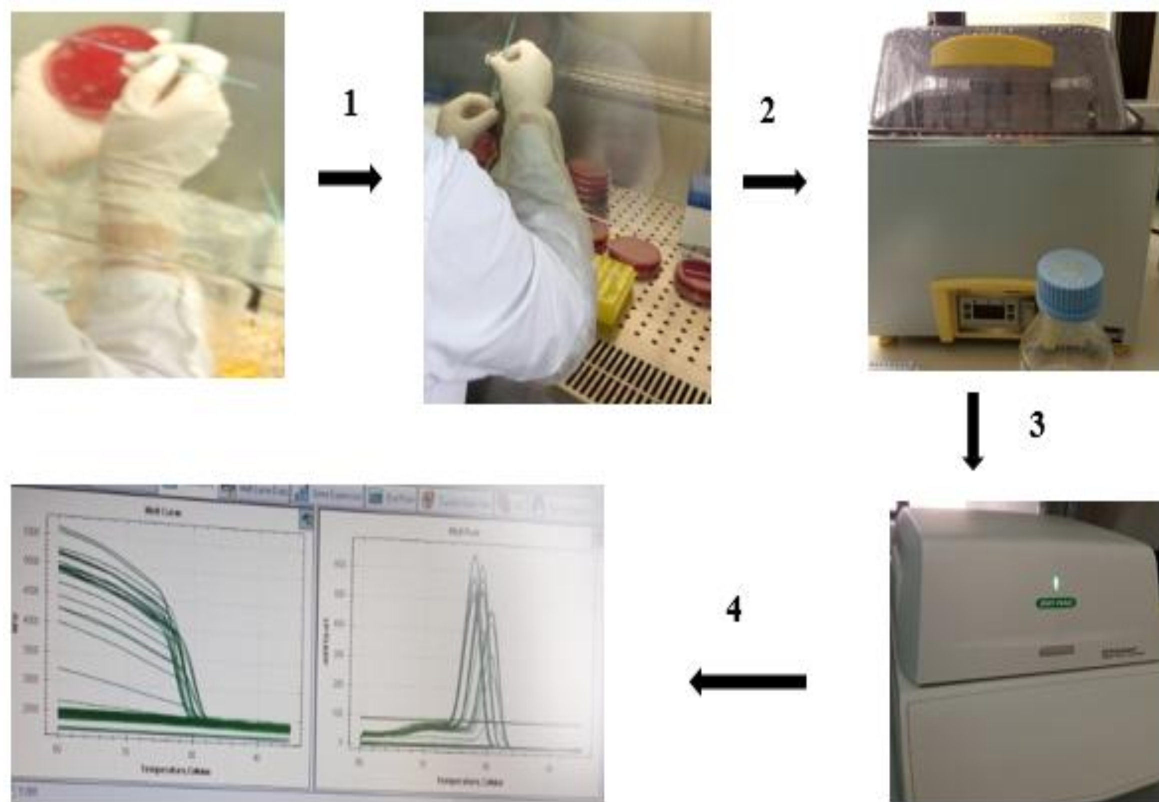

Figure 2.2.5: Pictorial presentation of DNA extraction and real time PCR assay.

Table 2.2.2 Primers used for PCR amplification

Sl. No.	Primer name	Sequence (5'-3')	Specificity	Length (bp)
1.	Ba 813 R1 Ba 813 R2	TTAATTCACTTGCAACTGATGGG AACGATAGCTCCTACATTTGGAG	Chromosome	152
2.	Pag 23 Pag 24	CTACAGGGGATTTATCTATTCC ATTGTTACATGATTATCAGCGG	Protective antigen, Plasmid pX01	151
3.	Cap (C) 57 Cap (C) 58	ACTCGTTTTTAATCAGCCCG GGTAACCCTTGTCTTTGAAT	Capsule, Plasmid pX01	264

Dhaka University Institutional Repository

2.2.1.4 PHRANA (Progressive hierarchical resolving assays using nucleic acids)

In this study to investigate the diversity of *B. anthracis* isolates, the hierarchical fingerprinting system PHRANA was applied. Thus, canSNP analysis was conducted as a preliminary test to confirm the origin of strains. Subsequently, multiple-loci variable number tandem repeat analyses (MLVA) were performed which exhibits greater resolving power than canSNP analysis and allows differentiation between closely related strains of the same canSNP group (Keim et al., 2004). For a finer genotype resolution, isolated strains were subjected to SNR analyses, which can show a high rate of mutation and allows verification of SNR minimal genetic differences within the same genotype.

2.2.1.5 Canonical single nucleotide polymorphism (CanSNP) analysis

CanSNP assay is a phylogenetic approach to identify SNPs that efficiently partition bacterial strains into genetic groups consistent with their recognized population structure (Keim et al., 2004; Keim et al., 2000; Pearson et al., 2004). Following the method described by Van Ert et al. (2007a) we were able to reveal the group of strains isolated from our samples and detect to which conserved group or lineage they belonged. Phylogenetic analyses, as well as information on isolate frequencies and global geographic distribution, facilitate the overview on the global diversity and historical transmission patterns of this pathogen (Van Ert et al., 2007a).

Since this test subdivides all of the *B. anthracis* isolates into three previously recognized major lineages (A, B and C), with further subdivisions into one of 12 distinct sub-lineages or sub-groups, each DNA extracted from our samples that had positive to PCR analysis, was processed for CanSNP identification using 13 TaqMan-Minor Groove Binding (MGB) allelic discrimination assays with oligonucleotides and probes as described by Van Ert et al. (2007a) for each of the 13 canonical SNPs. Each 10 µL reaction contained 1XTaqMan Genotyping Master Mix (Applied Biosystems), 250 nM of each probe, and 600 nM each of forward and reverse primers. To every reaction mixture was gradually added 10 ng template DNA. For all assays, thermal cycling parameters used were: 95°C for 10 minutes, followed by 40cycles at 95°C for 15 seconds, and 60°C for 1 minute. The results obtained by CanSNPs were compared to the recognized 12sub-lineage or sub-groups (Van Ert et al., 2007a).

Dhaka University Institutional Repository

2.2.1.6 Multiple-locus variable number tandem repeat analysis (MLVA) with 15 VNTRs

Molecular typing of *B. anthracis* strains is based on MLVA. The method is based on two approaches described firstly by Keim et al. (2000) (who identified 8 loci to genotype *B. anthracis*) and subsequently compiled together with the addition of 7 new loci, into a multiple-locus VNTR analysis with the final protocol described by Van Ert and associates, who identified the genotyping of *B. anthracis* (Van Ert et al., 2007a). All the VNTRs markers provide high levels of discrimination among different isolates also monitoring for the presence or absence of the plasmids as well as for plasmid-based variation. All isolates were subjected to 15-loci MLVA analysis using *B. anthracis* reference strain Ba001/1FG previously analyzed by Van Ert et al. (2007a) with designation number A0280ITA and then by Keim et al. (2004) with designation number K0021.

Seven PCR reactions amplify 15 VNTRs loci present at the level of the chromosome (vrrA, vrrB1, CG3, vrrB2, vntr19, vrrC1, vrrC2, mvntr32, vntr12, vntr35, vntr23) and at the level of plasmids (vntr16, vntr17, pxO1, pxO2). MLVA test provided the preparation of two singleplex and five multiplex reactions, in a final volume of 15 µL. Each reaction mixture contained: 1X PCR reaction buffer (Qiagen); 3 mM $MgCl_2$, 0.2 mM for each dNTPs; 1UI Hot Star Plus Taq DNA polymerase (Qiagen) and appropriate concentrations of each primer (singleplex 1: vrrC1, 0.2 µM; singleplex 2: vrrC2, 0.2 µM; multiplex 1: vrrA, 0.2 µM; vrrB1, 0.2 µM and CG3, 0.4 µM; multiplex 2: vrrB2, 0.25 µM; pXO2, 0.1 µM; pXO1, 0.3 µM; multiplex 3: vntr12, 0.25 µM; vntr19,0.2 µM; vntr35, 0.2 µM; multiplex 4: vntr16, 0.25 µM; vntr23, 0.2 µM; multiplex 5: vntr17, 0.1 µM; vntr32, 0.4 µM); and 2 µl of DNA.

The PCR thermocycling program for two singleplex and multiplex 1 and 2 was the same: 95°C for 5 minutes; 35 cycles at 94°C for 30 seconds, at 60°C for 30 seconds, and 72°C for 30 seconds. The final step was at 72°C for 5 minutes.

The amplification programs for three other multiplex PCR were: 95°C for 5 minutes, 35 cycles to 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 45 seconds and 72°C for 5 minutes (multiplex 3); 95°C for 5 minutes, 35 cycles at 94°C for 30 seconds, 56°C for 45

seconds, 72°C for 1 minute, and 72° for 5 minutes (multiplex 4); 95°C for 5 minutes, 35 cycles at 94°C for 30 seconds, 59°C for 45 seconds, 72°C for 1 minute and 72°C for 5 minutes (multiplex 5).

The MLVA PCR products were diluted 1:80 and subjected to capillary electrophoresis on an ABI Prism 3130 genetic analyzer (Applied Biosistems Inc.) with 0.25µL GeneScan 1200, and sized by Gene Mapper 4.0 (Applied Biosistems Inc.).

2.2.1.7 Single nucleotide repeat (SNR) analysis

To obtain deeper information about the genetic diversity within a single outbreak, SNR analysis gives a fine scale of resolution among isolates belonging to the same genotype. Single nucleotide repeats, also named mononucleotide-nucleotide repeats, are a type of VNTR (Variable Number Tandem Repeat) with a high rate of mutation (6.0 x 10^{-4} mutations per generation) present within the genome of *B. anthracis*.

In this study, we applied the modified SNR technique described by Kenefic and associates (Kenefic et al., 2008a). To identify, four SNRs were set up in two different kind of reactions mixture for multiplex 1 and a singleplex 1 in a final volume of 12.5 µl contained 1X PCR Buffer (Qiagen), 3.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 1 IU Hot Star Taq Plus DNA polymerase (Qiagen), 2 µl of DNA and appropriate concentrations of fluorescent-labelled forward and reverse primers (multiplex 1: HM1(CL33), HM13(CL35), 0.2 µM; HM6 (CL12), 0.1 µl; singleplex 1: HM2 (CL10), 0.2 µM). For both reactions, PCR cycle provided the following amplification process: 95°C for 5 minutes, 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minutes. Amplified SNR PCR products were diluted 1:80 and subjected to capillary electrophoresis on ABI Prism 3130 Genetic Analyzer (Applied Biosistems Inc.) with 0.25 µL GeneScan 120 LIZ, and sized by Gene Mapper 4.0 (Applied Biosistems Inc.).

2.2.2 Whole genome sequencing of *B. anthracis*

This study was carried out in collaboration with the Chittagong Veterinary and Animal Sciences University, the University of Dhaka (Bangladesh) and the Bundeswehr Institute of Microbiology, Munich, Germany.

2.2.2.1 Collection of soil samples

Following two reports on anthrax outbreaks in humans and bovine animals, as published in ProMED-mail on May 17 (ProMED-mail archive no. 20130517.1720541) and September 7 2013 (ProMED-mail archive no. 20130907.1929746), we visited the reported areas located at two sub-districts, namely Tangail Sadar (Dhaka division) near Tangail and Shahajadpur (Rajshahi division) in the north of Sirajganj, respectively, between July 6 and September 27 2013 and collected samples from different cattle farms in the reported areas. These sites had a history of sudden death of at least one bovine in the recent past with onset of convulsions or collapsing, with or without previous reported fever similar to what has been described earlier (Biswas et al., 2012). The cattle farms from which environmental samples were collected are referred hereafter as case farms and all of these have not been previously reported for anthrax cases by mass-media in the country or by any global electronic based surveillance. Additional samples were collected from diverse sampling sites in the regions of Tangail and Sirajganj (Figure 2.2.6) including soil, water, food and other environmental samples as well as swabs from animal bones or carcasses. In the Tangail region, samples from an animal burial site in a watered forest and from two stables of previously diseased cows were collected, in the Sirajganj region samples from a carcass-containing water pit and from a stable of a previously diseased cow were drawn, respectively.

Figure 2.2.6: Map of Bangladesh with locations of sampling sites for whole genome sequencing study. Stars indicate where samples were drawn in the regions of Tangail (A) and Sirajganj (B). Map of Bangladesh from http://www.worldofmaps.net.

2.2.2.2 Isolation of *B. anthracis* from soil samples and inactivation

To detect *B. anthracis* from samples, the GABRI method was used (Fasanella et al., 2013a). The GABRI method is a sensitive method for isolation of *B. anthracis* spores from soil particles as it disrupts hydrophobic interactions between spores and the adhering solid matrices. After incubation at 37°C for 24 h on selective solid media (Tomaso et al., 2006) suspected colonies lacking hemolysis were picked and streaked on Columbia blood agar (Merck KGaA). In general, bacteria were inactivated (for DNA extraction) after a single passage on blood agar in order to minimize in vitro genetic changes. For each isolate a few colonies were scraped off the agar plate and transferred into a 1.5 ml reaction tube filled with 500 µl 2% (v/v) Terralin PAA (Schülke & Mayr) and the cell pellet carefully resuspended by pipetting up and down. The reaction tube was completely filled up with 2% (v/v) Terralin PAA and incubated for 30 min for inactivation of *B. anthracis* endospores which are vulnerable to peracetic acid, the active agent of Terralin PAA (Jacob et al., 2012). Following centrifugation at 6000 x g for 2 minutes the supernatant was removed and the pellet resuspended in 1 ml phosphate-buffered saline (PBS). After two more washing steps with 1 ml PBS the pellet was stored at -20°C until further use. Work involving live *B. anthracis* was performed in a biosafety level 3 laboratory (BSL-3) within a class III safety cabinet (Glove box). Inactivated preparations were transferred to a BSL-2 laboratory for further analysis.

2.2.2.3 Extraction of DNA from inactivated culture material

For isolation of DNA from inactivated bacterial suspensions, the DNeasy Blood and Tissue Kit (Qiagen) was used as described in the manufacturer's manual for Gram-positive bacteria with the following minor changes. After cell wall lysis with lysozyme, 1 µl of RNase A (Qiagen) was added to each reaction tube and the mix was incubated at 37°C for another 10 min. Time for lysis with proteinase K was doubled to 60 min and elution was done twice (150 µl and 100 µl, respectively) into the same tube using sterile nuclease free Milli-Q water instead of Buffer AE. The DNA concentrations of each eluate were quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher) according to the supplier's protocol. DNA solutions were stored at -20°C until further use.

2.2.2.4 Diagnostic real-time PCR for chromosomal and plasmid markers of *B. anthracis*

The specific chromosomal marker *dhp61* (BA_5345) was used for identification of *B. anthracis* as described by (Antwerpen et al., 2008). Real-time PCR for detection of *capC* (pXO2) and *pagA* (pXO1) were performed according to (Turnbull, 2008). Assays were conducted using either Light Cycler 2.0, Light Cycler 480 II (Roche) or the Strata gene MX 3000P (Agilent Technologies). Data analysis was performed with the respective associated instrument software.

2.2.2.5 Analysis of canSNPs by Melt Mismatch Amplification Mutation Assay (Melt-MAMA)

CanSNP assay inquiry is a phylogenetic approach to identify SNPs that efficiently partition bacterial strains into genetic groups consistent with their recognized population structure (Keim et al., 2004; Pearson et al., 2004; Van Ert, et al., 2007a). For canSNP typing of the *B. anthracis*-isolates mismatch amplification mutation assays (Melt-MAMA) were performed (Birdsell et al., 2012). In short, for every canSNP assay two previously genotyped *B. anthracis* isolates were used as controls carrying either the ancestral or derivedallele, respectively. The 20 µl reactions contained 1 × LightCycler FastStart DNA Master SYBR Green-I, 4 mM $MgCl_2$, about 5 ng of template DNA and primer mixes specific for each assay (Birdsell et al., 2012). Real-time PCR was run on a Light Cycler 2.0 or Light Cycler 480 II (Roche). Thermal profiles used for amplification and subsequent melting curve analysis were as published previously (Birdsell et al., 2012).

2.2.2.6 Multi locus VNTR (variable number of tandem repeats) analysis (MLVA)

MLVA is a standard genotyping method for *B. anthracis* (Van Ert et al., 2007a). For the time being MLVA is still valuable because most of the long and highly-repetitive VNTRs cannot be determined by whole genome sequencing approaches relying on DNA-libraries yielding relatively short (<600 bases) read lengths. Therefore, DNA fragment lengths of fluorescently labeled amplicons covering complete VNTR loci were analyzed by capillary gel electrophoresis. In order to amplify the 31 different VNTR loci, five conventional multiplex PCRs were carried out as described previously (Keim et al., 2000; Lista et al., 2006; Beyer et al., 2012) with minor changes in primer concentrations.

PCR products were diluted 1:10 in water. A volume of 2 µl of each dilution and 0.5 µl of size standard Genescan 1200 LIZ (Applied Biosystems) were added to 17.5 µl HiDi formamide (Applied Biosystems) and incubated at 95°C for 5 min to denature DNA. Fragment lengths were determined on a 3130 Genetic Analyzer (Applied Biosystems) and the associated GeneMapper software.

2.2.2.7 Ion Torrent next generation whole genome sequencing

DNA-Library preparation for whole genome sequencing with the Personal Genome Machine (PGM, Thermo Fisher) required 100-1000 ng of high quality DNA in 50 µl nuclease free water. Where required, eluates from genomic extractions were concentrated to 2-20 ng/µl. For this a Concentrator Plus (Eppendorf) was used to evaporate water from aqueous solutions. Whole genome sequencing of DNA of *B. anthracis*-isolates was carried out with the Ion PGM (Thermo Fisher). To this end, library preparations were made according to the Ion Xpress Plus Fragment Library Preparation Guide (Thermo Fisher) using a sonication method using the Bioruptor system UCD-200 (Thermo Fisher) and subsequent end-repair (Thermo Fisher). The following settings of the Bioruptor were used to obtain fragment sizes of approx. 400 bp: time On/OFF: 3 × 15 min, 0.5 min/µM, min, Power level L (low). The 2100 Bioanalyzer (Agilent Technologies) was used for quality control after end-repair. Size selection and adapter ligation was achieved following the instructions of the Agilent High Sensitivity DNA Quick Start Guide (Agilent Technologies). Final DNA concentrations of the libraries were measured using the Qubit dsDNA HS Assay Kit (Thermo Fisher) and the DNAs were diluted to 44.2 pM. Emulsion PCR and enrichment of template positive Ion Sphere Particles were performed according to the Ion PGM Template OT2 400 Kit (Thermo Fisher). Chip loading was carried out according to the manual for the Ion 318 Chip Kit v2 (Thermo Fisher) in combination with Ion PGM Sequencing 400 Kit-chemistry (Thermo Fisher).

2.2.2.8 Analysis of whole genome sequencing data - SNP calling

Sequencing output files were mapped to the reference genome *B. anthracis* Ames ancestor (GeneBank: chromosome: AE017334.2; pXO1 AE017336.2; pXO2: AE017335.3) using bowtie2 (Langmead and Salzberg, 2012). A Variant Call File (VCF) was generated by SAMtools (Li et al., 2009) for every isolate containing all variations compared to the reference genome including quality and coverage information. A python

script was used to identify all single base variations that were both present in more than 75% of the reads and marked as high-quality bases. This analysis was carried out twice, once with a minimum coverage depth for variations of 15 and once with a minimum of 3. In order to avoid false negative as well as false positive results, the output data of the two approaches were compared. All results that indicated a SNP in one isolate compared to others were re-checked by manual inspecting the respective BAM files using Tablet software (Milne et al., 2013). The complete genome of isolate *B. anthracis* strain “Tangail-1” has can be accessed under accession number CP015779 (chromosome), CP015777 (pXO1) and CP015778 (pXO2).

2.2.2.9 Interrogation for branch-specific SNPs by high resolution melting analysis (HRM-SNP)

In order to determine the distribution of branch-specific SNPs obtained by whole genome sequencing, HRM-SNP assays were designed surrounding representative SNP positions, since base pair exchange from A + T to G + C or vice versa result in differences in melting temperatures of short amplicons. HRM-SNP primer sequences for real-time PCR-assays are listed in Table 2.2.3. Primer oligonucleotides were designed with the Primer-BLAST tool of NCBI (Ye et al., 2012) using the *B. anthracis* Ames ancestorgenome (GenBank: AE017334) as a reference template.

All SNPs were tested in 10 µl singleplex reactions containing 0.4 µM of the respective primer pair (B1, B2, NL/B1, A02/B1-NL and A02/A), 3 mM $MgCl_2$, approx. 1 ng of template DNA and 1 × LightCycler 480 High Resolution Melting Master Mix (Roche). Amplification and melting curve analysis was carried out on the LightCycler 480 II (Roche) using the following thermal profile for amplification: 95°C for 10 min, 45 cycles at 95°C for 10 s, 55°C (HRM-SNP B1: 52°C) for 20 s, 72°C for 10 s. The thermal profile used for melting curve analysis was for all samples: 95°C for 1 min, 60°C for 1 min and a final heating step to 95°C with a ramp rate of 0.02°C/s and continuous quantification at 530 nm.

Table 2.2.3 SNPs and respective primer sequences used for HRM-SNP

SNP	Position*	Variation	Forward primer (5'-3')	Reverse primer (5'-3')	Reference
A02/B1	59522	C to T	GCAAACGATTCGTGTTTCTATTATT	ACCTGCGATTGAACCTAGCA	This work
A02/B2	549428	G to A	AGCGGGAAGATTTGCGCTTA	GAGCGATACTCTGTTTCATTCGT	This work
A02/B1+NL	3766679	G to A	ATCCGCGTAACGCATTTCAA	AGTCGGCAGTTGCAGATTTAC	This work
A02/B1-NL	4248098	G to A	CGTAAACGATTCAACGATCATCT	GCGGAAATACATTACATGACTCC	**Derzelle et al. (2015a,b)**
A02/A	4961938	G to A	TTGTTAACGCAACATCTCTACGC	CATTTAATACGCCACCAATTACG	**Derzelle et al. (2015a,b)**

*in relation to the Ames Ancestor reference genome (AE017334.2)

2.2.3 Genotypes and subgenotypes of *B. anthracis* circulating in Bangladesh

The genotypes and subgentypes within the genotypes that are circulating in Bangladesh were illustrated by comaparing the genotypes and subgenotypes identified from the present study with those previously reported from the country in published literature.

2.3 Immunogenicity of anthrax vaccine strain used in Bangladesh

2.3.1 Source of anthrax vaccine

The anthrax vaccine used in this study was locally prepared by the Livestock Research Institute (LRI), Mohakhali, Dhaka. The master-seed of this vaccine is living spores of the non-capsulated attenuated Sterne F-24 strain of *B. anthracis* originated at Australia.

2.3.2 Serum sample collection

Blood sera from seven vaccinated and six non-vaccinated cattle were randomly collected from Sirajganj and Chittagong district of Bangladesh. A blood sample from an animal was collected in a single 15 ml falcon tube and allowed to clot for 30 min. The tube was centrifuged at 1,500 x *g* for 10 min. The sera were collected in sterile tubes and placed on dry ice for transportation. Each sample was aliquoted and stored at -80°C.

2.3.3 Immunological tests on laboratory animal

Ten mice were used for assessing immunogenicity of the anthrax vaccine. To generate antibodies against the fusion protein, 85 µg of the purified whole cell extract was injected into five female BALB/c mice by intraperitoneal injection. The mice were boosted another two times at 3-week intervals with 85 µg purified protein of whole cell extract. Sera were collected after 48 days from the first dose of injection and stored at -80°C. Antigens prepared from anthrax vaccine strain (Sterne strain F-24) were checked against different animal (Cattle (vaccinated and non-vaccinated) and mice (immunized and non-immunized)) sera using immune-blotting experiments (Hanson et al., 2006). For controls, sera from unimmunized mice were used.

2.3.4 Culture of bacteria by using anthrax vaccine strain

A total of 5 ml vaccine was taken in eppendorf tube and centrifuged at 3000 rpm for 10 minutes. Then the supernatant was removed. The sediment was washed by PBS (1ml) for 3 times and was used for bacteriological culture. The anthrax bacterial sediment was cultured onto nutrient agar plates. Six to eight colonies depending on the size were mixed thoroughly in sterile saline water (4 ml). Then 2 ml of this suspension was added to 50 ml broth and incubated in shaker incubator (120 rpm) at 37^0C for 24 hours. Stringent

personal protections were taken to culture the anthrax vaccine strain. The cultures in broth and agar of the vaccine strain were autoclaved and safely disposed of.

2.3.5 Preparation of whole cell extract

On obvious growth after 24 hours of incubation, the medium was centrifuged at 8,000 rpm for 10 minutes. For centrifugation, media was transferred to 50 ml falcon tubes at equal volumes/mass to prevent damage to the rotor. The centrifuge was started and waited in front of it till its speed reached to the desired level. After centrifugation, the culture soup was discarded (50 ml collected in 50 ml falcon tube), autoclaved and washed away. The cells were washed with 1 ml PBS and vortexed. After vortexing, the cells were transferred in an eppendorf tube containing PBS. Cells were sonicated using a sonicator, set at power 30, total time 10 min, pulsar 50%. Sonicated materials were centrifuged at 12,000 rpm for 10 min. Millipore ultrafilter (0.45 µm sizes) was used for removing unwanted particles such as inorganic matter like dust or organic matter like bacteria from the whole cell extract (WCE). Then 1 mg/ml protease inhibitor was added, alliquoted and kept at -20^{0}C until use. All the works of this study conducted for the isolation and identification of *B. anthracis* from anthrax vaccine were done following the methods of the Manual for Laboratory Diagnosis of Anthrax" by WHO (2003), the "OIE Terrestrial Manual, 2008 (OIE, 2008b) and the "Basic Laboratory Protocols for the Presumptive Identification of *B. anthracis*" by CDC (2001).

2.3.6 Bio-Rad protein assay

Materials

Bio-Rad dye reagent

Bovine Serum albumin (BSA)

Whatman filter paper # 1

Filtered distilled deionized water

Procedure

Protein concentration of the all the samples were estimated by Bio-Rad protein assay, which is based on the Bradford method (Bradford, 1976). It involved the addition of an acidic dye solution, and subsequent measurement at 595 nm with a spectrophotometer

(Shimadzu, Japan). Dye reagent was diluted so that 1 part dye reagent was mixed with 4 parts deionized water. The diluted dye reagent was then filtered through Whatman # 1 filter (or equivalent) to remove particulates. Five dilutions of a protein standard (BSA) were prepared for the preparation of standard curve against average absorbance values. The linear range of the assay for BSA is 0.2 to 0.8 mg/ml. 100 µl of each standard along with sample solution was pipetted into a clean, dry test tube. Protein solutions were assayed in duplicate. 0.5 ml of diluted dye reagent was added to each tube for at least 5 minutes. Absorbance was measured at 595 nm. The sample was incubated at room temperature for no more than 1 hour so as to avoid the increase of absorbance over time. Whole cell extrac's protein was determined from the standard curve.

2.3.7 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The following materials and reagent were used in SDS-PAGE:

a) 30% acrylamide (50 ml)
b) 0.5 M Tris HCl pH 6.8 (100 ml)
c) 1.5M Tris HCl pH 8.8 (200 ml)
d) 10% SDS (50 ml)
e) 10% Ammonium persulphate (10% APS-5 ml)
f) TEMED
g) Butanol saturated water
h) 12.5% separating gel
i) Stacking gel
j) Preparation of running buffer
k) 2X sample buffer
l) 0.1% BPB (Bromo phenol blue, loading dye/tracking dye)
m) Coomassie blue stain
n) Destaining solution

Procedure of SDS-PAGE

At first, one half of the Bio-Rad Protein II mini electrophoresis unit was opened, glasses were placed (larger glass to face the researcher) and the lower end was sealed by 2% agarose. Rest of 2% agarose was kept for future use. After proper sealing, the instrument was kept for agarose solidification for 10 min. After this duration, the sealing was

checked for any leakage with distilled water. On successful sealing, distilled water was poured off. Then the larger glass was marked after placing the comb. The mark was almost 1 cm below the lower end of the comb.

After mixing and degasing, the lower gel materials were placed into the chamber between the glasses (along the corner) by a micropipette without forming any bubble. The mixture was poured up to a bit higher than the mark. Butanol saturated water was added to the chamber up to top and left for 45 min. Solidification of the gel was confirmed by refractive index and residual materials in the beaker. After confirmation, butanol saturated water was poured or soaked off.

Before the lower gel solidified, upper gel materials was prepared except adding 10% APS and TEMED. After removing butanol saturated water, 10% APS and TEMED were added to the mixture and then placed into the chamber till it fills up. The comb was placed between the glasses. The gel was allowed to become solidified for about 45 min.

In the mean time, heated sample (20 µl) and sample buffer (20 µl) mixture were prepared followed by addition of 5 µl BPB. Each well (20 µl) contained >15µg of protein. The protein marker was also be prepared in the form of 5 µl diluted (10 times diluted- 5 µl concentration Marker+ 45 µl DW) marker + 5 µl 2X sample buffer+5 µl BPB.

After solidification, the comb was removed, the half of the unit was added to the rest half and placed into the tank. The running buffer was added to the tank and in the chamber filled in such a way that the bottom of the gel did not contain any bubble. The tank was tilted if required. The main chamber was fully loaded with running buffer. Then the stained samples and the marker (15-20 µl) were added in the specific wells and a note was kept. Then the roof of the tank was placed by matching wire colors. The other ends of the wires were connected to the power supply with volts free and regulated current (mA). The power supply was set at 20-22 mA. In case of smiling effect, flow of current was decreased to 18 mA. The total descent of the sample did take about 1 hour. When sample descent was complete, the power supply was disconnected, running buffer was poured off into the bottle and the gel containing unit was detached and drowned in a water containing tray. Spacers were removed first, then one of the glasses, then cut off

Dhaka University Institutional Repository

upper gel and agarose, marked the marker containing corner and then removed the other glass. The resultant gel was stained with Coomasiae blue R-250 for about 15 min on a shaker. The destaining was done with 7% acetic acid on a shaker overnight.

2.3.8 Transfer to nitrocellulose membrane

Materials

Nitrocellulose membrane (NCM)

Transfer buffer

Whatman no. 1 filter paper

Scotch-Brite pad and

Cathod grid

Procedure

Proteins resolved by SDS-PAGE were electrophoretically transferred to an NCM by using a modification of the procedure (Towbin et al., 1979) for SDS- containing gels. The gel was removed from the SDS-PAGE chamber as stated above and transferred to the Western blot chamber. At first, the foams, predetermined shape and size of the filter papers (4 pics) and NCM were soaked in transfer buffer. A sandwitch was made consisting sequentially of anode grid, Scotch-Brite pad, sheet of Whatman no. 1 filter paper, nitrocellulose membrane (NCM), polyacrylamide slab gel, sheet of Whatman no. 1 filter paper, Scotch-Brite pad and cathode grid. The sandwitch was placed in a Western blot tank.

The Western blot tank was filled with transfer buffer up to the height enough to drown the foams but not above the chamber. Then chamber was closed with load and connected to power supply. The power supply was set to ampere free, volt to 10 V for overnight transfer. On the next morning, after turning the power off, transfer buffer was poured into the bottle, followed by removal of Western blot box from the tank. The NCM was transferred to a tray containing amido black and stained. After marking the desired protein band with a sharp pencil, and cutting off the marker band and the center of the band, the piece of NCM was destained with distilled water and cut into very small piece and kept in sterile eppendorf and then kept at -20^{0}C.

Dhaka University Institutional Repository

2.3.9 Western blotting assay

Materials

Nitrocellulose membrane (NCM)

Blotting buffer, stored at 4^0 C

PBS

0.1% Tween 20 in 1X PBS

2% skim milk in 1X PBS

Primary antibody

i) Bovine sera

ii) Mice sera

Secondary antibody

i) Antibovine HRP conjugate (Santa Cruz Biotechnology, Inc., USA)

ii) Antimice AP conjugate (Sigma, USA)

Substrate

i) Horseradish peroxidase (HRP) substrate (Sigma, USA)

ii) Alkaline phosphatase (AP) substrate (Sigma, USA)

Procedure

Western blotting was performed by probing the blocked membrane with a primary antibody (bovine and mice) that recognized a single or more specific proteins or epitope on a group of proteins (e.g., SH2 domain or phosphorylated tyrosine) of anthrax vaccine. After separating native or denatured proteins by SDS-PAGE, the proteins were then transferred to the nitrocellulose membrane for detection using antibodies specific to the target protein.

To block the nonspecific binding, 2% (w/v) non-fat dry milk in phosphate buffered saline (PBS) was added on the membrane. After blocking the blot was washed 3 times with distilled water for 10 minutes. Fifteen ml falcon tubes were used for each sample. The membrane transferred into the falcon tube. Three ml skim milk with 30 µl primary antibody (1^0 Ab) solution (bovine serum/mice serum) was added separately into the NCM containing falcon tube and kept in a 4^0 C shaker for 1 hour for blocking. After incubation the blot was washed 3 times by 0.1% Tween 20. Five ml 0.1% Tween 20 was

Dhaka University Institutional Repository

added on NCM and shaked for 3-5 min and then washed by PBS for 1 min to remove 0.1% Tween 20. The membrane reblocked by secondary antibody (2^0 Ab) (dilution 1:10000 in skim milk). Twenty ml secondary antibody containing skim milk was added on the membrane and kept in a 4^0 C shaker for 1 hour. In this study, antibovine Horseradish peroxidase (HRP) conjugate (Santa Cruz Biotechnology, Inc., USA) and antimice Alkaline phosphatase (AP) conjugate (Sigma, USA) were used for bovine and mice, respectively. After 1 hour incubation, the blot was washed 3 times again by 0.1% Tween 20 and PBS as previously described. Finally, prepared substrate and added on the membrane for the detection of the target/ desired protein. Here, Horseradish peroxidase (HRP) substrate (Sigma, USA) and Alkaline phosphatase (AP) substrate (Sigma, USA) were used for HRP conjugate and AP conjugate, respectively.

Dhaka University Institutional Repository

Results

3.0 Results

3.1 Case-control study

3.1.1 Spatial distribution of case farms

The geographical locations of the case farms are indicated in the Figure 2.1.1 showing a map of Bangladesh. Except two, all (41/43) were clustered in three districts namely Pabna, Sirajganj, and Tangail, situated in the adjoining areas where the two great trans-boundary rivers in South Asia Ganges (Padma in Bangladesh) and Brahmaputra (western branch called Jamuna in Bangladesh) have conversed. The apparently clustering of the case farms however occupied seven upazilas (sub-districts) under the three districts mentioned. Due to very closer geographical positions, locations of some of the case farms appear to be overlapped, and thus are not well-distinctive in the figure. News on two outbreaks was not reported in any mass media or web sites of the concerned departments in the country. They were identified on the basis of the results of laboratory investigations done on the remnants of organ and/or soil samples collected from the farmyards where recent bovine animals had suddenly died and such events were reported by the local people while collecting information from the reported case farms in the same villages.

3.1.2 Population statistics of the case and control farms

Population statistics of the case and control farms are presented in Table 3.1.1. The median number of cattle observed on the day of survey in the case or in the control farms were four, ranging from 2 to 26 and 1 to 22 in the case and control farms, respectively.

3.1.3 Risk factors

The variables assessed and the results of the matched-pair analysis are presented in Table 3.1.2. Out of the 46 variables assessed only four had $p<0.05$. Of them the factor, “Sick animal on farm or a nearby farm slaughtered in the recent past” had the strongest point estimate of effect (odds ratio (OR) 14.0, $p=0.002$), although the 95% confidence interval (CI) of the effect was wider (2.1 - 591.0). According to the strength of point effect the other two risk factors were, “history of heavy raining occurred in the area in the last two

Dhaka University Institutional Repository

weeks" (OR 11.0, 95% CI 1.6- 473.5, p=0.010) and " Dead animal disposed of into nearby water body" (OR 8.0, 95% CI 1.1 - 354.0, p=0.046.

After matched-pair analysis eight variables with p<0.20 were considered for entering into the conditional logistic regression analysis and their effects in the initial model can be seen in Table 3.1.3. However, only three variables with p<0.05 retained in the final model as independent risk factors (Table 3.1.4). According to the probability of risk-association they were Sick animal on farm or a nearby farm slaughtered in the recent past (SAS) (OR 12.2, 95% CI 1.6 - 93.4, p=0.016), History of heavy raining occurred in the last 2 weeks (HHR) (13.1, 95% CI 1.2 - 147.1, p=0.037) and Disposing of dead animal into nearby water body (OR 11.9, 95% CI 1.0 - 145.3, p=0.052).

Table 3.1.1 Population statistics of the case and control farms enrolled in the study for the assessment of risk factors associated with anthrax in cattle in Bangladesh, 2010-2014

Farm Status			**Animal No.**		
	N	**Max**	**Min**	**Mean**	**Median**
Case	43	26	2	5.81	4
Control	43	22	1	5.35	4

Dhaka University Institutional Repository

Table 3.1.2 Matched-pair analysis of risk factors for anthrax in cattle in Bangladesh (N=43 pairs: 43 case farms, 43 control farms, 2010-2015)

Risk factor		N(+/-)	N(-/+)	OR (95% CI)*	P value
Farmer's education	Primary	6	9	0.7 (0.2-2.1)	0.606
	Secondary	10	7	1.4 (0.5-4.4)	0.628
	Higher Secondary	0	1	-	1.000
	Above	1	0	-	1.000
Farmer's occupation	Agriculture	7	8	0.9 (0.3-2.8)	1.000
	Animal Husbandry	0	2	-	0.480
	Service	3	1	3.0 (0.2-157.5)	0.617
	Business	6	5	1.2 (0.3-5.0)	1.000
Spatial	Plain land	1	4	0.3 (0-2.5)	0.371
	Coastal area	0	0	-	-
	Marshy land	0	0	-	-
	Flooded area	10	4	2.5 (0.7-11.0)	0.181
	Drought area	0	0	-	-
	Hilly area	0	0	-	-
Location of the farm	Near by a road	1	0	0	1.000
	Near by a river	2	3	0.7 (0.1-5.8)	1.000
Housing system	Old pattern/kacha	6	4	1.5 (0.4-7.2)	0.752
	Half building/semi paka	2	8	0.3 (0-1.3)	0.114
	Building/paka	0	1	0	1.000
Floor type	Animals kept tethered on wet muddy area	6	5	1.2 (0.3-5.0)	1.000
	Animal kept tethered on dry place	5	6	0.8 (0.2-3.3)	1.000
Feeding system	Mostly stall feeding	3	2	1.5 (0.2-18.0)	1.000
	Some grazing opportunity (only at homestead)	2	2	1.0 (0.1-13.8)	0.617
	Free grazing	9	3	3.0 (0.8-17.2)	0.149
	Feeding animals with uprooted and unwashed grass	9	9	1.0 (0.4-2.8)	0.814
	Grazing field recently flooded	2	1	2.0 (0.1-118.0)	1.000
	Dry grazing field	0	0	-	-
Feeding system	Feeding animals with water hyacinth	6	1	6.0 (0.67-276.0)	0.131

Table 3.1.2 (Continued)

Risk factor		N(+/-)	N(-/+)	OR (95% CI)*	P value
Feeding system	Feeding animals with feed added with bonemeal	0	0	-	-
	Grass collected from places where animal carcasses are disposed of in dry season	0	0	-	-
Source of drinking water	Canal/pond	1	1	1.0 (0-78.5)	0.480
	Clean/muddy	1	0	0	1.000
	RiverS	0	0	-	-
	Clean/muddy	0	0	-	-
	Tube- well	1	1	1.0 (0-78.5)	0.480
	Supply water	0	1	0	1.000
Sick animal on farm or a nearby farm slaughtered in the recent past	-	14	1	14.0 (2.1-591.0)	0.002
Types of disposal of dead animal in the area	Buried	24	0	0	0
	Burning	0	0	0	0
	Into near by water body	8	1	8.0 (1.1-354.0)	0.046
Proximity of burial place	Near to household	4	0	0	-
	Far from household	26	0	0	0
History of heavy raining occurred in the last 2 weeks	-	11	1	11.0 (1.6-473.5)	0.010
History of recently increased blood sucking insects population (HBP)	-	1	4	0.3 (0-2.5)	0.371
Vaccination time	Before outbreak	5	6	0.8 (0.2-3.3)	1.000
	During outbreak	10	4	2.5 (0.7-10.9)	0.181

OR, Odds ratio; CI, confidence interval; N (+/-), number of exposed case and unexposed control pairs; N (-/+), number of unexposed case and exposed control pairs; *, matched-pair analysis using McNemar’s test.

Table 3.1.3 Multivariable analysis of risk factor associated with anthrax affecting cattle in smallholdings in Bangladesh (Initial model)

Variable	OR	95% CI	P value
Flooded area	3.6	0.3-45.2	0.318
Half building /semi paka housing	1.5	0.2-16.0	0.712
Free grazing	1.5	0.2-13.7	0.730
Feeding animals with water hyacinth	10.3	0.3-371.4	0.202
Sick animal on farm or a nearby farm slaughtered in the recent past	15.6	1.5-159.7	0.021
Disposing of dead animal into nearby water body	33.0	0.9-1149.7	0.053
History of heavy raining occurred in the last 2 weeks	23.1	0.9-600.5	0.059
Anthrax vaccination during disease outbreak	4.3	0.4-44.0	0.224

Logistic regression; initial model with 8 variables entered; $\chi^2(8)$ for likelihood ratio test 35.21; $p<0.001$; No. of observation = 86; OR = Odds ratio; CI=confidence interval .

Table 3.1.4 Multivariable analysis of risk factor associated with anthrax affecting cattle in smallholdings in Bangladesh (Final model)

Variable	OR	95%CI	P value
Sick animal on farm or a nearby farm slaughtered in the recent past	12.2	1.6-93.4	0.016
Disposing of dead animal into nearby water body	11.9	1.0-145.3	0.052
History of heavy raining occurred in the last 2 weeks	13.1	1.2-147.1	0.037

Logistic regression; $\chi^2(3)$ for likelihood ratio test 27.44; $p<0.001$; No. of observation = 86; OR = Odds ratio; CI=confidence interval .

3.2 Molecular characterization of *B. anthracis*

3.2.1 Molecular characterization of *B. anthracis* by using 15 vntr loci

Among the 169 samples tested, 12 were positive to RT-PCR analysis: 10 soil and 2 bone samples. For each positive sample few colonies for PHRANA Assay were chosen randomly.

3.2.1.1 Canonical single nucleotide polymorphism (canSNP)

CanSNP analyses for the phylogenetic clustering showed that all isolates belonged to the lineage A major subgroup A.Br. 001/002 (Figure 3.2.1), which represent the same group circulating in China and other countries in South-East Asia. Furthermore, the A lineage isolates are widely distributed and are found in all over the world; probably this is due to the fact that the A lineage genotype have a better fitness and adaptability (Van Ert et al., 2007a).

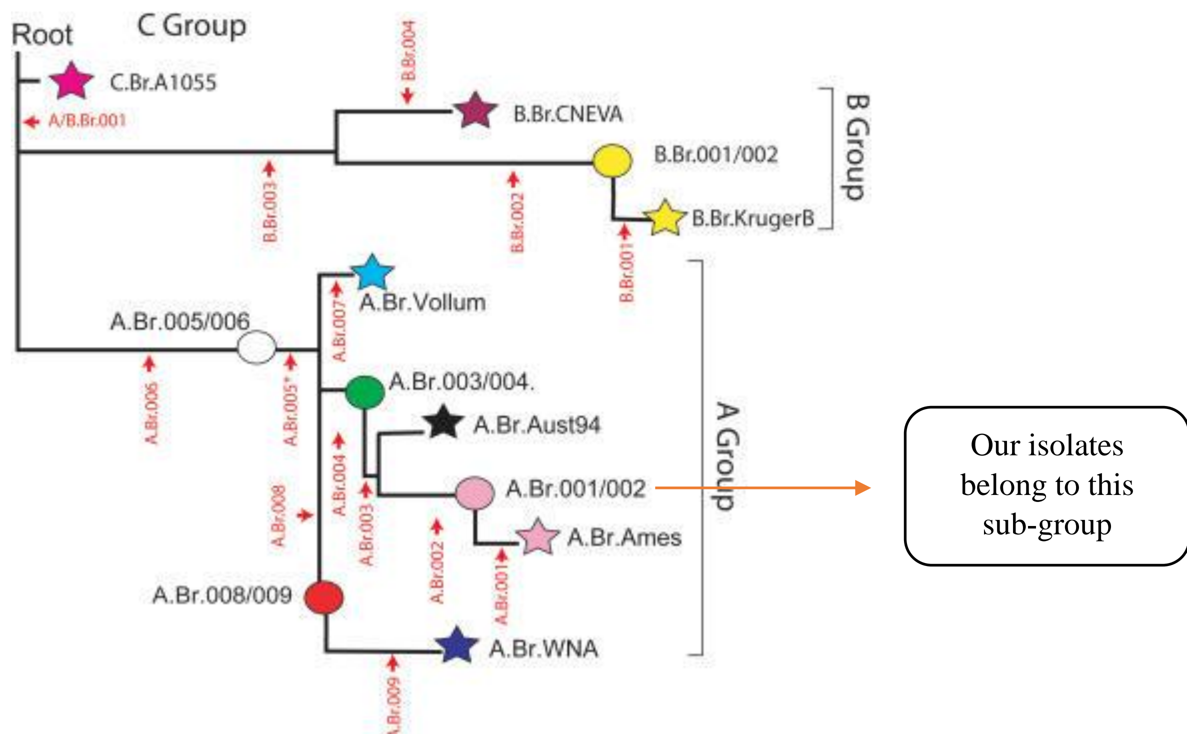

Figure 3.2.1: *B. anthracis* sub-grouping through canSNP analysis (Source: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1866244/figure/pone-0000461-g001/).

3.2.1.2 Multiple-locus variable number tandem repeat with 15 VNTRs

The MLVA-15 loci analysis of the isolates demonstrated the presence of four genotypes that were named GT1/Ban, GT2/Ban, GT3/Ban, GT4/Ban, in the district of Sirajganj and genotype GT2/Ban also present in the district of Tangail (Table 3.2.1) (Figure 3.2.2). The data reported in this study confirmed the presence of two genotypes (GT1/Ban and GT3/Ban, which corresponded respectively to genotypes GT/Kambel and GT/Chu) already identified from a previous study conducted in the district of Sirajganj and had also shown the existence of two new genotypes, named as GT2/Ban and GT4/Ban, not previously encountered (Fasanella et al., 2013b).

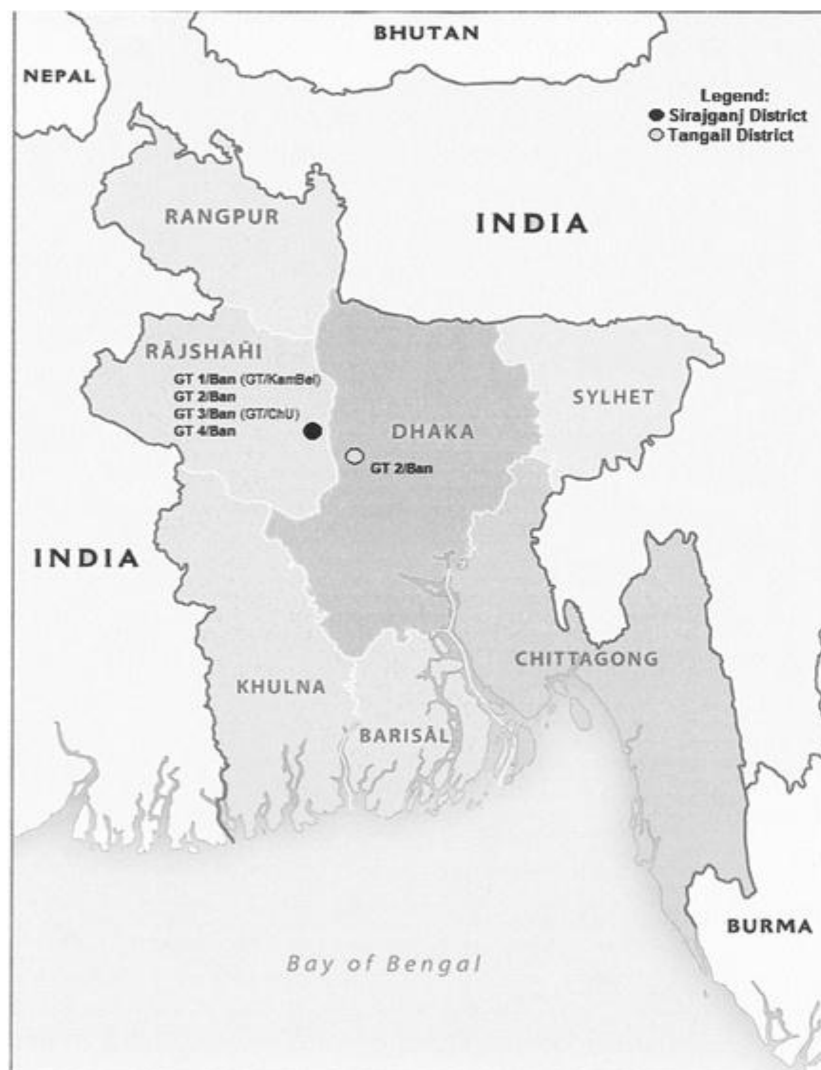

Figure 3.2.2: Map of Bangladesh indicating the genotype distribution of *B. anthracis*.

Dhaka University Institutional Repository

Table 3.2.1 MLVA 15- VNTRs results

District	No. Isolates	VNTRs observed (bp)															MLVA 15 GT	Fasanella et al. (2013b) GT
		vrra	vrrb1	vrrb2	vrrc1	vrrc2	cg3	pXO1	pXO2	vntr12	vntr16	vntr17	vntr19	vntr23	vntr32	vntr35		
Sirajganj	13	306	223	154	584	522	153	127	133	110	135	381	90	182	378	116	GT 1/Ban	GT/KamBel
Sirajganj	5	306	223	154	584	522	153	121	133	110	135	381	90	182	378	116	GT 2/Ban	NEW
Tangail	18																	
Sirajganj	14	295	223	154	584	522	153	127	133	110	135	381	90	182	563	116	GT 3/Ban	GT/ChU
Sirajganj	4	306	223	154	584	522	153	127	133	110	135	381	90	182	563	116	GT 4/Ban	NEW

3.2.1.3 Single nucleotide repeats

SNR assay highlighted the existence of four subgenotypes within the GT1/Ban, two subgenotypes within the GT3/Ban, while the GT2/Ban and GT4/Ban showed both one subgenotype (Table 3.2.2).

Table 3.2.2 SNR results

District	Genotype (GT)	SubGt	Sample	No. Isolates	SNR considered			
					HM1 CL33/pXO2	**HM2 CL10/Chrom**	**HM6 CL12/Chrom**	**HM13 CL35/pXO2**
Sirajganj	GT 1/Ban	*subGt1*	*17 - soil*	9	82	111	90	118
		subGt2	*17- soil*	1	83	111	90	118
		subGt3	61- soil	2	84	111	90	118
		subGt4	101- soil	1	82	110	90	118
	GT 2/ Ban	subGt5	21 - bone	5	82	109	90	119
	GT 3/Ban	*subGt6*	79- soil	7	78	107	91	118
			81- soil	6	78	107	91	118
		subGt7	*81- soil*	1	78	106	91	118
	GT 4/Ban	subGt8	77 - soil	4	82	109	92	119
Tangail	GT 2/ Ban	subGt5	87 - bone	5	82	109	90	119
			87 - soil	3	82	109	90	119
			91- soil	1	82	109	90	119
			93- soil	7	82	109	90	119
			95- soil	2	82	109	90	119

Dhaka University Institutional Repository

3.2.2 Whole genome sequencing of *B. anthracis*

3.2.2.1 *B. anthracis* isolation from environmental samples and initial genotyping

All non-hemolytic candidate strains retrieved by the modified GABRI method (Fasanella et al., 2013a) from Bangladesh samples were identified as *B. anthracis* by diagnostic real-time PCR on three markers *dhp61* (chromosomal), *pagA* (pXO1) and *capC* (pXO2), harboring both virulence plasmids (pXO1 and pXO2). The geographic attribution of these isolates within Bangladesh is presented in Table 3.2.3. All isolates belonged to the A. Br. 001/002 canSNP lineage.

Next, we performed multiple-loci variable number of tandem repeat (VNTR) analysis (MLVA). All isolates were screened for variations in their 31 MLVA markers (Table 3.2.4) and assigned to five similar MLVA genotypes. The majority of fragment length variations were observed in the markers bams5, bams15, bams21, bams24 and bams31 which are part of the MLVA-31 typing scheme (Beyer et al., 2012).Additional fragment length variations occurred in markers VNTR16, VNTR19-2 and VNTR35 which are part of MLVA-15 (Van Ert et al., 2007b).

A panel of diverse *B. anthracis* A.Br.001/002 branch strains was analyzed alongside the new Bangladesh strains. Interestingly, MLVA-31 typing of Chinese isolates of the A.Br.001/002 did not provide further clues on the potential origin of Bangladeshi strains. Conversely, we detected suspicious similarities between some European strains and the new strains from Bangladesh. Thus, MLVA-genotype data for the most similar Central European strains were also included in Table 3.2.4 to demonstrate the strains' potential close relationship with strains from Tangail and Sirajganj districts.

Table 3.2.3 Strains of *B. anthracis* from Bangladesh, Germany and the Netherlands used for analysis in this study belonging to canSNP-group A.Br.001/002

Name	Origin	Region	Country	Sampling date	Additional Information
CVI127491 -V08551	Bull	Winsum	The Netherlands	01.07.1991	carcass opened on farm, contamination of premises
CVI128268	Bull			14.08.1991	additional cases from the same farm
CVI131185	Beef Cattle			19.10.1991	
CVI131959**	Dairy Cattle			28.10.1991	
CVI132064-1**	Cattle			30.10.1991	
A163		Aulendorf	Germany	2008	
A40	Cattle	Giessen		Unknown	
Tangail-1*	Soil	Tangail	Bangladesh	16.09.2013	same stable of one diseased cow
Tangail-2*				26.09.2013	
Tangail-3*				26.09.2013	stable of a diseased cow
Tangail-4/1*				26.09.2013	animal burial site in a flooded forest
Tangail-4/2*				26.09.2013	
Sirajganj-1/1*	Soil	Sirajganj	Bangladesh	27.09.2013	sampled from a water pit with carcass
Sirajganj-1/2*				27.09.2013	
Sirajganj-2*				09.07.2013	stable of a diseased cow

Strains isolated from Bangladeshi samples are labeled with an asterisk (*); whole genome sequences were performed for all strains marked in bold letters. Strains from (Derzelle et al., 2015b) were re-sequenced because no genomic data has been released as part of the original publication (**).

Table 3.2.4 Results of MLVA-31 typing.

Strain	Country	bams1	bams3	bams5	bams13	bams15	bams30	bams24	bams31	bams21	pX01	VNTR12	pX02	VNTR23	VNTR32	VNTR16	VNTR19-2	VNTR35
Ames		490	548	300	823	418	733	599	774	660	121	113	137	192	562	269	90	112
CVI 127491-V08551	The Netherlands	296	578	378	869	612	677	599	647	660	**124**	113	133	180	385	140	90	112
CVI 128268																		
CVI 131959																		
CVI 131185											127							
CVI 132064-1																		
A163	Germany	296	578	378	**878**	612	677	599	647	660	127	113	133	180	385	140	90	112
A40											**133**	**111**						
Tangail-1	Bangladesh	296	578	378	869	612	677	599	647	660	127	113	133	180	385	140	90	112
Tangail-2																		
Tangail-3																		
Tangail-4/1																		
Tangail-4/2																**269**	**93**	**106**
Sirajganj-1/1	Bangladesh	296	578	**339**	869	**576**	677	599	647	660	127	113	133	180	385	140	90	112
Sirajganj-1/2										**400**								
Sirajganj-2								**553**	**667**	**360**								

Markers not differing from Ames reference strain are not shown in this table, namely bams22 (711bp), bams23 (633 bp), bams25 (392 bp), bams28 (498 bp), bams34 (509 bp), bams44 (420 bp), bams51 (498 bp), bams53 (234 bp), CG3 (153 bp), vrrA (307 bp), vrrB1 (224 bp), vrrB2 (149 bp), vrrC1 (567 bp), vrrC2 (522 bp), and VNTR17 (386 bp). Alleles differing from the genome-sequenced Bangladesh strain “Tangail-1” are indicated in **bold** letters.

3.2.2.2 Whole genome sequencing and assembly

For high-resolution genotyping, three strains from the collections of the Germany-based collaborating institution (A163) and The Netherlands (CVI 131959, CVI 132064-1) along with the five new strains from Bangladesh (Table 3.2.3) were genome sequenced using the Ion Torrent personal genome machine. Unmapped reads were assembled using the velvet-algorithm (Zerbino and Birney, 2008) and resulting contigs were analyzed.

3.2.2.3 SNP discovery and analysis suggest two Bangladeshi cluster related to strains from Central Europe

Comparative analysis of the genomic sequences of five Bangladeshi, one German, and two additional published Dutch *B. anthracis* isolates was carried with *B. anthracis* Ames Ancestor as outlying reference. From this dataset 262 non-homoplasic chromosomal SNPs were identified and these SNPs were used to infer the phylogeny of the Bangladeshi isolates in comparison with Central European strains of the A.Br.001/002 canSNP sublineage as pre-selected by MLVA-31. Figure 3.2.3 shows a maximum parsimony tree generated from this chromosomal SNPs analysis. The Bangladeshi strains fall into two distinct sub-groups B1 and B2 which are separated by 67 SNPs. With no genomic data available from Derzelle et al. (2015b) strains CVI 131959 and CVI 132064-1 were re-sequenced.

Notably, the Dutch isolates (CVI 131959 and CVI 132064-1) are clearly separated from B2, whereas the distance between B1 and the Dutch strains is 46 SNPs. German strain A163 is separated by 22 SNPs from the Bangladeshi B1/Dutch branching node (Figure 3.2.3). A sub-dataset of SNPs located on both virulence plasmids was also analyzed yielding 16 and 10 SNPs for pXO1 and pXO2, respectively). However, SNP differences in this plasmid data-set lack sufficient resolution for meaningful phylogenetic analysis.

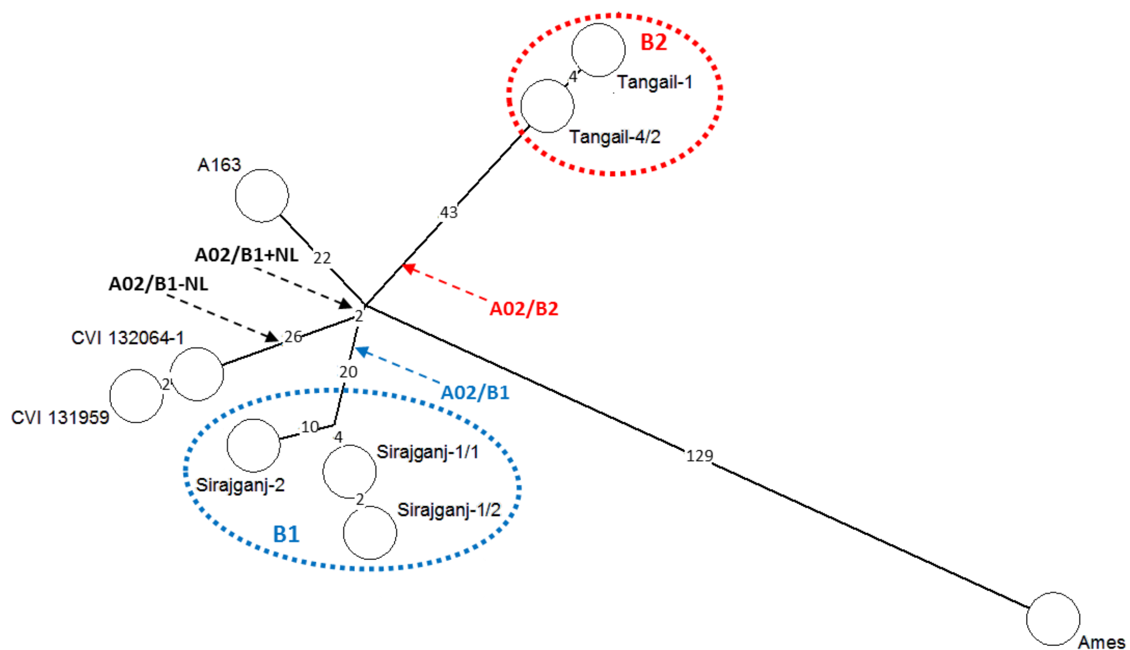

Figure 3.2.3: Maximum parsimony tree derived from chromosomal SNP analysis of Bangladeshi strains and their close relatives. Position of five Bangladeshi, two Dutch and one German *B. anthracis* strains based on 262 chromosomal SNP-positions are shown. Two distinct Bangladeshi regional clusters for Sirajganj and Tangail are indicated by dotted circles, B1 and B2, respectively. The length of each branch is proportional to the number of SNPs identified between strains. The Ames Ancestor chromosome (AE017334.2) served as an outlying reference. Arrows indicate the position of new (SNP A02/B1+NL, SNP A02/B1 and SNP A02/B2) or previously published SNPs (specific for canSNP A.Br.001/002 subgroup A02 SNP A02/A, SNP A02/B1-NL (Derzelle et al., 2015b).

Table 3.2.5 Results of HRM-SNP typing

Strain	Country	A02/A*	A02/B1-NL*	A02/B1+NL	A02/B1	A02/B2
Ames Ancestor		ancestral (G)	ancestral (G)	ancestral (G)	ancestral (C)	ancestral (G)
CVI 127491-V08551 CVI 128268 CVI 131959 **CVI 131185** **CVI 132064-1**	The Netherlands	ancestral (G)	derived (A)	derived (A)	ancestral (C)	ancestral (G)
A163 A40	Germany	ancestral (G) derived (A)	ancestral (G)	ancestral (G)	ancestral (C)	ancestral (G)
Tangail-1 Tangail-2 Tangail-3 Tangail-4/1 **Tangail-4/2**	Bangladesh	ancestral (G)	ancestral (G)	ancestral (G)	ancestral (C)	derived (A)
Sirajganj-1/1 **Sirajganj-1/2** **Sirajganj-2**	Bangladesh	ancestral (G)	ancestral (G)	derived (A)	derived (T)	ancestral (G)

*published as canSNP A02/A and A02/B1-NL by Derzelle et al. (2015b). Indicated in **bold** are strains with genomes sequenced in this study.

3.2.2.4 HRM-SNP analysis verifies the relationships of the genome sequenced strains

HRM SNP-PCR derived from our new genome data (Table 3.2.5) and from Derzelle et al. (2015b) were used to interrogate and confirm representative SNP positions (Table 3.2.5) on branches separating the Bangladesh B1 and B2 lineage as well that leading to the Dutch strains (Derzelle et al., 2015b). As expected the Dutch strains exhibited the ancestral allele for SNP A02/A and were derived for A02/B1-NL. The Bangladesh strains showed the ancestral alleles for these both SNPs regardless their belonging group, B1 or B2. Interestingly, German strain A40, which was not genome sequenced, exhibited the derived state for SNP A02/A, similar to strain CVI-un1 from Derzelle et al. (2015b), whereas genome sequenced strain A163 had the ancestral allele. Analysis of SNP B1+NL confirmed that the Dutch strains (Derzelle et al., 2015b) and our strains from Bangladesh group B1 (Table 3.2.5) share the derived allele, whereas the German and Bangladesh B2 strains were ancestral for this marker. Finally, specific SNPs for each Bangladeshi group B1 (SNP A02/B1) and B2 (SNP A02/B2) confirm positions of all analyzed B1 and B2 strains to their respective clade and separated the German and Dutch strains which exhibited the ancestral allele for these both markers. Thus, HRM-SNP analysis verified the relationships of the genome sequenced strains and amended this with information from the MLVA-typed but not genome-sequenced strains from this study.

3.2.3 Genetic identities of *B. anthracis* in Bangladesh: A comaparison with the results of this and other two previous studies

Based on the laboratory evidence from CDIL and CDC, Chakraborty et al. (2012) identified seven confirmed animal anthrax cases, five in cattle and two in goats. *B. anthracis* was isolated at CDC from vesicular swabs of three cases, two from the first outbreak in 2009 and one from the outbreaks in August 2010. MLVA was performed for *B. anthracis* isolated from human and animal specimens at CDC. The MLVA-8 genotype of 2009 isolates confirmed that animals and humans were infected by isolates of the same genotype (Table 3.2.6).

Fasanella et al. (2013b) recovered *B. anthracis* from soil samples and turbinate bones on six farms. They isolated *B. anthracis* from 16/20 samples, for a total of 99 isolates. The

MLVA on 15 VNTRs on the isolates showed the presence of three different novel genotypes, which they labelled as Gt/Kha, Gt/ChU, and Gt/KamBel (Table 3.2.6 and Table 3.2.7).

The present study demonstrated the presence of four genotypes that were named GT1/Ban, GT2/Ban, GT3/Ban, GT4/Ban in the district of Sirajganj and genotype GT2/Ban in the district of Tangail (Table 3.2.6). Of the genotypes two, GT1/Ban and GT3/Ban)were identical to genotypes GT/Kambel and GT/Chu identified in Sirajganj by Fasanella et al. (2013b) but the other two, GT2/Ban and GT4/Ban were identified for the first time in Bangladesh.

The canonical SNP analysis showed that all the isolates belonged to the major lineage A, sublineage A.Br.001/002 according to the worldwide distribution of *B. anthracis* clonal lineages (Van Ert et al., 2007a).The SNR analysis of Fasanella et al. (2013b) showed the presence of two SNR types (SubGt-1, SubGt-2) in 97/99 isolates and was able to detect the presence of two isolates with different SNR-loci type polymorphisms (SubGt-1a, SubGt-2a) within the MLVA genotypes Gt/Kha and Gt/KamBel (Table 3.2.8). The results of SNR assay from this study however revealed the existence of four subgenotypes within genotype GT1/Ban, two subgenotypes within GT3/Ban, and only one subgenotype each in genotype GT2/Ban and GT4/Ban (Table 3.2.8). These subgenotypes mentioned above were never reported before this study.

Table 3.2.6 Comparison among the genotypes of Bangladeshi *B. anthracis* isolates so far studied including those from the present study by using 8 VNTR loci

District	Outbreak year	Laboratories	MLVA type	No. of isolates	VNTRs observed bp								Reference
					Vrra	vrrb1	vrrb2	vrrc1	vrrc2	pXO1	pXO2	cg3	
Sirajganj	2009	CDC	GT152		313	229	162	554	532	132	141	158	Chakraborty et al. (2012)
	2010		GT155		313	229	162	554	532	129	141	158	Fasanella et al. (2013b)
	2010	IZSPB	Gt/KamBel or Gt_1/Sir	48	306	223	154	584	522	127	133	153	
	2010		Gt/Chu or Gt_3/Sir	18	295	223	154	584	522	127	133	153	
	2010, 2013		Gt_1/Sir	13	306	223	154	584	522	127	133	153	Rume et al. (2016); Fasanella et al. (2013b)
	2013		Gt_2/SirTan*	5	306	223	154	584	522	121	133	153	Rume et al. (2016)
	2014		Gt_3/Sir	14	295	223	154	584	522	127	133	153	Rume et al. (2016); Fasanella et al. (2013b)
Tangail	2014		Gt_2/SirTan*	18	306	223	154	584	522	121	133	153	Rume et al. (2016)

*Newly identified genotype

Table 3.2.7 An overview on the genotypes of *B. anthracis* circulating in Bangladesh, as observed using MLVA 15 VNTR loci on the isolates from this and other two previous studies

District	Outbreak year	Laboratories	MLVA type	No. of isolates	VNTRs observed bp															Reference
					Vrra	vrrb1	vrrb2	vrrc1	vrrc2	pXO1	pXO2	cg3	Vntr 12	Vntr 16	Vntr 17	Vntr 19	Vntr 23	Vntr 32	Vntr 35	
Sirajganj	2009	CDC	GT152		313	229	162	554	532	132	141	158	-	-	-	-	-	-	-	Chakraborty et al. (2012)
	20l0		GT155		313	229	162	554	532	129	141	158	-	-	-	-	-	-	-	
	2010	IZSPB	Gt/KamBel	48	306	223	154	584	522	127	133	153	110	135	381	90	182	378	116	Fasanella et al. (2013b)
	2010		Gt/Chu	18	295	223	154	584	522	127	133	153	110	135	381	90	182	563	116	
	2010		Gt/Kha	33	295	223	154	584	522	127	133	153	110	135	381	90	182	563	109	
	2010, 2013		Gt_1/Sir	13	306	223	154	584	522	127	133	153	110	135	381	90	182	378	116	Rume et al. (2016); Fasanella et al. (2013b)
	2013		Gt_2/SirTan*	5	306	223	154	584	522	121	133	153	110	135	381	90	182	378	116	Rume et al. (2016)
	2014		Gt_3/Sir	14	295	223	154	584	522	127	133	153	110	135	381	90	182	563	116	Rume et al. (2016); Fasanella et al. (2013b)
	2014		Gt_4/ Sir*	4	306	223	154	584	522	127	133	153	110	135	381	90	182	563	116	Rume et al. (2016)
Tangail	2014		Gt_2/SirTan*	18	306	223	154	584	522	121	133	153	110	135	381	90	182	378	116	Rume et al. (2016)

*Newly identified sub-genotypes

Table 3.2.8 Comparisons on the Single Nucleotide Repeats (SNR) results of the *B. anthracis* isolates from Bangladesh investigated from this study and other two previous studies

District	Laboratories	Outbreak year	MLVA type	SNR type	No. isolates	SNR considered				Reference
						HM1	HM2	HM6	HM13	
Sirajganj	CDC	2009	GT152							Chakraborty et al. (2012)
		2010	GT155							
	IZSPB	2010	Gt/Kha	SubGT-1	32	79	108	91	118	Fasanella et al. (2013b)
		2010		SubGT-1a	1	80	108	91	118	Fasanella et al. (2013b)
		2010	Gt/KamBel	SubGt-2	47	83	110	90	118	Fasanella et al. (2013b)
		2010	or Gt_1/Sir	SubGt-2a	1	83	110	91	118	Fasanella et al. (2013b)
		2013		subGt1*	9	82	111	90	118	Rume et al. (2016)
		2013		subGt2*	1	83	111	90	118	Rume et al. (2016)
		2010		subGt3*	2	84	111	90	118	Rume et al. (2016)
		2014		subGt4*	1	82	110	90	118	Rume et al. (2016)
		2013	Gt_2/SirTan	subGt5*	5	82	109	90	119	Rume et al. (2016)
		2010	Gt/Chu or	SubGT-1	18	79	108	91	118	Fasanella et al. (2013b)
		2014	Gt_3/Sir	subGt6*	13	78	107	91	118	Rume et al. (2016)
		2014		subGt7*	1	78	106	91	118	Rume et al. (2016)
		2014	Gt_4/ Sir	subGt8*	4	82	109	92	119	Rume et al. (2016)
Tangail		2014	Gt_2/SirTan	subGt5*	18	82	109	90	119	Rume et al. (2016)

*Newly identified sub-genotypes

3.3 Anthrax vaccine immunogenicity

3.3.1 Isolation and identification of anthrax bacteria (*B. anthracis*) from anthrax vaccine

In nutrient agar media, the colony characteristics of anthrax vaccine bacteria were flat or slightly convex with irregular edges (Figure 3.3.1). Anthrax vaccine bacterial sediments were also inoculated in nutrient broth. In nutrient broth, anthrax vaccine bacteria produced turbidity or cotton wool like growth.

Figure 3.3.1: Creamy white colonies showing growth of the vaccine strain used in this study on nutrient agar after overnight incubation at 37°C.

3.3.2 Protein estimation

The results of protein estimation are presented in Table 3.3.1, and in Figure 3.3.2 showing the standard curve of the protein estimated.

Table 3.3.1 Protein estimation of the sample (whole cell extract of the anthrax vaccine, Sterne strain F-24 of *B. anthracis*)

Stage	**Concentration gradient (mg/ml)**				**Sample**
	0.2	**0.4**	**0.6**	**0.8**	
1st	0.308	0.496	0.645	0.737	0.674
2nd	0.126	0.227	0.371	0.458	0.304
3rd	0.138	0.220	0.654	0.719	0.695
Average	0.1906	0.314	0.556	0.638	0.557

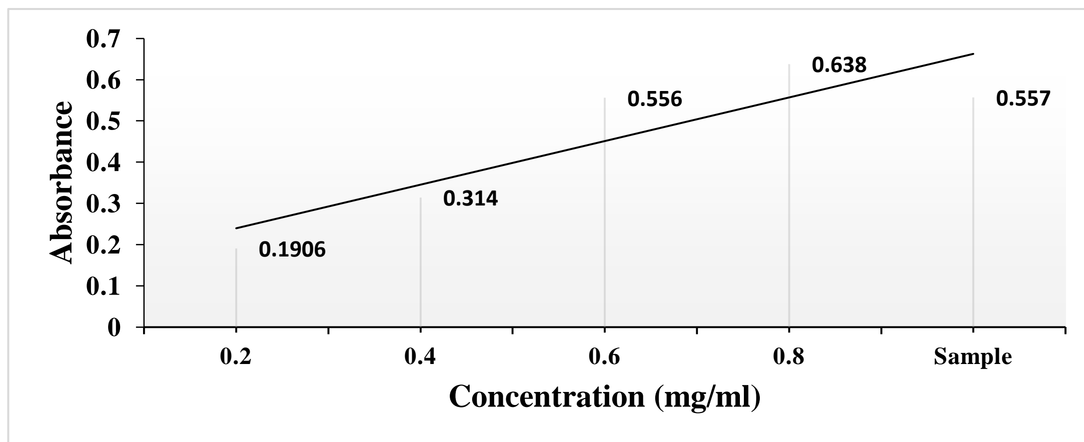

Figure 3.3.2: Standard curve of protein (BSA).

3.3.3 SDS-PAGE

Figure 3.3.3 is presented with migration patterns of whole cell-extract A and B proteins. Numerous proteins, ranging from 120 to 26 kDa were detected, having stained with Coomassie Brilliant Blue.

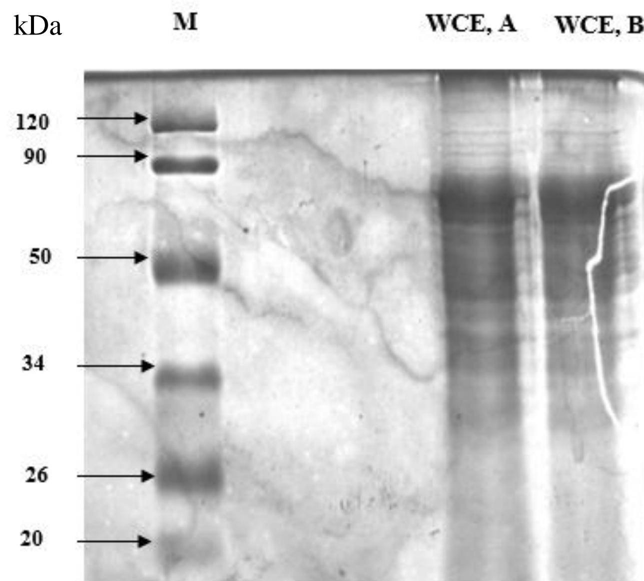

Figure 3.3.3: SDS-PAGE maps showing migration patterns of the whole cell extract proteins of the anthrax vaccine (Sterne F-24) used in the study, having the gel stained with Coomassie Brilliant Blue. Lane M: unstained protein molecular weight marker, Lanes WCE, A and WCE, B: whole cell extract proteins of the vaccine strain Sterne F-24.

3.3.4 Western blot.

In this study, Western blotting assays were done for Sterne F-24 whole cell proteins using sera from vaccinated, non vaccinated cattle, immunized and non immunized mice. Figures 3.3.4a, 3.3.4b and 3.3.4c are portrayed with the results of Western blot using the sera from vaccinated cattle, non vaccinated cattle, immunized and non immunized mice, respectively. Lanes 1, 2, 3, 5 and 6 of Figure 3.3.4a, and lanes of 11, 12 and 13 of Figure 3.3.4b represent the results of vaccinated sera from cattle, while lanes 4, 7 and 8 of Figure 3.3.4a and lanes 9 and 10 of Figure 3.3.4b represent the results of sera from non-vaccinated cattle. Lanes 14 and 15 of Figure 3.3.4 care illustrate the results of sera from immunized mice and lanes 16 and 17 of the same illustrate the results of sera from non immunized mice.

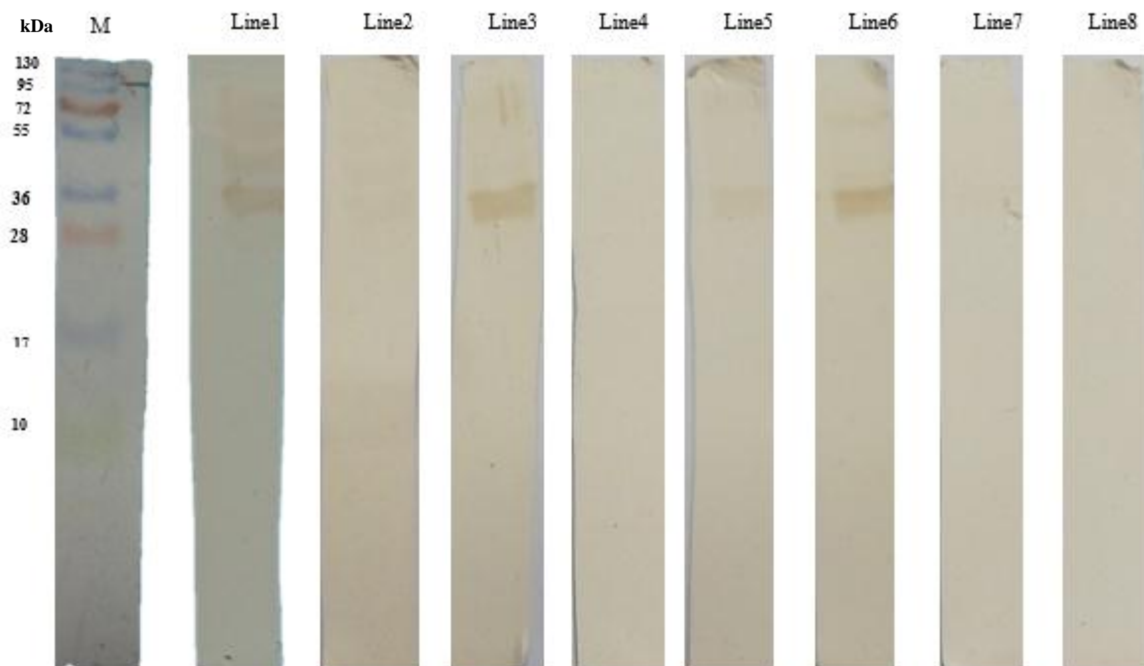

Figure 3.3.4a: Western blotting assays for Sterne F-24 whole cell proteins using sera from vaccinated cattle (Lanes 1, 2, 3, 5 and 6) and sera from non-vaccinated cattle (Lanes 4, 7 and 8). M: unstained protein molecular weight marker.

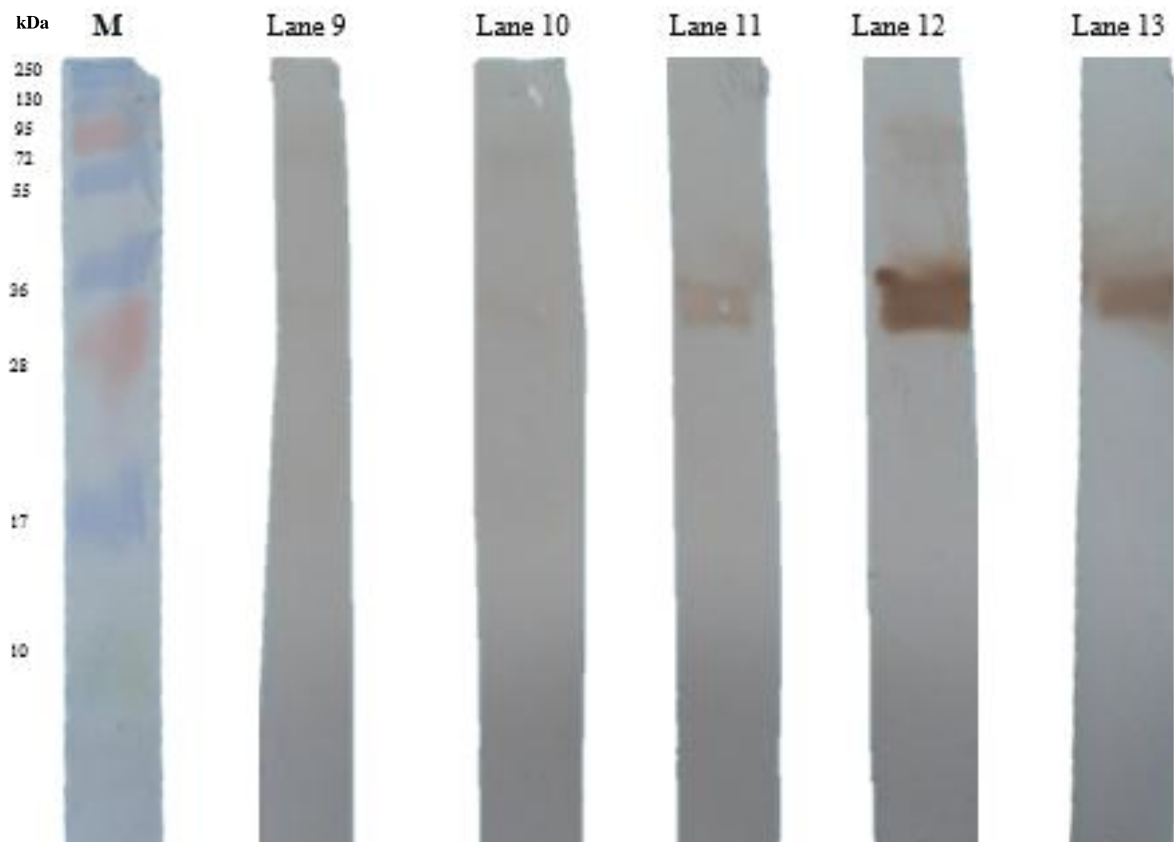

Figure 3.3.4b: Western blotting assays for Sterne F-24 whole cell proteins using sera from vaccinated cattle (Lanes 11, 12 and 13) and sera from nonvaccinated cattle (Lanes 9 and 10). M: unstained protein molecular weight marker.

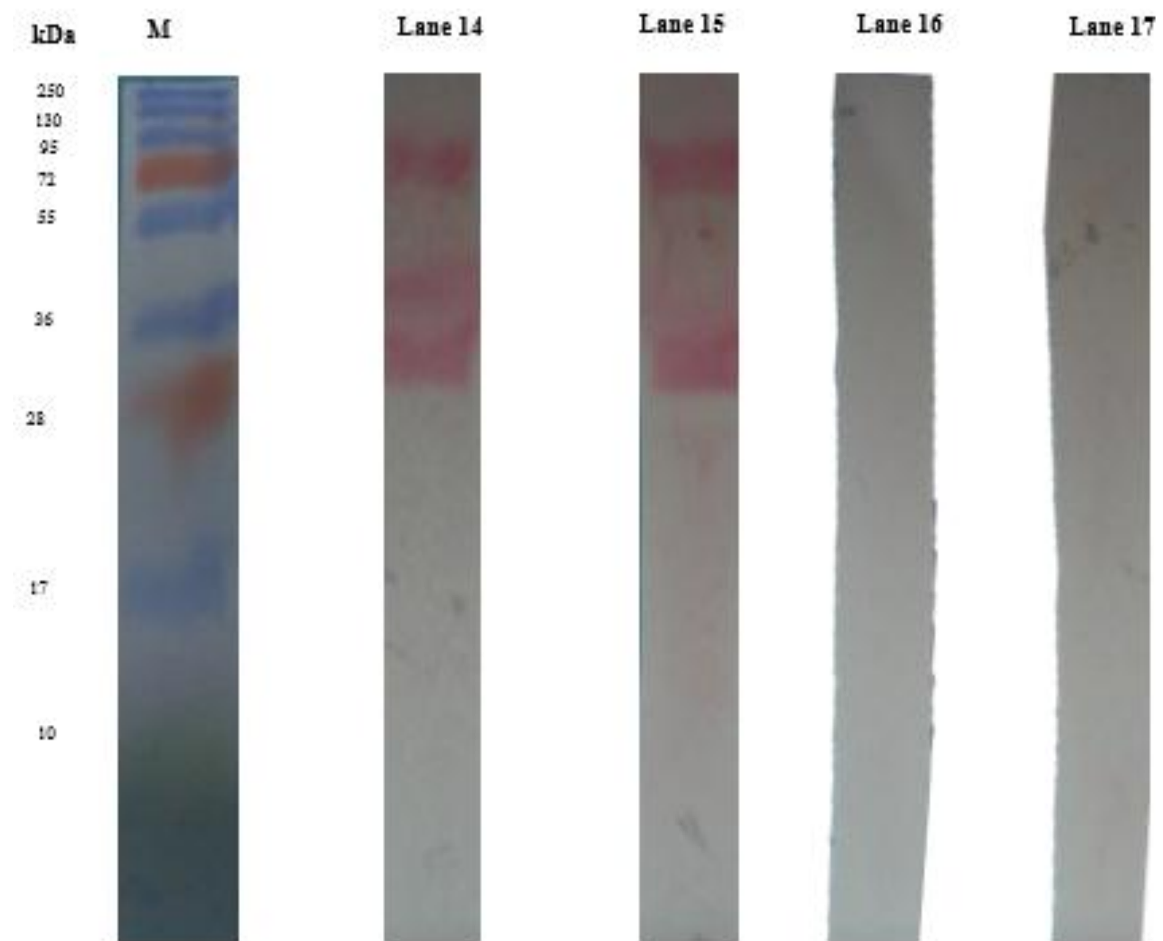

Figure 3.3.4c: Western blotting assays for Sterne F-24 whole cell proteins using sera from mice vaccinated with the whole cell extract proteins of the Sterne F-24 strain (Lanes 14 and 15) and sera from non-vaccinated mice (Lanes 16 and 17). M: unstained protein molecular weight marker.

Western blot analysis was carried out to characterize antigenic proteins associated with inducing proteins in vaccinated and non vaccinated cattle, and immunized and non-immunized mice. The results revealed that no protein bands were detectable in sera of non-vaccinated cattle and control mice. But clear bands about 36 kDa-sized proteins were visible in all the vaccinated cattle. Bands of 35-83 kDa-sized proteins were detected from sera of hyper-immunized mice.

Dhaka University Institutional Repository

Discussion

Dhaka University Institutional Repository

4.0 Discussion

4.1. Case control study

Unprecedented epidemic trend of anthrax outbreaks affecting both bovine animals and humans were recorded in the districts of Pabna and Sirajgong in monsoon months of two consecutive years, 2009 and 2010 (Ahmed et al., 2010; Biswas et al., 2012; Chakraborty et al., 2012). Any such temporal peak of outbreaks was not noticed in the period of the present study that ended in 2014. Following the mass media and/or available surveillance reports, we were able to identify only 40 outbreaks of anthrax across the country, and the other three were identified by testing remnants of organ and/or soil samples collected from adjacent farms reportedly had a history of sudden mortality. Such a low number of outbreaks over a long period of observation indicate the fact that anthrax outbreaks in animals in Bangladesh are predominantly reported when humans are also inflicted due to slaughtering and butchering of clinically diseased animals for meat. However, clustering of 41 out of the 43 outbreaks in only three adjacent districts Pabna, Sirajganj and Tangail lying at the merging site of the rivers Padma and Jamua suggests that the area remains as an “anthrax hot-spot” in Bangladesh, as were seen in the years 2009 and 2010 (Ahmed et al., 2010; Biswas et al., 2012; Chakraborty et al., 2012).

Seldom media reports on anthrax affecting bovine animals in Bangladesh do not disclose its true prevalence, which might be much higher because a retrospective study conducted on veterinary field data submitted from the entire country during the period 1 January 2010 to 31 December 2012 revealed 5937 animal cases of anthrax (Mondal and Yamage, 2014). The shortcoming of these data was that the occurrences of the cases were not confirmed by laboratory testing. Thus, cases of bovine anthrax occurred yearly meeting any of the three criteria set for this study were very limited.

During the peak of anthrax outbreaks in July-September 2010, a case-control study was conducted previously by enrolling only 15 cases (Biswas et al., 2012) and the study identified “Feeding animals with water hyacinth (*Eichhornia crassipes)*” an independent risk factor. In the present study, on the results of univariable analysis (Table 3.1.2) its association with anthrax was nearly significant (p=0.131), qualifying the variable to be entered into the multivariable analysis. Probably, due to small sample size it was not

found an independent risk factor in the end. However, the present study identified three new risk factors.

Being fearful of economic loss, farmers sometimes slaughter their severely clinically affected bovine animals for selling of meat unknowing the danger of transmission of *B. anthracis* to humans. Such slaughtering places, which are usually the farmyards for moribund animals, are not properly decontaminated, but rinsed with water to remove the blood and slaughter-associated wastes, and butchering wastes are often disposed of into nearby ditches, bodies of water, or on open fields, contaminating the environment (Islam et al, 2013). *B. anthracis* can produce spores in the said contaminated places and live in this dormant stage for years together. While feeding on the recently contaminated patches of grass/vegetation on such farm yards other animals of the same farm or nearby farms can be infected, could be the probable reason of finding "Sick animal on farm or a nearby farm slaughtered in the recent past" an independent risk factor. In the molecular study, we identified the same sub-genotype (lineage A major subgroup A. Br 001/002 GT2/Ban subGT5) of the organism from some remnants of bone and the soil samples from the same farmyard of a case farm (Rume et al., 2016). In North Dakota, USA, death of an animal on a neighboring premise was found to be a significant predictor of anthrax occurrence on a premise (Mongoh et al., 2008a).

It should be again noted that a control farm was matched with its case farm from a distantly located area of the case farm but within the same sub-district. This was for ensuring the freedom of the control farm from being exposed to any kind of environmental materials contaminated with emanations of the case farm. In this study, heavy raining was meant for short periods of intense rainfall. Most cases enrolled in the study occurred in the rainy season (July-October). Heavy rainfall variability in short time periods within an area of a sub-district is although not a frequently observed phenomenon, but can be seen in monsoon season in Bangladesh. And the results of this study showed that about 25% of the cases this variation occurred, indicating it an independent risk factor for bovine anthrax in Bangladesh. Heavy rainfall washes off the topsoil exposing the anthrax bacilli spores underneath that could be accumulated on certain spots on the ground through water runoffs. Cattle could be infected by feeding

Dhaka University Institutional Repository

soil-adhered new fresh shoots of grass grown on such places and their roots containing infective dose of anthrax bacilli spores (Dragon and Rennie, 1995).

Locations of farms in a river basin and flooding were suspected of having contributory roles in anthrax outbreaks in North Dakota, USA and in Sweden (Mongoh et al., 2008a; Lewerin et al., 2010). In a previous study in Bangladesh, it was found that farmers disposed of dead animals into nearby water (ICDDR,B, 2009). Dumping carcasses in the water may lead to contamination of the water. Taking or were offered to muddy water from such water bodies containing anthrax bacilli spores animals could be infected. Spores of *B. anthracis* have a high buoyant density, which provides an opportunity for them to adhere to vegetation as the vegetation resurfaces during evaporation (Dragon and Rennie, 1995). Floating and decomposed carcass could also contaminate the surrounding vegetation, which if was fed to animals, could also contribute to infection.

Animals in 11% (5/43) of the case farms appeared to have been vaccinated before the reported outbreaks compared with 14% (6/43) control farms, indicating low vaccine coverage. But why this vaccinated proportion of case farms was not protected from the disease is difficult to understand. However, the exact dates of the vaccinations were unknown. The Sterne strain of *B. anthracis* is used for immunization and should be administered to livestock in a dose containing up to10 million viable spores (Hambleton et al., 1984). Animals vaccinated >6 months ago might not retain sufficient immunity because Sterne strain vaccine induces immunity that typically lasts for just under 1 year (FAO, 2001). Six months apart from each other two doses of vaccine against anthrax might be ideal for anthrax-prone area (FAO, 2001).

Insufficient vaccine production might be the reason for weak vaccine coverage against anthrax even in the anthrax hot-spot in Bangladesh. The mitigations of the risks identified from the study might be helpful in reducing its incidence across the country. Awareness building among the farmers by the field veterinarians, their supporting staff and the non-governmental workers on the deadly threats being posed to their own lives and livestock from slaughtering of clinically sick animals is important to stop this bad practice. After a heavy rainfall, animals should not be allowed to feed on freshly shoots of grass, particularly grown on farmyard and grazing fields where animals were recently

Dhaka University Institutional Repository

slaughtered or dead animals were disposed of. Any dead animal must not be disposed of into open water or on field, but buried on a dry land sufficiently away from any water body following deep burial method under veterinary supervision.

4.2 Molecular characterization of *B. anthracis*

4.2.1 Molecular characterization of *B. anthracis* by using 15 VNTR loci

B. anthracis is considered one of the bacteria with a high degree of genetic homogeneity and this feature makes it difficult to discriminate among the bacterial strains. The phenomenon of high genetic homogeneity is motivated by the high spore survival capacity which has allowed *B. anthracis* to multiply a relatively limited number of times during its evolution.

This study was conducted analyzing 169 samples (73 soil, 1 tissue, 4 bone and 91 bone meal samples) collected from 12 different districts of Bangladesh. The sampling was carried out from 2012 to 2015. This study reveals to be an important tool for the active surveillance based on the monitoring of suspected anthrax foci.

For this reason, it was important to collect different kind of samples in order to inspect the reason of anthrax transmission, control the spread of the disease, and consequentially reduce the phenomenon. Thus, a total of 91 bone meal samples were collected from the cattle feed importer or wholesaler agent from 17 Upazila/Thanas of 10 districts of Bangladesh (Table 2.2.1) to detect the association between anthrax transmission and imported cattle feed (bone meal). Furthermore, as several outbreak reports reveal that the disease is most prevalent in Sirajganj and nearby districts like Tangail and Pabna (Ahmed et al., 2010; Biswas et al., 2012) a total of 73 soil samples were collected from 9 Upazila/Thanas of these three districts, randomly picking soil from homestead or burial sites with or without having the history of anthrax cases (Table 2.2.1). All of the 91 bone meal samples were negative and the results might nullify the hypothesis of anthrax transmission through imported cattle feed. On the other hand, 10 soil samples were positive out of 73. Here, each soil sample represented individual farm of the village with or without having the previous history of anthrax outbreak. After the isolation of *B. anthracis*, biomolecular assays allowed to find out the genetic diversity among the

isolates of the same and different samples and to depict the persistence of the organism in the environmental samples rather than the calculation of prevalence of the disease.

Using the CanSNP analysis, it has been possible to understand the phylogenetic origin of isolates and confirm that all the strains analyzed in this study, belonged to A.Br. 001/002, typical of strains circulating in China and other countries in South-East Asia.

MLVA is a standard tool for *B. anthracis* genotyping. This molecular technique has proved to be useful for molecular typing of *B. anthracis* is the analysis of VNTR sequences. These are short nucleotide sequences, tandemly repeated and in a variable number of copies which give rise to length polymorphisms easily detectable by the PCR technique. The several loci analyzed, such as hypervariable regions, can be used for discrimination between different strains. The analysis of these hypervariable regions with methods such as MLVA is a valuable tool for studying the diversity, evolution and molecular epidemiology of *B. anthracis*. Because of the high homoplasy of VNTR loci, MLVA utility is limited by the difficulties in understanding how these genotypes are related to each other. Nevertheless, MLVA represents a valid method for gaining an overall view on the genetic diversity of *B. anthracis* within a country. The MLVA analysis of the isolates revealed the presence of four genotypes: GT1/Ban, GT2/Ban, GT3/Ban and GT4/Ban. All the samples with these genotypes came from Sirajganj district, in Nothern Bangladesh. In particular, GT/2 Ban has been found also in Tangail district, geographically very close to Sirajganj. Gt1/Ban differed from GT2/Ban, in the size of pXO1, from GT3/Ban in relation to vrra and vntr32 and from GT4/Ban for the variation in locus vntr32. GT1/Ban was already identified in a previous study by Fasanella et al. (2013b) and it was named as GT/KamBel, that took the name from the area from which they were isolated: Kamarkhand and Belkuchi. GT3/Ban was also identified in the same study and called GT/ChU because the genotype was the same in Chitulia village and Ullapara subdistrict. GT2/Ban and GT4/Ban resulted as new genotypes, earlier undiscovered (Table 3.2.1) (Figure 3.2.2).

For what concerns the SNR analysis, it has been demonstrated a high discriminatory power among all the isolates with 8 SNR types detected. These findings confirm the ability of SNRs to mutate rapidly, suggesting the presence of mutational step during the

multiple replications. In our study, this hypothesis is strengthened by presence of two different sub-genotypes in the same sample: subGT1 and subGT2 identified within the GT1/Ban deriving from a unique soil sample. This occurs also for subGT6 and subGT7 within GT3/Ban, discovered in isolates coming from a distinct soil sample (Table 3.2.2).

Thus, in this context, the SNR analysis detecting sub-genotypes, allows one to understand how many variations are in the same genotype, paving the way to further investigations that might explain which other causes intervene in the genetic variations of *B. anthracis*. The presence of genetic variations identified using MLVA and SNR analysis allows us to understand that the diversity within the A.Br. 001/002 group arises from the evolution of a strain ecologically established and not recently introduced.

In the present study the high percentage of positive soil samples allows us to consider soil as one of the major source for the spread of *B. anthracis* and for the animal infection. The practice of burial of dead animals and the improper removal of infected carcasses are the mainstay of causes that determine the contamination and persistence of bacteria in the environment.

The negative results of bone meal samples probably nullify the hypothesis of anthrax transmission through the imported cattle feed supplement; may be a good news for the cattle feeds trades of Bangladesh.

4.2.2 Whole genome sequencing of *B. anthracis*

MLVA data are notoriously difficult to extract from whole genome projects since sequence alignments often fail because of their partially long repetitive DNA-structures. Typically MLVA is used in a series of progressive hierarchical resolving assays for subtyping *B. anthracis* following canSNP-typing because of MLVA’s higher resolution power (Keim et al., 2004). MLVA genotyping has rather limited resolution that does not provide phylogenetically useful information. Thus, this technique more and more becomes superseded by interrogating for genome-wide phylogenetically informative SNPs.

Phylogenetic analysis using chromosomal SNP analysis reveal the presence of two distinct Bangladeshi regional clusters of *B. anthracis* genotypes for Sirajganj and Tangail, B1 and B2, respectively, a novel finding of this present study.

Separated by 67 SNPs, the Bangladeshi strains fall into two distinct sub-groups B1 and B2. The Bangladesh strains showed the ancestral alleles for these both SNPs regardless of their genotypic sub-group. The Dutch isolates compared with the Bangladeshi isolates are clearly separated from B2, whereas the distance between B1 and the Dutch strains is 46 SNPs and between the German strain A163 and B1 is 22 SNPs. Because of close relatedness between B1 and the European strains mentioned, there might be a probability of transmission of this genotype from South or Southeast Asian countries to central Europe or vice versa. However, the source of such introduction into South eastern and South Asian countries including Bangladesh is difficult to verify because meta-data on the origins of older European strains are often fragmentary and MLVA-data for individual markers (MLVA-31) of many strains in collections are largely not published (Derzelle and Thierry, 2013).

4.2.3 Circulating genotypes of *B. anthracis*: comparisons with other previous studies

Using MLVA 8 VNTR loci Chakraborty et al. (2012) reported two genotypes (GT 152 and GT 155) by investigating samples collected from 2009 to 2010. According to that report, the MLVA-8 genotype identified from outbreaks in 2010 differed from the isolates of outbreaks occurred in 2009, and the difference was by one locus (pXO1). Difference in the causal genotypes suggests that the outbreaks in the years 2009 and 2010 were unlikely to be attributed to the spread of the same strain from one place to another. Later, by MLLVA 8 VNTR loci Fasanella et al. (2013b) identified two new genotypes (Gt/KamBel or Gt_1/Sir and Gt/Chu or Gt_3/Sir) in the isolates isolated from the areas of outbreaks reported in 2010. Using increased number of VNTRs, the genotypes identified were discriminated further into more subgenotypes (but data on whole genome sequence of five strains from Bangladesh: 3 from Sirajgonj and 2 from Tangail are only available from this present study. These genomic data clearly indicate that two specific genotypic clusters of the organisms are circulating in these two areas. They might have evolved in those areas with slow natural mutation over time.

Dhaka University Institutional Repository

4.3 Anthrax vaccine immunogenicity

Anthrax has been a major cause of death in grazing animals and an occasional cause of death in humans for thousands of years. The control of anthrax in animals and humans depends on its prevention in livestock, principally cattle, sheep, and goats. Since the late 1800s there has been an exceptional international history of anthrax vaccine development. Due to animal vaccinations, the rate of infection has dropped dramatically. Most anthrax vaccines for animals utilize the *B. anthracis* strain 34F2, developed by Max Sterne in 1937 (Sterne, 1939a) which lacks genes for capsule formation but still produces the toxin (pXO1+/pXO2–), responsible for the induction of protective antibodies.

The officials of the Livestock Research Institute explained that the Government of Bangladesh has two laboratories that produce anthrax vaccine that contains F-24 strain of *B.anthracis* (Roy et al. 2013). Although these two laboratories have a target production of 5.1 million vaccine doses annually, their average annual vaccine production for the last 6 years (July 2004 to June 2010) was 3.8 million doses, whereas the country's total ruminant population is approximately 48.7 million (Tareque and Chowdhury, 2010). Therefore, there has been consistent shortage of anthrax vaccine in this country. Furthermore, the immunogenic properties and efficacy of this local vaccine are not well known. Only a few studies on immunological assessment of this anthrax vaccine have been conducted in cattle and goats (Dipti et al., 2013; Roy et al., 2013). It is worth noting that the immunogenicity of local vaccine should be evaluated and control strategy should be setup accordingly to confront the remerging challenge of anthrax. Therefore in this study, the immunogenicity of the anthrax vaccine was assessed between vaccinated and non vaccinated cattle and also between the sera of the hyperimmunize and non-immunize mice by using immunoblotting assay.

In this study, the advantage of immunological analysis was taken to assess the local vaccine efficacy. The spores are crucial to infection and persistence of *B. anthracis*, and the spores and vegetative cells can elicit a humoral immune response in the course of bacterial infections. Therefore, we used the vegetative form of *B. anthracis* vaccine strain Sterne F-24 to identify immunogenic proteins that might augment the protective

Dhaka University Institutional Repository

efficacy of the local vaccines. In this study, the efficacy of the vaccine against *B. anthracis* was evaluated in the mice model.

In this study, the protein concentration of whole cell extract was 0.6mg/ml. Multiple proteins with different molecular weight ranging from 120 to 26 kDa were detected from whole cell extract of the anthrax vaccine. By Western blot analysis, no bands were detected in non-vaccinated cattle and the control mice. But bands were detected of about 36 kDa proteins from all the vaccinated cattle. In case of hyperimmune mice, bands were detected with 83 to 35kDa proteins. In a study by Liu et al. (2013), immunoproteomics was used to screen the immunogenic spore and vegetative proteins of *B. anthracis* vaccine strain A16R. Eleven and 45 immunogenic proteins were identified in the spores and vegetative cells, respectively. To verify their immunogenicity, 12 of the identified proteins were selected to be expressed, and the immune sera from the mice vaccinated by the 12 expressed proteins, except one (BA0887), had a specific Western blot band with the A16R whole cellular lytic proteins. According to Liu et al. (2013), Gap-2 gene is responsible for the production of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein having the molecular weight of 35974. This protein is found both in spore and vegetative cells. The molecular weight of our studied protein of the vaccinated cattle and hyperimmune mice was about 36 kDa, which is similar or may be related to BA5369 found by Liu et al. (2013). Shin et al. (2007) assessed the pathogenicity and immunogenicity of *Streptococcus iniae* and found that GAPDH, fructose bisphosphate and enolase had high immunogenicity. It has been reported that GAPDH (BA5369) can bind various mammalian proteins, such as lysozyme, fibronectin and cytoskeletal proteins myosin and actin (Lei et al., 2000; Len et al., 2004; Nakamura et al., 2004). Therefore, it can be concluded that GAPDH plays an important role in adhesion to host cells and their colonization. Delvecchio and associates concluded that GAPDH is involved in spore germination, energy metabolism and strain growth (Delvecchio et al., 2006). On the other hand, an 83 kDa was identified in the hyperimmune mice sera but not in the vaccinated cattle sera. This 83 kDa protein could be related to the protective antigen (PA), which also has got a molecular weight of 83 kDa. PA is an important component of the anthrax toxin and this protein plays a major role in anthrax immunity after both immunization and infection (Shlyakhov et al., 1997). Of the three proteins, EF, LF and PA, only PA elicits antibodies that are protective against the disease (Vodkin and

Leppla, 1983; Singh et al., 1989). This immunity is thought to occur as a result of neutralizing the activity of the anthrax toxin (Ezzell and Abshire, 1988). Antibodies to PA will either block the protein from binding to host cell receptors or once bound will block the action of furin cleavage. Either situation renders PA biologically inactive. Without active PA bound to the cell, EF and LF cannot enter the cell. Thus, the anthrax toxin's influence on the host is halted (Singh et al., 1998). Since PA is the only antigen known to induce protective antibodies against anthrax, therefore, the protein has become the main focus of anthrax vaccine research (Ivins and Welkos, 1988; Turnbull, 1991). PA, when produced in the absence of LF and EF, has been shown to be capable of producing effective protection both as a purified protein and when used in a recombinant or attenuated vaccine (Turnbull, P. 1991). However, protection studies have shown that high antibody titers to PA do not correlate with level of protection (Turnbull et al., 1986; Ezzell and Abshire, 1988). In fact, the veterinary live spore vaccine produced from the Sterne strain of *B. anthracis*, gives better and more prolonged protection against infection by the bacterium than merely adjuvanted PA even though antibody levels induced are much lower (Sterne, 1937; Sterne, 1939b; Ezzell and Abshire, 1988; Turnbull, 1991; Shlyakhov et al., 1997).

In the present study, our local anthrax vaccine strain has the immunogenic properties to produce anti anthrax IgG antibody both for vaccinated cattle and hyperimmune mice. But the anti-anthrax IgG antibody against PA, a key component of the vaccine is absent in vaccinated sera of cattle but present in hyperimmune mice. It may be due to difference in pXO1 VNTR locus size. The pXO1 VNTR locus size of Sterne strain is 129 bp (Jung et al., 2012) and the pXO1 VNTR locus sizes of Bangladeshi field isolates are 121 bp (Rume et al., 2016), 127 bp (Rume et al., 2016; Fasanella et al., 2013b), 129 bp (Fasanella et al., 2013b) and 132 bp (Chakraborty et al., 2012). Due to the difference of pXO1 molecular structure between Stern strain and Bangladeshi field isolates, local vaccine might not produce sufficient protection against the new genotypes of the organism circulating in Bangladesh. Whether this genetic mismatching between the vaccine strain and the strains circulating in the country is contributing to vaccine failure needs to be studied further. The protective effect of a single dose of this vaccine is said to last for 9 to12 months so annual boosters are recommended in endemic areas (Sterne, 1939b). Also, a single dose of Sterne vaccine may not be sufficient to ensure protective

Dhaka University Institutional Repository

immunity in the animal to last for a year, and more than one initial dose of the Sterne vaccine may be necessary (Turnbull et al., 2004; Mongoh et al., 2008b; WHO, 2008). Antibodies against *B. anthracis* toxins were detected in unvaccinated herbivores that had grazed in anthrax risk areas (Fasanella et al., 2003), consistent with sub-clinical exposure to anthrax bacilli or spores during grazing or through fly bites. The protective effect of such low-level immune responses is unclear. Even the immune responses to vaccination may be insufficient to protect animals if the environmental concentration of spores is very high; immune memory to the Sterne-type animal vaccines may be poor and regular vaccinations are required in high endemic or epidemic areas, together with prompt diagnosis and appropriate carcass destruction (Hudson et al., 2008). Our results on the immunogenic properties of anthrax vaccine in cattle suggest that animal should be boosted for another two times at 2-3 week intervals for the development of antibody against protective antigen.

To intensify the fight against the disease, the improvement of the Sterne vaccine is essential, because while it is valid in programs of disease control, in emergency situations only 80% of vaccinated animals are protected starting from 7 days from vaccination (Fasanella et al., 2008). In addition, the Sterne vaccine is a live attenuated vaccine and therefore cannot be used in animals during antibiotic therapy. A cheap, improved, livestock vaccine is needed that can be used with antibiotics. Additionally, the sufficient supply of vaccine, trained personal for vaccination and effective vaccine strategy should be ensured in anthrax prone areas to control the disease.

Finally, the present study unravels the antigenic vegetative proteins of *B. anthracis* of the local anthrax vaccine that may be potential candidates in the future for therapeutics and vaccines development. The immunologic approach is a valuable tool for studying and identifying immunogenic proteins for use in vaccine development. This study would help to make documentation on anthrax vaccine, providing information for prevention and control strategies against anthrax and also help to develop a framework for strengthening the capacity to confront the threats of anthrax in Bangladesh.

Dhaka University Institutional Repository

References

Dhaka University Institutional Repository

References

Ahmed B, Sultana Y, Haque MM, Karim MR, Rahman D. 2001. *Bacillus anthracis*: an agent of biological weapon. JOPSOM. **20:** 45-50.

Ahmed BN, Sultana Y, Fatema DSM, Ara K, Begum N, Mostanzid SM, Jubayer S. 2010. Anthrax: An emerging zoonotic disease in Bangladesh. Bangladesh J Med Microbiol. **4:** 46-50.

Ahsan MM, Khan MFR, Rahman MB, Hassan J, Chowdhury SMZH, Parvej MS, Jahan M, Nazir KHMNH. 2013. Investigation into *Bacillus anthracis* spore in soil and analysis of environmental parameters related to repeated anthrax outbreak in Sirajganj, Bangladesh. Thai J Vet Med. **43:** 449-454.

Aikembayev AM, Lukhnova L, Temiraliyeva G, Meka-Mechenko T, Pazylov Y, Zakaryan S, Denissov G, Easterday WR, Van Ert MN, Keim P, Francesconi SC, Blackburn JK, Hugh-Jones M, Hadfield T. 2010. Historical distribution and molecular diversity of *Bacillus anthracis*, Kazakhstan. Emerg Infect Dis. **16:** 789-796.

Alegria-Schaffer A, Lodge A, Vattem K. 2009. Performing and optimizing western blots with an emphasis on chemiluminescent detection. Methods Enzymol. **463:** 573-599.

Antelmann H, Scharf C, Hecker M. 2000. Phosphate starvation-inducible proteins of *Bacillus subtilis*: proteomics and transcriptional analysis. J Bacteriol. **182:** 4478-4490.

Antwerpen MH, Zimmermann P, Bewley K, Frangoulidis D, Meyer H. 2008. Real-time PCR system targeting a chromosomal marker specific for *Bacillus anthracis*. Mol Cell Probes. **22:** 313-315.

Bales ME, Dannenberg AL, Brachman PS, Kaufmann AF, Klatsky PC, Ashford DA. 2002. Epidemiologic response to anthrax outbreaks: field investigation, 1950-2001. Emerg Infect Dis. **8:** 1163-1174.

Bangladesh Bureau of Statistics (BBS). 2013. 2012 Statistical Yearbook of Bangladesh. Agriculture: Crops, Livestock, Forestry and Fishery, 32:125-126. http://www.bbs.gov.bd/WebTestApplication/userfiles/Image/SubjectMatterDataIndex/YB-2012.pdf, access 05/11/2015.

Bangladesh Agricultural Research Council (BARC) Bangladesh. 2010. Agricultural Research Priority: Vision-2030 and Beyond (Sub-sector: Livestock). Bangladesh Agriculture Research Council, Farmgate, Dhaka.

Bartlett J, Inglesby T, Borio L. 2002. Management of anthrax. Clin Infect Dis. **35:** 851-857.

Beall FA, Taylor MJ, Thorne CB. 1962. Rapid lethal effect in rats of a third component found upon fractionating the toxin of *Bacillus anthracis.* J Bacteriol. **83:** 1274-1280.

Beatty ME, Ashford DA, Griffin PM, Tauxe RV, Sobel J. 2003. Gastrointestinal anthrax: review of the literature. Arch Intern Med. **163:** 2527-2531.

Beveridge WIB. 1983. Anthrax. In: Animal Health in Australia. Bacterial diseases of cattle, sheep and goats. Canberra: Australian Government Publishing Service. 14-19.

Beyer W, Bellan S, Eberle G, Ganz HH, Getz WM, Haumacher R, Hilss KA, Kilian W, Lazak J, Turner WC, Turnbull PC. 2012. Distribution and molecular evolution of *Bacillus anthracis* genotypes in Namibia. PLoS Negl Trop Dis. **6:** e1534.

Birdsell DN, Pearson T, Price EP, Hornstra HM, Nera RD, Stone N, Gruendike J, Kaufman EL, Pettus AH, Hurbon AN, Buchhagen JL, Harms NJ, Chanturia G, Gyuranecz M, Wagner DM, Keim PS. 2012. Melt analysis of mismatch amplification mutation assays (Melt-MAMA): a functional study of a cost-effective SNP genotyping assay in bacterial models. PLoS ONE. **7:** e32866.

Biswas PK, Islam MZ, Shil SK, Chakraborty RK, Ahmed SS, Christensen JP. 2012. Risk factors associated with anthrax in cattle on smallholdings. Epidemiol Infect. **140:** 1888-1895.

Dhaka University Institutional Repository

Blackburn JK, Goodin DG. 2013. Differentiation of springtime vegetation indices associated with summer anthrax epizootics in west Texas, USA, deer. J Wildl Dis. **49:** 699-703.

Blake MS, Johnston KH, Russell-Jones GJ, Gotschlich EC. 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal Biochem. **136:** 175-179.

Blanc HW. 1890. Anthrax: the disease of the Egyptian plagues. New Orleans Med Surg J. **18:** 1-25.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem. **72:** 248-254.

Brossier F, Weber-Levy M, Mock M, Sirard J. 2000. Role of toxin functional domains in anthrax pathogenesis. Infect Immun. **68:** 1781-1786.

Brossier F, Mock M. 2001. Toxins of *Bacillus anthracis*. Toxicon. **39:** 1747-1755.

Burnette WN. 1981. Western blotting: electrophoretic transfer of proteins from dodecyl sulphate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem. **112:** 195-203.

Canas B, Pineiro C, Calvo E, Lopez-Ferrer D, Gallardo JM. 2007. Trends in sample preparation for classical and second generation proteomics. J Chromatogr. **1153:** 235-258.

Castellanos-Serra L. Paz-Lago D. 2002. Inhibition of unwanted proteolysis during sample preparation: evaluation of its efficiency of its efficiency in challenge experiments. Electrophoresis. **23:** 1745-1753.

Cataldi A, Mock M, Bentancor L. 2000. Characterization of *Bacillus anthracis* strains used for vaccination. J Appl Microbiol. **88:** 648-654.

Celli J, Chasteelier C, Franchini DM, Pizarro-Cerda J, Moreno E, Gorvel JP. 2003. *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J Exp Med. **198:** 545-556.

Centers for Disease Control and Prevention (CDC). 2001. Update: investigation of anthrax associated with intentional exposure and interim public health guidelines, October 2001. MMWR Morb Mortal Wkly Rep.50: 889-893.

Centers for Disease Control and Prevention (CDC). 2009a. Questions and answers about anthrax. (http://www.bt.cdc.gov/agents/anthrax/faq/, accessed on 30-11-2009).

Centers for Disease Control and Prevention (CDC). 2009b. Anthrax: Comprehensive CDC information about bioterrorism and related issues. (http://www.anthrax.osd.mil, accessed on 02-12-09).

Chabot DJ, Scorpio A, Tobery SA, Little SF, Norris SL, Friedlander AM. 2004. Anthrax capsule vaccine protects against experimental infection. Vaccine. **23:** 43-47.

Chakraborty A, Khan SU, Hasnat MA, Parveen S, Islam MS, Mikolon A, Chakraborty RK, Ahmed BN, Ara K, Haider N, Zaki SR, Hoffmaster AR, Rahman M, Luby SP, Hossain MJ. 2012. Anthrax outbreaks in Bangladesh, 2009-2010. Am J Trop Med Hyg. **86:** 703-710.

Cherwonogrodzky J. 1993. *Brucella* antigens-old dogmas, new concepts. Rev Lat-Am Microbiol. **35:** 339-344.

Cheville N, Stevens M, Jensen A, Tatum F, Halling S. 1993. Immune responses and protection against infection and abortion in cattle experimentally vaccinated with mutant strains of *Brucella abortus*. Am J Vet Res. **54:** 1591-1597.

Chitlaru T, Ariel N, Zvi A, Lion M, Velan B, Seffarman A, Elhanany E. 2004. Identification of chromosomally encoded membranal polypeptides of *Bacillus anthracis* by a proteomic analysis: prevalence of proteins containing S-layer homology domains. Proteomics. **4:** 677-691.

Dhaka University Institutional Repository

Cimons M. 1998. DoD move forward with anthrax vaccination program. Am Soc Microbiol News. **64:** 128-129.

Cloeckaert A, Weynants V, Godfroid J, Verger J, Grayon M, Zygmunt M. 1998. O-polysaccharide epitopic heterogenicity at the surface of *Brucella* spp. studied by enzyme-linked immunosorbent assay and flow cytometry. Clin. Diag. Lab Immun. **5:** 862-870.

Cloeckaert A, Grayon M, Grepinet O, Boumedine K. 2003. Classification of *Brucella* strains isolated from marine mammals by infrequent restriction site- PCR and development of specific PCR identification tests. Microbes Infect. **5:** 593-602.

Coldham NG, Woodward MJ. 2004. Characterization of the *Salmonella typhimurium* proteome by semi-automated two dimensional HPLC-mass spectrometry: detection of proteins implicated in multiple antibiotic resistance. J Proteome Res. **3:** 595-603.

Compton J (ed.). 1987. Military Chemical and Biological Agents: chemical and toxicologic properties. pp. 354-361.

Corbel MJ. 1997. Brucellosis: an overview. Emerg Infect Dis. **3:** 213-321.

Cosivi O, Corbel M. 1998. WHO consultation on the development of new/improved brucellosis vaccines. Biologicals. **26:** 361-363.

Coulson N, Fulop M, Titball R. 1994. *Bacillus anthracis* protective antigen, expressed in *Salmonella typhimurium* SL3261, affords protection against anthrax spore challenge. Vaccine. **12:** 1395-1401.

Curtis A, Heath S, Hugh-Jones M. 2005. GIS Investigations of Epizootics: The Limitations of Surveillance Data. In: Majumdar SK, Huffman J, Brenner F, Panah IA (eds.). Wildlife Diseases: Landscape Epidemiology. Spatial Distribution and Utilization of Remote Sensing Technology. The Pennsylvania Academy of Science, Easton, PA. pp. 459-474.

Dhaka University Institutional Repository

Daffonchio D, Cherif A. Borin S. 2000. Homoduplex and heteroduplex polymorphisms of the amplified ribosomal 16S-23S internal transcribed spacers describe genetic relationships in the "*Bacillus cereus* group". Appl Environ Microbiol. **66:** 5460-5468.

Dai Z, Koehler T. 1997. Regulation of anthrax toxin activator gene (atxA) expression in *Bacillus anthracis*: temperature, not CO2/bicarbonate, effects AtxA synthesis. Infect Immun. **65:** 2576-2582.

Davis L, Kuehl M, Battey J. 1994. Basic methods in molecular biology. 2nd edition. Appleton & Lange, Norwalk, Connecticut.

Delvecchio VG, Connolly JP, Alefantis TG, Walz A, Quan MA, Patra G, Ashton JM, Whittington JT, Chafin RD, Liang X, Grewal P, Khan AS, Mujer CV. 2006. Proteomic profiling and identification of immunodominant spore antigens of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*. Appl Environ Microbiol. **72:** 6355-6363.

Derzelle S, Girault G, Kokotovic B, Angen O. 2015a. Whole genome-sequencing and phylogenetic analysis of a historical collection of *Bacillus anthracis* strains from Danish cattle. PLoS One. **10:** e0134699.

Derzelle S, Girault G, Roest HI, Koene M. 2015b. Molecular diversity of *Bacillus anthracis* in the Netherlands: Investigating the relationship to the worldwide population using whole-genome SNP discovery. Infect Genet Evol. **32:** 370-376.

Dipti M, Rashid MM, Ferdoush MJ, Roy P, Khan MAHNA, Hossain MM. 2013. Morphological and immunological characterization of anthrax vaccine in cattle. Bangladesh J Vet Med. **11:** 43-49.

Dixon TC, Meselson M, Guillemin J, Hanna PC. 1999. Anthrax. N Engl J Med. **341:** 815-826.

Dragon DC, Rennie RP. 1995. The ecology of anthrax spores: tough but not invincible. Can Vet J. **36:** 295-301.

Dhaka University Institutional Repository

Dragon DC, Elkin BT, Nishi JS, Ellsworth TR. 1999. A review of anthrax in Canada and implications for research on the disease in northern bison. J Appl Microbiol. **87:** 208-213.

Dragon DC, Rennie RP. 2001. Evaluation of spore extraction and purification methods for selective recovery of viable *Bacillus anthracis* spores. Lett Appl Microbiol. **33:** 100-105.

Duesbery N, Webb C, Leppla S, Gordon V, Klimpel K, Copland T, Ahn N, Oskarsson M, Fukasawa K, Paul K, Vande Wonde G. 1998. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science. **280:** 734-737.

Durmaz R, Doganay M, Sahin M, Percin D, Karahocagil MK, Kayabas U, Otlu B, Karagoz A, Buyuk F, Celebi O, Ozturk Z, Ertek M. 2012. Molecular epidemiology of the *Bacillus anthracis* isolates collected throughout Turkey from 1983 to 2011. Eur J Clin Microbiol Infect Dis. **10:** 2783-2790.

Durrheim DN, Freeman P, Roth I, Hornitzky M. 2009. Epidemiologic questions from anthrax outbreak, hunter valley, Australia. Emerg Infect Dis. **15:** 840-842.

Dutz W, Kohout-Dutz E. 1981. Anthrax. Int J Derm. **20:** 203-206.

Easterday WR, Van Ert MN, Simonson TS, Wagner D., Kenefic LJ, Allender CJ, Keim P. 2005. Use of single nucleotide polymorphisms in the *plcR* gene for specific identification of *Bacillus anthracis*. J Clin Microbiol. **43:** 1995-1997.

Ezzell J, Abshire T. 1988. Immunological analysis of cell-mediated antigens of *B. anthracis* . Infect Immun. **56:** 349-356.

Fasanella A, Losito S, Trotta T, Adone R, Massa S, Ciuchini F, Chiocco D. 2001. Detection of anthrax vaccine virulence factors by polymerase chain reaction. Vaccine. **19:** 4214-4218.

Dhaka University Institutional Repository

Fasanella A, Losito S, Adone R, Ciuchini F, Trotta T, Altamura SA, Chiocco D, Ippolito G. 2003. PCR assay to detect *Bacillus anthracis* spores in heat-treated specimens. J Clin Microbiol. **41:** 896–899.

Fasanella A, Tonello F, Garofolo G, Muraro L, Carattoli A, Adone R, Montecucco C. 2008. Protective activity and immunogenicity of two recombinant anthrax vaccines for veterinary use. Vaccine. **26:** 5684-5688.

Fasanella A, Scasciamacchia S, Garofolo G. 2009. The behaviour of virulent *Bacillus anthracis* strain AO843 in rabbits. Vet Microbiol. **133:** 208-209.

Fasanella A, Garofolo G, Galante D, Quaranta V, Palazzo L, Lista F, Adone R, Jones MH. 2010a. Severe anthrax outbreaks in Italy in 2004: considerations on factors involved in the spread of infection. New Microbiol. **33:** 83-6.

Fasanella A, Galantea D, Garofoloa G, Hugh Jones M. 2010b. Anthrax undervalued zoonosis. Vet Microbiol. **140:** 318-331.

Fasanella A, Di Taranto P, Garofolo G, Colao V, Marino L, Buonavoglia D, Pedarra C, Adone R, Hugh-Jones M. 2013a. Ground Anthrax *Bacillus* Refined Isolation (GABRI) method for analyzing environmental samples with low levels of *Bacillus anthracis* contamination. BMC Microbiol. **13:** 167.

Fasanella A, Garofolo G, Hossain MJ, Shamsuddin M, Blackburn JK, Hugh-Jones M. 2013b. Bangladesh anthrax outbreaks are probably caused by contaminated livestock feed. Epidemiol Infect. **141:** 1021-1028.

Fatema TZ. 2011. Standardization of an ELISA protocol for the detection of IgG antibody in cattle against anthrax vaccine. MS Thesis submitted to Dept. of Pathology, BAU, Bangladesh. pp. 45-55.

Fellows PF, Linscott MK, Ivins BE, Pitt ML, Rossi CA, Gibbs PH, Friedlander AM. 2001. Efficacy of a human anthrax vaccine in guinea pigs, rabbits, and rhesus macaques against challenge by *Bacillus anthracis* isolates of diverse geographical origin. Vaccine. **19:** 3241-3247.

Dhaka University Institutional Repository

Firdaus RM, Ahmad HA, Sharum MY, Azizi N, Mohamed R. 2005. ProLysED: an integrated database and meta-server of bacterial protease systems. Appl Bioinformatics. **4:** 147-150.

Flick-Smith HC, Eyles JE, Hebdon R, Waters EL, Beedham RJ, Stagg TJ, Miller J, Alpar HO, Baillie LW, Williamson ED. 2002. Mucosal or parenteral administration of microsphere-associated *Bacillus anthracis* protective antigen protects against anthrax infection in mice. Infect Immun. **70:** 2022-2028.

Food and Agriculture Organization (FAO) of the United Nations. 2001. Anthrax in Animals (http://www.fao.org/ag/magazine/0112sp.htm), Rome. Accessed 5 December 2010.

Fox MD, Kaufmann AF, Zendel SA, Kolb RC, Songy CG Jr, Cangelosi DA, Fuller CE. 1973. Anthrax in Louisiana, 1971: epizootiologic study. J Am Vet Med Assoc. **163:** 446-451.

Fraser C, Mays A (eds.). 1986. In: The Merck Veterinary Manual. pp. 639-645.

Friedlander AM. 1986. Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. J Biol Chem. **261:** 7123-7126.

Friedlander AM, Welkos SL, Pitt ML, Ezzell JW, Worsham PL, Rose KJ, Ivins BE, Lowe JR, Howe GB, Mikesell P, et al. 1993. Postexposure prophylaxis against experimental inhalation anthrax. J Infect Dis. **167:**1239-1242.

Freidlander AM. 1997. Anthrax. In: Sidell FR, Takafuji ET, Franz DR (eds.). Medical aspects of chemical and biological warfare. Office of the Surgeon General, Washington, D.C. pp. 467-478.

Fritz DL, Jaax NK, Lawrence WB, Davis KJ, Pitt ML, Ezzell JW, Friedlander AM. 1995. Pathology of experimental anthrax in the rhesus monkey. Lab Invest. **73:** 691-702.

Dhaka University Institutional Repository

Garofolo G, Ciammaruconi A, Fasanella A, Scasciamacchia S, Adone R, Pittiglio V, Lista F. 2010. SNR analysis: molecular investigation of an anthrax epidemic. BMC Vet Res. **6:** 11.

Garofolo G, Serrecchia L, Corro M, Fasanella A. 2011. Anthrax phylogenetic structure in Northern Italy. BMC Res Notes. **4:** 273.

Gates CC, Elkin B, Dragon D. 2001. Anthrax. In: Williams ES, Barker IK (eds.). Infectious Diseases of Wild Mammals. 3rd Ed. Iowa State University Press. Ames, IA. pp. 396-412

Gaur R, Gupta P, Banerjea A, Singh Y. 2002. Effect of nasal immunization with protective antigen of *Bacillus anthracis* on protective immune response against anthrax toxin. Vaccine. **20:** 2836-2839.

Gorg A, Weiss W, Dunn MJ. 2004. Current two-dimensional electrophoresis technology for proteomics. Proteomics. **4:** 3665-3685.

Gracey JF, Collins DS, Huey RJ. 1999. Meat Hygiene, 10th edition. Philadelphia: W. B. Saunders. pp. 768.

Greenfield R, Bronze M. 2003. Prevention and treatment of bacterial diseases caused by bacterial bioterrorism threat agents. Drug Discov Today. **8:** 881-888.

Guidi-Rontani C, Duflot E, Mock M. 1997. Anthrax lethal toxin-induced mitogenic response of human T-cells. FEMS Microbiol Lett. **157:** 285-289.

Gupta S, Ghosh T. 2001. Gene expressivity is the main factor in dictating the codon usage variation among the genes in *Pseudomonas aeruginosa*. Gene. **273:** 63-70.

Gupta, P., S.M. Waheed, R. Bhatanagar. 1999. Expression and purification of the recombinant protective antigen of *Bacillus anthracis*. Prot. Express. Purif. **16:** 369-376.

Gutmann DS, Dykhuizen DE. 1994. Clonal divergence in *Escherichia coli* as a result of recombination, not mutation. Science. **266:** 1380-1383.

Haines BW, Klein F, Lincoln RE. 1965. Quantitative assay for crude anthrax toxins. J Bacteriol. **89:** 74-83.

Halperin G, Marcus H. 2001. Application of recovery tests in validation of immunoassays for assessing the immunogenicity of *B. anthracis* PA vaccine. PDA J Pharm Sci Technol. **55:** 150-161.

Hambleton P, Carman JA, Melling J. 1984. Anthrax: the disease in relation to vaccines. Vaccine. **2:** 125-132.

Hanna PC, Ireland JA. 1999. Understanding *Bacillus anthracis* pathogenesis. Trends Microbiol. **7:** 180-182.

Hanson JF, Taft SC, Weiss AA. 2006. Neutralizing antibodies and persistence of immunity following anthrax vaccination. Clin Vac Immun. **13:** 208-213.

Hassan J, Rahman MB, Chowdhury SMZH, Rabidas SK, Parvej MS, Nazir KHMNH. 2015. ELISA based anthrax antibody titer in cattle induced by locally prepared anthrax vaccine originated from Sterne F-24 strain in Bangladesh. Microbes and Health. **4:** 36-38

Hawkes R, Niday E, Gordon J. 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal Biochem. **119:** 142-147.

Hicks CW, Sweeney DA, Cui X, Li Y, Eichacker PQ. 2012. An overview of anthrax infection including the recently identified form of disease in injection drug users. Intensive Care Med. **38:** 1092-1104.

Hilleman M. 2002. Overview: cause and prevention in biowarfare and bioterrorism. Vaccine. **20:** 3055-3067.

Dhaka University Institutional Repository

Hoffmaster AR, Fitzgerald CC, Ribot E, Mayer LW, Popovic T. 2002. Molecular subtyping of *Bacillus anthracis* and the 2001 bioterrorism-associated anthrax outbreak, United States. Emerg Infect Dis. **8:** 1111-1116.

Hudson MJ, Beyer W, Bohm R, Fasanella A, Garofolo G, Golinski R, Goossens PL, Hahn U, Hallis B, King A, Mock M, Montecucco C, Ozin A, Tonello F, Kaufmann SH. 2008. *Bacillus anthracis*: balancing innocent research with dual-use potential. Int J Med Microbiol. **298:** 345-364.

Hugh-Jones ME. 1999. 1996-97 Global anthrax report. J Appl Microbiol. **87:** 189-191.

Hugh-Jones M. 2006. "Distinguishing between natural and unnatural outbreaks of animal diseases," in Biological disasters of animal origin: the role and preparedness of veterinary and public health services. OIE Sci Tech Rev. **25:** 173-186.

Hugh-Jones M, Blackburn J. 2009. The ecology of *Bacillus anthracis*. Mol Aspects Med. **30:** 356-367.

Iacono-Connors L, Welkos S, Ivins B, Dalrymple J. 1991. Protection against anthrax with recombinant virus-expressed protective antigen in experimental animals. Infect Immun. **59:** 1961-1965.

Ibrahim K, Brown G, Wright D, Rotschafer J. 1999. *Bacillus anthracis*: medical issues of biologic warfare. Pharmacotherapy. **19:** 690-701.

Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Friedlander AM, Hauer J, McDade J, Osterholm MT, O'Toole T, Parker G, Perl TM, Russel PK, Tonat K. 1999. Anthrax as a biological weapon-medical and public health management. JAMA. **281:** 1735-1745.

Inglesby TV, O'Toole T, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Friedlander AM, Gerberding J, Hauer J, Hughes J, McDade J, Osterholm MT, Parker G, Perl TM, Russell PK, Tonat K. 2002. Anthrax as a biological weapon, 2002: updated recommendations for management. JAMA. **287:** 2236-2252.

International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). 2009. Cutaneous anthrax outbreaks in two districts of North-Western Bangladesh, August-October, 2009. Health Sci Bul. **7:** 1-8.

International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). 2011. Recurrent animal and human anthrax outbreaks in Bangladesh: Improved vaccination strategies needed. Health Sci Bul. **9:** 8-14.

Islam S, Hossain MJ, Mikolon A, Parveen S , Khan MSU, Haider N, Chakraborty A, Titu AMN, Rahman MW, Sazzad HMS, Rahman M, Gurley ES, Luby SP. 2013. Risk practices for animal and human anthrax in Bangladesh: an exploratory study. Infect Ecol Epidemiol. **3:** 10.3402/iee.v3i0.21356.

Ivins BE, Welkos SL. 1986. Cloning and expression of the *Bacillus anthracis* protective antigen gene in Bacillus subtilis. Infect Immun. **54:** 537-542.

Ivins B. 1988. The search for a new-generation human anthrax vaccine. Clin Immun Newsletter. **9:** 30-32.

Ivins BE, Welkos SL. 1988. Recent advances in the development of an improved, human anthrax vaccine. Eur J Epidemiol. **4:** 12-19.

Ivins BE, Welkos SL, Little SF, Crumrine MH, Nelson GO. 1992. Immunization against anthrax with *Bacillus anthracis* protective antigen combined with adjuvants. Infect Immun. **60:** 662-668.

Ivins B, Fellows P, Nelson G. 1994. Efficacy of a standard human anthrax vaccine against *B. anthracis* spore challenge in guinea pigs. Vaccine. **12:** 872-874.

Ivins B, Fellows P, Pitt L, Estep J, Farchaus J, Friedlander A, Gibbs P. 1995. Experimental anthrax vaccines: efficacy of adjuvants combined with protective antigen against an aerosol *Bacillus anthracis* spore challenge in guinea pigs. Vaccine. **13:** 1779-1784.

Dhaka University Institutional Repository

Jacob D, Sauer U, Housley R, Washington C, Sannes-Lowery K, Ecker DJ, Sampath R, Grunow R. 2012. Rapid and high-throughput detection of highly pathogenic bacteria by Ibis PLEX-ID technology. PLoS ONE. **7:** e39928.

Jones CT, Hunt RD, King NW. 1997. Veterinary Pathology. 6th Edn. William and Wilkins, USA. p. 415.

Jung KH, Kim SH, Kim SK, Cho SY, Chai JC, Lee YS, Kim JC, Kim SJ, Oh HB, Chai YG. 2012. Genetic populations of Bacillus anthracis isolates from Korea. J Vet Sci.13(4):385-93.

Kanafani ZA, Ghossain A, Sharara AI, Hatem JM, Kanj SS. 2003. Endemic gastrointestinal anthrax in 1960s Lebanon: clinical manifestations and surgical findings. Emerg Infect Dis. **9:** 520-525.

Kanaya S, Yamada Y, Kudo Y, Ikemura T. 1999. Studies of codon usage and tRNA genes of 18 unicellular organisms and quantification of *Bacillus subtilis* tRNAs: gene expression level and species- specific diversity of codon usage based on multivariate analysis. Gene. **238:** 143-155.

Karlin S, Barnett M, Campbell A, Fisher R, Mrazek J. 2003. Predicting gene expression levels from codon biases in a-proteobacterial genomes. Proc Natl Acad Sci. **100:** 7313-7318.

Keim P, Price LB, Klevytska AM, Smith KL, Schupp JM, Okinaka R, Jackson PJ, Hugh-Jones ME. 2000. Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. J Bacteriol. **182:** 2928-2936.

Keim P, Smith KL, Keys C, Takahashi H, Kurata T, Kaufmann A. 2001. Molecular investigation of the Aum Shinrikyo anthrax release in Kameido, Japan. J Clin Microbiol. **39:** 4566-4567.

Keim P, Van Ert MN, Pearson T, Vogler AJ, Huynh LY, Wagner DM. 2004. Anthrax molecular epidemiology and forensics: using the appropriate marker for different evolutionary scales. Infect Genet Evol. **4:** 205-213.

Kenefic LJ, Beaudry J, Trim C, Daly R, Parmar R, Zanecki S, Huynh L, Van Ert MN, Wagner DM, Graham T, Keim P. 2008a. High resolution genotyping of *Bacillus anthracis* outbreak strains using four highly mutable single nucleotide repeat markers. Lett Appl Microbiol. **46:** 600-603.

Kenefic LJ, Beaudry J, Trim C, Huynh L, Zanecki S, Matthews M, Schupp J, Van Ert M, Keim P. 2008b. A high resolution four-locus multiplex single nucleotide repeat (SNR) genotyping system in *Bacillus anthracis*. J Microbiol Methods. **73:** 269-272.

Keppie J, Harris-Smith PW, Smith H. 1963. The chemical basis of the virulence of *Bacillus anthracis*. IX. Its aggressins and their mode of action. Br J Exp Pathol. **44:** 446-453.

Khanna H, Singh Y. 2001. War against anthrax. Mol Med. **7:** 795-796.

Kim C, Oh Y, Lee T. 1997. Codon opitimization for high-level expression of human erythropoietin (EPO) in mammalian cells. Gene. **199:** 293-301.

Klein F, DeArmon IA, Lincoln R, Mahlandt BG, Fernelius AL. 1962. Immunological studies of anthrax. II. Levels of immunity against *Bacillus anthracis* obtained with protective antigen and live vaccine. J Immunol. **88:** 15-19.

Koehler T, Dai Z, Kaufman-Yarbray M. 1994. Regulation of the *Bacillus anthracis* protective antigen gene: CO2 and a trans-acting element activate transcription from one of two promoters. J Bacteriol. **176:** 586-595.

Koehler TM. 2002. *Bacillus anthracis* genetics and virulence gene regulation. Curr Top Microbiol Immunol. **271:** 143-164.

Dhaka University Institutional Repository

Kreidl P, Stifter E, Richter A, Aschbachert R, Nienstedt F, Unterhuber H, Barone S, Huemer H P, Carattoli A, Moroder L, Ciofi Degli Atti ML, Rota MC, Morosetti G, Larcher C. 2006. Anthrax in animals and a farmer in Alto Adige, Italy. Euro Surveill. 11(7).

Krishnanchettiar S, Sen J, Caffrey M. 2002. Expression of purification of the *Bacillus anthracis* protective antigen domain 4. Prot Express Purif. **27:** 325-330.

Kurien BT, Scofield RH. 2003. Protein blotting: a review. J Immunol Methods. **274:** 1-15.

Kurien BT, Scofield RH. 2006. Western blotting. Methods. **38:** 283-293.

Kurien BT, Scofield RH. 2009. A brief review of other notable protein detection methods on blots. In: Kurien BT, Scofield RH (eds.). Methods in molecular biology, protein blotting and detection.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227:** 680-685.

Laforce FM. 1978. Woolsorters' disease in England. Bull NY Acad Med. **54:** 956-963.

Lei B, Mackie S, Lukomski S, Musser JM. 2000. Identification and immunogenicity of group A Streptococcus culture supernatant proteins. Infect Immun. **68:** 6807-6818.

Len Ac, Harty DW, Jacques NA. 2004. Proteome analysis of *Streptococcus mutans* metabolic phenotype during acid tolerance. Microbiology. **150:** 1353-1366.

Lewerin SS, Elvander M, Westermark T, Hartzell LN, Norstrom AK, Ehrs S, Knutsson R, Englund S, Andersson AC, Granberg M, Backman S, Wikstrom P, Sandstedt K. 2010. Anthrax outbreak in a Swedish beef cattle herd-1st case in 27 years: Case report. Acta veterinaria Scandinavica. **52:** 7.

Dhaka University Institutional Repository

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. 2009. The sequence alignment/map format and SAMtools. Bioinformatics. **25:** 2078-2079.

Lincoln RE, Walker JS, Klein F, Rosenwald AJ, Jones WI Jr. 1967. Value of field data for extrapolation in anthrax. Fed Proc. **26:** 1558-1562.

Lindeque PM, Turnbull PC. 1994. Ecology and epidemiology of anthrax in the Etosha National Park, Namibia. Onderstepoort. J Vet Res. **61:** 71-83.

Lista F, Faggioni G, Valjevac S, Ciammaruconi A, Vaissaire J, le Doujet C, Gorge O, De Santis R, Carattoli A, Ciervo A, Fasanella A, Orsini F, D'Amelio R, Pourcel C, Cassone A, Vergnaud G. 2006. Genotyping of *Bacillus anthracis* strains based on automated capillary 25-loci multiple locus variable-number tandem repeats analysis. BMC Microbiol. **6:** 33.

Liu X, Wang D, Ren J, Tong C, Feng E, Wang X, Zhu L, Wang H. 2013. Identification of the immunogenic spore and vegetative proteins of *Bacillus anthracis* vaccine strain A16R. PLoS ONE. **8:** e57959

Lucey D. 2005. *Bacillus anthracis* (anthrax). In: Mandell G, Bennett J, Dolin R. (eds.). Mandell, Douglas, and Bennett's principles and practice of infectious diseases. Philadelphia, PA: Churchill Livingstone pp. 2485-2491.

MacPhee DJ. 2010. Methodological considerations for improving western blot analysis. J Pharmacol Toxicol Methods. **61:** 171-177.

Marques MA, Espinosa BJ, Xavier da Silveira EK, Pessolani MC, Chapeaurouge A, Perales J, Dobos KM, Belisle JT, Spencer JS, Brennan PJ. 2004. Continued proteomic analysis of *Mycobacterium leprae* subcellular fractions. Proteomics. **4:** 2942-2953.

Dhaka University Institutional Repository

Mayer TA, Bersoff-Matcha S, Murphy C, Earls J, Harper S, Pauze D, Nguyen M, Rosenthal J, Cerva D Jr, Druckenbrod G, Hanfling D, Fatteh N, Napoli A, Nayyar A, Berman EL. 2001. Clinical presentation of inhalational anthrax following bioterrorism exposure: report of 2 surviving patients. JAMA. **286:** 2549-2553.

Mikesell P, Ivins BE, Ristroph JD, Dreier TM. 1983. Evidence for plasmid-mediated toxin production in *Bacillus anthracis.* Infect Immun. **39:** 371-376.

Milne I, Stephen G, Bayer M, Cock PJ, Pritchard L, Cardle L, Shaw PD, Marshall D. 2013. Using Tablet for visual exploration of second-generation sequencing data. Brief Bioinform. **14:** 193-202.

Minett FC. 1950. Sporulation and viability of *B. anthracis* in relation to environmental temperature and humidity. J Com Pathol. **60:** 161-176.

Mizrahi A (ed.). 1990. Bacterial vaccines. In: Advances in Biotechnological Processes. pp. 105-122.

Mohan RW, Ali SM. 1948. Some aspect of anthrax in Bengal. Indian J Vet Sci. **8:** 1-15.

Mongoh MN, Dyer NW, Stoltenow CL, Khaitsa ML. 2008a. Risk factors associated with anthrax outbreak in animals in North Dakota, 2005: A retrospective case-control study. Public Health Reports. **123:** 352-359.

Mongoh MN, Dyer NW, Stoltenow CL, Hearne R, Khaitsa ML. 2008b. A review of management practices for the control of anthrax in animals: the 2005 anthrax epizootic in North Dakota-case study. Zoonoses Public Health. **55:** 279-290.

Mondal SP, Yamage M. 2014. A retrospective study on the epidemiology of anthrax, foot and mouth disease, haemorrhagic septicaemia, peste des petits ruminants and rabies in Bangladesh, 2010-2012. PLoS ONE. **9:** e104435.

Moore ER, Mihaylova SA, Vandamme P, Krichevsky MI, Dijkshoorn L. 2010. Microbial systematics and taxonomy: relevance for a microbial commons. Res Microbiol. **161:** 430-438

Murshidul A. 2012: Epidemiological investigation of anthrax in Bangladesh. Dept. of Microbiology, BAU, Bangladesh. pp 23-48.

Mustafa AH. 1984. Isolation of anthrax bacillus from an Elephant in Bangladesh. Vet Rec. **114:** 590.

Nakamura T, Hasegawa T, Torii K, Hasegawa Y, Shimokata K, Ohta M. 2004. Two-dimensional gel electrophoresis analysis of the abundance of virulent exoproteins of group A streptococcus caused by environmental changes. Arch Microbiol. **181:** 74-81.

Ndyabahinduka DG, Chu IH, Abdou AH, Gaifuba JK. 1984. An outbreak of human gastrointestinal anthrax. Ann Ist Super Sanita. 20: 205-208.

Nishi JS, Ellsworth TR, Lee N, Dewar D, Elkin BT, Dragon DC. 2007. Northwest Territories. An outbreak of anthrax (*Bacillus anthracis*) in free-roaming bison in the Northwest Territories, June-July 2006. Can Vet J. **48:** 37-38.

O'Farrell PH. 1975. High resolution two-dimensional electrophoresis of proteins. J Biol Chem. **250:** 4007-4021.

Oaks JL, Gilbert M, Virani MZ, Watson RT, Meteyer CU, Rideout BA, Shivaprasad HL, Ahmed S, Chaudhry MJ, Arshad M, Mahmood S, Ali A, Khan AA. 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. Nature. **427:** 630-633.

Okinaka RT, Cloud K, Hampton O, Hoffmaster AR, Hill KK, Keim P, Koehler TM, Lamke G, Kumano S, Mahillon J, Manter D, Martinez Y, Ricke D, Svensson R, Jackson PJ. 1999. Sequence and organization of pXO1, the large *Bacillus anthracis* plasmid harboring the anthrax toxin genes. J Bacteriol. **181:** 6509-6515.

Dhaka University Institutional Repository

Pain D, Cunningham AA, Donald PF, Duckworth JW, Houston DC, Katzner T, Parry-Jones J, Poole C, Prakash V, Round P, Timmins R. 2003. Causes and effects of temporospatial declines of Gyps vultures in Asia. Conserv Biol. **17:** 661-671.

Papaparaskevas J, Houhoula DP, Papadimitriou M, Saroglou G, Legakis NJ, Zerva L. 2004. Ruling out *Bacillus anthracis*. Emerg Infect Dis. **10:** 732-735.

Pearson T, Busch JD, Ravel J, Read TD, Rhoton SD, U'Ren JM, Simonson TS, Kachur SM, Leadem RR, Cardon ML, Van Ert MN, Huynh LY, Fraser CM, Keim P. 2004. Phylogenetic discovery bias in *Bacillus anthracis* using single-nucleotide polymorphisms from whole-genome sequencing. Proc Natl Acad Sci USA. **101:** 13536-13541.

Pezard C, Weber M, Sirard J, Berche P, Mock M. 1995. Protective immunity induced by *B. anthracis* toxin-deficient strains. Infect Immun. **63:** 1369-1372.

Pimental RA, Christensen KA, Krantz BA, Collier RJ. 2004. Anthrax toxin complexes: heptameric protective antigen can bind lethal factor and edema factor simultaneously. Biochem Biophys Res Commun. **322:** 258-262.

Pitt M, Little S, Ivins B, Fellows P, Barth J, Hewetson J, Gibbs G, Dertzbaugh M, Friedlander A. 2001. *In vitro* correlate of immunity in a rabbit model of inhalational anthrax. Vaccine. **19:** 4768-4773.

Prakash V. 1999. Status of vultures in Keoladeo National Park, Bharatpur, Rajasthan, with special reference to population crash in Gyps species. J Bom Nat Hist Soc. **96:** 365-378.

Prescott LM, Harley JP, Klein DA. 1999. Microbiology, 2nd ed. ButterworthHeinemann, United States.

Price LB, Hugh-Jones ME, Jackson PJ, Keim P. 1999. Genetic diversity in the protective antigen gene of *Bacillus anthracis*. J Bacteriol. **181:** 2358-2362.

Prince A. 2003. The host response to anthrax lethal toxin: unexpected observations. J Clin Invest. **112:** 656-658.

Rajalakshmi A, Renugadevi TS, Gunasekaran S. 2010. Effect of anthrax spore vaccine on cattle - a spectroscopic approach. Int J Pharm Sci Rev Res. **3:** 19-22.

Rao G R, Padmaja J, Lalitha M K, Rao P V, Gopal K V, Kumar HY, Mohanraj P. 2005. An outbreak of cutaneous anthrax in a non-endemic district-Visakhapatnam in Andhra Pradesh. Indian J Dermatol Venereol Leprol. **71:** 102-105.

Rasko DA, Altherr MR, Han CS, Ravel J. 2005. Genomics of the *Bacillus cereus* group of organisms. FEMS Microbiol Rev. **29:** 303-329.

Rath A, Glibowicka M, Nadeau VG, Chen G, Deber CM. 2009. Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. Proc Natl Acad Sci U S A. **106:**1760-1765.

Read TD, Peterson SN, Tourasse N, Baillie LW, Paulsen IT, Nelson KE, Tettelin H, Fouts D, Eisen J A, Gill SR, Holtzapple EK, Økstad OA, Helgason E, Risltone J, Wu M, Kolonay JF, Beanan MJ, Dodson RJ, Brinkac LM, Gwinn M, DeBoy RJ, Madpu R, Daugherty SC, Durkin AS, Haft DH, Nelson WC, Peterson JD, Pop M, Khouri HM, Raduna D, Benton J L, Mahamoud Y, Jiang L, Hance IR, Weidman JF, Berry KJ, Plaut RD, Wolf AW, Watkins KL, Nierman WC, Hazen A, Cline R, Redmond C, Thwaite JE, White O, Salzberg SL, Thomason B, Friedlander AM, Koehler TM, Hanna PC, Kolstø AB, and Fraser CM. 2003. The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. Nature. **423:** 81-86.

Read TD, Salzberg SL, Pop M, Shumway M, Umayam L, Jiang L, Holtzapple E, Busch JD, Smith KL, Schupp JM, Solomon D, Keim P, Fraser CM. 2002. Comparative genome sequencing for discovery of novel polymorphisms in Bacillus anthracis. Science. **296:** 2028-2033.

Dhaka University Institutional Repository

Redmond C, Baillie Lw, Hibbs S, Moir AJ, Moir A. 2004. Identification of proteins in the exosporium of *Bacillus anthracis*. Microbiology. **150:** 355-363.

Reuveny S, White MD, Adar YY, Kafri Y, Altboum Z, Gozes Y, Kobiler D, Shafferman A, Velan B. 2001. Search for correlates of protective immunity conferred by anthrax vaccine. Infect Immun. **69:** 2888-2893.

Rose AL. 1940. The period of incubation of anthrax in cattle. Austral Vet Jour. **16:** 214-215.

Roy P, Rashid MM, Ferdoush MJ, Dipti M, Chowdury MGA, Mostofa MG, Roy SK, Khan MAHNA, Hossain MM. 2013. Biochemical and immunological characterization of anthrax spore vaccine in goat. Bangladesh J Vet Med. **11:** 151-157.

Rume FI, Affuso A, Serrecchia L, Rondinone V, Manzulli V, Campese E, Di Taranto P, Biswas PK, Ahsan CR, Yasmin M, Fasanella A, Hugh-Jones M. 2016. Genotype analysis of *Bacillus anthracis* strains circulating in Bangladesh. PLoS One. **11:** e0153548.

Saile E, Koehler TM. 2006. *Bacillus anthracis* multiplication, persistence, and genetic exchange in the rhizosphere of grass plants. Appl Environ Microbiol. **72:** 3168-3174.

Samad MA, Hoque ME. 1986. Anthrax in man and cattle in Bangladesh. J Trop Med Hyg. **89:** 43-45.

Samad MA. 2008. Animal husbandry and veterinary science, Vol. 2 (1st edn). LEP publication no. 11, BAU, Mymensingh campus, Bangladesh. pp. 1192-96.

Samad MA. 2011. Public health threat caused by zoonotic diseases in Bangladesh. Bangl J Vet Med. **9:** 95-120.

Shin GW, Palaksha KJ, Kim YR, Nho SW, Kim S, Heo GJ, Park SC, Jung TS. 2007. Application of immunoproteomics in developing a *Streptococcus iniae* vaccine for olive flounder (*Paralichthys olivaceus*). J Chromatogr B Analyt Technol Biomed Life Sci. **849:** 315-322.

Shlyakhov E, Rubinstein E, Novikov I. 1997. Anthrax post-vaccinal cell-mediated immunity in humans: kinetics pattern. Vaccine. **15:** 631-636.

Shury TK, Frandsen D, O'Brodovich L. 2009. Anthrax in free-ranging bison in the Prince Albert National Park area of Saskatchewan in 2008. Can Vet J. **50:** 152-154.

Singh Y, Chaudhary V, Leppla S. 1989. A deleted variant of *B. anthracis* protective antigen is non-toxic and blocks anthrax toxin action *in vivo*. J Biol Chem. **264:** 19103-19107.

Singh Y, Ivins B, Leppla S. 1998. Study of immunization against anthrax with the purified recombinant protective antigen of *B. anthracis*. Infect Immun. **66:** 3447-3448.

Sirisanthana T, Brown AE. 2002. Anthrax of the gastrointestinal tract. Emerg Infect Dis. **8:** 649-651.

Smith KA. 2012. Louis Pasteur, the father of immunology? Front Immunol. **3:** 68.

Smith KL, De Vos V, Bryden H, Price LB, Hugh-Jones M, Kleuytska A, Price LB, Keim P. 2000. *Bacillus anthracis* diversity in Kruger National Park. J Clin Micro. **38:** 3780-3784.

Stein CD. 1947b. Anthrax in animals and its relationship to the disease in man. Ann. NY Acad Sci. **48:** 507-534.

Sterne M. 1937. Variation in *Bacillus anthracis*. Onderstepoort J Vet Sci Anim Ind. **8:** 271-349.

Sterne M. 1939a. The immunization of laboratory animals against anthrax. Onderstepoort J Vet Sci Anim Ind. **13:** 313-317.

Sterne M. 1939b. The use of anthrax vaccine prepared from avirulent (unencapsulated) variants of *B. anthracis*. Onderstepoort J Vet Sci Anim Ind. **13:** 307-312.

Stevens M, Olsen S, Pugh G, Brees D. 1995. Comparison of immune responses and resistance to brucellosis in mice vaccinated the *Brucella abortus* 19 or RB51. Infect Immun. **63:** 264-270.

Stratilo CW, Lewis CT, Bryden L, Mulvey MR, Bader D. 2006. Single-nucleotide repeat analysis for subtyping *Bacillus anthracis* isolates. J Clin Microbiol. **44:** 777-778.

Tareque AMM, Chowdhury SMZH. 2010. Agricultural research priority: vision-2030 and beyond. Dhaka: Bangladesh Agricultural Research Council.

Tomaso H, Bartling C, Al Dahouk S, Hagen RM, Scholz HC, Beyer W, Neubauer H. 2006. Growth characteristics of *Bacillus anthracis* compared to other *Bacillus* spp. on the selective nutrient media Anthrax Blood Agar® and Cereus Ident Agar®. Syst Appl Microbiol. **29:** 24-28.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences USA. **76:** 4350-4354.

Turnbull P, Leppla S, Broster M, Quinn C, Melling J. 1988. Antibodies to anthrax in humans and guinea pigs and their relevance to protective immunity. Med Microbiol Immun. **177:** 293-303.

Turnbull PC, Broster MG, Carman JA, Manchee RJ, Melling J. 1986. Development of antibodies to protective antigen and lethal factor components of anthrax toxin in humans and guinea pigs and their relevance to protective immunity. Infect Immun. **52:** 356-363.

Dhaka University Institutional Repository

Turnbull PCB, Barman JA, Lindeque DM, Joubert F, Hubschle OJB, Snoeyenbos GS. 1989. Further progress in understanding anthrax in Etosha National Park. Mdoqua. **16:** 93-104.

Turnbull PCB, Quinn CP, Hewson R, Stockbridge MC, Melling J. 1990. Protection conferred by microbially-supplemented UK and purified PA vaccines. Salisbury Med Bull. **68S:** 89-91.

Turnbull PC. 1991. Anthrax vaccines: past, present and future. Vaccine. **9:** 533-539.

Turnbull PC. 1998. Guidelines for the surveillance and control of Anthrax in human and animals. 3rd edition. pp. 51-61.

Turnbull PCB, Sirianni NM, LeBron CI, Samaan MN, Sutton FN, Reyes, AE, Peruski LF. 2004. MICs of Selected Antibiotics for *Bacillus anthracis, Bacillus cereus, Bacillus thuringiensis,* and *Bacillus mycoides* from a Range of Clinical and Environmental Sources as Determined by the Etest. J Clin Microbiol. **42:** 3626-3634.

Turnbull PC. 2008. World Health Organization. Anthrax in humans and animals. In: (Editor) PT, editor. Geneva (CH): WHO Press.

Uchida I, Hornung J, Thorne C, Klimpel K, Leppla S. 1993. Cloning and characterization of a gene whose product is a trans-activator of anthrax toxin synthesis. J Bacteriol. **175:** 5329-5338.

Valli K, Schneerson R, Kubler-Kielb J, Liu TV, Dai ZD, Leppla SH, Yergey A, Backlund P, Shiloach J, Majadly F, Robbins JB. 2003. Poly (gamma-Dglutamic acid) protein conjugates induce IgG antibodies in mice to the capsule of *Bacillus anthracis*: a potential addition to the anthrax vaccine. Proceedings-of-the-National-Academy-of-Sciences-of-the-United-States-of America. **100:** 8945-8950.

Dhaka University Institutional Repository

Van Ert MN, Easterday WR, Huynh LY, Okinaka RT, Hugh-Jones ME, Ravel J, Zanecki SR, Pearson T, Simonson TS, U'Ren JM, Kachur SM, Leadem-Dougherty RR, Rhoton SD, Zinser G, Farlow J, Coker PR, Smith KL, Wang B, Kenefic LJ, Fraser-Liggett CM, Wagner DM, Keim P. 2007a. Global genetic population structure of *Bacillus anthracis*. PLoS One. **2:** e461.

Van Ert MN, Easterday WR, Simonson TS, U'Ren JM, Pearson T, Kenefic LJ, Busch JD, Huynh LY, Dukerich M, Trim CB, Beaudry J, Welty-Bernard A, Read T, Fraser CM, Ravel J, Keim P. 2007b. Strain-specific single-nucleotide polymorphism assays for the *Bacillus anthracis* Ames Strain. J Clin Microbiol. **45:** 47-53.

Van Ness GB. 1971. Ecology of anthrax. Science. **172:** 1303-1307.

Vijaikumar M, Thappa DM, Karthikeyan K. 2002. Cutaneous anthrax: an endemic outbreak in South India. J Trop Pediatr. **48:** 225-226.

Vodkin M, Leppla S. 1983. Cloning of the protective antigen gene of *Bacillus anthracis*. Cell. **34:** 693-697.

Vos D, Trotta L, Altamura C, Fasanella M. 2002. Anthrax vaccines for veterinary use. Obiettivi-e-Documents-Veterinari. **23:** 7-11.

Wang J, Ying T, Wang H, Shi Z, Li M, He K, Feng E, Wang J, Yuan J, Li T, Wei K, Su G, Zhu H, Zhang X, Huang P, Huang L. 2005. 2-D reference map of *Bacillus anthracis* vaccine strain A16R proteins. Proteomics **5:** 4488-4495.

Welkos SL, Keener TJ, Gibbs PH. 1986. Differences in susceptibility of inbred mice to *Bacillus anthracis.* Infect Immun. **51:** 795-800.

Welkos SL. 1991. Plasmid-associated virulence factors of non-toxigenic (pXO1-) *Bacillus anthracis.* Microb Pathog. **10:** 183-198.

Dhaka University Institutional Repository

Whitford HW. 1979. Anthrax. In: Stoenner H, Kaplan W, Torten M , eds. CRC Handbook Series in Zoonoses, Section A.: Bacterial, Rickettsial and Mycotic Diseas. Boca Raton, Florida: CRC Press, Inc. **1:** 31-66.

Woese CR. 1994. There must be a prokaryote somewhere-microbiologys search for itself. Microbiol Rev. **58:** 1-9.

World Health Organization (WHO). 1997. Disease outbreak in Kenya: WHO does not recommend travel restrictions. Press release WHO/101, 30 December 1997.

World Health Organization (WHO). 2003. Manual for laboratory diagnosis of anthrax. New Delhi, WHO Regional Office for South-East Asia (SEAHLM-371).

World Health Organization (WHO). 2008. Anthrax in humans and animals - 4th ed. I. World Health Organization. II. Food and Agriculture Organization of the United Nations. III. World Organization for Animal Health: WHO Press, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

World Organization for Animal Health (OIE). 2008a. Anthrax. In: The Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Paris: OIE, pp. 135-144.

World Organization for Animal Health (OIE). 2008b. OIE Terrestrial Manual 2008. OIE listed diseases and other diseases of importance to international trade. Part-2, Chapter 2.1.1.-Anthrax. pp. 135-144.

World Organization for Animal Health (OIE). 2010. World Animal Health Information Database. Available: http://www.oie.int/wahis/public. php?WAHIDPHPSESSID =adce2e623aeedddaa4b 858ff84f59f. Accessed August 12, 2010.

World Organization for Animal Health (OIE). 2011. World Animal Health information database (WAHID) Interface (http://web.oie.int/wahis/public.php?page=home), Paris. Accessed 23 August 2011.

Dhaka University Institutional Repository

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. **13:** 134.

Zerbino DR, Birney E. 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. **18:** 821-829.

Dhaka University Institutional Repository

Appendix

Dhaka University Institutional Repository

Appendix-1

Media composition

The composition of the media used in this thesis work are given below.

Columbia agar

Ingredients	Amount
Peptone	23 gm
Starch	1 gm
Nacl	8-18 (1) gm
Water	1 lit

TSMP (Trimethoprime-Sulphomethaxazole Polymyxin B) agar media

Ingredients	Amount
Columbia agar	950 (945 ml)
Trimethoprime-Sulphomethaxazole solution	1ml
Polymyxin B solution	0.5ml
Defibrinated blood from sterile mutton	50ml

Suppliment for TSMP agar media

i) Defibrinated blood from sterile mutton 5%

ii) Trimethoprime-Sulphomethaxazole solution

  a) Trimethoprim -16 mg

  b) Sulphomethaxazole -80 mg

  c) Methanol -5 ml

iii) Polymyxin B solution

  a) Polymyxin B -300000 UI

  b) Water -5ml

Dhaka University Institutional Repository

Nutrient agar media

Ingredients	Amount (g/l)
Peptic digest of animal tissue	5.000
Sodium chloride	5.000
Beef extract	1.500
Yeast extract	1.500
Agar	15.000
Water	1 lit

Nutrient broth media

Ingredients	Amount (g/l)
Peptone	10.000
Beef extract	10.000
Sodium chloride	5.000
Water	1 lit

Tryptose Phosphate Broth

Ingredients	Amount (g/l)
Tryptose	20.000
Dextrose	2.000
Sodium chloride	5.000
Disodium phosphate	2.500
Water	1 lit

Appendix-II

The preparation of buffer and solutions used in this thesis work are given below.

Tris-HCl

121.1 g tris-base was dissolved in 800 ml of distilled water. The pH was adjusted to the desired value by adding concentrated HCl, and the final volume was made up to 1lit with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

Phosphate buffered saline (PBS)

PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 2 g of KH_2PO_4 in 800 ml of distilled water. pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1 lit by distilled water. The solution was sterilized by autoclave and stored at room temperature.

Preparation of standard bovine serum albumin (BSA) for protein estimation

1 mg/1 ml concentration (3-4 ml) solution was made in a 15 ml falcon tube. This solution was divided in the following concentration gradient (0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml and 0.8 mg/ml) and kept in eppendorf tubes-

a) For 0.2 mg/ml preparation, 200 µl BSA solution and 800 µl water were taken in the eppendorf tube.
b) For 0.4 mg/ml preparation, 400 µl BSA solution and 600 µl water were taken in the eppendorf tube.
c) For 0.6 mg/ml preparation, 600 µl BSA solution and 400 µl water were taken in the eppendorf tube.
d) For 0.8 mg/ml preparation, 800 µl BSA solution and 200 µl water were taken in the eppendorf tube.

Each concentration gradient solution was transferred to another set of eppendorf tubes, containing 20 µl in each tube.

Preparation of reagent for protein estimation

The reagent (BioRad dye) was diluted 5 times (2.5 ml dye+10 ml distilled water) and filtered through Whatman filter paper and collected in a 15 ml falcon tube. A volume of 1 ml of prepared reagent was dispensed in each eppendrof tube (1 blank, 4 standard, 2 1:10 dilution of sample and 2 samples) and incubated at room temperature for 15 min after vortex.

Preparation of blank

20 µl of distilled water was dispensed in an eppendrof tube to prepare the blank.

Preparation of sample

1:10 dilution of sample (10 µl+90 µl) was prepared in an eppendrof tube and 40 µl was transferred to 2 eppendrof tubes as duplicated at a volume of 20 µl each.

Preparation of stock solutions for SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

a) 30% acrylamide (50 ml): 14.5 gm acrylamide + 0.5g bis acrylamide+ dH_2O upto 50 ml were stored at 4^0C. As the powder is neurotoxic, mask was used during handling.

b) 30% acrylamide (50 ml): 14.5 gm acrylamide + 0.5 g bis acrylamide+ dH_2O upto 50 ml were stored at 4^0C. As the powder is neurotoxic, mask was used during handling.

c) 0.5 M Tris HCl pH 6.8 (100 ml): 6.57 g [0.5 x M.W. (121.14)/10] of Tris base was taken in 70 ml dH_2O, mixed, brought the pH to 6.8 with concentrated HCl, then brought the total volume of the solution up to 100 ml mark by adding dH_2O and stored at 4^0C.

d) 1.5M Tris HCl pH 8.8 (200 ml): 36.34 g [1.5 x M.W. (121.14)/5] of Tris base was taken in 150 ml dH_2O, mixed, brought the pH to 8.8 with concentrated HCl, then brought the total volume of the solution up to 200 ml mark by adding dH_2O and stored at 4^0C.

Dhaka University Institutional Repository

e) 10% SDS (50 ml): 5 g SDS + dH_2O upto 50 ml mark. The solution was stored at room temperature.

f) 10% Ammonium persulphate (10% APS-5 ml): 0.5 g APS was taken and dH_2O upto 5 ml mark was added and mixed. 0.5 ml was alequoted in each eppendrof tube and stored at -20^0C.

g) TEMED: This was readymade and stored at room temperature.

h) Butanol saturated water: 50 ml dH_2O was taken in a beaker with a magnetic stirrer. Butanol was kept adding to water provided that the machine was turned on. Butanol was stopped adding when the solution got saturated. It was stored at 4^0C.

Composition of 12.5% separating gel

Ingredients	Amount
dH_2O	1.6 ml
1.5M Tris HCl pH 8.8	1.25 ml
30% acrylamide	2.1 ml
10% SDS	0.1 ml

Composition of stacking gel

Ingredients	Amount
dH_2O	2.137 ml
0.5M Tris HCl pH 6.8	937 µl
30% acrylamide	625 µl
10% SDS	37 µl

Dhaka University Institutional Repository

Composition of running buffer

Ingredients	Amount
Tris base	3.0 g
Glycerine	14.4 g
10% SDS	10 ml
dH_2O	1000 ml

Composition of sample buffer

Ingredients	Amount
0.5M Tris HCl pH 6.8	0.4 ml
10% SDS	0.4 ml
2-mercaptoethanol	0.04 ml
Glycerol	0.4 ml
dH_2O	0.76l

Preparation of 0.1% BPB (Bromo phenol blue, loading dye/tracking dye)

At first, 2 ml of 50% glycerol solution was made by mixing 1 ml glycerol with 1 ml dH_2O. Then 50% glycerol was added in 2 mg of bromo phenol blue upto 2 ml mark and stored at 4^0C. 5 µl of 0.1% BPB was added per sample.

Running electrophoresis

Electrophoresis was carried out at 15-20 mA. Time depended upon the descent of the materials.

Staining the gel after electrophoresis

At first, 200 ml 7% acetic acid solution was made by dissolving 14 ml acetic acid in 186 ml dH_2O. 100 ml was used as destaining solution and the rest was used to prepare 0.1% Coomassie blue solution as the stainer of the gel. To prepare this solution, 100 mg of the dye was dissolved by adding 7% acetic acid upto the 100 ml mark. Solution was stored at 4^0C.

Destaining of the gel

Destaining of the gel was done by 7% acetic acid.

Ponceau S staining buffer

0.2% (w/v) Ponceau S

5% glacial acetic acid

Composition of lower gel for western blot

Ingredients	Amount
dH_2O	2.4ml
1.5M Tris HCl pH 8.8	1.9 ml
30% acrylamide	3.15 ml
10% SDS	150 µl
10% Ammonium persulphate (APS)	45 µl
TEMED	12.75µl

Composition of transfer buffer

Ingredients	Amount
dH_2O	1 liter
Methanol	250 µl
Tris base	3.6 gm
Glycerine	18 gm

Composition of AP substerate (Sigma, USA)

Ingredients	Amount
50 mM Tris HCL (pH- 9.14)	10 ml
Fast Red TR (Sigma)	20 mg
AS-MX Napthol Phosphate	10 mg

Dhaka University Institutional Repository

Composition of HRP substerate (Sigma, USA)

Ingredients	Amount
PBS	100 ml
DAB	60 mg
30% H_2O_2	60 µl

Dhaka University Institutional Repository

Appendix-III

Western blot or dot procedure

The procedures for western blot or dot used in this study were as follow: