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Technical note: Development of multiplex PCR assays for the molecular characterization of *Streptococcus uberis* strains isolated from bovine mastitis

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ABSTRACT

Streptococcus uberis is an important causative agent for clinical and subclinical mastitis in dairy cattle. The aim of this study was to develop 2 multiplex PCR assays (mPCR) for the simultaneous detection of virulence factors and housekeeping genes for use when investigating the genetic variability and distribution of *Strep. uberis* virulence factors. The *tuf*, *cpn60*, *pauA*, *sodA*, *sua*, *oppF*, and *gapC* genes were grouped in assay 1 (mPCR1) and the *hasA*, *hasB*, and *hasC* genes were included in assay 2 (mPCR2). The detection limits were 11.8 pg and 5.9 pg of DNA for mPCR1 and mPCR2, respectively. The 2 mPCR assays were validated with 56 *Strep. uberis* strains isolated from mastitis milk samples collected from different bovine herds in northern Italy. Results revealed that *gapC* and *oppF* were detected in 98.2% of the strains, whereas *sua* and *hasC* genes were detected in 94.6 and 89.2% of the strains, respectively. The most common pattern was *gapC*+, *oppF*+, *cpn60*+, *sua*+, *sodA*+, *pauA*+, *tuf*+, *hasA*+, *hasB*+, and *hasC*+, which appeared in 59% of the strains analyzed. The molecular assays developed in the present study represent a powerful tool for the evaluation of virulence pattern distribution in *Strep. uberis* strains associated with intramammary infections.
Key words: *Streptococcus uberis*, virulence factor, mastitis, multiplex PCR

Technical Note

Mastitis is a common disease in dairy cattle and the cause of important economic losses for the dairy industry (Liang et al., 2017). Among mastitis pathogens, *Streptococcus uberis* can colonize different dairy environments (Zadoks et al., 2005) and is implicated in clinical and subclinical IMI during lactation and the dry period (Reinoso et al., 2011), representing a potential risk factor for dairy cattle. It has been estimated that *Strep. uberis* is responsible for 14 to 26% of clinical mastitis cases in Canada, the United States, and the Netherlands, and it is the main cause of clinical mastitis in New Zealand and Australia (Collado et al., 2018). It has also been identified as being responsible for a large part of clinical mastitis in several European countries such as Belgium, Germany, Italy, and the UK (Bradley et al., 2007; Krömker et al., 2014). The control of this environmental microorganism can be particularly problematic (Boonyayatra et al., 2018). *Streptococcus uberis* has several virulence genes; for example, the hyaluronic acid capsule genes (*hasA*, *hasB*, and *hasC*; Ward et al., 2001), the plasminogen activator A gene (*pauA*; Rosey et al., 1999), and the *Strep. uberis* adhesion molecule gene (*sua*; Almeida et al., 2006). These genes all contribute to making this microorganism contagious, as well as having a role in its pathogenicity. Many studies (Yuan et al., 2014; Perrig et al., 2015; Loures et al., 2017) have shown high prevalence of these virulence genes in *Strep. uberis* strains harvested from several regions of the world. The *pauA* and *sua* genes seem to be highly conserved across *Strep. uberis* strains (Perrig et al., 2015), and have been chosen as target genes for detection in milk by PCR assays (Gillespie and Oliver, 2004). Other virulence factors such as *gapC* (Pancholi and Fischetti, 1993), which encodes glyceraldehyde-3-phosphate dehydrogenase, or *oppF* (Smith et al., 2002), which is involved in the acquisition of essential amino acids from milk during bacterial growth, have previously been studied for their association with pathogenesis in IMI. A clear understanding of these virulence genes as key factors for mastitis development is still lacking (Boonyayatra et al., 2018). From previous studies (Perrig et al., 2015; Reinoso et al., 2015), it appears that in any given herd, only a limited group

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of *Strep. uberis* strains colonize the bovine mammary gland, resulting in cow-to-cow infection, whereas other strains appear to be less suited for this environment (Tassi et al., 2013). Different techniques have been used to discriminate individual strains of *Strep. uberis*. Random amplified polymorphic DNA (RAPD; Wieliczko et al., 2002), pulsed-field gel electrophoresis (PFGE; Reinoso et al., 2015) and multilocus sequence typing (MLST; Pullinger et al., 2006) have all been developed for epidemiological and genotypic studies. Species-specific PCR assay targeting the 16S rRNA gene was used by Hassan et al. (2001) to unambiguously detect *Strep. uberis* from a phenotypically identical species, *Strep. parauberis*. This PCR protocol had been widely used to identify *Strep. uberis* isolated from mastitis cases before the genotyping methods listed above. Multiplex PCR (**mPCR**) is an additional molecular tool for epidemiological studies and rapid characterization of *Strep. uberis* strains isolated from bovine mastitis milk (Boonyayatra et al., 2018).

The aim of this study was to develop 2 low-cost and fast mPCR assays for the simultaneous detection of 10 genes. Included, from the Italian dairy herds, were virulence factors and housekeeping genes helpful for investigating the genetic variability of *Strep. uberis* and the distribution of its virulence factors in isolates. The virulence factors included in these 2 mPCR assays are widely studied (Parin et al., 2017; Boonyayatra et al., 2018) and are thought to represent key factors in the invasion process of mammalian epithelial tissue for *Strep. uberis*.

To optimize the mPCR protocol, the reference *Strep. uberis* ATCC 9927 strain (LGC Promochem, Middlesex, UK) was used. The mPCR assays were then validated on 56 isolates from composite subclinical mastitis milk samples. These samples were collected between January 2016 and August 2017 from 12 bovine herds in northern Italy. Those farms were chosen because of their large herd size, a monthly incidence of *Strep. uberis* mastitis of 5 to 6%, availability of reliable health records, and access to microbiological diagnosis of milk samples at the University of Milan. The samples were collected aseptically and the isolation of *Strep. uberis* strains was performed by plating 10 μL of bovine mastitis milk onto trypticase soy agar (TSA) containing 5% sheep blood and 0.1% esculin (bioMérieux, Marcy l'Étoile, France). Plates were then incubated aerobically for 24 to 48 h at 37°C. Preliminary identification of *Strep. uberis* was based on colony morphology, esculin splitting, and catalase testing (National Mastitis Council, 2017). All streptococcal isolates were identified at species level by the API 20 Strep (bioMérieux). Genomic DNA was extracted from pure cultures, as previously described by Cremonesi et al. (2006), without a pretreatment step, using a method based on the combination of a chaotropic agent, guanidium thiocyanate, with silica particles to obtain bacterial cell lysis and nuclease inactivation. The concentration of the purified DNA was determined using NanoDrop 2000 (ThermoFisher Scientific Inc., Waltham, MA) and stored at −20°C until further use. The target genes of *Strep. uberis* included in the mPCR were *gapC*, *oppF*, *cpn60*, *sua*, *sodA*, *pauA*, *tuf*, *hasA*, *hasB*, and *hasC*. All details concerning the oligonucleotides, including product size, primer sequences, and GenBank accession numbers, are summarized in Table 1. All primers of this study were designed using Primer 3 software (http://primer3.ut.ee/), except for primer *cpn60*, which was previously described (Dmitriev et al., 2006). The *in silico* specificity was checked by using the BLAST software tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The primers were synthesized by ThermoFisher Scientific. The oligonucleotides were chosen based on similar melting temperatures to use the same amplification protocol with minimal interactions, resulting in different-sized products distinguishable by agarose gel electrophoresis. Using these criteria, 2 mPCR assays were optimized: *gapC*, *oppF*, *cpn60*, *sua*, *sodA*, *pauA*, and *tuf* were grouped in mPCR1 and *hasA*, *hasB*, and *hasC* in mPCR2.

Initially, both mPCR assays were set up using genomic DNA extracted from the reference *Strep. uberis* ATCC 9927 strain. The mPCR assays, prepared in 0.2-mL tubes, were performed in a 25-μL volume with *sodA* primers at 1.6 μ*M* and all other primers at 0.8 μ*M* (ThermoFisher Scientific, Milan, Italy), PCR master mix 10× (AccuPrime, Invitrogen, Minneapolis, MN), 1 U of AccuPrime *Taq* DNA Polymerase (Invitrogen), and 2 μL of DNA (~40 ng/μL). Amplifications were carried out in a thermocycler (Applied Biosystems, Foster City, CA) with the following program: 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 56°C for 1 min, 68°C for 1 min, and finally a step of 72°C for 7 min. The amplified PCR products were visualized simultaneously by standard gel electrophoresis in a 3% agarose gel (GellyPhor, Euroclone, Milan, Italy), stained with ethidium bromide (0.05 mg/mL; Sigma Aldrich, Milan, Italy). A molecular size marker (100-bp DNA ladder; Finnzymes, Espoo, Finland) was loaded in each agarose gel. The DNA bands were visualized on a UV transilluminator (BioView Ltd., Nes Ziona, Israel). The sensitivity of the 2 mPCR assays was tested using a 2-fold serial dilution of the ATCC 9927 reference strain genomic DNA from 96 ng to 1.5 pg. The PCR products obtained by the 2 mPCR assays were analyzed and quantified using the Agilent BioAnalyzer

Table 1. Multiplex PCR primers used in this study, including gene targets, amplicon size, primer sequences (For, forward; Rev, reverse), and gene sequence accession numbers

Multiplex PCR	Target gene	Amplicon size (bp)	Primer sequence	GenBank accession number	Reference
Reaction 1	*gapC*	505	For: 5′-GCTCCTGGTGGAGATGATGT-3′ Rev: 3′-AACCGTAAGCCATACCGATG-5′	GU392494	This study
	oppF	454	For: 5′-TCAGAGATATTGTTGCTGAAGGA-3′ Rev: 3′-GGCTCTGGAATTGCTGAAAG-5′	GU392621	This study
	cpn60	400	For: 5′-TCGCGGTATTGAAAAAGCAACAT-3′ Rev: 3′-TGCAATAATGAGAAGGGGACGAC-5′	AF485804	Dmitriev et al. (2006)
	sua	350	For: 5′-GCAACATTGGCACCTACAAA-3′ Rev: 3′-GCAGCTGTTACCTCGTCAGA-5′	LN885239.1	This study
	sodA	280	For: 5′-TGATAAAGAAACAATGACCCTTCA-3′ Rev: 3′-TGCATCAAAAGAACCAAATGC-5′	GU392754.1	This study
	pauA	205	For: 5′-TGACGAGTTTCGAAAAATTGC-3′ Rev: 3′-ACCGAGTTCTTTTCCGGATT-5′	KT006562.1	This study
	tuf	143	For: 5′-TCCTTCTTTCACGCCAAGTT-3′ Rev: 3′-GTCATCACCTGGGAAATCGT-5′	GU392973	This study
Reaction 2	*hasA*	599	For: 5′-AAATGGCTTTGGAGACCAAG-3′ Rev: 3′-CAACACTTGGTGTGGCTAATAA-5′	AM946015.1	This study
	hasB	400	For: 5′-CGATCAAGCATTTAGGGATG-3′ Rev: 3′-AGCCTCTGCTGAACCCATAA-5′	AJ242946	This study
	hasC	193	For: 5′-AGGCTTAGGGGATGCTGTTT-3′ Rev: 3′-GGATACGTCATCGTGAGGAAC-5′	AJ400707	This study

2100, applying the DNA 500 LabChip kit (Agilent Technologies, Palo Alto, CA).

Using the ATCC reference strain, the mPCR assays were successfully optimized and the desired amplicons were obtained in both reactions, as shown in Figure 1. In reaction 1, 7 bands with sizes of 505, 400, 369, 350, 280, 205, and 143 bp, corresponding to *gapC*, *oppF*, *cpn60*, *sua*, *sodA*, *pauA*, and *tuf* genes, were distinguished without interactions among them, (Figure 1A). In reaction 2, 3 bands with sizes of 599, 400, and 193 bp were obtained, corresponding to *hasA*, *hasB*, and *hasC* genes, respectively (Figure 1B). As shown in lane 1 (Figure 1A and 1B), the primer concentrations used in the single reaction resulted in approximately equal yields for all amplification products. To check the assays' sensitivity and verify similar results between multiplex and simplex PCR, 2-fold serial dilutions were tested starting from the *Strep. uberis* ATCC 9927 genomic DNA reference strain. The sensitivity of the 2 mPCR assays was 23.5 and 11.8 pg, for mPCR1 and mPCR2, respectively, corresponding to approximately 20 cfu/mL for both reactions (Figure 2), which confirms that the standardized assays allowed amplification of all putative and known virulence-associated genes of *Strep. uberis*. The limit of detection (**LOD**) of the 2 mPCRs developed in this work was comparable to those of previous studies. For example, Wang and Liu (2015) optimized a loop-mediated isothermal amplification (LAMP)-PCR for mastitis pathogens, including *Strep. uberis*, with an LOD of 0.1 pg of DNA. Phuektes and coworkers (2001) reported an LOD of 50 pg for their mPCR assay to identify *Strep. uberis*, whereas Shome et al. (2011) improved the sensitivity of an mPCR assay for the detection of mastitis pathogens including *Strep. uberis* and reached an LOD of 10 fg.

All 56 *Strep. uberis* isolates harbored at least one virulence-associated gene and all were positive for *tuf*, *sodA*, and *cpn60* genes. The mPCR assays detected the *gapC* and *oppF* genes in 98.2% of the strains, whereas the *sua* gene was found in 53 strains (94.6%). These virulence genes were commonly detected among the *Strep. uberis* strains, as previously described (Reinoso et al., 2011; Parin et al., 2017; Boonyayatra et al., 2018). The higher distribution of these genes among the *Strep. uberis* strains involved in clinical and subclinical mastitis is due to their role in *Strep. uberis* growth in milk (*oppF*; Smith et al., 2002) or to their involvement in virulence (*gapC* and *sua* genes; Boonyayatra et al., 2018).

The *hasC*, *hasA*, and *hasB* genes were detected in 50 (89.2%), 48 (85.7%), and 47 (83.9%) isolates, whereas the *pauA* gene was detected in 45 (80.3%) isolates. These results concur with previous studies (Reinoso et al., 2011; Boonyayatra et al., 2018) that reported a higher frequency of *hasC* than of *hasA* and *hasB*. As previously published by Reinoso et al. (2011) and Boonyayatra et al. (2018), differences in frequency among these 3 genes arise from the fact that the hyaluronic acid capsule (coded by *hasABC* genes) of *Strep. uberis* may not have a primary role in mammary gland

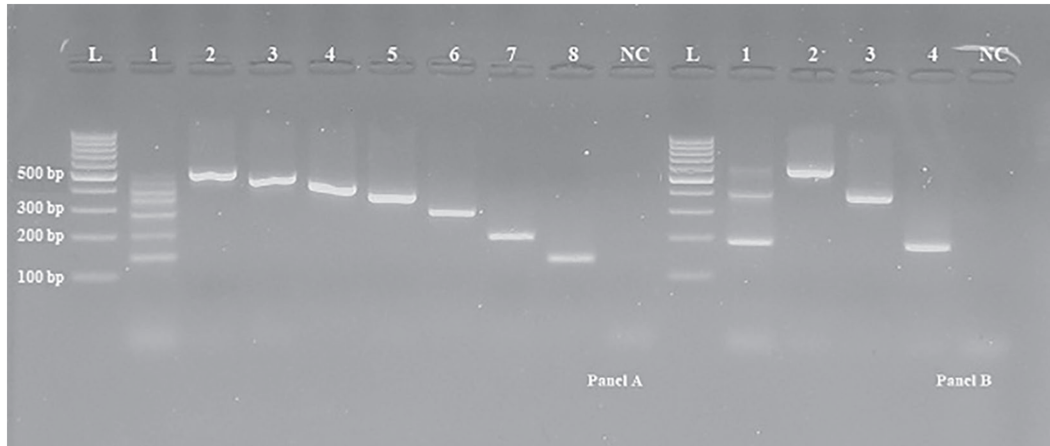

Figure 1. Optimization of multiplex (m)PCR reactions using genomic DNA of *Streptococcus uberis* ATCC 9927 strain. (A) Lane 1 = mPCR1 with all 7 genes; lanes 2–8 = uniplex PCR reactions for each gene: *gapC* (505 bp), *oppF* (454 bp), *cpn60* (400 bp), *sua* (350 bp), *sodA* (280 bp), *pauA* (205 bp), *tuf* (143 bp), respectively; lane L: 100-bp DNA ladder; lane NC = negative control. (B) Lane 1 = mPCR2 with 3 genes; lanes 2–4 = uniplex PCR reactions for each gene: *hasA* (599 bp), *hasB* (400 bp), and *hasC* (193 bp), respectively; lane L = 100-bp DNA ladder; lane NC = negative control.

Figure 2. Sensitivity of multiplex (m)PCR analysis of different DNA amounts derived from *Streptococcus uberis* ATCC 9927 strain: (A) reaction 1, (B) reaction 2. The concentration of each DNA fragment was calculated using the Agilent 2100 Bioanalyzer software (Agilent Technologies, Palo Alto, CA). The gel-like image shows the mPCR results obtained using 96.4 ng (lane 1), 48.2 ng (lane 2), 24.1 ng (lane 3), 12.0 ng (lane 4), 6.0 ng (lane 5), 3.0 ng (lane 6), 1.5 ng (lane 7), 0.75 ng (lane 8), 0.4 ng (lane 9), 0.2 ng (lane 10), 94.1 pg (lane 11), 47.1 pg (lane 12), 23.5 pg (lane 13), 11.8 pg (lane 14), 5.9 pg (lane 15), 2.9 pg (lane 16), and 1.5 pg (lane 17) of DNA from the reference strain; lane 18: negative control; lane L = DNA 500 ladder.

infection. Also, noncapsulated *Strep. uberis* isolates can induce mastitis by resistance to neutrophil phagocytosis (Field et al., 2003).

The distribution of virulence-associated genes revealed 12 virulence profiles, labeled A to N (Figure 3). The most common pattern was *gapC*+, *oppF*+, *cpn60*+, *sua*+, *sodA*+, *pauA*+, *tuf*+, *hasA*+, *hasB*+, and *hasC*+ (A profile) where *cpn60*, *sodA*, and *tuf* genes are housekeeping genes (Zadoks et al., 2005). Profile A included 59% (33/56) of isolates and was prevalent in 50% of the herds analyzed (6/12). The remaining 41% (23/56) of isolates were distributed in 11 profiles with 14% of isolates presenting pattern B (*gapC*+, *oppF*+, *cpn60*+, *sua*+, *sodA*+, *tuf*+, *hasA*+, *hasB*+, *hasC*+; 7%) or C (*gapC*+, *oppF*+, *cpn60*+, *sua*+, *sodA*+, *pauA*+, *tuf*+, *hasB*+, *hasC*+; 7%). Although a small group of isolates was analyzed, our results showed a large genetic variability of *Strep. uberis* isolates, as previously published by Boonyayatra et al. (2018) and Reinoso et al. (2011) for 88 and 78 isolates, respectively. Some of these genes, such as *sua*, *pauA*, and *gapC*, encode virulence factors involved in the survival of the microorganism in the host environment, in its evasion of host tissue, and in its internalization in mammary gland cells, suggesting that isolates with pattern A could be more virulent and have a greater probability of causing mastitis (Reinoso et al., 2011; Boonyayatra et al., 2018). Further studies should be carried out on more isolates to reinforce

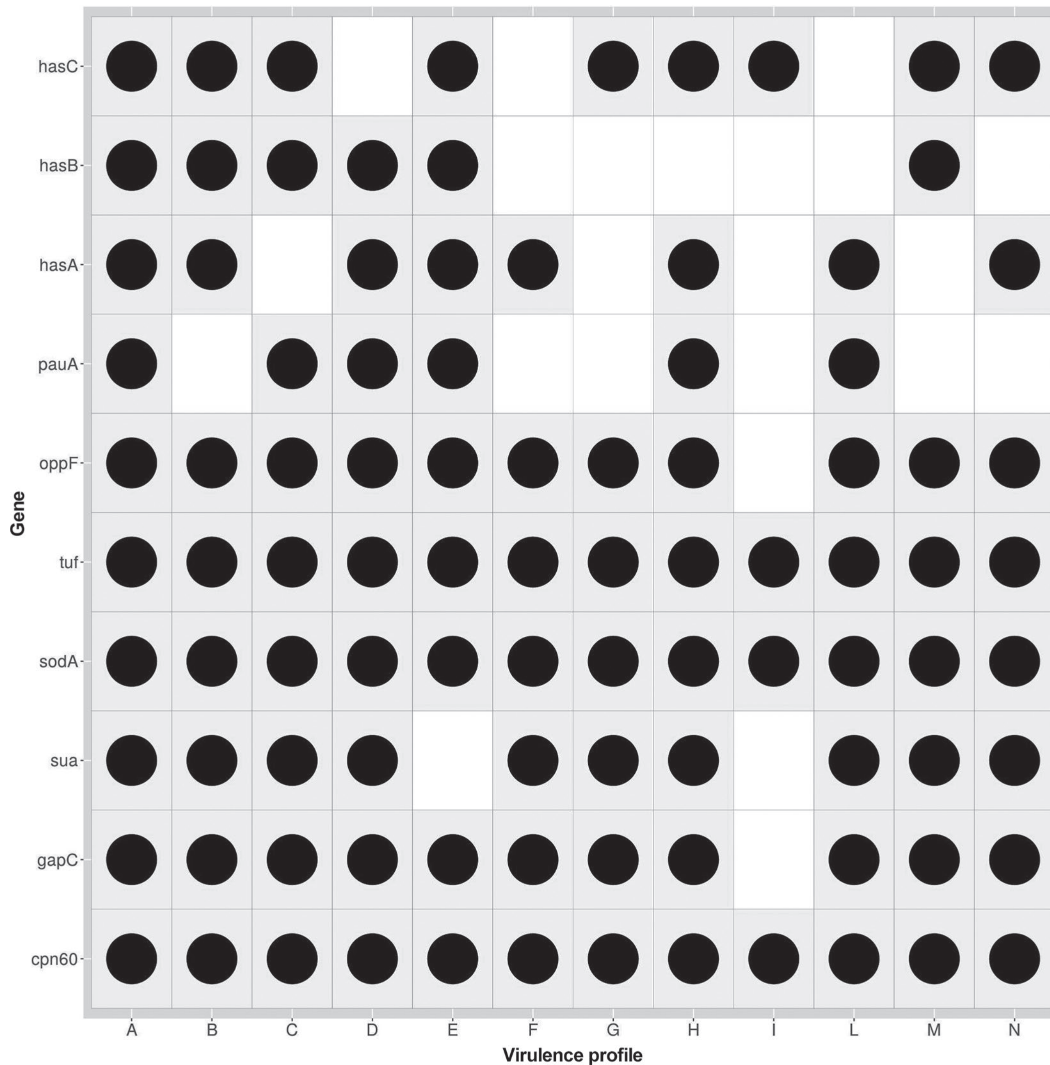

Figure 3. The distribution of genes investigated in virulence profiles. Each profile is indicated by letters from A to N and the presence of a given gene is shown by a black circle. Profile A was found in 59% of isolates (33/56); profiles B and C were found in 7.1% of the isolates (4/56); profile D was found in 5.4% of the isolates (3 out of 56); profiles E, F, G, and H were each found in 3.5% of the isolates (2/56); and profiles I, L, M, and N were each found in 1.7% of the strains (1/56).

these findings. Finally, to our knowledge, only a few studies have described mPCR assays for the molecular characterization of *Strep. uberis* strains (Parin et al., 2017; Boonyayatra et al., 2018). Boonyayatra et al. (2018) analyzed 11 genes in 3 different mPCR reactions that used 3 different annealing temperatures, whereas Parin et al. (2017), after species identification by the 16S rRNA gene, detected 10 virulence genes in a single multiplex amplification reaction. They probably could not distinguish between the *hasB* and *hasC* virulence factors, because these 2 genes have amplification products of the same size (300 bp). The mPCR assays developed in the present study used some housekeeping genes—*cpn60*, *soda*, and *tuf*—in order to unambiguously distinguish *Strep. uberis* from *Strep. parauberis*, a genetically closely related species, avoiding the need for any additional genotyping steps. It may be possible to analyze the same virulence genes, previously described, by reducing the time of analysis and the cost of the assay. These assays used only one amplification program, making the approach less costly and faster than previously described methods (Parin et al., 2017; Boonyayatra et al., 2018). This approach will potentially be very useful for the characterization of *Strep. uberis* in epidemiological studies, offering the ability to quickly obtain relevant information on the pathogenicity of isolates and progression of herd infections for a disease of growing concern.

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