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Original Article

Molecular Characterization of Foot-and-Mouth Disease Virus Type O from Wild Pig in Bangladesh

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Foot-and-mouth disease (FMD) is a disease of all non-avian livestock animals which costs direct and indirect economic burden of 6.5-21 billion USD per year worldwide in endemic countries. In Bangladesh, FMD is endemic and mainly caused by foot-and-mouth disease virus (FMDV) serotypes O, A and Asia1. Among FMD susceptible animals cattle, sheep and goat were reported to be more susceptible to this virus, whereas, pig was reported as amplifier of the virus. To date there is no epidemiological data in Bangladesh defining the circulation of FMDV in pig population. This investigation first reports the circulation of FMDV in wild pigs of Bangladesh, its molecular characterization and genotyping. To pursue this, tissue sample from ruptured vesicles of mammary gland was collected from wild pig suspected to be infected with FMD followed by RT-PCR amplification, sequencing and phylogenetic study of the VP1 gene, which is the most variable region of FMDV genome. The virus was identified as FMDV serotype O and phylogenetically clustered together with India 2001 (Ind2001) lineage under middle-east, south Asia (ME-SA) topotype. Within the clade of Ind2001 lineage, FMDV from pig formed a sub-clade with 2013 sequences of cattle which indicates the phylogenetic relatedness of the virus from pig with circulatory virus in cattle population of Bangladesh in 2013. Pair-wise local alignment of the FMDV type O VP1 sequence of pig with other local cattle FMDV type O VP1 sequences of 2012 and 2013 supported the phylogenetic affiliation of the pig FMDV with the circulatory virus in cattle population of Bangladesh in 2013, whereas nucleotide sequences of cattle FMDV VP1 of 2012 were found to be 7-8% divergent compared to pig FMDV VP1. Phylogenetic and pair-wise alignment data conclusively revealed that (i) homologous circulation of the FMDV type O (Ind2001 lineage) occurs in both animal traits; and (ii) FMDV type O VP1 sequence of pig origin is distantly related to 2012 local FMDV type O VP1 sequences of cattle origin, but closely related to 2013 local cattle FMDV type O VP1.

Keywords: Bangladesh, Pig, Cattle, FMDV, Serotype, ME-SA, Ind2001

Introduction

Occurrence of a virus in animal disease, foot-and-mouth disease (FMD) was first reported 118 years earlier[1], but still it is considered as one of the key threats to livestock industries as well as a constraint to international trade in livestock products worldwide. FMD is a contagious disease for all non-avian livestock animals, rarely in human[2-3]. It is an acute, systematic disease that affects mainly cloven-hoofed animals of domestic and wild origin[2]. The etiological agent, foot-and-mouth disease virus (FMDV), is a prototypic member of *Aphthovirus* genus under *Picornaviridae* family having a single stranded positive sense RNA genome[3]. The FMDV is grouped into seven distinct serotypes on the basis of immunological cross reaction, assigned as serotype O, A, Asia1, C, south Asian territories (SAT 1-3)[4-5]. Vaccination is one of the effective arms to control this disease but this practice becomes perplexing due to the lack of cross-protection among different serotypes, vaccine escape within same serotype and high mutation rate and quasi-species dynamics of the RNA genome of the virus[5-7]. Moreover the multiplicity of the host range makes the situation worse towards approaches to eradicate this virus. It is affirmed that more than 70 species of wild animals besides domesticated cloven-hoofed animals like cattle, swine, sheep, and goats are susceptible to this disease[3]. Besides it has been also established that FMDV rapidly replicates and spreads within susceptible animals mainly via aerosol transmission[3-4,8]. So far aerosol transmission is considered as major risk during FMD outbreaks and preceding reports clearly documented that, cattle and sheep are particularly susceptible to aerosolized virus while pig produce the highest level of aerosolized virus during infection[9].

FMD is endemic in Bangladesh and remains a major threat to the livestock industry due to continuously increasing incidences and evolution of novel circulatory FMDV strains as well as vaccine escape mutants. Our laboratory studied epidemiology of FMDV in cattle population and conclusively reported the

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association of FMDV type O, A and Asia1with recent FMD outbreak in Bangladesh during 2012-2014[10-13].The molecular epidemiology of FMDV in cattle population of Bangladesh like the previous established works have been based on comparing the sequences of the genome region coding for the outer capsid polypeptide, VP1[7]. VP1 protein along with other structural proteins (VP2, VP3, and VP4) of FMDV is involved in capsid assembly and stability, virus binding and antigenicity[3,7]. VP1 is the most variable region of the FMDV genome and the seven serotypes of FMDV cluster into distinct genetic lineages with approximately 30-50% difference in the VP1 gene[7].

Among the FMD susceptible animals cattle, sheep and goat were reported to be more susceptible to this virus[9], whereas buffalo was considered as carrier[14] and pig was reported to be the amplifier of thevirus[9]. To date there is no epidemiological data in Bangladesh defining the circulation of the FMDV in pig necessary to interpret conclusively whether the same lineage of FMDV serotype is circulatory among pig and cattle population. This study reports the molecular characterization of FMDV circulating in wild pig of Bangladesh.

Materials and Methods

Sample collection

Tissue sample from ruptured vesicles of mammary gland of wild pig was collected from Rangamati district of Bangladesh on 25th of October 2013. The sample was transported to the Microbial Genetics and Bioinformatics Laboratory (http://microbialgen.du.ac.bd/) within 20 hours maintaining cold chain (4 ºC) and stored at –80ºC until tested. The name tag for the sample was assigned according to three letter country code, District, Upazilla, Number of entry followed by collection year thus having ID BAN/RA/Sa-189/2013 (Bangladesh/Rangamati/Sadar-189/2013).

Sample preparation and RNA extraction

Tissue sample preparation and total RNA was extracted in Maxwell® 16 system (Promega, USA) using Maxwell® 16 total RNA purification kit (Promega, USA) according to manufacturer's instruction. Approximately 70 mg of tissue was homogenized after addition of about 462 µl lysis buffer (66 µl/10 mg tissue) in automated Maxwell® 16 System until completely lysed. From the tissue lysate total RNA was extracted according to manufacturer's instruction and eluted to a final volume of 300 µl nuclease free water. The extracted RNA was subjected to reverse transcription PCR (RT-PCR) immediately after extraction.

Complementary DNA (cDNA) synthesis

The extracted RNA was reverse transcribed into complementary DNA (cDNA) by using ImProm-II™ reverse transcription system (Promega, USA) using both hexameric random primer and oligodT primer. The primer and template RNA combination was pre-heated at 70ºC followed by incubation at 4ºC until reverse transcription started. Reverse transcription was carried out in thermal cycler (Applied Biosystem, USA) at 25ºC for 5 minutes of annealing followed by extension at 42ºC for 60 minutes and inactivation of reverse transcriptase at 70ºC for 15 minutes. The final product of reverse transcription was subjected to subsequent PCR screening and stored at –20ºC.

PCR amplification of VP1 gene and automated cycle sequencing

PCR amplification of the VP1 gene of FMDV was carried out with primer set 16F/16R[10]usingGoTaq® PCR master mix (Promega, USA). The PCR cycling parameter was performed in thermal cycler (Applied Biosystem, USA) including an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1.5 minutes and a final extension at 72°C for 10 minutes. The 5 ìl PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide, and visualized under gel documentation (AlphaImager HP System, ProteinSimple, USA). PCR amplicon was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and purified PCR product was subjected to automated cycle sequencing reaction using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems®, USA) according to manufacturer's instruction with the same primers (16F, 16R) used in the PCR reaction and analysis of the data was performed in ABI Genetic Analyzer (Applied Biosystems®, USA).

Sequence analysis and phylogeny

The raw sequences were assembled in SeqMan ver. 7.0 (DNASTAR, Inc., USA). Comparison of the assembled sequence with other entries from NCBI GenBank using BLAST search (blastn algorithm[15]) was performed to reveal the serotype level identification of the sample of pig origin. Phylogeny reconstruction was executed in MEGA 5.2 to access the phylogenetic relatedness among FMDV from pig origin with other FMDV serotypes and lineages belonging to different hosts mainly cattle. To perform this, reference nucleotide sequences was retrieved from NCBI nucleotide database. Multiple sequence alignment (codon alignment) of the FMDV from pig origin and prototypic sequences were performed with ClustalW algorithm built in MEGA 5.2[16]. To obtain the optimum nucleotide substitution model, according to BIC (Bayesian Information Criterion) scoring, model selection analysis was computed[17]. Neighbour-joining tree was constructed based on the best nucleotide substitution model with 1000 bootstrap replication value to evaluate the robustness (biologists feel comfort to mention 'reliability') of the constructed tree.

Pair-wise alignment of the nucleotide sequence of FMDV VP1 gene (pig origin, BAN_RA_Sa-189_2013) and other FMDV type O VP1 prototype sequences from outbreak in cattle population of Bangladesh respectively in 2012 (GenBank accession number KC795947, KC795956-KC795959, KJ175178-KJ175180) and 2013 (GenBank accession number KJ175176, KJ175181-KJ175185) was performed using Align Sequences Nucleotide BLAST program.

The FMDV VP1 nucleotide sequence from pig origin was submitted in NCBI GenBank under accession number KJ175177.

Result and Discussion

Characterization of foot-and-mouth disease virus from pig

RT-PCR amplification of the partial VP1 region confirmed the presence of Foot-and-Mouth Disease Virus (FMDV) in pig symptomized by the presence of fluid-rich vesicle in mammary gland. About 416 bp amplicon was generated using VP1 specific primer pair which fully complies with the known positive samples of cattle origin (Figure 1). Sequencing and subsequent BLAST search identified the FMDV of pig origin as serotype O. The statistical significance of the BLAST outcome generally expressed as Expect value (E-value) was 0, which diminished the chance of falsified identification of the FMDV serotype based on the sequence of VP1[18]. FMDV type O sequence of pig origin was 99% identical to that of cattle FMDV type O VP1 sequences of Bangladesh, indexed as accession number KJ175176, KJ175183-KJ175185.

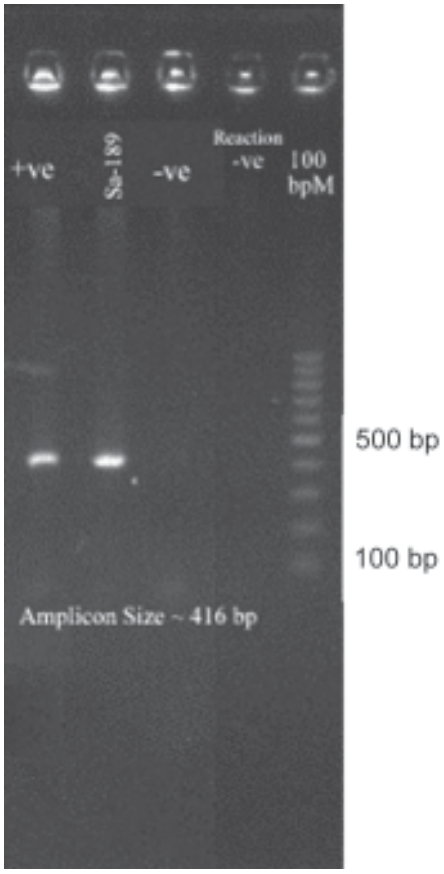

Figure 1. *RT-PCR amplification of the complementary DNA (cDNA) from FMDV of pig origin. A 416 bp amplicon was observed. Here +ve and –ve represents known positive and known negative FMDV samples respectively. Sa-189 resembles the FMDV samples from pig assigned as BAN/RA/Sa-189/2013. Reaction –ve means no template control. The 100bp ladder was originated from Bioneer, USA.*

Phylogenetic study of FMDV type O from pig

To define the topotype and lineage of the FMDV type O from pig, Neighbour-Joining tree was constructed with representative topotype and lineage sequences from NCBI GenBank. It was found that Pig FMDV type O clustered within India 2001 (Ind2001) lineage under Middle-East South Asia (ME-SA) topotype of FMDV type O (Figure 2). Moreover VP1 sequence of pig origin formed a sub-clade with 2013 VP1 sequences of FMDV type O of cattle origin within monophyletic clade of Ind2001, which indicates that VP1 sequence of pig was phylogenetically closely related to 2013 VP1 sequences from cattle, whereas more divergent from 2012 VP1 sequences of cattle origin. This particular Ind2001 lineage was reported to be in circulation among cattle population since 2009 in Bangladesh[10,19]. So it can be anticipated that the same lineage of FMDV type O (Ind2001) is circulatory in both pig and cattle population. This is a start up work and more pig samples need to analyze in future to support the scientific statement coined here in this study. However, it is necessary to determine possible route of virus transmission, whether virus transmits from pig to cattle or vice-versa. Further scientific study is necessary to unveil the viral transmission route.

In earlier studies it was documented that pig is the best amplifier of the disease whereas cattle and sheep are the most susceptible[9] animals; therefore a large number of pig population needs to be assessed through epidemiological and molecular methods to point out the route of aerosol transmission from pig to cattle conclusively. In Bangladesh although pig population is not subject to commercial farming practice, it may still serve as a source of disease transmission to cattle. The present report describes the circulation of the same lineages of FMDV type O in both cattle and wild-pig in Bangladesh which is a concern for FMD management considering the route of FMDV transmission.

VP1 sequence variation from FMDV of cattle origin

Pair-wise local alignment of the FMDV type O VP1 sequences of pig and cattle (reported in 2012 and 2013) of Bangladesh revealed the divergence of the FMDV type O VP1 of pig from sequences of 2012 whereas significant correlation existed with 2013 sequences. The percentile value of divergence of the FMDV type O VP1 of pig was 7-8% compared to 2012 sequences, but that value was 2-6% in case of two sequences of 2013 which was collected at the beginning (March) of 2013 (Table 1). Another four 2013 sequences of VP1 from cattle (collected on September and October,2013) were close to the VP1 sequence of pig giving a divergence value as low as 1% (Table 1). Pair-wise alignment data clearly indicated the proximate agreement of the FMDV type O VP1 sequence of pig with most of the 2013 VP1 sequences of cattle which revealed the co-circulation of the antigenetically related FMDV type O strain in both animal traits during 2013.

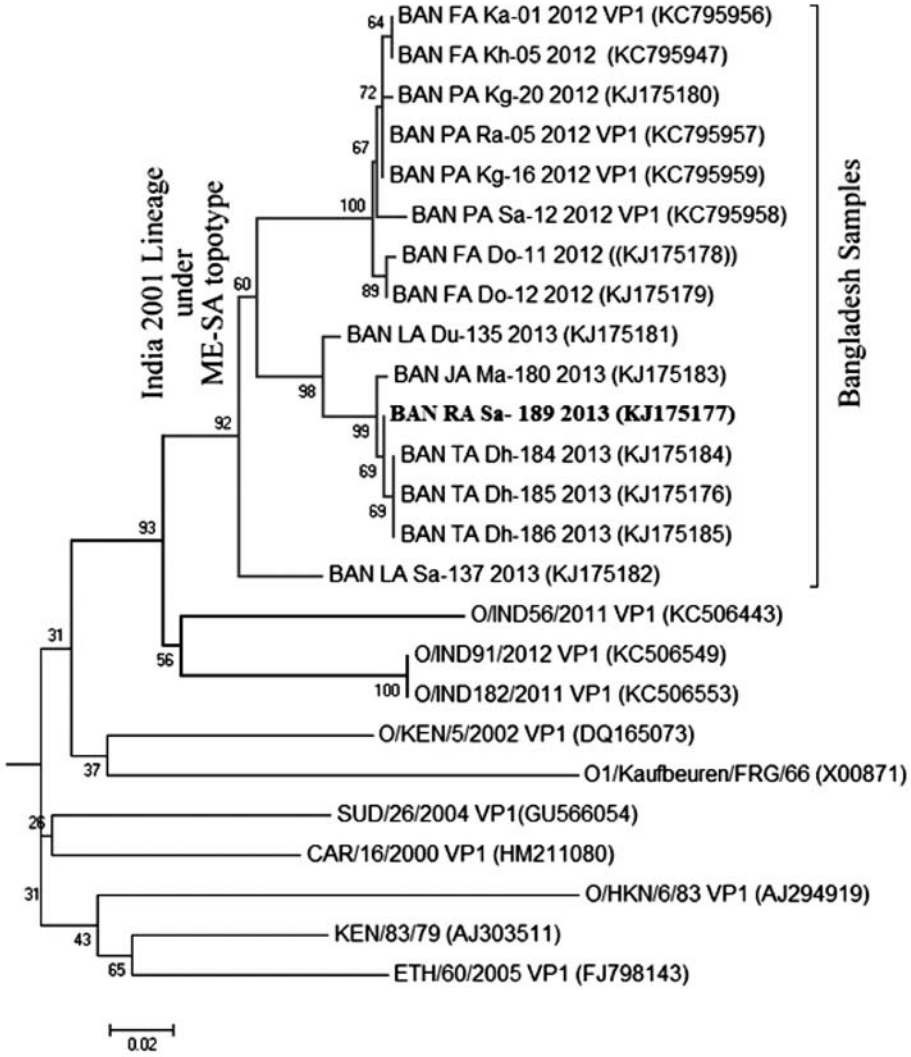

Figure 2. *Neighbor-Joining tree based on VP1 sequences of FMDV type O showing significant agreement among FMDV type O of pig and cattle origin. Here FMDV type O VP1 sequence from pig (shown as bold) clustered within the Bangladesh samples belonging to India 2001 lineage under Middle East South Asia (ME-SA) topotype while FMDV type O sequences from other lineages of ME-SA topotype and other FMDV type O topotypes branched out earlier. This phylogeny was re-constructed in MEGA 5.2.*

Table 1. *Nucleotide sequence variation among FMDV type O VP1 of pig and cattle origin*

FMDV type O VP1 sequences of	Nucleotide sequence variation from FMDV type O VP1 of pig origin		
Bangladesh (cattle origin)	Variation (%)	Query coverage (%)	E-Value
BAN_FA_Kh-05_2012 (KC795947)	7	95	0
BAN_FA_Ka-01_2012 (KC795956)	8	89	2e-172
BAN_PA_Ra-05_2012 (KC795957)	7	86	2e-172
BAN_PA_Sa-12_2012 (KC795958)	7	87	1e-170
BAN_PA_Kg-16_2012 (KC795959)	7	85	5e-169
BAN_TA_Dh-185_2013 (KJ175176)	1	100	0
BAN_FA_Do-11_2012 (KJ175178)	8	99	0
BAN_FA_Do-12_2012 (KJ175179)	8	99	0
BAN_PA_Kg-20_2012 (KJ175180)	7	99	0
BAN_LA_Du-135_2013 (KJ175181)	2	99	0
BAN_LA_Sa-137_2013 (KJ175182)	6	99	0
BAN_JA_Ma-180_2013 (KJ175183)	1	98	0
BAN_TA_Dh-184_2013 (KJ175184)	1	98	0
BAN_TA_Dh-186_2013 (KJ175185)	1	98	0

Conclusion

In this study it was found that FMDV type O belonging to Ind2001 lineage is circulating simultaneously in cattle and pig population of Bangladesh in 2013. Moreover the divergence pattern among 2012 and 2013 cattle FMDV type O sequences with FMDV VP1 of pig sequence encourages us to point at high mutation and quasi-species dynamics of the RNA genome of FMDV type O that may contribute to the emergence of new viral strain with time. Authors suggest continuous monitoring of FMDV in both cattle and pig population throughout the year to reduce possible route of aerosol transmission as a measure of management of this disease in Bangladesh.

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