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Epidemiological Diversity, Isolation, and Molecular Characterization of Circulating Contagious Ecthyma Virus (Orf Virus) in Goats of Bangladesh

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Abstract

ORF virus (ORFV), which belongs to the Poxviridae family, is the source of the extremely contagious disease known as Contagious Ecthyma (CE) or Orf in sheep and goats. In Bangladesh, CEV continually exists in goats and is associated with mortality and weight loss. To extract, identify, and characterize the circulating ORFV from goats suspected of having CE, a study was conducted. Based on the age, sex, and season of the goats, a total of 100 CE suspect samples were collected from goats in various parts of Bangladesh, such as Dhaka, Rajshahi, Bandarban, Meherpur, Mymensingh, and Chuadanga. After that, DNA was extracted, and a PCR test was run to amplify the ORFV VIR and GIF genes. Isolation of the virus was performed using Lamb testicular cell (LTC) for detecting these two genes. The univariate linear regression model showed a higher prevalence in Mymensingh district (70%) and subsequently lower prevalence in Bandarban districts (50 %). The prevalence was higher in the younger age group of 6–12 years (77.27%), with female goats (63.49%) and the winter season (75.61%) being the most susceptible. Three to seven days post inoculation (dpi), rounding and detaching appearance were seen in PCR-positive samples of ORFV with two isolates (GIF and VIR) in primary lamb testicular cells (LTC). Sequencing of these two isolates results in 99-100% homology to each other and our circulating isolates are closely related with some Indian and Chinese variants. In conclusion, it can be stated that contagious ecthyma (Orf) is prevalent in Bangladesh and more genomic analysis should be performed for control.

Keywords: Contagious ecthyma, Prevalence, isolation, Goat, Bangladesh.

Introduction

The Orf virus, which is a member of the genus Parapoxvirus of the family Poxviridae, is the source of the highly contagious viral disease known as contagious ecthyma (CE) in goats and sheep [1]. Goats, sheep, alpacas, camels, reindeer, deer, pronghorn antelope, wapiti, and seal squirrels are among the animals affected by CE, also known as Orf, infectious pustular dermatitis, or scabby mouth [2]. With surgical intervention, the condition mostly affects the lips, breasts, gums, and oral cavity, resulting in a tumor-like appearance [3]. The ubiquitous nature of the virus is found in Norway [4], China [5], India [6], Iraq [7], Ethiopia [3], Argentina [8], USA [9] Pakistan [10], and also in Bangladesh [11]. CE is a common widespread viral disease of small ruminants affecting all age groups and both sexes [3, 6]. In some areas of Bangladesh morbidity is about 23.89% and mortality may reach up to 1.02% [12]. Despite the low fatality rates associated with self-limiting ORFV infections, major outbreaks have been known to have mortality rates as high as 20–90% [13]. But CE also has low mortality and high morbidity; nonetheless, in young animals, mortality is significant because they cannot suckle [14]. Based on 2.167 million afflicted sheep, an English report estimated that production losses and treatment costs could reach $10 million and $4.62 per head, respectively, indicating serious economic problems [15].

The major way that the infectious ecthyma virus is spread is via direct contact with infected animals, and the virus can live for many years in the environment [2]. Infected animals are also the source of transmission of the virus due to abrasions around the buccal cavity during the feeding of dry and spiny fodder [16]. CE or ORFV contains

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different virulence factors and envelop proteins namely highly immunogenic B2L genes, GIF gene that is an apoptosis inhibitor, IL-10-like gene, and vascular endothelial growth factors (VEGF) [1, 17]. ORFV mainly encodes for 132 genes including high GC content with 138,000bp long genetic elements. [1,18].

Viruses of it mainly remain in desiccated scabs for a long time and it is also resistant to soil and weather conditions. As it is a viral disease and treatment is not very effective, vaccination may prevent it better [19]. Vaccine production for the orf virus is now a burning demand to reduce the morbidity and mortality of goats and sheep thus reducing the tremendous losses in our economy. Under the present immunological pressure, genetic changes in the dominant strains might not, however, shield the host from reinfection with inactivated and attenuated vaccinations [19, 20]. To choose vaccine strains and modify immunization protocols, information on regional pandemic strains is desperately needed. Furthermore, there is a chance that attenuated vaccinations will cause virulence reversion, viral propagation, cross-species and transregional transmission of ORFV, and co-prevalence of various strains [20, 21]. These results highlight the need for more thorough and organized epidemiological data and genetic ORFV information to create better preventative and control measures for the CE/ORFV virus circulating in Bangladeshi goats. Thus, the purpose of this work was to determine the prevalence and risk factors for CEV in Bangladeshi goats as well as to isolate, identify, and characterize it molecularly.

Material and Methods

Study sites and period

The study was conducted with the collection of suspected samples from Dhaka, Rajshahi, Bandarban, Meherpur, Mymensingh, and Chuadanga districts in Bangladesh (Fig. 1) from July 2020 to June 2022.

Samples and methods of sampling

From the research locations, a total of 100 dried scabs, papular secretions, and crust samples from goats suspected of having CEV were gathered (Fig. 2). A questionnaire was used to gather some information through in-person interviews with the owners regarding locations, age, sex, and seasons. After that, every sample was gathered aseptically, put into sterile, individual falcon tubes with PBS, labeled appropriately, and stored in an ice box. The samples were then sent right away to the Bangladesh Livestock Research Institute's Small Ruminants Research Laboratory in Savar, Dhaka. Before being used, the field samples were either processed right away or kept at -80°C.

Preparation of inoculum

Using sterile scissors and forceps, about 25 mg of the sample was chopped. It was then triturated and homogenized with phosphate-buffered saline (pH 7.4) in a sterile pestle and mortar, and then centrifuged at 1500 rpm for 15 minutes. After that, the supernatant was gathered and kept for later use at -80°C.

Isolation of CEV in LTC

The 25 cm^2 tissue culture flask was used to cultivate LTC cells, and the flask was watched for three to four days. Once the cell monolayers were discovered, two flasks were infected with the previously prepared, antibiotic-treated inoculum, while one flask was preserved as a control. Penicillin (10,000 UI/ml) and streptomycin (100 lg/ml) were introduced after an hour of incubation at 37°C in a maintenance medium (Eagle MEM, Sigma, USA) without fetal calf serum (FCS). For a maximum of five to seven days after vaccination, the flask was incubated at 37 °C and checked every day to see if any cytopathic effects (CPE) had been shown [17]. The test sample was conducted alongside a negative control. To identify the virus from the culture supernatant, PCR was performed.

Identification of CEV by PCR

DNA extraction and PCR

Following the manufacturer's recommendations, DNA was extracted from clinical samples (scabs, crusts) and cell culture isolated virus using a DNA extraction kit (Monarch® Genomic DNA Purification Kit, New England, USA). Genomic DNA was amplified using VIR-UP 5'-ATGGCCTGCGAGTGCG-3'; VIR-DN: 5'-GAAGCTGATGCCGCAGTTGT-3' (Li et. al 2013) and GIF5: GCT CTA GGA AAG ATG GCG TG; GIF 6: GTA CTC CTG GCT GAA GAG CG [9]. The PCR was run in a reaction volume of 25µl. 12.75µl of GoTaq Master Mix, 1µl of forward and 1µl of reverse primer (each 10 pmol), 5.25µl of nuclease-free water, and 5µl of template DNA made up the reaction mixture. First, denaturation at 95^0C for five minutes was followed by 35 cycles of denaturation at 940C for fifteen seconds, annealing at 620C for fifteen seconds, and extension at 72^0C for seventy-five seconds. For 20 minutes, the last extension was run at 72^0C. To analyze the DNA band, the 1% agarose gel electrophoresis was performed using ethidium bromide and pictured under a UV transilluminator (GEP-UV1, Infitek, China). A 30-minute runtime at 100 volts was used.

Sequencing and phylogeny analysis

PCR-positive culture fluids were considered for the sequencing of GIF and VIR genes, respectively. The PCR products were purified by PureLinkTM Quick PCR Purification kit (ThermoFisher, USA). Then the purified PCR products were sent to the Molecular Division, National Institute of Biotechnology, Bangladesh for both forward and reverse direction of partial sequencing of GIF and

VIR genes using Sanger sequencing. Obtained raw sequences from cycle sequencing were read by BioEdit 7.2 and proofread by aligning with reference genome derived from the GenBank database. The final consensus sequences were made from the bidirectional sequences in FASTA file format which were then BLAST searched to check maximum similarity. The final sequences of the FASTA format were compared with the published sequences of CEV of respected genes deposited in the GenBank database. Next, the sequences were aligned by the MUSCLE algorithm in MEGA 11 [21] with other related sequences collected. The phylogenetic tree of both viruses was constructed using the software package MEGA X (37). Then these aligned homogenous sequences were exported in FASTA file format and mega file format for diversity and phylogenetic analysis. Subsequently, phylogenetic trees based on nucleotide sequences were constructed for the CEV using the Neighbor-Joining methods with 1,000 bootstrap replicates in MEGA11 and edited in Fig Tree v1.4.4. The sequences were submitted to the NCBI GenBank database under assigned accession numbers OR452355.1, OR452356.1, OR452357.1, OR452358.1, and OR452360.1.

Statistical analysis

The record of the animal data like age, sex, location, and seasons were analyzed for the determination of prevalence rate, 95% CI, and odds ratio (OR) by univariate linear regression in STATA 18 software (Stata Corp, USA).

Results

Prevalence of Contagious ecthyma (CE) on different parameters regarding locations, age, sex, and seasons showed a higher prevalence in Mymensingh (70%, 95% CI: 34.75-93.32) on which lower prevalence showed in Meherpur (50%, 95% CI: 18.70-81.29). Statistically, the results revealed that there is a significant difference in different areas with 95% confidence intervals (Table 1). Sex-wise prevalence showed that female goats are more prone to CE than male goats. Among 63 female goats, 40 (63.49%, 95% Cl: 50.40-75.26) were found positive whereas among 37 male goats 17 (45.95%, 95% CI: 29.48-63.07) were found positive (Table 1). So, there is a significant difference between the male and female goats. Considering the age, the goats with the highest prevalence was found in the age group 6 to 12 months whereas the lowest prevalence was found in the age group of 1 to 3 years. Subsequently, the prevalence rate in the age group 1 to 3 months was 45.71% (95% CI: 28.82-63.35) whereas >3 to 6 months was 62.50%, (95% CI: 35.43-84.80), >1-3 years 50%, (95% CI: 21.09-78.90), and > 3 years 53.33 % (95% CI: 26.58-78.73) respectively. The statistical analysis shows that goats get more infection during the winter season (75.61%95% CI: 59.69-87.63) than in two other seasons of summer (50%, 95% CI: 31.88-68.11) and in the rainy season (60%, 95% CI: 19.40-57.63).

DNA fragments of CEV were amplified using primer pairs of specific sizes and identified as effective for amplifying CE viral DNA that gives the band at 549bp and 400bp (Figure 3). Granulocyte-macrophage-colony-stimulating factor (GM-CSF) and interleukin-2 inhibition factor (GIF) were expressed by the target genes GIF5 and GIF6. Three days post-inoculation (dpi) in LTC were confirmed to be PCR-positive samples following confirmation of isolation in cell cytopathic effect (CPE) (Figure 4b). Nearly all of the cells started to round and separate at 7 dpi (Figure 4c). These virus-containing cells were then removed and kept at -800C for additional attenuation and propagation. The virus was then confirmed again using PCR with the real band size. The isolate under study is found to be closely related to Bangladeshi ORFV isolates, as shown by phylogenetic analysis using neighbor-joining methods. The VIR and GIF genes of the Bangladeshi isolates showed all the members of ORFV clustered into different distinct groups (Figs. 5a and 5b). The GIF coding gene sequences (OR452357.1/ORFV/Goat/Bangladesh/BLRI-05/ 2021, OR452359.1/ORFV/Goat/ Bangladesh/ BLRI-02/2021 and OR452360/ORFV/Goat/ Bangladesh/ BLRI-01/2021) generated in the present study showed a 99.98 to 99.99% similarity of nucleotide among them. It was found that 99.97 to 99.99% and 99.99 to 100% were identical to the nucleotide sequences of India and China, respectively (Figure 6a). For the VIR coding gene sequences (OR452355/ORFV/Goat/Bangladesh/BLRI-01/2021 and OR452356/ORFV/Goat/Bangladesh/BLRI-02/2021), it was revealed that 99.98% similarity of NT between the isolates, whereas the isolates were 99.94 to 99.97% similar to other isolates of India (Fig. 6b).

Discussion

Contagious ecthyma is a viral disease that affects young small ruminants globally and can lead to major health issues [3]. Due to a lack of immunizations and preventative measures, CE is an endemic disease in Bangladesh and makes individuals more susceptible. The present study prevails the recent data with the prevalence of this disease with different parameters and also with molecular detection of isolates and phylogenetic analysis of strain of contagious ecthyma virus. In this study prevalence rate of contagious ecthyma found in Bangladesh was 57 %. Regarding the age of goats, 6 to 12 months old goats (77.27%) were more prone to be affected by contagious ecthyma which can be compared with [12] where 41.42% of goats aged between 7-12 months were prevalent with contagious ecthyma. Considering the other age group from 1-3 years (50%) was less susceptible than 3-6 months

(62.50%) which can be compared with the findings of [12] where 2 months of age goat showed 24.29%, 2-3 years showed 8.57% and >3 years showed 8.57% prevalence with contagious ecthyma. Other similar studies also stated that young goats <1 year old were more prone to CE than other age group [4, 22, 23].

Season-wise variation was also observed in this study where contagious ecthyma in Bangladesh mainly occurs during the winter season with extensive lesions on the mouth of goats. In our study, the most vulnerable season for the occurrence of the disease is winter where 75.61% of goats were affected, whereas 50% were in summer and 37.04% in the rainy season, and this finding can be compared with other author's findings [23] where authors found a higher prevalence of CE in the winter season compared to other seasons. This finding may also be compared with [12] where 78.57% of cases occur during the winter season than other seasons. However, these findings are contrary to some researchers who found a higher prevalence of CE during the early summer [2]. This could happen due to differences in agroecological zones and different breeds used for the study. Three days post inoculation (dpi) in LTC were found to be PCR-positive samples following confirmation of isolation in cell cytopathic effect (CPE) [17]. CPE is made up of intracytoplasmic or intranuclear aggregation as well as the rounding and detaching of cells [11]. PCR-positive samples produced a VIR gene band of 549 bp, which was consistent with the results of another researcher [24]. In contrast, GIF genes produced a 400 bp band that was consistent with the findings of previous researchers [9, 11, 25, 26].

Bangladeshi isolates showed higher similarities percentage with Indian isolates (VIR) whereas other isolates (GIF) showed higher similarities percentage with China and Pakistani isolates. Phylogenetic analysis of VIR and GIF genes formed clustered with Indian and China isolates compared with the other parapoxviruses. The OR452355.1/ORFV/ Goat/Bangladesh/BLRI-01/2021 isolates grouped with Indian isolates whereas OR452357/ORFV/Goat/Bangladesh/BLRI-05/2021 of GIF genes grouped with China KF726847.1/ORFV/ Goat/ China. Hypothetically the possible route of transmission of this virus for entrance is extremely difficult to predict. The importation of animals may be the possible route for the entrance of ORFV in Bangladesh. For this reason, close observation with special monitoring should be implemented in the near future.

Conclusion

Contagious ecthyma (CE) is circulating in different areas of Bangladesh. Winter season and young goats are more vulnerable to CE. Female goats are more susceptible than male goats. Sequence analysis and phylogenetically two isolates of contagious ecthyma virus have a close relationship and genetic variation with China and Indian isolates. The whole genome sequence should be performed for the in-depth genetic information of CEV circulating in Bangladesh as well as a potential vaccine development is recommended. Additionally, nationwide action should be taken for the prevention and control of CE for protecting the goat industry.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

This research study was affirmed by the Animal Experimentation and Ethics Committee of Bangladesh Livestock Research Institute (Reference number: AEEC/BLRI00110/2023).

Authors Contribution

M.S.A.: Conceptualization, designing the study, laboratory test, supervision, and critical review; **So.A.:** Data and sample collection, laboratory test, epidemiological data analysis and interpretation, and drafted and revised the manuscript. **M.H.R.:** Sample collection, laboratory test, epidemiological data analysis, assisted in writing the manuscript, revised the manuscript, and critical review; **M.Z.A.:** Gene sequence data and epidemiological analysis and interpretation, revised the manuscript, and critical review; **S.A.:** Supervision, funding acquisition, and reviewed the manuscript. All authors have read, reviewed, and approved the published version of the manuscript.

TABLE 1. Prevalence of contagious ecthyma virus from suspected field sample

Variable	Category	No. of samples tested	No. of Positive (%)	95% CI	SE	OR
Location	Dhaka	30	15 (50)	31.29-68.70	0.091	1
	Rajshahi	15	10 (66.67)	38.38-88.17	0.121	1.33
	Bandarban	20	12 (60)	36.05-80.88	0.109	1.20
	Meherpur	10	5 (50)	18.70-81.29	0.158	-
	Mymensingh	10	7 (70)	34.75-93.32	0.144	1.40
	Chuadanga	15	8 (53.33)	26.58-78.73	0.128	1.06
Age	1-3 Months	35	16 (45.71)	28.82-63.35	0.084	1
	>3-6 Months	16	10 (62.50)	35.43-84.80	0.121	1.36
	>6-12 Months	22	17 (77.27)	54.62-92.17	0.089	1.69
	>1-3 Year	12	6 (50)	21.09-78.90	0.144	1.09
	>3 Year	15	8 (53.33)	26.58-78.73	0.128	1.166
Sex	Male	37	17 (45.95)	29.48-63.07	0.081	1
	Female	63	40 (63.49)	50.40-75.26	0.060	1.383
Season	Summer	32	16 (50)	31.88-68.11	0.088	1.350
	Rainy	27	10 (37.04)	19.40-57.63	0.092	1
	Winter	41	31 (75.61)	59.69-87.63	0.067	2.041
Total		100	57 (57%)	46.71-66.80	0.049	

SE- Standard error; OR- Odds Ratio, CI- Confidence interval

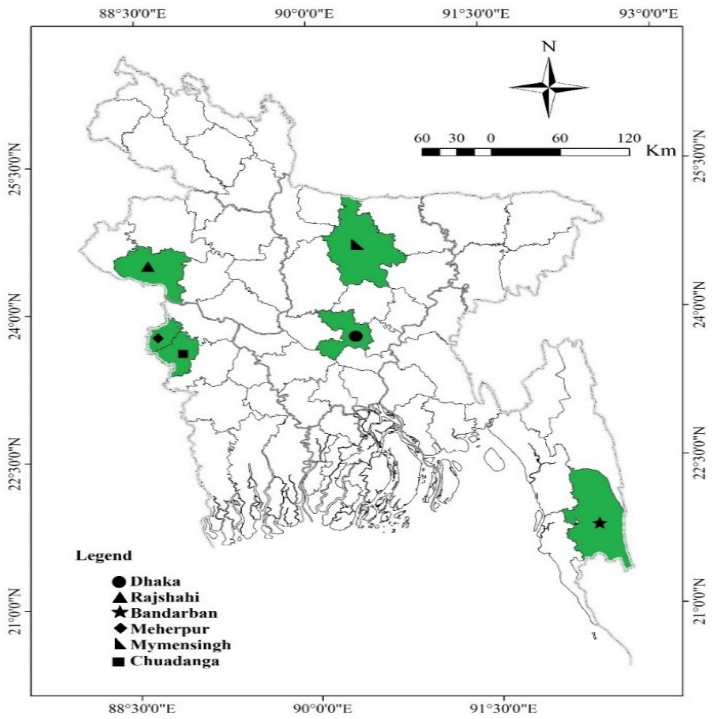

Fig. 1. Location of sampling areas of Contagious ecthyma virus.

Fig.2. Typical clinical signs (red circle) of Contagious ecthyma-affected goats (udder, leg and mouth).

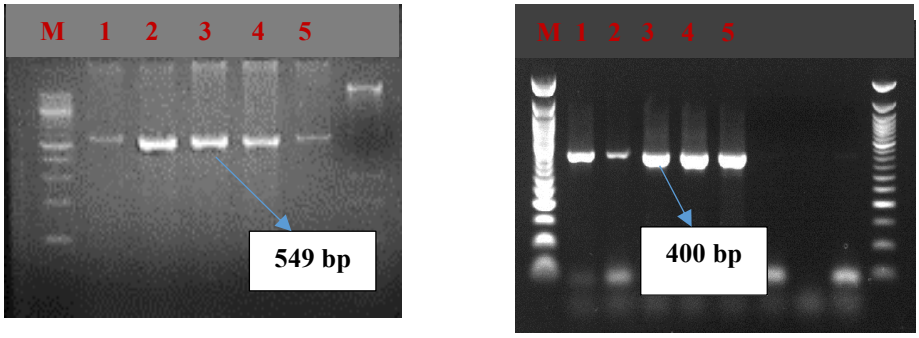

Fig. 3. Amplifications of VIR gene (549 bp) with 100bp ladder marker (M) (left) and GIF gene (400 bp) with 50bp ladder marker (M) (right) of contagious ecthyma virus.

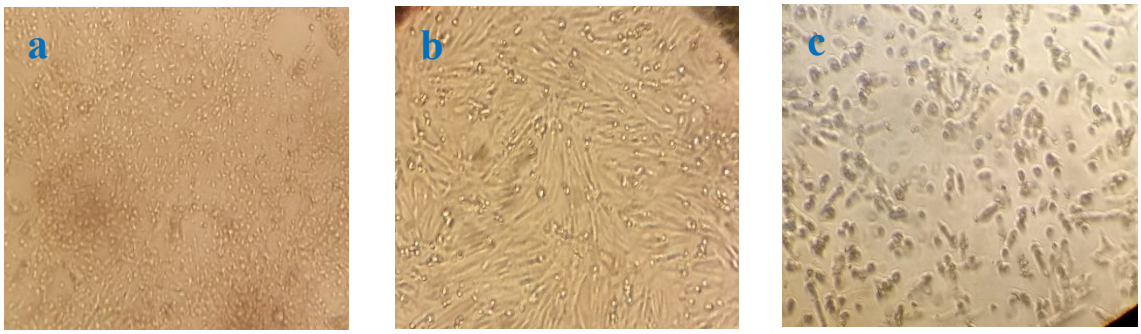

Fig. 4. Culture of contagious ecthyma virus (CEV) in LTC. (a) Shows the normal lamb testicular cell, (b) shows characteristic CPE at 3 DPI of CEV, and (c) shows characteristic CPE at 7 DPI of CEV.

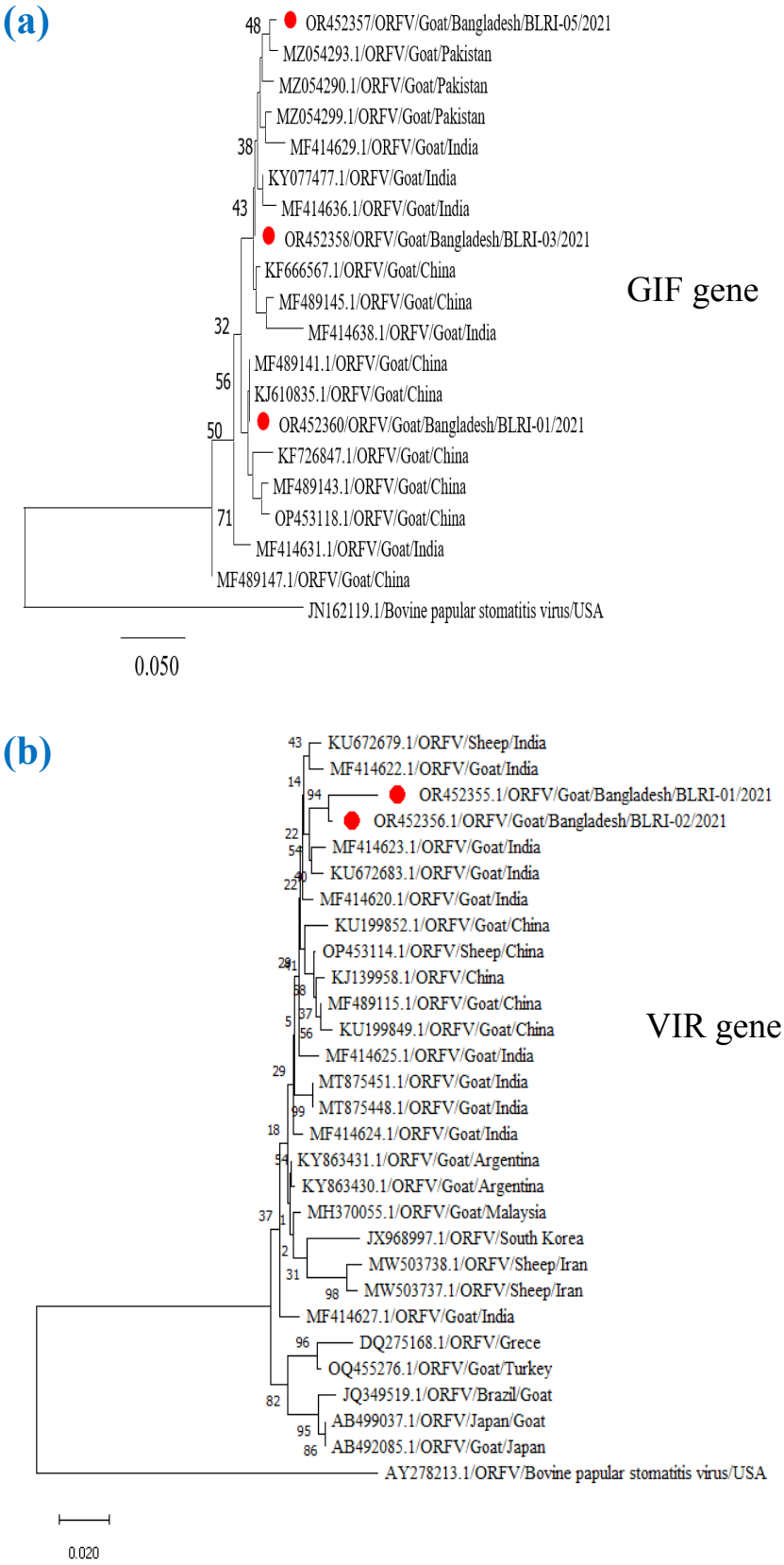

Fig. 5. Phylogenetic relationship of GIF and VIR gene of contagious ecthyma virus. The tree was prepared using the neighbor-joining method. The red circle indicates isolated for this study.

Fig. 6. Similarity percent and divergence of the contagious ecthyma virus GIF and VIR genes isolated from Bangladesh in this study in comparison to other contagious ecthyma viruses.

		Divergence															
	Percent similarity		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
BLRI-01/2021		1		0.01	0.02	0.01	0.02	0.02	0.00	0.00	0.01	0.01	0.02	0.02	0.02	0.01	0.01
BLRI-03/2021		2	99.99		0.01	0.00	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.01	0.01	0.01	0.00
BLRI-05/2021		3	99.98	99.99		0.01	0.01	0.01	0.02	0.02	0.02	0.03	0.01	0.02	0.02	0.01	0.01
KY077477.1/India		4	99.99	100.00	99.99		0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.01	0.01	0.00
MZ054299.1/Pakistan		5	99.98	99.99	99.99	99.99		0.01	0.02	0.02	0.02	0.03	0.02	0.01	0.01	0.01	0.01
MZ054290.1/Pakistan		6	99.98	99.99	99.99	99.99	99.99		0.02	0.02	0.02	0.03	0.02	0.01	0.02	0.01	0.01
MF489141.1/China		7	100.00	99.99	99.98	99.99	99.98	99.98		0.00	0.01	0.01	0.02	0.02	0.02	0.01	0.01
KJ610835.1/China		8	100.00	99.99	99.98	99.99	99.98	99.98	100.00		0.01	0.01	0.02	0.02	0.02	0.01	0.01
MF489143.1/China		9	99.99	99.98	99.98	99.98	99.98	99.98	99.99	99.99		0.01	0.03	0.03	0.03	0.01	0.02
OP453118.1/China		10	99.99	99.98	99.97	99.98	99.97	99.97	99.99	99.99	99.99		0.02	0.03	0.03	0.02	0.02
MF489145.1/China		11	99.98	99.99	99.99	99.99	99.98	99.98	99.98	99.98	99.97	99.98		0.02	0.02	0.02	0.01
MF414636.1/India		12	99.98	99.99	99.98	100.00	99.99	99.99	99.98	99.98	99.97	99.97	99.98		0.02	0.02	0.01
MF414629.1/India		13	99.98	99.99	99.98	99.99	99.99	99.98	99.98	99.98	99.97	99.97	99.98	99.98		0.02	0.01
MZ054293.1/Pakistan		14	99.99	99.99	99.99	99.99	99.99	99.99	99.99	99.99	99.99	99.98	99.98	99.98	99.98		0.01
KF666567.1/China		15	99.99	100.00	99.99	100.00	99.99	99.99	99.99	99.99	99.98	99.98	99.99	99.99	99.99	99.99	

(a) Pairwise comparison of GIF gene

		Divergence														
	Percent similarity		1	2	4	5	6	7	8	9	10	11	12	13	14	15
BLRI-01/2021		1		0.022	0.065	0.074	0.086	0.068	0.036	0.066	0.032	0.040	0.040	0.042	0.035	0.049
BLRI-02/2021		2	99.98		0.044	0.054	0.065	0.048	0.019	0.048	0.017	0.020	0.020	0.022	0.021	0.030
AB499037.1/Japan		4	99.93	99.956		0.010	0.043	0.052	0.048	0.048	0.049	0.045	0.038	0.043	0.035	0.036
JQ349519.1/Brazil		5	99.93	99.946	99.99		0.037	0.054	0.055	0.050	0.054	0.052	0.041	0.050	0.038	0.039
DQ275168.1/Grece		6	99.91	99.935	99.957	99.963		0.061	0.061	0.055	0.061	0.054	0.048	0.056	0.043	0.050
JX968997.1/S. Korea		7	99.93	99.952	99.948	99.946	99.939		0.039	0.044	0.039	0.046	0.035	0.035	0.023	0.039
KU672679.1/India		8	99.96	99.981	99.952	99.945	99.939	99.961		0.036	0.013	0.017	0.017	0.011	0.021	0.022
MW503738.1/Iran		9	99.93	99.952	99.952	99.95	99.945	99.956	99.964		0.042	0.042	0.038	0.040	0.039	0.046
MF414623.1/India		10	99.97	99.983	99.951	99.946	99.939	99.961	99.987	99.958		0.018	0.018	0.017	0.019	0.028
MF414625.1/India		11	99.96	99.98	99.955	99.948	99.946	99.954	99.983	99.958	99.982		0.015	0.020	0.019	0.024
MF414624.1/India		12	99.96	99.98	99.962	99.959	99.952	99.965	99.983	99.962	99.982	99.985		0.020	0.011	0.017
MF414622.1/India		13	99.96	99.978	99.957	99.95	99.944	99.965	99.989	99.96	99.983	99.98	99.98		0.017	0.022
MH370055.1/Malaysia		14	99.97	99.979	99.965	99.962	99.957	99.977	99.979	99.961	99.981	99.981	99.989	99.983		0.017
MF414627.1/India		15	99.95	99.97	99.964	99.961	99.95	99.961	99.978	99.954	99.972	99.976	99.983	99.978	99.983	

(b) Pairwise comparison of VIR gene

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