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Utility of CSP typing to sub-type clinical *Aspergillus fumigatus* isolates and proposal for a new CSP type nomenclature

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ABSTRACT

CSP typing is a newly developed sub-typing strategy that employs comparative DNA sequence analysis of the 12-mer tandem repeat region of the AFUA_3G08890 gene. In order to allow standardization of analysis and exchange of results between laboratories, we propose a new nomenclature for individual CSP repeats as well as for CSP types. A collection of 209 clinical isolates of *Aspergillus fumigatus* recovered from various hospitals throughout The Netherlands was analyzed by using CSP typing and this newly proposed nomenclature. Eighteen different CSP types were recognized, positioning the CSP gene as a typing target between the relatively low discriminatory MLST loci and the highly discriminatory microsatellite markers. CSP typing may be a welcome addition to the existing molecular methods to study the diversity of *A. fumigatus* at the sub-population level. The results also show the presence of lineages of closely related CSP types within the *A. fumigatus* population, adding unique and valuable information about the population structure of *A. fumigatus*.

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1. Introduction

Molecular typing methods based on DNA sequences rather than those based on complex banding patterns are considered to be the most useful when it comes to sharing data between laboratories. This is primarily because of the universal language of DNA sequence data, and the increasing availability of DNA sequencers to clinical laboratories. Other advantages of such sequencing methods include the ease of performance and option of automation. Multi-locus sequence typing (MLST) aims to discriminate between various isolates based on DNA sequence variation in a set of housekeeping genes and is considered the reference method in sequence based typing strategies for many microbial species (Maiden, 2006). Previously, Bain et al. (2007) reported about a multi-locus sequence type (MLST) scheme for *Aspergillus fumigatus*. Each target in this scheme contained up to 5 allelic variants whereas the combined selection of 7 genes discriminated 30 sequence types in a collection of 100 *A. fumigatus* isolates from various origins. This number of genotypes is relatively low compared to other fungal species such as *Candida albicans* (Tavanti et al., 2005; Garcia-Hermoso et al., 2007). Apparently, the *A. fumigatus* population contains only little sequence variation rendering MLST based approaches less useful for strain discrimination for this fungus.

Recently however, Balajee et al. (2007) reported about a novel gene, AFUA_3G08990, encoding a putative cell surface protein (further referred to as the CSP gene) as a target for a single locus sequence typing strategy and employed this method to study the relationships between *A. fumigatus* isolates from several nosocomial outbreaks in North America. The CSP gene contains a repeat region with a repeat unit of 12 bp with a consensus translation of TSVP. In a study looking at variations in protein coding regions, Levdansky et al. (2007) also studied this gene but they focussed on another repeat region located some 140 bp downstream relative to the first repeat region. This second repeat region contains a repeat unit of 18 bp with a consensus translation of PGQPSV. They showed that the encoded protein is involved in the conidial germination process and adherence to extracellular matrix (Levdansky et al., 2007). Variations in both repeat regions are the result of copy number changes (as the result of insertions or deletions of one or more repeat units) and point mutations (Balajee et al., 2007; Levdansky et al., 2007).

Here we report on the application of CSP typing (focussing on the 12-mer repeat region) to sub-type a collection of 209 presumably unrelated clinical *A. fumigatus* isolates. We demonstrate the presence of CSP lineages of closely related CSP types within the *A. fumigatus* population recovered from The Netherlands. In addition we also propose a standardized nomenclature for individual repeats as well as for CSP types in order to facilitate portability and exchange of CSP based typing data.

2. Materials and methods

2.1. Isolates

All isolates in this study were of clinical origin and were collected from 14 hospitals and from different patients throughout The

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Table 1
Summary of CSP repeat sequences.

Repeat nr.	Repeat sequence
r01	ACTTCTGTCCCG
r02	ACTTCTGTCCCA
r03	ACTCAAAACGCG
r04	ACTTCAATCCCG
r05	ACTTTTGTCCCG
r06	ACTTCAGTCCCG
r07	ACTACTATTGTG
r08	ACTTTTCTCCCG
r09	ACTTCTGTTCCG

Netherlands in the year 2005 as part of a surveillance study. All isolates were identified as *A. fumigatus* by macroscopic and microscopic characteristics and by their ability to grow at 48 °C. Isolates were stored as a suspension of conidia at −80 °C in regular freezing broth supplemented with 12.5% v/v glycerol. Prior to DNA isolation, isolates were revived by scraping off part of the frozen stock and plating on Sabouraud agar plates followed by incubation at 30 °C until sporulation.

2.2. DNA isolation

DNA was isolated from freshly prepared suspensions of conidia as described before (de Valk et al., 2005). Briefly, conidia were subjected to mechanical lysis using a MagNA lyser (Roche Diagnostics, Almere, The Netherlands) and further processed using a MagNA Pure LC instrument (Roche Diagnostics) in combination with the MagNA Pure LC DNA Isolation Kit III as recommended by the manufacturer (Roche Diagnostics). DNA yield and purity were estimated by UV absorbance measurements.

2.3. CSP typing

Part of the CSP gene was amplified and sequenced using the following procedures. Amplification reactions of 25 µl contained 0.5 µM of each amplification primer (5′-TTGGGTGGCATTGTGCCAA-3′ and 5′-GAGCATGACAACCCAGATACCA-3′ from Eurogentec, Seraing, Belgium), 0.2 mM dNTP's, 2 mM $MgCl_2$, 1 U FastStart Taq DNA polymerase (Roche Diagnostics) and 1 µl of DNA in 1× reaction buffer. The cycling program consisted of a 10 min activation/denaturation step at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C. An additional incubation for 10 min at 72 °C was included before the reactions were cooled to room temperature. PCR products were purified using SPRI chemistry (AMPure; Beckman Coulter, Woerden, The Netherlands) and subjected to DNA sequence analysis in combination with the DYEnamic ET Dye Terminator Kit, MegaBACE (GE Healthcare, Diegem, Belgium) as recommended. Sequence reactions were purified by SPRI chemistry (CleanSeq; Beckman Coulter) and analyzed on a MegaBACE 500 automated DNA analysis platform (GE Healthcare) equipped with a 48 capillary array using standard procedures.

2.4. STR*Af* typing

All isolates were typed using a robust and highly discriminatory 9-marker microsatellite panel as described before (de Valk et al., 2007a,b, 2005).

3. Results

A total of 209 *A. fumigatus* isolates were analyzed using both the CSP typing and the STR*Af* typing method. In concordance with previous results, 100% typeability was achieved using both methods for all *A. fumigatus* isolates (Balajee et al., 2007; de Valk et al., 2005).

3.1. CSP typing

In order to facilitate a standardized approach of analysis of the CSP gene repeat region sequence, each distinct repeat sequence was assigned an arbitrary number (Table 1). Furthermore, each unique combination of CSP repeats was assigned a CSP type. This led to the recognition of 18 CSP types, each with very different prevalences (Table 2). In addition to analysis of the DNA sequence of the repeat region, we screened for DNA sequence variations upstream and immediately downstream of the repeat region. DNA sequence variations were found at codon positions −1, −14, −15 and at codon positions +1 and +2 relative to the start and end of the repeat region, respectively. For instance, all isolates with CSP types t07, t08, t13 and t15 contained 3 polymorphisms in the flanking region: whereas the consensus sequence at position −14 involved a GTC triplet, in these CSP types it involves a CTC triplet. In addition, codon +1 involved a CCG triplet (consensus CCA) and codon +2 is duplicated (CCTCCT versus CCT in the consensus sequence). Furthermore, all isolates belonging to CSP type t11 contained a GCG codon at position −15 (consensus GTG) and a CTC codon at position −14 (consensus GTC). Most of these DNA sequence variations did not result in the recognition of additional sequence types: these CSP types could already be discriminated from other CSP types based on the repeat sequence. However, in a low percentage of isolates with CSP type t04 (2 out of 38 isolates), DNA sequence variations in the flanking region at codon −1 (reading CCA versus CCG in all other isolates) lead to the recognition of an additional CSP (sub)type. Since this additional subtype can only be discriminated based on the variation in the flanking region of the repeat and not by the repeat region itself, these types are assigned as subtypes t04A and t04B, respectively.

3.2. STR*Af* typing

All isolates in this study were also analyzed by using a highly discriminatory typing assay based on a panel of 9 microsatellite markers (a.k.a. the STR*Af* assay, (de Valk et al., 2005)). STR*Af* typing

Table 2
Overview and prevalence of CSP types in The Netherlands.

CSP type	CSP repeat succession	Prevalence (%)	Previous type designations[a]
t01	01-01-01-01-05-03-01-06-03-07	37.3	31, 52, 61, 63
t02	01-01-02-03-04-05-03-01-06-03-07	10.0	1, 32, 66
t03	01-02-03-04-06-03-07	11.0	11, 34, 41, 44, 51, 62
t04	01-02-03-04-05-03-01-06-03-07	18.2	2, 12, 21, 33, 43, 53, 62
t05	01-01-01-03-01-06-03-07	9.1	23
t06	01-01-01-02-03-04-05-03-01-06-03-07	0.5	23, 24, 42
t07	01-02-03-04-05-03-04-05-03-01-06-03-07	0.5	
t08	01-01-01-02-03-04-05-03-04-05-03-01-06-03-07	3.3	
t09	01-01-01-01-01-05-03-01-06-03-07	1.9	
t10	01-01-01-05-03-01-06-03-07	1.0	
t11	01-01-08-03-01-06-03-07	1.9	
t12	01-01-01-01-01-02-03-04-05-03-01-06-03-07	0.5	
t13	01-01-02-03-04-05-03-04-05-03-01-06-03-07	0.5	
t14	01-01-01-01-02-03-04-05-03-01-06-03-07	2.9	64
t15	01-01-01-01-02-03-04-05-03-04-05-03-01-06-03-07	0.5	
t16	01-05-03-01-06-03-07	0.5	
t17	01-01-02-03-04-05-03-09-06-03-07	0.5	
t18[b]	01-01-05-03-01-06-03-07	0.0[b]	65

[a] Sequence types and data taken from Balajee et al. (2007) and reanalyzed according to the new proposal for nomenclature as described here.
[b] This CSP type was not found in this population but was retrieved from Balajee et al. (2007).

CSP-type	Codon -15	-14	-1	Repeat succession	Codon +1	+2	Lineage
t09	GTG	GTC	CCG	01-01-01-01-01-------------------05-03-01-06-03-07	CCA	CCT	A
t01	GTG	GTC	CCG	---01-01-01-01-------------------05-03-01-06-03-07	CCA	CCT	
t10	GTG	GTC	CCG	------01-01-01-------------------05-03-01-06-03-07	CCA	CCT	
t18	GTG	GTC	CCG	---------01-01-------------------05-03-01-06-03-07	CCA	CCT	
t16	GTG	GTC	CCG	------------01-------------------05-03-01-06-03-07	CCA	CCT	
t12	GTG	GTC	CCG	01-01-01-01-01-02-03-04----------05-03-01-06-03-07	CCA	CCT	B
t14	GTG	GTC	CCG	---01-01-01-01-02-03-04----------05-03-01-06-03-07	CCA	CCT	
t06	GTG	GTC	CCG	------01-01-01-02-03-04----------05-03-01-06-03-07	CCA	CCT	
t02	GTG	GTC	CCG	---------01-01-02-03-04----------05-03-01-06-03-07	CCA	CCT	
t04A	GTG	GTC	CCG	------------01-02-03-04----------05-03-01-06-03-07	CCA	CCT	
t04B	GTG	GTC	CCA	------------01-02-03-04----------05-03-01-06-03-07	CCA	CCT	
t03	GTG	GTC	CCG	------------01-02-03-04-------------------06-03-07	CCA	CCT	C
t05	GTG	GTC	CCG	------01-01-01----------------------03-01-06-03-07	CCA	CCT	D
t15	GTG	CTC	CCG	---01-01-01-01-02-03-04-05-03-04-05-03-01-06-03-07	CCG	CCTCCT	E
t08	GTG	CTC	CCG	------01-01-01-02-03-04-05-03-04-05-03-01-06-03-07	CCG	CCTCCT	
t13	GTG	CTC	CCG	---------01-01-02-03-04-05-03-04-05-03-01-06-03-07	CCG	CCTCCT	
t07	GTG	CTC	CCG	------------01-02-03-04-05-03-04-05-03-01-06-03-07	CCG	CCTCCT	
t11	GCG	CTC	CCG	---------01-01-------------------08-03-01-06-03-07	CCA	CCT	F
t17	GTG	GTC	CCG	---------01-01-02-03-04----------05-03-09-06-03-07	CCA	CCT	G

Fig. 1. Alignment of *A. fumigatus* CSP types and segregation of CSP types into CSP lineages. Variations in the flanking region of the repeat were found as indicated with a shaded background. CSP types were assigned based on their unique succession of repeat numbers. CSP (sub)types are types that can be distinguished solely based on differences in the flanking region (as is the case for t04A and t04B only). Lineages of CSP types consist of CSP types that differ only in the number of repetitions of the first repeat unit. Dashes indicate deletions of repeat units relative to other CSP types and were introduced to facilitate recognition of related CSP types.

yielded a total of 188 genotypes (results not shown). 173 genotypes were unique, 11 genotypes were found twice, 2 genotypes were found 3 times and 2 genotypes were found in 4 isolates. Isolates with identical STR*Af* types also had identical CSP types.

4. Discussion

CSP typing was introduced as an alternative single gene based sequence typing strategy to distinguish between *A. fumigatus* isolates. The diversity of sequence types found within the repeat region of the CSP gene indeed is much larger than for any of the markers previously reported in the MLST scheme for *A. fumigatus* (Bain et al., 2007) where a maximum of 5 different alleles were found per gene whereas CSP typing yielded 18 different CSP types rendering this gene an interesting target for single gene based sequence typing. In contrast, the microsatellite markers in the STR*Af* assay display up to 100 alleles (Klaassen, 2009). As a result, microsatellites are sometimes criticized for being too discriminatory, potentially leading to a loss of recognition of relationships between isolates. The CSP gene seems to be a well positioned typing target with a discriminatory power between the low discriminatory MLST markers and the highly discriminatory microsatellite markers and thus suitable for typing at the sub-population level. This is also reflected in the Simpsons' index of diversity (D). The value of D for the CSP gene was calculated at 0.800 versus 0.767 to 0.963 for the individual microsatellite markers in the STR*Af* assay. In contrast, the value of D for the individual loci in the MLST scheme ranges from 0.135 to 0.563 (calculated from ref. (Bain et al., 2007)). Furthermore, within the *A. fumigatus* genome, multiple other protein coding regions containing tandem repeats similar to the CSP gene have been identified (Levdansky et al., 2007) and the discriminatory power of these targets could be increased by combining multiple of such genes into one novel typing scheme.

In the original study, the authors evaluated the CSP typing method as a sub-typing tool where the various CSP sequences from *A. fumigatus* isolates were arbitrarily assigned a random sequence type number within a particular outbreak (Balajee et al., 2007). As a consequence, isolates from different outbreaks but with identical repeat region sequences were assigned different sequence types. For instance, sequence types with identical sequences but recovered in different outbreaks were assigned different numbers — 2, 12, 21, 33, 43, 53 and 62; these sequences, when reanalyzed with the present algorithm would be of sequence type t04, thus indicating that these sequences represent identical genotypes. Thus, the analysis method employed in the original manuscript would be ultimately confusing and does not contribute to a reliable and unambiguous exchange of results between labs. Adherence to a single sequence type assignment for isolates with identical repeat successions would be a more practical algorithm. A new nomenclature is therefore proposed for the assignment of sequence types based on sequence analysis of the *A. fumigatus* CSP gene repeat region. Basically, we used an approach similar to the widely used and successful approach for the *spa* gene of *Staphylococcus aureus* (Harmsen et al., 2003): each unique repeat sequence was arbitrarily numbered and each unique succession of repeat numbers was arbitrarily assigned a sequence type. The proposed new numbering of CSP types is primarily based on the sequence of the repeat region. Only in the case where DNA sequence variations in the flanking region would lead to the recognition of additional sequence types, subtypes could be defined by the addition of a syllable as proposed here for t04A and t04B. Expanding the analysis of the flanking region within the amplified region did not

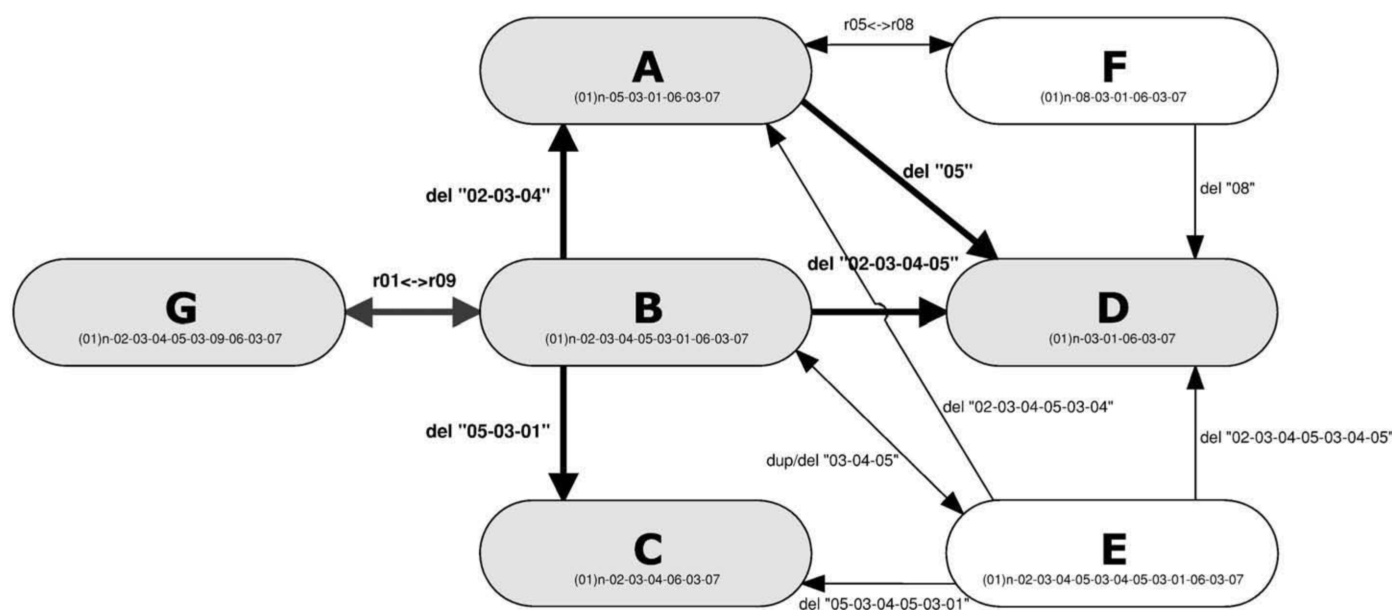

Fig. 2. Theoretical unrooted model of inter-lineage relationships based on analysis of the CSP repeat region. Lineages connected by a thick line could have evolved from each other by a single genetic event. Such an event could consist of the insertion or deletion of one repeat (or multiple repeats at the same time) or could consist of a point mutation in one repeat sequence leading to another repeat sequence. These lineages are identified with shaded boxes. Lineages connected by a thin line (clear boxes) require more than one genetic event. Although, the repeat succession could easily have changed as the result of a single genetic event, the presence of additional point mutations in the flanking region of the repeat region renders these options less likely and may indicate the existence of missing links (see text for details).

result in recognition of additional subtypes. We therefore recommend to restrict analysis of the flanking region to the sequences immediately flanking the repeat region as in Fig. 1 (e.g. including 45 bp upstream and 9 bp downstream of the repeat region).

With the limited numbers of different repeats that are found so far, conversion of the repeat region sequence data into repeat numbers can be quite readily performed simultaneously on multiple isolates by using the 'find and replace' option using a simple text processor. Sequence variations in the flanking region are easily identified by making multiple sequence alignments using widely available and free software programs. If larger numbers of repeats are found, a more sophisticated approach such as the 'spa-server' software currently in use for analysis of the *S. aureus spa* gene (Harmsen et al., 2003), might be a more suitable alternative.

The sequence diversity in the CSP gene is the combined result of insertions and deletions of individual or multiple repeat units (as the result of slipped-strand mispairing) as well as of point mutations. From the repeat succession it is obvious that certain genotypes appear to be much more related to each other than to the other genotypes. Previously, clusters of related sequences were identified by DNA sequence alignment and cluster analysis of the repeat region (Balajee et al., 2007). However, algorithms that are currently in use for DNA sequence alignments and cluster analysis were not designed to deal with tandemly repeated DNA elements where insertions and/or deletion of one or more repeat units may be much more common events than point mutations. In non-repetitive DNA elements, point mutations may contribute more to sequence diversity than insertions or deletions (especially in protein coding regions). In Fig. 1, an alternative alignment of CSP types is shown. For the above reasons, this alignment was made manually and favoured repeat insertion and deletions over point mutations. Based on this alignment, lineages of CSP types can be assigned that contain CSP types that appear to be more closely related to each other than to the remaining CSP types. For instance: CSP types t07, t08, t13 and t15 (grouped into lineage E) all contain a unique combination of sequence variations in the flanking region of the repeat (Fig. 1). Therefore, these types appear to be more closely related to each other than to the other CSP types. Compared to other CSP types, these types all possess the same repeat successions that differ only in the number of repetitions of the first repeat unit. It would therefore be assumed that the number of repetitions of this first repeat is much more subject to copy number changes than the remaining part of the repeat region. Based on this reasoning, other lineages of CSP types can be recognized that contain genotypes with a unique repeat succession and/or genotypes that also only differ in the copy number of the first repeat (assigned lineages A–G, respectively in Fig. 1).

It would be interesting to speculate on how these lineages of *A. fumigatus* might have evolved. Apart from the minor sequence polymorphism that was found in part of the isolates with CSP type t04, lineages A–D and G all have the same flanking region and could have evolved from each other as illustrated in Fig. 2. Lineages E and F are a little more complicated to fit into this picture. When solely based on analysis of the repeat region, lineage E could easily have evolved from lineage B by duplication of the repeat stretch '03-04-05' (or vice versa by deletion of this repeat stretch). Lineage F (t11) could have evolved from lineage D (t05) as the result of a change of r01 into r08 (or vice versa) but, likewise, it could also have evolved from lineage A (in particular t16) as the result of a mutation in r05 leading to r08. Since the latter change requires 2 point mutations instead of only 1, the first option would be more likely. However, if the flanking region of the CSP types in lineages E and F are also taken into account, both these options are suddenly less likely since they also require at least 2 additional nucleotide changes in the flanking region of the repeat. Since such changes are highly unlikely to result from a single genetic event, this indicates the existence of missing links with intermediate sequence types (for instance with only 1 of the changes in the flanking region). Possibly such missing links are recognized when other populations or when larger collections of isolates are screened. Thus, lineages E and F appear to be more distantly related to the other CSP lineages. Fig. 2 also clearly demonstrates that the more classical approach of sequence alignment and cluster analysis may not grasp the entire complexity of inter-lineage relationships that can be recognized visually. For a more definite assignment of inter-lineage relationships however, the stability of individual CSP types should be studied in more detail.

A noteworthy finding of this study was that the most common CSP types and lineages in The Netherlands (types t01–t05, Table 2) also

appear to be the most common CSP types in North America (Balajee et al., 2007), emphasizing the earlier observation that *A. fumigatus* has cosmopolitan distribution and lack clear geographically separated *A. fumigatus* populations. It has to be pointed here that in this study as well as in the study by Balajee et al. the *A. fumigatus* population analyzed may not be representative of a global population: the former study contained only clinical isolates and the latter study analyzed *A. fumigatus* isolates recovered from outbreaks. It would therefore be interesting to analyze multiple collections (both clinical and environmental) from different geographic regions. Furthermore, it was recently shown that CSP typing yields highly concordant results when performed in different settings (Hurst et al., 2009). This enables global comparisons of locally generated typing data, especially when a standardized nomenclature for CSP types is used.

In conclusion, a proposal for a standardized nomenclature of CSP repeats, types and lineages is presented. This nomenclature could greatly facilitate exchange of CSP typing data between laboratories. With this nomenclature, CSP typing may offer an entirely new perspective on the population structure of *A. fumigatus*.

5. Disclaimer

The findings and conclusions in this presentation are those of the author and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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