Implementation of molecular serotype-specific identification for *Streptococcus pneumoniae*

Danial A. Malik[1,2], Subathra Marimuthu[2], Holly Aliesky[2], Brigid Connelly[2], Leslie Wolf Parrish[1, 2]

1. University of Louisville School of Public Health & Information Sciences, Department of Epidemiology
2. University of Louisville School of Medicine, Division of Infectious Diseases

UNIVERSITY OF LOUISVILLE. SCHOOL OF PUBLIC HEALTH & INFORMATION SCIENCES

UNIVERSITY OF LOUISVILLE. | Division of Infectious Diseases, Department of Medicine

Abstract

Streptococcus pneumoniae (S. pneumoniae) **is a commensal human pathogen that is typically present in the upper respiratory tract of the host. The high burden of pneumococcal disease is mainly observed in elderly individuals, especially those with underlying comorbidities causing substantially high mortality rates. Since the introduction of pneumococcal conjugate vaccines (PCV7 & PCV13), the incidence of disease caused by *S. pneumoniae* have decreased, but invasive infections caused by PCV13-targeted serotypes are still a major public health concern due to pneumococcal resistance. The Quellung reaction remains the gold standard for serotyping, which is dependent on initial organism isolation for diagnosis of pneumococcal disease. Limitations of this approach include long incubation periods with respectively high specificity but lower sensitivity. Our Infectious Diseases Laboratory (IDL) implemented the Center for Disease Control (CDC) protocol to develop the real-time PCR for the molecular serotyping-specific identification of *S. pneumoniae* that proves to be highly sensitive, specific, and reproducible on isolates of *S. pneumoniae*. The overall aim for the development of this molecular specific assay for *S. pneumoniae* is to improve the timely diagnosis of pneumococcal disease with serotype identification. This will lead to a better understanding of geographical distribution and shifts in prevalence over time. This in turn will aid in surveillance efforts for vaccine optimization and development, yielding a clearer assessment of vaccine on disease burden.**

Background

Pneumococcal pneumonia is a preventable disease that is caused by the bacterium *Streptococcus pneumonaie.* According to the CDC, in the United States, roughly 1.5 million adults are hospitalized annually due to pneumonia, and 41,601 deaths occur for those hospitalized [1]. It is suspected that more than 50% of children and roughly 30% of adults are asymptomatic carriers leading to higher transmission rates [2]. More than 100 different serotypes of pneumococcus exist, causing a wide range of clinical manifestations classified by their severity, invasiveness, anti-microbial susceptibility, community burden, hospitalization rates, vaccine coverage, and vaccine resistance. There has been a great emphasis on serotypes 3, 6, 7, 14, 18, 19, and 23, which are implicated as being the most common cause of Community-Acquired Pneumonia (CAP) hospitalization rates [3, 4]. With current trends of pneumococcal diseases, it is projected that the organism (without any interventions) will cause a doubling of hospitalization rates between the years 2020 to 2040, which is projected to result in $9 billion in healthcare costs in the United States alone [3,5]. The high associated morbidity and mortality burden caused by pneumococcal diseases calls for immediate attention to golden standard diagnostic and surveillance measures. Those that are commercially available oftentimes fail to meet the need for prevention and intervention due to the lack of timely diagnosis and classification of serogroups by quick antigen test [3].

Objectives

- **Describe and compare a molecular serotype real-time PCR for identification of *Streptococcal pneumoniae* to other gold-standard clinical diagnostic measures.**
- **Analyze and assess the detection of *Streptococcus pneumoniae* to demonstrate the sensitivity, specificity, and reproducibility of the molecular serotype-specific identification test.**
- **Evaluate whether molecular serotyping of *Streptococcus pneumoniae* can be dependable and reliable for prevalence, disease burden, and/or surveillance purposes.**

Materials and Methods

Using the CDC protocol, we identified panels, set up total of seven panels of *S.pneumoniae*

Panel 1: *S.pneumoniae* serotype group targets – 3, 7F/7A, & 19A

Panel 2: *S.pneumoniae* serotype group targets – 6C/6D, 12F/12A/12B/44/46, & 22F/22A

Panel 3: *S.pneumoniae* serotype group targets – 15A/15F, 23A, & 33F/33A/37

Panel 4: *S.pneumoniae* serotype group targets – 1, 11A/11D, & 16F

Panel 5: *S.pneumoniae* serotype group targets – 4, 6A/6B/6C/6D, & 9V/9A

Panel 6: *S.pneumoniae* serotype group targets – 14F, 18C/18F/18B/18A, & 19F

Panel 7: *S.pneumoniae* serotype group targets – 2, 5, & 23F

Reaction		Primer	Primers ordered through IDT (As per CDC paper)	
			Oligo ID/Primer name	Oligo Sequence
1	1	3-F (817283)	CCACTAAAGCTTTGGCAAAAGAAA	
		3-R (817284)	CCCGAACGTAAAGCTTCTTCA	
		3-pb YY (02823)	/5YAKYEL/TTGTAGACCGCCCCACAAT/ZEN/TCATTTTGT/3IABkFQ/	
	2	7F/7A-F (817293)	ATGAAGGCTTTGGTTTGACAGG	
		7F/7A-R (817294)	ATTCTCGCCATCAATTGCATATTC	
		7F/7A-pb TEX615 (817315)	/5TEX615/ACACCACTATAGGCTGTTGAGACTAACGCACA/3IABQSP/	
	3	19A-F (1019212)	CGCCTAGTCTAAATACCA	
		19A-R (1019213)	GAGGTCAACTATAATAGTAAGAG	
		19A-pb FAM (1019183)	/56-FAM/TATCAATGAGCCGATCCGTCACTT/3IABkFQ/	
2	4	6C/6D-F (1001589)	TTGGGATGATTGGTCGTATTAG	
		6C/6D-R (1001590)	CTCTTCAATTAGTTCTTCAGTTCG	
		6C/6D-pb FAM (1002498)	/56-FAM/CCA+CGC+AAT+TCG+CCATC/3IABkFQ/	
	5	12F/12A/12B/44/46-F (902281)	GCACCCACGGGTAAATATTCTAC	
		12F/12A/12B/44/46-R (902282)	CAACTAAGAACCAAGGATCCACAG	
		12F/12A/12B/44/46-pb TEX615 (817319)	/5TEX615/TGCCCACCACACACCAGGTCCAGGT/3IABRQSP/	
	6	22F/22A-F (821384)	TCTATTAAATATCCATTGGAATTGAAACG	
		22F/22A-R (821385)	TCGCAATTGAAGACCACATAAACTG	
		22F/22A-pb YY (02824)	/5YAKYEL/TCCGTAATT/ZEN/CGCTTTATGGGCACATTCTCCA/3IABkFQ/	
3	7	15A/15F-F (1011034)	AATTCCCTATAACTCATTGAGATAG	
		15A/15F-R (1011035)	CCATAGGAAGGAAATAGTATTTGTTC	
		15A/15F-pb TEX615 (1013070)	/5TEX615/CCC+GCA+AAC+TCT+GTCCT/3IABRQSP/	
	8	23A-F (1011015)	CTCCCCTCCATTACCGCATTTGG	
		23A-R (1011016)	TGAAGAAAGTCCTGTTTGTGAACC	
		23A-pb FAM (1013071)	/56-FAM/AGCTAGAACT/ZEN/CCCACACTCCCTACTCCCA/3IABRQSP/	
	9	33F/33A/37-F (1010951)	CGAACTCGTCAGCAACTATACG	
		33F/33A/37-R (1010952)	CGTTCTATAGACCGTCTGAAATACC	
		33F/33A/37-pb YY (1013072)	/5YAKYEL/CCCCAAATAGGACT/ZEN/TTTCTGCCATGCCAAA/3IABKFQ/	
4	10	1-F (817279)	TTTCATCCCTATGTGTGGTATAG	
		1-R (817280)	GCTTTAGAAGGTAGAGTTATACAAC	
		1-pb FAM (819001)	/56-FAM/TGC+CAA+AGC+CAG+CCAT/3IABkFQ/	
	11	11A/11D-F (821382)	AAATCGTTTCGATATGCTTTGTTTGG	
		11A/11D-R (821383)	AGTCCTAACTTGTAAAACTTGATTATGAG	
		11A/11D-pb TEX615 (1004313)	/5TEX615/ATTCCAACTTCTCCCAATTTCTGCCACGG/3IABRQSP/	
	12	16F-F (821386)	TAATGTTATGACCTTGGTAATCTTCCC	
		16F-R (821387)	TCCCAAAGGATAATCCAATACTTTTAGAAG	
		16F-pb YY (1005203)	/5YAKYEL/AGCCATAAGTCTT/ZEN/CCAAATGCTTAACCGCT/3IABKFQ/	
5	13	4-F (817285)	GCTTCTGCTGTAACTGTTGTGC	
		4-R (817286)	CACCACCATAGTAACCAAAGTTCC	
		4-pb TEX615 (1004311)	/5TEX615/TTCCACAAAAGAAGAGCCTACAGGTAACCCCA/3IABRQSP/	
	14	6A/6B/6C/6D-F (1005960)	GTTTGCACTAGAGTATGGGAAGG	
		6A/6B/6C/6D-R (1005961)	TAGCCTTTATGAAAACATTTGATTAGCG	
		6A/6B/6C/6D-pb FAM (1006435)	/56-FAM/TGTTCTGCCCT/ZEN/GAGCAACTGGTCTTGTATC/3IABKFQ/	
	15	9V/9A-F (817295)	AGGTATCCTATATACTGCTTTAGG	
		9V/9A-R (817296)	CGAATCTGCCAATATCTGAAAG	
		9V/9A-pb YY (819002)	/5YAKYEL/ACA+CAT+TGA+CAA+CCGCT/3IABKFQ/	
6	16	14-F (817299)	AGAGTGTATGAGGAATCC	
		14-R (817300)	ATATATCTACTGTAGAGGGAAT	
		14-pb FAM (819003)	/56-FAM/CGCCAAGTAACAT/ZEN/TTCCATTCCATT/3IABKFQ/	
	17	18C/18F/18B/18A-F (703368)	TCGATGGCTAGAACAGATTTATGG	
		18C/18F/18B/18A-R (703369)	CCATTGTCCTGTAAGACCATTC	
		18C/18F/18B/18A-pb YY (817317)	/5YAKYEL/AGGGAGTTGAATCAACCTATAATTTCGCCCC/3IABKFQ/	
	18	19F-F (1004362)	TGAGGTTAAGATTGCTGATCG	
		19F-R (1004363)	CAGGAATGAAACTCCAATAAAAG	
		19F-pb TEX615 (1004938)	/5TEX615/CGC+ACT+GTC+AAT+TCACCTTC/3IABRQSP/	
7	19	2-F (817281)	TGTTATCCCATATAAGAACCGAGTGT	
		2-R (817282)	AAAATTAACCCAAAAGCTATCCAA	
		2-pb YY (02822)	/5YAKYEL/TTGCAATTT/ZEN/CAATTTTTTTGCCCCAATCTC/3IABkFQ/	
	20	5-F (817287)	CATGATTTATGCCCTCTTGCAA	
		5-R (817288)	GACAGTATAGAAAAAGCAAGGCTAA	
		5-pb FAM (902140)	/56-FAM/TCTTCTTCTCAT/ZEN/CGTTTCCGCATGCTTTT/3IABkFQ/	
	21	23F-F (817307)	GACAGCAACGACAATAGTCATCTC	
		23F-R (817308)	TCCATCCCCAACCTAAGCAGCACTTC	
		23F-pb TEX615 (1005206)	/5TEX615/ATTGTGTCCAT/TAO/AACCCTTCGTCGTATTTCCAAAG/3IABRQSP/	
SPC	22	MHV-RNA-F	CGGTGTTAGCGGTTTTGCTG	
		MHV-RNA-R	GCCACTCCGTTTGTTTGAGGCCA	
		MHV-RNA-Pr-TYE665	/56-TAM/TATGTGAAGTCCAAGGTCGGAAATTACCGACTG/3IABkRQSp/	

Table 1: Each panel has a set of 3-4 *S.pneumoniae* serogroups categorized by the CDC protocol, and the sequence of each serogroup primer that targets the lytAgene. A total of seven panels, along with forward transcription (F), reverse transcription (R), and internal control (MHV). These oligonucleotide panel are manufactured in IDT's GMP Manufacturing suite , which is compliant with cGMP/ under the FDA's QSR 21 CFR Part 820 requirements as it is applicable to oligo manufacturing and certified to ISO13485:2003. Primers are HPLC purified and resuspended to 100uM in IDTE pH 8.0 in manufacturing facility itself and shipped to University of Louisville by frozen overnight shipment. Primers stored in -20 degree C after receiving at UofL ID lab.

Prepare fresh inoculum using each serogroup in serial dilution (10-fold), plate, count CFU

- Dilution 1-Take 6 colonies using loop and swirl into 2mL saline. **(Above countable range)**
- Dilution 2-200ul of Dilution 1 +1800uL of Saline **(Above countable range)**
- Dilution 3-200ul of Dilution 2 +1800uL of Saline-plate 5uL of inoculum into 2 sides of the plate
- Dilution 4-200ul of Dilution 3 +1800uL of Saline-plate 5uL of inoculum into 2 sides of the plate **(8900CFU/mL)**
- Dilution 5-200ul of Dilution 4 +1800uL of Saline-plate 5uL of inoculum into 2 sides of the plate (**900CFU/mL)**
- Dilution 6-200ul of Dilution 5 +1800uL of Saline-plate 5uL of inoculum into 2 sides of the plate (**200CFU/mL) LOD**
- Dilution 7-200ul of Dil 6 +1800uL of Saline-plate 5uL of inoculum into 2 sides of the plate (**100CFU/mL)**
- Dilution 8-200ul of Dilution 7 +1800uL of Saline **(No growth)**
- Dilution 9-200ul of Dilution 8 +1800uL of Saline **(No growth)**
- Dilution 10-200ul of Dilution 9 +1800uL of Saline **(No growth)**

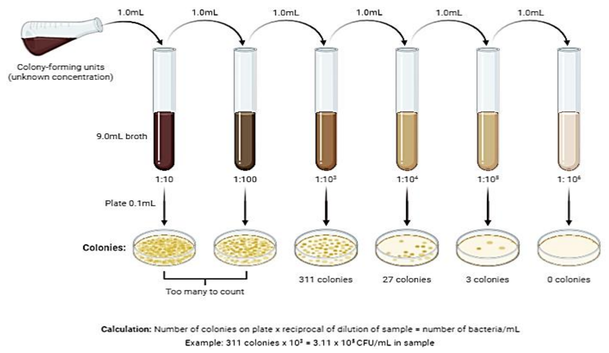

Figure 1:. Prepare fresh serogroup inoculum using 6 colonies (plate streaked 40 hrs. before) in 2mL saline for a serial dilution (10-fold) for a total of 10. This step was also was used for quantifying colonies forming units (CFU) in each dilution so that we could estimate which dilution is going to be the maximum and minimum limit of detection (LOD) based on CFU and number of colonies needed to detect at the lowest and highest concentration during our validation analysis on the RT-PCR using Luminex ARIES system. Each dilution was plated on a Sheep Blood Agar plate in somewhat anaerobic conditions using CO_2 GasPak at 37ºC for 24 hours.

Validation

Dynamic range: Limit of Detection (LOD) for all organism (1:10, 1:100, 1:1,000, 1:10,000…)
Accuracy: Ideally three (3) of each serotype.
Precision: One (1) of each serotype run on different days/different personnel

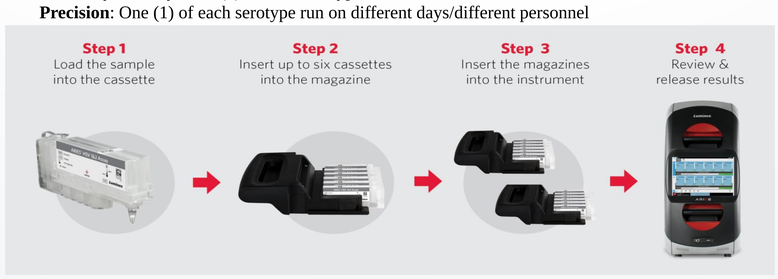

Figure 2: Using Luminex ARIES® The Limit of Detection was initiated by transferring 4 µL serogroup Panel 1-7 primers/probes pool to Exo ReadyMix. Connected the ReadyMix tube containing primers to the cassette. Loaded 200µL of prepared *S. pneumoniae* isolate dilution into the cassette and ran the TaqMan PCR assay. This was done for each dilution in each serogroup, which equates to 10 dilutions for each serogroup, in triplicates over the course of three different days by three different personnel to characterize accuracy and precision of the test.

Results

Sample ID	MHV RNA Pr-TAMRA CT cycle	Sero-group 19A-FAM CT cycle	Sero-group 3-YY CT cycle	Sero-group 7F/7A-Tex615 CT cycle
	AVG (SD)	AVG (SD)	AVG (SD)	AVG (SD)
SEROGROUP 3, DILN 2	29.80	20.03 (2.32)	28.63 (4.55)	22.63 (0.25)
SEROGROUP 3, DILN 3	31.00 (2.39)	23.03 (1.86)	26.80 (0.72)	25.63 (0.51)
SEROGROUP 3, DILN 4	30.60 (0.80)	26.03 (1.46)	25.93 (3.44)	28.40 (0.90)
SEROGROUP 3, DILN 5	32.40 (0.90)	29.90 (0.95)	35.20 (0.66)	31.93 (1.44)
SEROGROUP 3, DILN 6	33.00 (1.73)	35.57 (1.46)	37.20 (0.50)	36.57 (0.64)
SEROGROUP 3, DILN 7	33.10 (1.45)	38.10 (2.12)		38.10 (0.42)
SEROGROUP 3, DILN 8	33.43 (1.10)	37.40 (0.00)		
SEROGROUP 3, DILN 9	32.70 (0.52)			

Table 2: Each serogroup in each panel (1-7) available in the IDL was tested using primers following CDC protocol. The Ct values helped distinguish the LOD for each serogroup which varied per serotype. Each serogroup had MHV as an internal control, which was amplified in all dilutions. Dilutions 1-2 were usually very close to maximum cutoff, while dilutions 8-10 were typically too low in concentration for detection; hence, most LOD fell between dilutions 6 and 7.

Panel/Reaction	*S.pneumoniae* sero-group target Primers	LOD in ARIES Luminex	Ct value for LOD
1	3	SG 3=Dilution 6 (200CFU/mL)	37.20±0.50
	7F/7A	SG 7A= Dilution 6 (150CFU/mL)	36.57±0.74
	19A	SG 19A= Dilution 6 (4450CFU/mL)	31.53±0.31
2	6C/6D	SG 6C =Dilution 6 (1250CFU/mL)	35.70±1.13
	12F/12A/12B/44/46	SG 12F=Dilution 5 (300 CFU/mL)	35.07±0.51
		SG 46C=Dilution 6 (30 CFU/mL)	34.4±0.50
	22F/22A	SG 22F=Dilution 7 (300 CFU/mL)	33.97± 0.29
3	15A/15F	SG 15A=Dilution 7 (100 CFU/mL)	32.73±1.10
	23A	SG 23A=Dilution 7 (300 CFU/mL)	35.13±0.21
	33F/33A/37	SG 33F= Dilution 7 (300 CFU/mL)	35.70±0.85
		SG 37= Dilution 7 (50 CFU/mL)	36.33±0.35
4	1	SG 1= Dilution 7 (900CFU/mL)	38.33±1.94
	11A/11D	SG 11A=Dilution 8 (120 CFU/mL)	31.55±0.07
	16F	SG 16F=Dilution 6 (4500 CFU/mL)	29.10±0.44
5	4	SG 4=Dilution 7 (4250 CFU/mL)	29.97±1.40
	6A/6B/6C/6D	SG 6B=Dilution 6 (3000 CFU/mL)	32.77±1.63
		SG 6C=Dilution 5 (6600 CFU/mL)	34.30±0.95
	9V/9A	SG 9V= Dilution 6 (4600 CFU/mL)	29.13±0.70
		SG 9A= Dilution 6 (4600 CFU/mL)	29.40±0.66
6	14-F	SG 14=Dilution 6 (14350 CFU/mL)	31.87±1.64
	18C/18F/18B/18A	SG 18=Dilution 5 (above countable range) (dilution 6 range is 9800CFU/mL)	25.03±0.40
	19F	SG 19F Dilution 5 (15250 CFU/mL)	26.80±0.46
7	2	SG 2 is dilution 6 (2100 CFU/mL)	27.87±0.42
	5	SG 5 Dilution 7 (6250CFU/mL)	34.97±3.06
	23F	Isolate not available	
SPC	MHV-RNA		

Table 3: Limit of Detection for all the serogroups that were tested on Luminex ARIES. This along with quantification of CFU per dilution for each serogroup enables us to calculate how much organism was needed for testing and how sensitive the test is based on these Ct values. These cut off values are used as reference for different samples types which include blood, plasma, serum, sputum, cerebrospinal fluid, and urine.

Conclusion

Ramirez et al. looked community acquired pneumonia in the city of Louisville with hopes to determine whether the PCV-23 vaccine if effective in prevention of poor disease outcomes. The analysis was done using 3,686 patients, of which 608 patients had PPSV23-serotype CAP, while 3078 had non-PPSV23-serotype CAP. The study adjusted for comorbidities and covariates and found that in the overall cohort the effectiveness of the PPSV23 vaccine for the prevention of hospitalized PPSV23-serotype CAP was 14%. When analyzing by subgroups of ages, the authors found that PPSV23 in 65 and older was only 2% effective. The age 65 and older is of particular interest because current CDC guidelines and recommendations call for anyone over the age of 65 to receive PPSV23, and current vaccination guidelines suggest a booster dose of the PPSV23 vaccine 5 years after the initial dose. This study is important to highlight, because it demonstrated real-time surveillance of individuals that were hospitalized in the city of Louisville. The one limitation of this study was that the study failed to note which serogroups were most commonly circulating, or how many cases fell under each serotype of *S.pneumoniae.* This gave us the impetus to implement the molecular serotyping assay in the Division of Infectious diseases for future population-based epidemiologic studies.

Future Direction

The future direction of this project is to test true positive and true negative human specimens for clinically diagnosed patients to further validate sensitivity, specificity, positive predictive value, and negative predictive value so the test is fully functional, and can serve as an important milestone in surveillance measures for CAP. The test would in return provide real-time data to further enhance current vaccine development and characterization towards circulating serotypes within a given community. The city of Louisville has been considered a good sampling city, that closely represents population and demographic data nationally. With this test, the authors hope to provide the city of Louisville with instrumental diagnostic tools to enhance clinical prognostics, and adequate treatment, with a turn around time of 2 hours from specimen preparation to results.

Acknowledgments

Authors want to acknowledge and personally thank Laura Schindler, Sabrena Garr, and Jennifer Wick, for taking direct mentorship of a Masters student by being an instrumental support to the student by providing him valuable time, effort, and experience as a practicum site. Furthermore, the authors would like to acknowledge the Division of Infectious Diseases at the University of Louisville School of Medicine, and Chief of the Division Dr. Forest Arnold for providing support, specimens, and the opportunity to fund the project for implementation.

References

1. Kim, L., McGee, L., Tomczyk, S., & Beall, B. (2016). Biological and epidemiological features of antibiotic-resistant streptococcus pneumoniae in pre- and Post-Conjugate vaccine eras: A united states perspective. *Clinical Microbiology Reviews*, *29*(3), 525–552. https://doi.org/10.1128/cmr.00058-15
2. Lee, J., Yoon, Y., Kim, E. J., Lee, D., Baek, Y., Takano, C., Chang, B., Iijima, T., Kilgore, P. E., Hayakawa, S., Hoshino, T., Kim, D. W., & Seki, M. (2021, February 16). *23-valent polysaccharide vaccine (ppsv23)-targeted serotype-specific identification of streptococcus pneumoniae using the loop-mediated isothermal amplification (LAMP) method*. PloS one. Retrieved April 24, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7886117/
3. Brooks, L. R., & Mias, G. I. (2018). Streptococcus pneumoniae's virulence and host immunity: Aging, diagnostics, and prevention. *Frontiers in Immunology*, *9*. https://doi.org/10.3389/fimmu.2018.01366
4. Centers for Disease Control and Prevention. (2021, July 23). *Streptococcus lab resources and protocols*. Centers for Disease Control and Prevention. Retrieved April 24, 2022, from https://www.cdc.gov/streplab/pneumococcus/resources.html
5. Chandler, T., Furmanek, S., Carrico, R., Balcom, D., Arnold, F., & Ramirez, J. (2022). 23-valent pneumococcal polysaccharide vaccination does not prevent community-acquired pneumonia hospitalizations due to vaccine-type streptococcus pneumoniae. *Microorganisms*, *10*(3), 560. https://doi.org/10.3390/microorganisms10030560