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LETTER TO THE EDITOR

To be capsulated or not be capsulated: that is the GAS question

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To the Editor,

The capsule of *Streptococcus pyogenes* (Group A Streptococcus, GAS) is composed by hyaluronic acid, the same component of the extracellular matrix of higher animals, humans included. Its synthesis as a linear polymer of N-acetylglucosamine and glucuronic acid is mediated by three enzymes whose genes (*has*A, *has*B, and *has*C) are clustered in an operon [1, 2].

The most relevant is *has*A gene, encoding a membrane-associated enzyme, hyaluronan synthase, that promotes the formation of the hyaluronic acid polymer from the nucleotide sugar precursors. The *has*A gene is 1260 nucleotides long, coding for a 420 aminoacids protein. According to transcriptional studies [3], the start codon is a GTG triplet; nevertheless, many nucleotide entries identified in-frame ATG as the first codon, either upstream or downstream the GTG triplet, reporting longer or shorter deduced aminoacid sequences.

The *has*ABC operon is conserved among GAS strains but it is known that there are capsule-negative *emm* types that lack these genes, as *emm*4 and *emm*22 to which the newly emergent clonal lineage *emm*89 clade 3 has been added [4–6]. Indeed, a recent study has demonstrated that other two *emm* types (*emm*28 and *emm*87) cannot produce capsule because, even if they possess the *has*ABC operon, present frameshift point mutations in the *has*A gene, leading to a truncated and not functional enzyme [7]. Both *emm*28 and *emm*87 types present an adenine insertion in position 219 (insA219) or in position 56 (insA56) of *has*A gene, respectively. All available *emm*28 GAS genomes, whose date of isolation spans over decades, have different sources of isolation and a worldwide distribution, present the insA219 nonsense point mutation suggesting this is a fixed mutation (Table 1). Only one *emm*87 complete genome is available; nevertheless, the insA56 mutation was confirmed in 30 clinical *emm*87 isolates by Authors, supposing *emm*87 is an additional capsule-negative type [7]. We also checked for these point mutations in a collection of GAS strains (data in preparation) and confirmed the presence of both insA219 and insA56 mutations in our *emm*28 and *emm*87 isolates, respectively.

To ascertain if other *emm* types, even if possessing the *has*ABC operon, are possibly capsule-negative *emm* types, we made a BlastN search using as query sequence the complete *has*A gene of the *emm*12 MGAS2096 strain (NCBI Reference Sequence: NC_008023.1; pos.1840494-1841753; search done on July 3th, 2019; default parameters; max 500 target sequences).

We observed several unidentified point insertions/deletions, leading to open-reading frameshifts (delA19, insA19, insT48, delA193, insT219, delA406, delT463, delT971) plus one A/T nucleotide substitution at pos. 13 of the *has*A gene transforming the AAA codon in the TAA stop codon (Table 1). Many of them reside in mononucleotide stretches (poly-A or poly-T) that could act as hot spot mutation sites.

The delT463 mutation is present in all four deposited *emm*77.0 complete genomes isolated in UK, USA, and Australia (but not in TSPY165 genome that is *emm*77.4) as well as in *emm*77.0 strains of our GAS collection strongly supporting the hypothesis that also *emm*77.0 is an unidentified capsule-negative *emm* type.

The remaining open-reading frameshifts are possessed by less common *emm* types presenting only one or two reference genome representatives in the nucleotide collection (*emm*11, *emm*64, *emm*73, *emm*78, *emm*80), then hampering their evaluation as possible capsule-negative *emm* types. Conversely, we observed frameshift mutations only in one out of six *emm*75 completed reference genomes, then not representing a mutation fixed by a clonal expansion (Table 1). Also a recent whole-

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genome sequence-based characterisation of 1454 invasive GAS isolates recovered in 2015 by US Active Bacterial Core Surveillance found single *has*A-negative *emm*1, *emm*11, and *emm*12 isolates in the analysed bacterial set [8]. Noteworthy, we detected an *emm*22.8 genome, isolated from skin in India, possessing a mutated *has*A gene, contrasting the notion that all *emm*22 type isolates lack the *has*ABC operon [9].

Presumably because it is recognised by the host immune system as a self-antigen, hyaluronic acid capsule is poorly immunogenic but there is evidence it constitutes an important virulence determinant. The role of capsule in disease pathogenesis is suggested since early studies on GAS, when the overrepresentation of mucoid (highly encapsulated) *S. pyogenes emm* types strains to some clinical conditions as invasive disease and rheumatic fever, was noted [10, 11]. MTB313 and MTB314 GAS strains have been isolated from the same patient with meningitis and the different mucoid or not mucoid colony morphology relies on a frameshift mutation in the *has*A gene (Table 1).

The GAS capsule protects bacteria from complement-mediated phagocytic killing and is essential for full virulence in a variety of experimental infection models [2, 12]. It is also a ligand for attachment to the hyaluronic acid-binding glycoprotein CD44 which is expressed on human oropharyngeal keratinocytes and tonsillar epithelial cells inducing cytoskeletal rearrangements, disruption of intercellular junctions, and enhancement of group A streptococcal translocation across the epithelial barrier [13].

Streptococcus pyogenes capsule is, indeed, not covalently linked to the cell wall. In liquid culture, it is maximally expressed during exponential growth, then it declines to very low levels during the stationary phase and shed from the cell surface into the culture medium [1, 14, 15]. In vivo, the regulation of capsule expression may be a useful adaptation to survival in host environments. Changing levels of encapsulation are finely tuned by CovR/S two-component regulator system acting on *has* promoter that itself influences the rate of transcription by nucleotide polymorphism between *emm* types [1, 2].

In the asymptomatic throat carriage state, that is probably the dominant state of GAS interaction with the human host, the expression of many virulence factors responsible for the dissemination between individuals is decreased, including capsule production. It has been postulated that the carriage strains represent lineal descendants of the strain causing acute pharyngitis in the same individual, rather than new acquisition via transmission from another carrier. Indeed, GAS *emm*3 strains cultured serially from human throat after an acute pharyngitis developed mutations in the *has* operon that reduced or eliminated capsule biosynthesis, possibly offering an advantage to persistence on the pharyngeal mucosal surface [16].

Conversely, the same Authors found that the proportions of not encapsulated GAS types increased significantly as a cause of invasive disease and skin and soft-tissue infections in a paediatric hospital in years 2013–2017 while a similar significant shift among pharyngeal strains was not observed, lessening the notion that mutations negatively affecting capsule synthesis contribute to the phenotypic differences observed between carriage and invasive strains [7].

All *S. pyogenes* strains possess an extracellular hyaluronidase, coded by *hyl*A gene, that can degrade the hyaluronic acid capsule. This has raised the puzzle on why an organism would produce both a protective shield as the hyaluronic acid capsule and an enzyme capable of destroying that protection.

Indeed, it has been demonstrated that only capsule-negative *emm*4 and *emm*22 produce an active hyaluronidase while capsulated *emm* types present a non-synonymous single nucleotide substitution at position 199 of *hyl*A gene (resulting in the Asp-Val amino acid change) that compromises the enzymatic activity of hyaluronidase [17, 18]. This property is considered an evolutionary branch point for the adoption of a dual strategy in which group A streptococcal strains express either the anti-phagocytic hyaluronic acid capsule or hyaluronidase for degrading the host's extracellular matrix.

Newly identified not capsulated *emm*28, *emm*87, and *emm*89 clade 3 (and possibly *emm*77.0) possess the Asp-Val substitution in the hylA protein similarly to the capsulated strains, suggesting the inactivating mutations in the *has*ABC operon may have been occurred independently on a capsule-producing genetic background. How these *emm* types overcome the absence of both the above-mentioned host immune evasion systems being still capable of causing infection is worth of investigation. Alternative strategies that promote virulence have been reported for *emm*4, *emm*22, and *emm*89, but the biological role of capsule-negative GAS strains has still to be completely elucidated [19–24].

In conclusion, despite the fine tuning regulation on the *has*ABC operon for capsule production and the important role of capsule in life cycle of GAS, there is an increasing report of *emm* types presenting a permanent incapability to produce capsule. We focused our analysis on the polymorphism of *has*A gene that is considered essential for the production of GAS capsule; nevertheless, inactivating mutations in *has*B gene could also be present [16] (hasC product seems instead to be dispensable for capsule production due to the existence of another gene of UDP-glucose pyrophosphorylase in the genome), rendering the proportion of capsule defective *emm* types more prominent. If the frameshift mutations identified in the available complete reference genomes represent a reversible phase variation in the *has*A gene expression is not evaluable and considered improbable. Synonymous and not synonymous nucleotide substitutions in several *has*A genes of completed reference genomes have also been observed (Table 1) but how and if they affect the enzyme activity is unpredictable.

Whole-genome sequencing era will greatly contribute to identify GAS clinical isolates possessing nonsense nucleotide mutations in *has* operon genes; this analysis should be included in surveillance studies to define the extent capsule-negative *emm* types and their role in the GAS pathogenesis, hopefully stimulating further investigations on the strategies adopted by these *S. pyogenes emm* types to successfully exploit their niches within the human host.

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