Short Communication

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Designing Two Synthetic Constructs for Real Time PCR Detection of *Francisella tularensis* and *Ebola* Virus

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Abstract

Background: Generally, timely diagnosis of micro-organisms is very important to prevent many diseases. Many methods can detect micro-organisms like culture-based methods and molecular methods. The molecular methods are usually preferred because they provide fast and reliable results. In some cases, microbial strains are not accessible, and there is no safety to work with them; therefore, synthetic constructs which are designed according to the available sequences in databases can be used as a positive control for detection of them.

Methods: In this study, a synthetic construct was designed for molecular detection of *Francisella tularensis* (*F. tularensis*) and the *Ebola* virus by multiplex real-time PCR reaction. For this, sequences were taken from databases and then multiple alignments were done by software. Also, conventional PCR and two models of real-time PCR (SYBR green and TaqMan) were applied. Finally, multiplex real-time PCR was performed.

Results: The synthetic construct was designed and used for conventional PCR and multiplex PCR. The results of common PCR showed a single band at 148 *bp* and 167 *bp* in 1.5% agarose gel stained by ethidium bromide for *F. tularensis* and *Ebola* virus, respectively. Also, a dual-band at 148 and 167 *bp* was observed in multiplex PCR. Results of real-time PCR showed a limit of detection about 0.1 *pg* of plasmid/*µl*.

Conclusion: In conclusion, the designed construct can be used as a positive control for an accurate diagnosis of these micro-organisms without any biological danger for laboratory staff. So, this method is useful for diagnosis of these agents in food, water, and blood samples.

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Keywords: *Ebola* virus, Humans, Multiplex polymerase chain reaction, Real-time polymerase chain reaction

Introduction

Francisella tularensis (*F. tularensis*) and *Ebola* virus are highly-infectious elements belonging to family of *Francisellaceae* and *Filoviridae*, respectively [1,2]. *F. tularensis* is a gram-negative intracellular bacterium that causes zoonosis and tularemia or rabbit fever that are highly infectious [2]. These bacteria have two strains including type A strains which are found in America and type B strains which are found in the northern hemisphere [3]. However, these bacteria can survive several days in nature, but they need special conditions to grow up in microbial cultures in the laboratory. Although these bacteria are pathogenic, they can be dangerous for laboratory staff. *Ebola* virus is a non-segmented single-stranded RNA virus which causes hemorrhagic fever [4]. This virus has three species of *Undibugyo*, *Zaire*, and *Sudan*. The first outbreaks of diseases associated with *Ebola* appeared in 1976 in Nzara in Sudan and Yambuku in Congo [5]. According to these features, the existence of a diagnostic method for accurate and safe detection of these pathogens is necessary to prevent infection. There are many diagnostic methods for the detection of bacteria and viruses like culture-based methods, immunological methods, and molecular ones. Cell culture-based methods are

time-consuming and may be dangerous for laboratory staff who work on infectious elements. Immunological methods include Complement Fixation (CF) [6,7], Enzyme-Linked Immunosorbent Assay (ELISA) [8,9], and so on. These approaches use antibodies to detect the infectious micro-organisms. These are also time-consuming, because the detection is based on antibody titration and antibodies usually appear nearly 2 weeks after infection. Therefore, molecular methods are more preferred, because of fast and safe diagnosis and their accurate results. In molecular methods such as Polymerase Chain Reaction (PCR) [10-13], Loop-Mediated isothermal Amplification (LAMP) [14] Nucleic Acid Sequence-Based Amplification (NASBA) [15] and DNA micro-array [16-18], a specific region in the genomic area can be recognized. These approaches are safe because the genome of micro-organisms is used for experimental works and risk factors affecting laboratory personnel are controlled. Molecular methods like other methods have some problems; for example, LAMP methods for detection are very sensitive and due to their high sensitivity, there is a probability for false positive results or in PCR-based methods, thermocycler is necessary. But the basic problem in these methods is preparing the genome of micro-organisms. In some cases like *F. tularensis* and *Ebola* virus, micro-organism or genome of them is so rare in some countries like our country. Therefore, for solving the problem of diagnosis of infections caused by these pathogens, a new approach is introduced in this study. In this approach, conserved genomic area of micro-organism was selected and then synthesized and cloned to a plasmid vector for amplification [19]. In the current study, a synthetic construct containing a specific genomic area of pathogenic elements (*F. tularensis* and *Ebola*) was designed. This vector was used for a simple PCR reaction for a separate diagnosis of these elements and multiplex PCR reaction for the detection of these elements at the same time. Also, quantitative PCR reaction was applied due to its high specificity and sensitivity. For this study, fopA and whole-genome were chosen for the detection of *F. tularensis* and the *Ebola* virus, respectively.

Identification of two pathogens with different clinical symptoms by a positive control structure is the difference between this study and other studies. In fact, the purpose of designing this positive control structure is to obtain the necessary technical knowledge to design a safe positive control for very dangerous pathogens with a high probability of contamination when working with it. This positive control construct also reduces the likelihood of false positives when examining clinical specimens of the patient.

Materials and Methods

Synthetic construct designing

In this paper, a specific construct was designed for the fast detection of two micro-organisms containing *F. tularensis* and *Ebola* virus. For this, specific sequences as targets were chosen from NCBI/EMBL. After that, multiple alignments for the selected targets were done by 6Mega BLAST, BioEdit, AliView, and Clustal W software. In the next step, selected sequences were used in SnapGene offline software for designing a plasmid vector containing these sequences. In this step, for each target, two restriction sites were designed at the ends of each target as a simple method for cloning and subcloning the segments. Accordingly, XhoI restriction sites were added to the beginning of fopA segment of *F. tularensis* and SacI was added to the end of the *Ebola* segment and both segments were ligated to each other by an EcoRI restriction site. Also, a BamHI restriction site was designed in the middle of segment to prevent false positive results in case of suspicious template. However, the amplicon sizes were different in the construct as well as the actual size in the micro-organisms to prevent vague or false positive results. The designed constructs were shown in figure 1. The base of this pUC57 structure and both pathogen fragments in the relevant plasmid were artificially cloned and lyophilized from the relevant company.

Designing multiplex primers and hydrolysis probes for multiplex qPCR assay

Target sequences for the reliable detection of *F. tularensis* and *Ebola* virus were selected based on previous literature and sequences available in databases. Selected targets were *fopA* gene (AF097542) for *F. tularensis* and the whole-genome for *Ebola* virus.

Sequences retrieved from NCBI/EMBL were organized and aligned using 6 Mega BLAST software. Oligonucleotides for multiplex qPCR assays and conventional PCR assays were designed using the OligoAnalyzer online software. The design strategy for a multiplex qPCR assay was as follows. First, a hydrolysis probe and primer set were designed for each of the selected target sequences for these agents. Finally, the BLAST N analysis was performed to confirm the specificity of the target organisms.

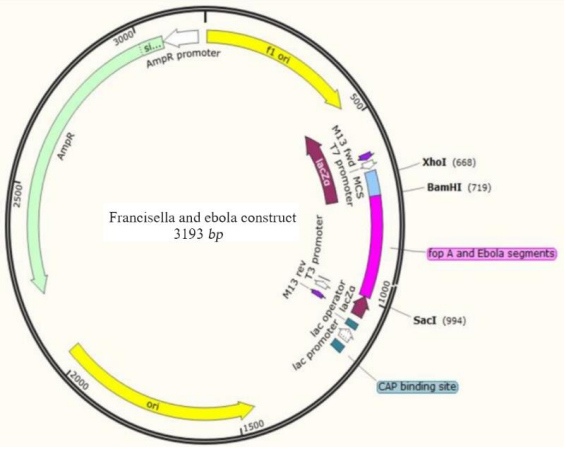

Figure 1. Designed construct in SnapGene software.

Construct synthesis and plasmid extraction

After target selection and construct designing, target segments were sent for synthesis of DNA segments and cloning to plasmid vectors then transforming to *Escherichia coli (E. coli)* DH5α. After synthesis of the segments, the bacteria were cultured overnight on LB-broth media containing ampicillin as a selection marker for carrier bacteria. In the next step, plasmid extraction was performed and the extracted constructs were used for PCR amplification.

Conventional PCR

The common PCR method was performed in a volume of 20 *µl*, containing 10 *µl* of PCR master mix (Amplicon, Denmark), 1 *µl* from each primer (10 *pM*), 4 *µl* of plasmid as a template (100 *pg*), and 4 *µl* of sterile water. PCR reaction was performed based on the following program: initial denaturation at 95 ℃ for 5 *min*, 30 cycles of denaturation at 94 ℃ for 30 *s*, primer annealing at 62 ℃ for 30 *s*, extension at 72 ℃ for 30 *s*, and final extension at 72 ℃ for 5 *min*. In the end, PCR products were analyzed by 2% agarose gel stained by ethidium bromide. Primer sequences and properties were shown in table 1.

Multiplex PCR reaction

The multiplex PCR assay was performed *via* designed primers as follows: 10 *µl* of PCR master mix (Amplicon, Denmark), 1 *µl* from each primer (10 *pM*), 4 *µl* of plasmid as a template (100 *pg*), and 4 *µl* of sterile water. In this step, 2 sets of primers were used according to the targets in the plasmid vector.

Development of real-time PCR

At first, monoplex real-time PCR was done for each segment. In the next step, multiplex real-time PCR was performed by SYBR green. For SYBR green-based qPCR reaction, a mixture with volume of 10 *µl* was prepared as follows: 5 *µl* of SYBR green, 0.5 *µl* from each primer (10 *pM*), 2 *µl* of plasmid vector as a template (100 *pg*), and 2 *µl* of sterile water. Finally, multiplex real-time PCR was performed using a designed TaqMan probe. The probes sequences were shown in table 1.

Sensitivity

The sensitivity of the reaction was tested by making a serial dilution from 100 *pg* to 0.1 *pg* of plasmid template for common PCR, multiplex PCR, and real-time PCR assays.

Results

Conventional PCR assay

Conventional PCR was performed to confirm the primer and constructs. Also, results showed a specific band at 148 *bp* and 167 *bp* for *F. tularensis* and *Ebola* virus, respectively. Also, the sensitivity of the reaction was analyzed using a serial dilution of an extracted plasmid. The results of conventional PCR were shown in figure 2.

Multiplex PCR assay and sensitivity of the reaction

After designing the primers and construct, a multiplex PCR reaction was performed. According to this, a dual-band at 148 and 167 *bp* was observed. Also, the sensitivity analysis of the reaction was done and the results were shown in figure 3.

SYBR green qPCR assay

After the common PCR was done and results confirmed the primers and designed constructs, the SYBR green PCR assay was performed and the results were shown in figure 4.

TaqMan qPCR assay

In the next step, the segments were used in a TaqMan qPCR reaction with a designed probe. The results were shown in figure 5.

Discussion

F. tularensis and *Ebola* virus are high-infectious elements that cause rabbit fever and *Ebola* hemorrhagic fever. Several methods may detect these microbes. One

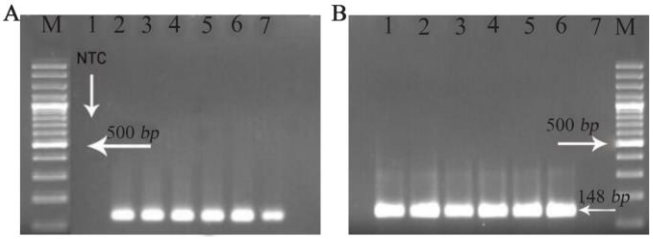

Figure 2. A) Analysis of the sensitivity of conventional PCR for *F. tularensis fopA* gene in 1.5% agarose gel stained by ethidium bromide. Lane (M) 100 *bp* DNA marker, lane (1) negative control, lane (2) 10 *ng* of DNA, lane (3) 1 *ng* of DNA, lane (4) 100 *pg* of DNA, lane (5) 10 *pg* of DNA, lane (6) 1 *pg* of DNA, lane (7) 0.1 *pg* of DNA . B) Analysis of the sensitivity of the PCR reaction for *Ebola* virus in 1.5% agarose gel stained by ethidium bromide. Lane (1) 10 *ng* of DNA, lane (2) 1 *ng* of DNA, lane (3) 100 *pg* of DNA, lane (4) 10 *pg* of DNA, lane (5) 1 *pg* of DNA, lane (6) 0.1 *pg* of DNA, lane (7) negative control and lane (M) 100 *bp* DNA marker.

Table 1. Primer and probes sequences and properties

Primer and probe	Sequence	Target organism	Amplicon size of construct	Amplicon size of micro-organism
F.T	TGCAGCTAATAATTTCATTGCTCC	*F. tularensi*	148 *bp*	110 *bp*
R.T	CTACACCTAAGTACCACTGGC			
Probe	TACTTATAGCGCTTTGACTAACAAGGACAATACTTG			
F.E	GATGCCAACGATGCTGTGA	*Ebola*	167 *bp*	129 *bp*
F.T	TGCAAGAGGATGGAGACGAA			
Probe	CAGTGGCTCAAGCTCGTTTTTCAGGTC			

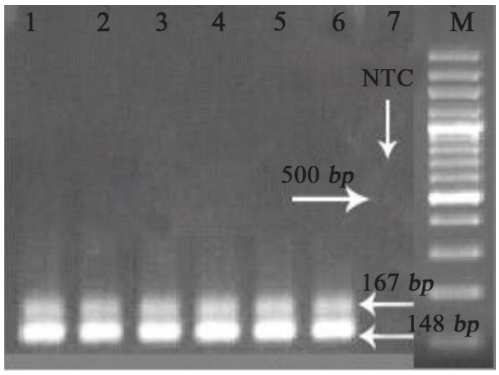

Figure 3. Analysis of the multiplex PCR and sensitivity of the reaction in 1.5% agarose gel stained by ethidium bromide. From left to right, lane (1) 10 *ng* of DNA, lane (2) 1 *ng* of DNA, lane (3) 100 *pg* of DNA, lane (4) 10 *pg* of DNA, lane (5) 1 *pg* of DNA, lane (6) 0.1 *pg* of DNA, lane (7) negative control and lane (M) 100 *bp* DNA marker.

of the most common diagnostic methods for bacterial detection is culturing, but this approach is not proposed in some cases like *F. tularensis*, because of infectious and fastidious growth conditions. However, there are many serological methods like ELISA, IFA, CFT, and so on for the common detection of both bacterial and viral infections in humans and animals. Berdal *et al* studied the serological methods such as rapid immunochromatography, ELISA, and the common PCR for diagnosis of *F. tularensis* after the outbreak in Norway in 2009 [20]. One of the most important problems in these approaches is that they are time-consuming and need special laboratory conditions like the current cases that required BSL3 conditions [21]. Immunological methods work on IgG and IgM antibodies although antibodies may appear 2-3 weeks after the infection. However, these methods are not suitable in some cases like food poisoning, because they are time-consuming. For ex-

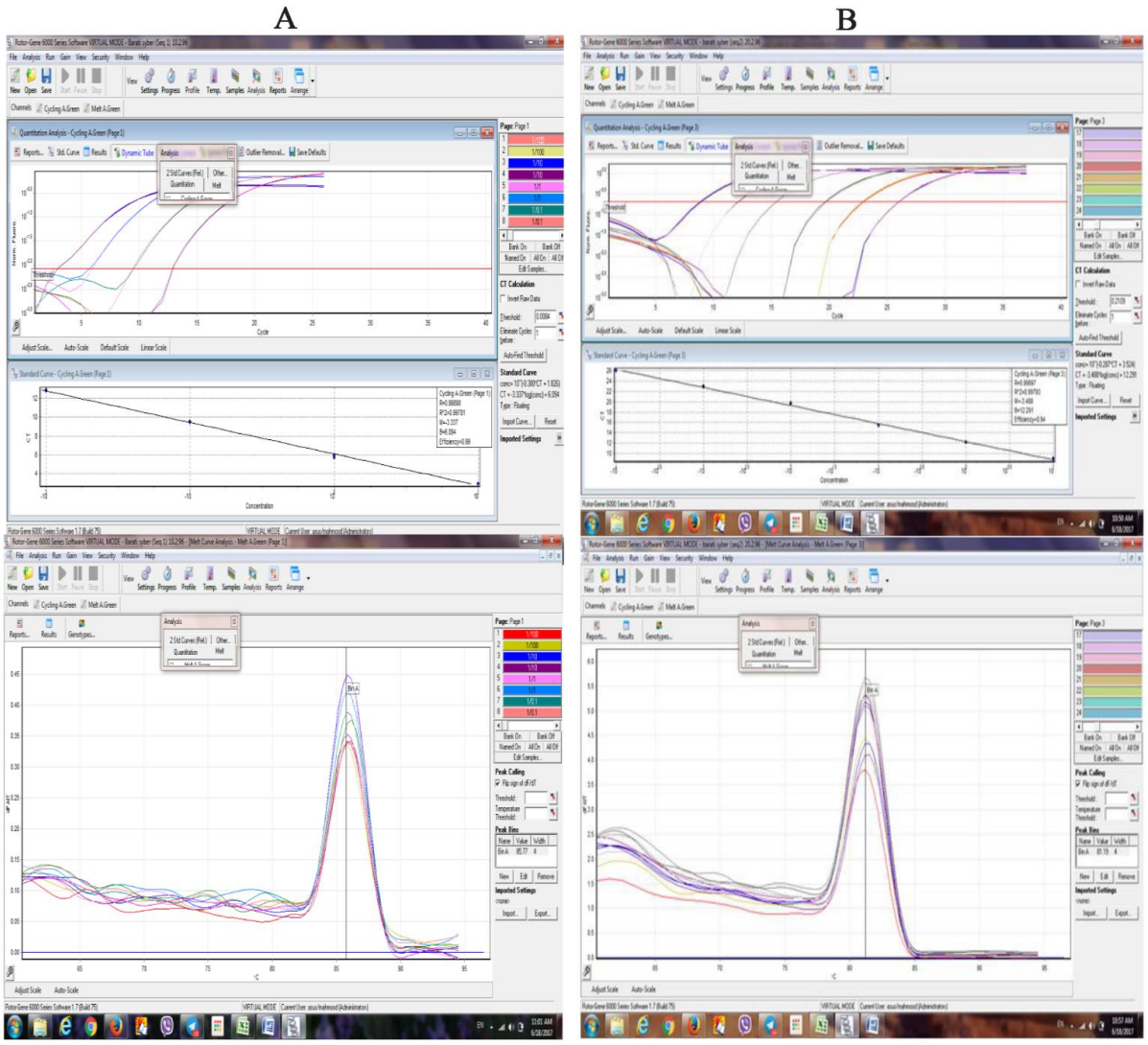

Figure 4. A) Analysis of SYBR green real-time PCR of *fopA* gene of *F. tularensis* using Corbett Rotor-Gene 6000. A serial dilution from 100 *pg* to 0.1 *pg* of plasmid was examined. B) Analysis of SYBR green real-time PCR of *Ebola* virus using Corbett Rotor-Gene 6000. A serial dilution from 100 *pg* to 0.1 *pg* of plasmid was examined.

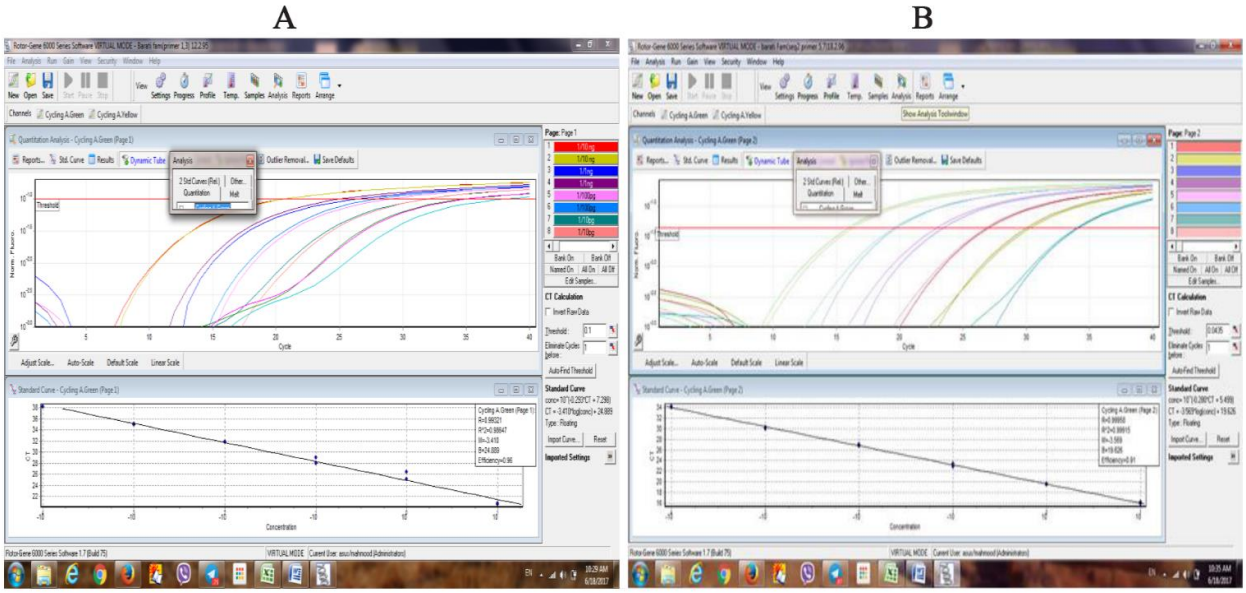

Figure 5. A) Analysis of TaqMan real-time PCR of *fopA* gene of *F. tularensis* using Corbett Rotor-Gene 6000. A serial dilution from 100 *pg* to 0.1 *pg* of plasmid was examined. B) Analysis of TaqMan real-time PCR of *Ebola* virus using Corbett Rotor-Gene 6000. A serial dilution from 100 *pg* to 0.1 *pg* of plasmid was examined.

ample, in these cases, the resulting rate and the reliability of the reaction is very important because the health of water and foods is vital in human life. In past years, the invention of the thermocycler and the appearance of molecular biology approaches established advanced progress in the field of diagnosis and molecular typing of microbes. In this study, a positive control was designed for the rapid and reliable detection of *F. tularensis* and the *Ebola* virus by multiplex real-time PCR reaction. Primers and probes were designed for an accurate diagnosis of the target organisms. In this work, TaqMan probes were used to increase the diagnosis range of different strains. There are so many designed constructs with different goals like detection or identification of some microbes in laboratories or producing subunit vaccines against pathogens. For example, Caasi *et al* designed a positive control for PCR reaction of Barley yellow dwarf virus, Soil-borne wheat mosaic virus, Triticum mosaic virus, and Wheat streak mosaic virus [22]. Also, Pourmahdi *et al* studied the molecular diagnosis of *Yersinia pestis* and *F. tularensis* by multiplex PCR in a synthetic construct and reported the sensitivity of about 36×10 -3 *ng/µl* [23]. In another study, Sohni *et al* designed an internal amplification control for the *Bacillus anthracis rpoB* gene, detected by TaqMan real-time polymerase chain reaction and reported a limit of detection about 5 *fg/µl* [19]. In 1996, Junhui *et al* studied the PCR detection of *F. tularensis* and they compared the sensitivity of the PCR method with the culture-based method to detect bacteria. They chose 3 sets of primers for PCR reaction [24]. In a previous study, Euler *et al* studied real-time PCR detection of *F. tularensis* by a new detection approach. They used a recombinase polymerase for isothermal real-time PCR amplification and they chose Tul4 as a target for amplification. Also, they reported a sensitivity of about 102 molecules for their diagnostic method [25]. In the case of detection of the *Ebola* virus, Towner *et al* diagnosed *Ebola* virus by RT–PCR in BSL4 conditions and they reported a limit of detection about 3-38 RNA copies for their method [26]. In another study, Weidmann *et al* studied molecular detection of *Zaire Ebola* virus, *Ebola Sudan* virus and *Marburg* virus (MBGV) by a RT-PCR assay [27].

Conclusion

According to the previous study in the field of detection of these micro-organisms and the reported data, our new designed construct and multiplex real-time PCR may be useful for a safe detection of these microbes simultaneously. These tools could be used as diagnosis kits for a rapid and safe detection of these microbes in contaminated or suspicious food and water or dairy products. Also, it could be used in laboratory to prevent the contamination without the need for BSL 3 and 4 laboratory conditions which leads to reduction of laboratory costs.

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