1. Context

In the context of the rapidly evolving COVID-19 epidemic, the coming season with circulation of several respiratory viruses, the evolution of the type of tests available and the possible limitations in testing and sampling capacity, an update of the testing strategy is necessary, in order to use the available resources in the best way, potentially with inclusion of new techniques. Therefore we revised the existing evidence and other countries’ strategies on pooling, saliva testing, RT-LAMP, rapid antigen test and self-swabbing of nasal, throat and nasopharyngeal samples in order to make recommendations about the conditions in which these techniques can be used. Additionally, a recommendation for multiplex testing for the coming season of respiratory viruses, is included.

2. Pooling of samples

2.1. BACKGROUND POOLING

Pooling of samples implies mixing of samples (before or after RNA extraction) and performing the first diagnostic test on this mixture of samples.

Several pooling strategies can be used, a recent news report published in Nature (1) gives an overview of 4 pooling strategies that are used or evaluated in the COVID-19 pandemic (a schematic overview can be found in Annex 1).

- **Method 1**: involves two rounds of testing, where each sample of a positive pool is retested individually, most straightforward, used in Wuhan with up to 5 samples per pool, most efficient if prevalence is ≤1% according to researchers.
- **Method 2**: involves three or more rounds of testing, where pools with positive samples are further divided into smaller pools and then are tested individually or re-divided in smaller pools. Not time efficient, needs several rounds before obtaining final results for positive samples.
- **Method 3**: involves two rounds of testing (and if needed an extra round for individual testing), in the second round samples are tested in multiple overlapping groups. Difficult pooling scheme, will be trialed in Rwanda.
- **Method 4**: involves one round of testing, where samples are distributed into a matrix of overlapping groups, fast but very complex pooling scheme (but apps being developed), being trialed in India and Israel (with promising results according to the article).

The benefits of pooling are:

- Can save reagents, costs and time.
• Allows for high throughput.

The possible drawbacks are:

• Efficiency decreases at higher prevalences/test positivity ratios.
• Sensitivity decreases due to dilution of viral RNA (theoretically increase of 1 Ct for 2-fold dilution). The bigger the pool, the greater the decrease in sensitivity.
• Can imply longer time till test result (especially for samples in a positive pool that needs retesting in case of method 1-3). However, because throughput is extensively increased, this is likely to be counterbalanced in the context of large scale screening in low prevalence settings.
• Pooling schemes can be complex (method 3 and 4).
• Sample mix up and contamination can more easily happen.

CDC has issued an interim guidance on the use of pooling procedures for diagnostic, screening and surveillance testing, recommending pooling only to be used in areas or situations where the number of positive test results is expected to be low (for example in areas with a low prevalence of SARS-CoV-2 infections) (2).

ECDC has issued a technical report on Methodology for estimating point prevalence of SARS-CoV-2 infection by pooled RT-PCR testing. They present a pooling methodology for point prevalence studies, including an PooledTesting R package to help identify optimal study design. The tool requires user defined operational limits (maximum pool size, maximum number of pools to test, maximum number of individual samples) and a hypothetical prevalence of the disease in the target population (3).

Some implementations of pooling worldwide:

• China has used pooling up to 5 samples (method 1) to test the population of Wuhan (1).
• Nebraska (US) has used pooling of 5 samples (method 1), but discontinued when prevalence rose above 10% (4).
• Israel has used pooling of 8 samples (method 1) for the routine testing of nasopharyngeal swab samples from screened asymptomatic healthcare personnel, employees of essential industries, and residents and employees of nursing homes (5) and is evaluating method 4.
• In Germany, pools ranging in size from four to 30 samples were used to test nursing home residents and staff (6).

2.2. NON-EXHAUSTIVE OVERVIEW OF STUDIES ON POOLING

Evaluations of method 1:

• Yelin et al. evaluated method 1 with pooling after RNA extraction and found a sensitivity of 96% (when using a cut of Ct of 40) for a 16-sample pool with 1 positive sample (observing an increase of 1.24 in Ct for each dilution by a factor of 2). They also evaluated pooling before RNA extraction, and found that successful results were obtained for 8-sample pools with 1 positive sample, with an average increase in Ct of 2.9. (7)
• Lohse et al. evaluated method 1 (unclear if extraction before or after pooling) and found that over a range of pool sizes (4-30 samples), Ct values in pools were up to 5 higher. They found that, even with Ct values of individual samples up to 34, positive pools could still be confidently identified. (8)
• Sahajpal et al. evaluated method 1 with pooling before RNA extraction. 940 samples (of which 6 positive) were tested in 94 pools of 10 samples resulting in 148 reactions to test 940 samples. The authors state that Ct values in the pool- and individually tested samples were comparable. (9)
• Ben-Ami et al. evaluated method 1 with pooling before RNA extraction. They found that test results were not significantly affected when using pools of 8 samples. They also evaluated method 3 and found that this strategy is more efficient when prevalence is higher than 2%. Additionally, they created a tool (https://github.com/matanseidel/pooling_optimization) to help choose the approach and pool size based on the prevalence. They implemented this strategy in Israel to test 26,576 samples from asymptomatic individuals. (5)
• Abdelhamid et al. used method 1 with pooling before RNA extraction. They tested 25 pools of 5 samples with 1 positive and found that all 25 pools were positive with Ct values within 0 and 5.03 Ct of the original individual specimens. They calculated that, with a prevalence below 10%, pooling can increase testing capability by at
least 69%. They calculated optimal pool size using a web-based application https://www.chrisbilder.com/shiny (4)

- Gupta et al. evaluated method 1 with pooling after RNA extraction. They found that in pools of 8 RNA samples the method can easily detect even up to a single positive sample with Ct value as high as 38. The study suggests the results of pool testing are not affected by number of positive samples in a pool. (10)

- Torres et al. evaluated method 1 (unclear if extraction before or after pooling) in 40 samples (10 positive in pools of 5 and 10 (both containing 1 positive)). They found that positive samples were detected in minipools of both sizes, as long as Ct values of the individual positive samples were <32 for the E gene and <35.2 for the RdRp gene. (11)

- The University of Liège is validating pooled testing on 3 samples (1 positive with varying Ct, and 2 negative samples) and found that all positive samples with Ct ≤34 are detected (unpublished data). Evaluation of the sensitivity of this pooling approach for specimens with higher CT is ongoing.

**Models to determine efficiency, method and/or ideal number of samples in a pool:**

- Aragon-Caqueo et al. developed a mathematical model to determine efficiency at different prevalences (test positivity ratios) for method 1. Their model predicts group sizes that range from 3 to 11 samples. They found that for a prevalence of 10% of positive tests, 40.6% of tests can be saved using pools of four subjects. For a 20% prevalence, 17.9% of tests can be saved using pools of three subjects. With prevalences higher than 20% the strategy loses effectiveness. They do state that further studies are needed to address how large these groups can be, without losing sensitivity on the RT-PCR. (12)

- Pilcher et al. evaluated method 1 and method 2 in a mathematical model. When the prevalence was >1%, simple pooling schemes and smaller pools (eg. 6:1 “minipools” for prevalence of 5%) were more comparable in efficiency to larger and/or more complex pooling schemes. Below 1% prevalence, larger minipools could be several-fold more efficient (in terms of results per test used) than 5:1 minipools. When prevalence was 0.1%, larger pools and particularly 3-stage pools were substantially more efficient. Below 1% prevalence, adding the intermediate pool stage generally resulted in much higher testing efficiency. They provide a free, publicly-available web calculator is provided to help inform laboratory decisions on SARS-CoV-2 pooling algorithms http://www.bios.unc.edu.vdicp.health.fgov.be:8080/~mhudgens/SARS-CoV-2.pooling.home.html (13)

- In a not yet peer reviewed paper, Pikovski et al. present a model to estimate the ideal pool size for method 1 and estimated it to be 4 samples in 1 pool. (14)

**2.3. CONCLUSION POOLING**

- Literature on pooling is promising.

- Pooling is more efficient at low prevalences/test positivity ratios (although one study suggests pooling is efficient up to a prevalence of 20%). With increasing prevalence/test positivity ratio, pooling will increasingly become less efficient and may lead to delayed test results (for positive pools, due to retesting of all individual samples).

- Pooling could be used in a context of shortages of capacities (reagents, human resources in lab). However, since such shortages are more likely to occur in a context of high virus circulation (and high seroprevalence), it will probably not be cost-effective.

- Optimal pooling size depends on:
  - Prevalence/test positivity ratio.
  - Pooling method used.
  - Ct values of positive samples.

- Optimal pooling strategy depends on:
  - Prevalence/test positivity ratio.
  - Acceptable complexity of pooling scheme.

- Sensitivity decreases with pooling, the extent (which is small according to literature) to which depends on the protocol used. A decrease of sensitivity will mainly impact samples with high Ct values (theoretically a 2-fold dilution of RNA increases the Ct with 1). Some studies suggest to use an alternative cut off when pooling.

- Particular caution is required with regards to pre and post analytical errors. The risk of cross-contamination indeed greater when using pooling approaches.
Pooling can be done before and after RNA extraction. The impact on sensitivity of both techniques is not clear from literature. Pooling before RNA extraction will additionally save extraction reagents and decrease the risk of contamination.

Several models and applications have been developed to determine the best pool size or pooling strategy.

Limited information is available on experiences with/capacity of pooling for COVID-19 in Belgian labs.

2.4. RECOMMENDATION POOLING

- Currently, it is not recommended to use pooling as a diagnostic tool for symptomatic individuals or contacts, since pooling reduces sensitivity to a certain extent, increases the risk of errors/contamination and is probably not efficient in reducing the use of capacities in case of shortages (when seroprevalence is probably high).
- Pooling could be used for screening of large asymptomatic populations expected to have a low prevalence. When screening in low risk asymptomatic populations (e.g. schools), a certain loss in sensitivity is acceptable, as those most contagious/superspreaders (i.e. lowest CT values) should still be detected. The impact of the reduced sensitivity will depend on the setting, being possibly more problematic in high risk settings (e.g. WZC/MRS) than in low-risk settings (e.g. schools).
- It is recommended that laboratories that implement pooling use a validated protocol (extension of the RT-PCR cycles may be considered. For development of a pooling protocol, the applications and methods developed and referred to in this document can be useful to determine the best pooling method and/or the number of samples in a pool. Deconstruction of the pools should also be well described to reduce risk of cross-contamination. The experience of veterinary departments and transfusion centers in developing pooling protocols should be used, as they have a lot of experience in pooling for mass screening.
- It is recommended to pool before RNA extraction to reduce the risk of contamination and limit the use of reagents.
- An overview of Belgian laboratories experienced with/capable of pooling for COVID-19 is needed.
- RIZIV/INAMI reimbursement of pooling is currently administratively not possible.
- In the context of platform bis, pooling for screening should be integrated in its ‘own’ flow.

See point 4. for a Note on pooling of saliva samples.

3. RT-PCR on saliva

3.1. BACKGROUND RT-PCR ON SALIVA

Since salivary glands are a potential target of infection due to high level of ACE2 expression on the epithelial cells of the oral mucosa, saliva testing instead of using nasopharyngeal swabs (NPS) for RT-PCR has been suggested as a way to:

- Overcome a possible shortage of swabbing material.
- Facilitate the sampling procedure.
- Decrease discomfort of sampling, likely leading to higher acceptability.
- Decrease exposure and requirement of health care workers (possibility of self-sampling).

The Netherlands will, according to several news websites, start testing children younger than 6 years old with saliva tests. (15)

In France, the Haute Autorité de Santé, recently concluded that there is still uncertainty around the performance of saliva tests as an alternative for nasopharyngeal swabs. They agreed for a ‘forfait
d’innovation’ which enables to have the necessary budget for a wide scale evaluation of these tests. (16)

Some laboratories report concerns about contamination through received saliva samples (not well closed, saliva out of the container).

### 3.2. NON-EXHAUSTIVE OVERVIEW OF STUDIES ON RT-PCR ON SALIVA

#### Systematic reviews and meta-analyses:
- In a not yet peer reviewed Belgian rapid systematic review, Peeters et al. concluded that the relative sensitivity of SARS-CoV-2 testing on saliva versus NPS was 0.97 (no significant difference; 95% CI=0.92-1.02). In the second outcome (incorporating more data), they estimated that a pooled proportion 86% (95% CI=77-93%) of NPS positive cases was also positive on saliva. They conclude that saliva could potentially be considered as an alternative sampling method. But that studies were often small and involved inclusion of subjects with insufficient information on clinical covariates. Most studies included patients who were symptomatic (78%, 911/1167). Therefore, additional and larger studies should be performed to verify the relative performance of saliva in the context of screening of asymptomatic populations and contact-tracing. (17)
- A not yet peer reviewed meta-analysis by Czumbel et al. found 91% (95%CI = 80%-99%) sensitivity for saliva tests and 98% (95%CI 89%-100%) sensitivity for NPS in previously confirmed COVID-19 infected patients, with moderate heterogeneity among studies. (18)

#### Other studies:
- A not yet peer reviewed Belgian study collected two saliva samples (saliva swab and saliva container) and NPS from more than 2000 people from triage centers. They found that for people with a low viral load (< 20 000 copies/ml nasopharyngeal transport medium), the correspondence between NPS and saliva samples is < 5%. For people with a medium to high viral load, the correspondence between NPS and saliva samples is high (up to 97%), and this for collection in a container (swabs performed less good) and irrespective of the symptomatic status. They concluded that saliva testing is not suitable for individual diagnosis of COVID-19 virus in symptomatic patients and high-risk contacts. But is likely to have value to identify asymptomatic individuals with medium to high viral load in the context of systematic screening campaigns. (19)
- Belgian data (unpublished) evaluated different methods for collecting saliva (Salivette, stimulated drooling and unstimulated drooling) and unstimulated drooling provides the best Ct results of RNAseP.
- Landry et al. tested NPS and saliva (collected in a container, not drink or eat 30 minutes prior to saliva collection) of 124 symptomatic individuals. Thirty-five were RT-PCR positive, 33 by NPS (sensitivity = 94.3 % (95 % CI 81.4%-99.0%)) and 30 by pure saliva (sensitivity = 85.7 % (95 % CI 70.6%-93.7%)), for an overall agreement of 117/124 (94.4 %). The Ct’s were significantly lower for NPS than for saliva. (20)
- Williams et al. tested the saliva (collected in a container, keep saliva in mouth for 1 to 2 min prior to collection) of 39 NPS-positive patients at a screening clinic. Thirty-three (84.6 %) of patients with positive NPS had SARS CoV-2 RNA detected in saliva. In 1/50 (2%) of saliva samples from patients with negative NPS were also positive. The Ct’s were significantly lower for NPS than for saliva. (21)
- Rao et al. tested the deep throat saliva (collected in a container, early morning saliva) and NPS among 217 individuals who tested positive for SARS-CoV-2 (NPS) in day 8-10 of their quarantine. At the moment of testing they were asymptomatic (no information about their symptom history available). In total, 160 persons still tested positive by either test. In 76/160 patients virus was detected in saliva but not in swab and in 11/160 virus was detected in swab but not in saliva. Among the 73 individuals with concordant results, the Ct-values were significantly lower in saliva. (22)
- Iwasaki et al. tested the saliva (collected in a container) and NPS among 10 COVID-19 patients and 66 patients suspected for COVID-19 and found a concordance rate of 97.4% (95%CI: 90.8-99.7). Viral load was equivalent at earlier time points but lower in saliva than in NPS samples at convalescent phase. (23)
- Azzi et al. tested saliva (drooling technique, collected in a container) 25 SARS-CoV-2 infected patients with severe or very severe disease (NPS-positive). All tested positive in the saliva test. (24)
- Guo et al. investigated the effect of throat washing on the detection of SARS-CoV-2 in 11 laboratory-confirmed COVID-19 participants (6 hospitalized, 5 discharged). They collected 24 paired throat washing and NPS (unclear in which sequence) and found that in 18/24 the results were concordant, the other 6 tested positive for throat washing, but negative for NPS. (25)
- Pasomsub et al. tested saliva samples (void of coughing, collected in a container), throat swabs and nasopharyngeal swabs of 200 symptomatic individuals under investigation (median onset of symptoms was 3 (2–7) days before). They estimated sensitivity and specificity was 84.2% (95% CI 60.4–96.6%) and 98.9% (95% CI 96.1--99.9%) when using NPs and throat swabs as the gold standard. (26)
Lai et al. tested 150 saliva samples (early morning deep throat saliva, DTS), 309 NPS or throat swabs and 104 sputum samples of 50 hospitalized (2 asymptomatic). They found DTS had the lowest overall RT-PCR positive rate (68.7% vs. 89.4% [sputum] and 80.9% [pooled NPS and throat swabs]), and the lowest viral RNA concentration (mean log copy/mL 3.54 vs. 5.03 [sputum] and 4.63 [pooled NPS and throat swabs]). (27)

In a not yet peer reviewed study Becker et al. simultaneously tested NPS and saliva specimens, collected with Orasure OM-505 Microbiome and OGD-610 DNA collection kits, of 88 persons from a community testing environment (non-hospitalized). With a Bayesian latent class model, they estimated the NPS sensitivity to be 98.9% (95% CI: 67.6%- 99.7%) and saliva sensitivity 69.2% (95% CI: 38.6%- 97.6%). In an additional study, they included 24 patients with prior positive COVID-19 results and found that overall, the sensitivity using the NPS samples was higher than for the saliva samples. (28)

In a not yet peer reviewed study, Wyllie et al. tested saliva (collected in a container, avoid food, water and brushing of teeth until the saliva sample was collected) and NPS from 44 hospitalized COVID-19 patients (multiple saliva samples) and 98 asymptomatic health care workers (NPS also self-collected for the latter group). They found that the sensitivity of saliva is comparable, if not superior to NPS in early hospitalization and is more consistent during extended hospitalization and recovery. Moreover, SARS-CoV-2 was detected in the saliva of two asymptomatic healthcare workers with negative NPS. (29)

In a not-yet peer reviewed study, Ott et al. investigated the stability of SARS-CoV-2 RNA and infectious virus detection from saliva without supplementation. RNA stability was tested over 2-25 days and at temperatures representing at-home storage and elevated temperatures which might be experienced when cold chain transport may be unavailable. They concluded that RNA in saliva from infected individuals is stable at 4°C, room temperature (~19°C), and 30°C for prolonged periods and found limited evidence for viral replication in stored saliva samples. They conclude that expensive saliva collection options involving RNA stabilization and virus inactivation buffers are not always needed, permitting the use of cheaper collection options. (30)

The University of Gent has collected 1400 saliva samples of mainly asymptomatic individuals in nursing homes (residents and personnel). Saliva was collected in a container after people were asked to mix 2ml water with saliva in their mouth and gargle (the latter only for personnel). They found a sensitivity <50% (preliminary unpublished data, will be communicated when analysis finalized). It will be further evaluated in collaboration with the University of Antwerp whether repeated testing increases the sensitivity.

The University of Antwerp participates in a study in nursing homes evaluating the difference between early morning saliva and saliva collected later in the day (saliva collected in a container). Although not statistically significant due to limited number of paired tests, current observations hints a preference towards early-morning saliva due to the lower SARS-CoV-2 E-gene Ct-value (mean Ct-value of 31.9 and 34.3 in early-morning and in-day saliva respectively) and a higher detection rate (4/5 vs 3/5 in included paired samples in early-morning and in-day saliva respectively) in this study. More paired samples should be tested to make a conclusion about the existence of a difference between early-morning and in-day saliva samples. Notably, the elderly experience a difficult saliva production, as seen with the large number of discarded samples due to insufficient sample volume (< 100 µl in 6/24 samples) and thick samples which could not be retested after dilution (< 200 µl in 2/24 samples). There was unwillingness or incapability of stimulation of saliva production using a chewing-gum. An alternative stimulator should be found if saliva will be used in this population.

3.3. CONCLUSION RT-PCR ON SALIVA

Studies evaluating saliva testing often have a limited sample size and differ regarding type of saliva (deep throat, early morning, void of coughing, throat washing, stimulated drooling vs. non-stimulated drooling, no eating/drinking/teeth brushing before…), collection method/device and timing of sampling, making it difficult to draw conclusions. The above mentioned study of University of Antwerp, will answer some of these questions (early morning vs. later collected saliva). It is expected that early morning samples will yield the best results (higher viral load).

Most studies suggest a sensitivity of saliva >80% (which is in line with the results of the meta-analyses), however some studies suggest a sensitivity below 70% (especially at low viral load and/or in asymptomatic individuals, albeit not all).

Recent studies showed higher sensitivity if early morning saliva is collected (before breakfast).

Most studies evaluating saliva testing studied symptomatic and hospitalized persons, few results of studies on asymptomatic persons are available. No studies included children.

In some cases, saliva yields a positive result, whereas the nasopharyngeal swab is negative, which could be due to differences in the kinetics of viral load in saliva as compared to nasopharyngeal swabs. The clinical/public health relevance of this is unknown.
• The biggest study so far (2000 people) concluded that correspondence between nasopharyngeal swabs and saliva (collected in a container) is up to 97% for viral loads above 20,000 copies/ml.
• Saliva testing with collection in a container works better than with saliva swabs.
• Limited data suggest that there is no need for expensive collection options involving RNA stabilization and virus inactivation buffers.
• Saliva could be an excellent sample in specific settings. However, in elderly people saliva is more difficult to collect.
• People will very likely accept more to be tested repeatedly with saliva samples than with nasopharyngeal swabs.

3.4. RECOMMENDATION RT-PCR ON SALIVA

• Currently, most studies suggest a lower sensitivity for RT-PCR on saliva than on nasopharyngeal swabs. Therefore saliva testing seems less suitable as a diagnostic tool for symptomatic individuals or contacts, and is presently not recommended. This should be reevaluated in some groups of individuals, when the abovementioned systematic reviews and meta-analysis will be published or if data on studies in Belgium are performed (but results should be available very quickly). Saliva testing could be an alternative in case of shortage of swabs, PPE, or overloaded medical staff collecting samples. For children < 6 years, saliva testing for diagnostic should also be considered, as alternative for nasopharyngeal swabs. The current RAG advice for these children is to only test in a limited number of situations. Using saliva tests, if satisfactory sensitivity compared with throat and/or nose swabs, should allow to test more children.
• Saliva testing could be used for screening of large groups of asymptomatic populations, to identify highly infectious individuals.
• When collecting saliva in a container, it is important that these containers do not pose any risk of contamination (no saliva outside of the container, container well closed etc…).
• More studies are urgently needed (and should be encouraged) to determine the best type of saliva collection method and optimal sampling time for diagnosis and screening of SARS-CoV-2, in different Belgian settings and age groups (nursing homes, schools, high risk groups, etc.).
• The performance of saliva testing for screening should be urgently compared with throat and nasal swabs collected by healthcare workers, as well as with self-collection methods, see also point 7.

See point 4. for a Note on pooling of saliva samples.

4. Note on pooling of saliva samples

Pooling of saliva samples combines the benefits of both strategies, however, sensitivity will inevitably be further decreased. There are limited data available on the performance of the combination of these two techniques, and further operational studies are highly needed.

Currently, we are aware of two Belgian studies regarding pooling of saliva samples.
• The University of Gent will evaluate pooled saliva testing (up to 20-60 samples per pool) for screening in nursing homes. The protocol is under development.
• The University of Liège is currently validating pooled saliva testing that will be used to screen staff and students. A container that inactivates the virus upon closing (inactivation buffer released upon screwing of cap) is used and early morning saliva is collected. The protocol can be shared.
5. RT-LAMP as an alternative for RT-PCR

5.1. BACKGROUND RT-LAMP

The current gold standard for molecular diagnosis of COVID-19 is based on the detection of SARS-CoV-2 RNA by real-time quantitative reverse transcription-polymerase chain reaction, but other techniques have been developed/adapted to SARS-CoV-2. One of these, the RT-LAMP (reverse-transcriptional loop-mediated isothermal amplification assay), combines reverse transcriptase, DNA polymerase, pH indicator, and primers to amplify RNA templates causing a drop in pH and, a color change detectable by the eye. RT-LAMP does not require thermal cycles.

The advantages of this technique are:
- simple equipment and techniques,
- low cost (estimated 2-4€ for reagents and <1000€ for equipment, no need for expensive qPCR machines),
- result, indicated by color change, is easily interpreted by naked eyes,
- could be used as point-of-care test with limited laboratory equipment needed.

The disadvantage is that it is not a tool for massive testing (tests done one my one). Experience in use of LAMP in Belgium is limited to other pathogens (Malaria, CMV, Cl. Difficile, Strepto A and Influenza). With Influenza, Belgium experience reports a risk of false positives.

The technique was commercially available for DNA targets before the pandemic and has been adjusted. Currently Abbott has a commercial test available in the US only, the ID Now COVID-19. This test has recently been improved to increase sensitivity (evaluation see below), but is not exported. Other commercially available RT-LAMP tests are also available (Enbiotech, ICGene), but no information on evaluations is available. The Federation of Belgian meat (FEDBEV) has asked for an evaluation of these tests for use in slaughterhouses and meat processing companies.

5.2. NON-EXHAUSTIVE OVERVIEW OF STUDIES ON RT-LAMP

Non-commercially available assays:
- Chow et al. developed an COVID-19-RT-LAMP with primers targeting a region across orf3a and E gene with a detection limit of 42 copies/reaction. They evaluated it on 223 respiratory samples positive for SARS-CoV-2 by qRT-PCR and 143 samples positive for other respiratory viruses. On NPS, they found a sensitivity of 95.07% (95% CI:0.92–0.98) and 98.21% (95% CI: 0.96–1.00) at 60 and 90 minutes respectively. None of the 143 samples with other respiratory viruses were positive by COVID-19-LAMP, showing 100% specificity. (31)
- Dao Thi et al. developed a two-color RT-LAMP using a primer set specific for the N gene. Evaluation on 768 RNA samples showed an overall specificity of 99.7% (95% CI: 98.9%-99.9%), and sensitivity for samples with Ct < 30 on RT-PCR of 97.5% (95% CI: 91.4%-99.3%). Almost all samples with Ct values between 30 and 40 scored negative (only 4 positive out of 36). (32)
- Lu et al. developed an RT-LAMP using a primer set specific for the N gene with a detection limit of 118.6 copies of SARS-CoV-2 RNA per 25 μL reaction. Evaluation on 56 clinical samples showed a concordance rate between both assays was 92.9%. Both RT-PCR and RT-LAMP missed 2 samples that were detected by the other test. The two samples missed by RT-LAMP had Ct values >38 in RT-PCR. (33)
- Lee et al. developed an RT-LAMP using a primer set specific for the N gene with a detection limit between 50 and 500 viral genome copies per reaction. Evaluation on 157 clinical specimens previously screened by E-gene RT-qPCR revealed assay sensitivity and specificity of 87 and 100%, respectively. (34)
- Jiang et al. developed an RT-LAMP using a primer set specific for the N gene with a detection limit of 500 copies/ml. Evaluation on 213 negative and 47 positive patients showed a high degree of specificity (99.5%), sensitivity (91.4%). (35)

Commercially available assays (Abbott ID NOW COVID-19):

[Continued on next page]
• Harrington et al. evaluated the Abbott ID Now COVID-19 on 524 samples (nasal and nasopharyngeal swabs) and found an overall agreement was 75% positive agreement (95% confidence interval [95% CI], 67.74%, 80.67%) and 99% negative agreement (95% CI, 97.64%, 99.89%) between IDNCOV and ACOV for all specimens tested. (36)
• An evaluation by Basu et al. of the Abbott ID Now COVID-19 found that, regardless of method of collection and sample type, Abbott ID Now COVID-19 had negative results in a third of the samples that tested positive by Cepheid Xpert Xpress when using nasopharyngeal swabs in viral transport media and 45% when using dry nasal swabs. (37)
• An evaluation by Smithgall et al. of the Abbott ID Now COVID-19 e overall positive agreement was 73.9% with ID Now. Negative agreement was 100%. ID Now and Xpert showed 100% positive agreement for medium and high viral concentrations (Ct value <30). However, for Ct values >30, positive agreement was 34.3% for ID Now. (38)

5.3. CONCLUSION ON RT-LAMP

• RT-LAMP techniques are not intended for massive testing.
• Several non-commercial RT-LAMP tests have been developed and the results are promising, suggesting RT-LAMP could be a faster and more affordable alternative to RT-PCR. The current place of such assays in clinical settings has still to be defined; No wide scale validation of these techniques has been done yet, the validations that are done are mainly done by the developers of the test, on a limited number of samples. Impact on sensitivity depends on exact protocol developed.
• The commercial Abbott ID now (not exported) has varying results in evaluations.
• No information was found about the performance of the commercially available Enbiotech and ICGene RT-LAMP assays.
• To our knowledge, RT-LAMP technology for COVID-19 is currently not used in Belgian labs, but the technique is used for several DNA-based targets.

5.4. RECOMMENDATION RT-LAMP

• Currently, it is not recommended to use RT-LAMP as a diagnostic tool for symptomatic individuals or contacts nor for screening of an asymptomatic population because of the lack of wide scale validation studies.
• However, since results of non-commercial assays are promising, developments in the area will be followed closely.
• An evaluation of the commercially available RT-LAMP tests (Enbiotech and ICGene) is recommended.

6. Rapid antigen testing

6.1. BACKGROUND RAPID ANTIGEN TESTS

Rapid antigen tests for diagnosis of covid-19 infection are performed on nasopharyngeal swabs, they have a decreased sensitivity but high specificity, implying that negative results should be retested with RT-PCR, while positive results allow immediate patient management.

In the RAG-advice of 02/04/2020 it was recommended to only use a rapid antigen test in situations where no RT-PCR could be performed and in situations with a 30-40% positivity ratio (i.e. high pre-test probability), since every negative sample needs to be retested with RT-PCR (due to low sensitivity of the test). Here we provide an update, based on current literature.

WHO, in an advice of 08/04/2020, does not currently recommend the use of antigen-detecting rapid diagnostic tests for patient care, although research into their performance and potential diagnostic utility is highly encouraged. (39)
FIND, the Foundation for Innovative New Diagnostics, a global non-profit organization, is leading a call for expressions of interest to accelerate the availability and manufacturing scale-up of rapid diagnostic tests for the detection of SARS-CoV-2 antigens, because of the current suboptimal performance of these tests. (40)

In their Guidelines on the Diagnosis of COVID-19, the Infectious disease society of America, does not include antigen tests for the diagnosis and it is stated that antigen detection tests may be on the horizon, but that it needs to be defined how they compare to RT-PCR. (41)

The CDC states that negative results from an antigen test may need to be confirmed with a PCR test prior to making treatment decisions or to prevent the possible spread of the virus due to false negative results. (42)

In the Netherlands, RIVM states that they are ’looking at’ other types of tests such as antigen tests. (43)

In France, the Haute Autorité de Santé, states that antigen tests are not recommended for clinical use due to their weak performance, especially at lower viral loads. (44)

In many low-income countries, the use of rapid antigen tests is scrutinized/investigated, to decrease the volume of high cost PCR. Indeed, its use could substantially reduce the need for PCR (no PCR needed if rapid antigen test is positive). In clinical practice, a highly specific test (even if poorly sensitive) has its value.

6.2. NON-EXHAUSTIVE OVERVIEW OF STUDIES ON RAPID ANTIGEN TESTS

- Mertens et al. report on the development and evaluation of the COVID-19 Ag Respi-Strip (Coris BioConcept, Gembloux, Belgium). In a retrospective multi-centric evaluation on aliquots of 328 nasopharyngeal samples, they found an overall sensitivity and specificity of 57.6 and 99.5%. (45)
- Lambert-Nicot et al. evaluated a rapid antigen diagnostic test, COVID-19 Ag Respi-Strip, for detection of the SARS-CoV-2 antigen in 138 nasopharyngeal secretions of which 94 (68.8%) were positive for SARS-CoV-2 by RT-PCR. Compared to that of RT-PCR, the specificity of the Ag test was 100% (95% CI: 91.8 to 100). Among the 94 RT-PCR-positive samples, the rapid test detected only 47 specimens, resulting in a sensitivity of 50.0% (95 CI: 39.5 to 60.5). (46)
- Scohy et al. evaluated a rapid antigen diagnostic test, COVID-19 Ag Respi-Strip testing 148 nasopharyngeal swabs. Amongst the 106 positive RT-qPCR samples, 32 were detected by the rapid antigen test, giving an overall sensitivity of 30.2%. All the samples detected positive with the antigen rapid test were also positive with RT-qPCR. (47)
- Mak et al. evaluated the Biocredit COVID-19 Ag test on respiratory samples collected from confirmed COVID-19 patients and found that the rapid antigen test detected between 11.1% (compared with sputum) and 45.7% (compared with nasopharyngeal aspirate and throat swab) of RT-PCR-positive samples from COVID-19 patients. (48)
- Loeffelholz et al. state that new approaches to try to concentrate antigen and amplify the detection phase are likely to be needed for these methods to have any clinical utility. (49)
- In a systematic review La Marca et al. state that the evaluation of these diagnostic tests has been limited, although direct antigen tests are being registered by several health authorities, the sensitivity of these tests is lower than that of RT-PCR. Their greatest utility if they come to fruition may be in symptomatic patients, when the viral load will be at its greatest, to enable accurate triage. (50)

6.3. CONCLUSION RAPID ANTIGEN TESTS

- Rapid antigen tests have low performance in diagnosis COVID-19 infection, with a sensitivity for the Coris Bioconcept COVID-19 Ag Respi-Strip of lower than 60% (some studies suggest as low as 30%).
- Rapid antigen tests offer consistently high specificity.
- Research and development is ongoing.
- A newly developed test BD Veritor reports a higher sensitivity (70%) but is not available in Europe yet.
6.4. RECOMMENDATION RAPID ANTIGEN TESTS

- The use of rapid antigen tests is generally not recommended and should only be used if no RT-PCR is available, or as a first diagnostic/screening test to isolate positive individuals if an RT-PCR can be performed only at a later time point (e.g. in hospitals without microbiology lab, for diagnosis or screening of admissions at night, GP practices). In this case, RT-PCR should anyway be performed as soon as possible on all samples with negative antigen test.
- More studies are needed to evaluate the sensitivity of detecting highly infectious people (useful in context of screening), instead of comparing results to PCR for all viral loads. However, rapid antigen tests are time consuming (one by one), and therefore not suitable for large screenings.
- Studies should also address the usefulness and cost-effectiveness of rapid antigen tests for the clinical practice/immediate management in GP practices.

7. Self-collected nose, throat and nasopharyngeal swabs

7.1. BACKGROUND SELF-COLLECTED SWABS

Additionally to self-collection of saliva, implementation of self-swabbing could save the use of personal protective equipment and decrease work load and exposure of health care workers. In some cases, self-swabbing/self-collecting could potentially be an alternative for saliva sampling in screening settings.

7.2. NON-EXHAUSTIVE OVERVIEW OF STUDIES ON SELF-COLLECTED SWABS

- McCulloch et al. compared self-collected midnasal swab performance with clinician-collected nasopharyngeal swabs in 185 participants. They found that Cycle thresholds of home swabs were positively correlated with clinician swabs (correlation coefficient, 0.81; P < .001). Four of 5 false-negative home swabs had a Ct greater than or equal to 33. In a sensitivity analysis of all swabs with Ct less than or equal to 32, sensitivity of home swabs was 95%. (51)
- In a not-yet peer reviewed study, Demmer et al. evaluated self-sampling in 489 symptom-free health care workers. Over 95% of participants reported a willingness to repeat a self-collected nasopharyngeal swabs in the future for either clinical or research purposes. 24% preferred a provider collected-swab, 57% preferred self-collection and 19% reported no preference. No comparison was done with not-self-sampled swabs and no case of SARS-CoV-2 infection was found. (52)
- Waghmare et al. compared self-sampling with flocked nasal swabs and self-sampling with foam nasal swabs in 15 patients with respiratory symptoms for the detection of 11 respiratory viruses. They found that self-sampling with foam swabs was better tolerated than with flocked swabs. Agreement between samples collected by foam and flocked swabs from the same nostril was generally high, particularly with high viral load samples, with no evidence of higher yield with one method versus another. No comparison was done with not-self-sampled swabs. (53)
- Valentine-Graves et al. assessed willingness and feasibility of patients to self-collect three diagnostic specimens (saliva, oropharyngeal swab (OPS) and dried blood spot (DBS) card) while observed by a clinician through a telehealth session. Of the 153 US adults enrolled, A large majority of participants (>84%) reported that collecting, packing and shipping of saliva, OPS, and DBS specimens were acceptable. Nearly nine in 10 (87%) reported being confident or very confident that the specimens they collected were sufficient for laboratory analysis. (54)
- Guest et al. assessed quality of oropharyngeal swabs collected during a telehealth video appointment while clinical observers watched and documented the suitability of the collection in 159 participants. Of the enrolled participants, 153/159 (96.2%) returned their kits and 146/151 (96.7%) of the samples were of sufficient quality for submission for laboratory testing, 100% of these had cycle threshold values for RNA-PCR testing for SARS-CoV-2. (55)
- In a not yet peer reviewed study, Kojima et al. assessed the performance of self-collected oral fluid swab specimens with and without clinician supervision, clinician-supervised self-collected mid-turbinate (nasal) swab specimens, and clinician-collected nasopharyngeal swab specimens in 45 participants. They found that clinician-supervised self-collected oral fluid swab specimens detected 26 (90%) of 29 infected individuals,
clinician-supervised self-collected nasal swab specimens detected 23 (85%) of 27, clinician-collected posterior nasopharyngeal swab specimens detected 23 (79%) of 29, and unmonitored self-collected oral fluid swab specimens detected 19 (66%) of 29. Despite nasopharyngeal swabs being considered the gold standard, 4 participants tested negative by clinician-collected nasopharyngeal swab and positive by the 3 other specimen types. Additionally, false negative results by each sample type were seen to generally not overlap. (56)

- Wehrhahn et al. evaluated performance of self-collection of nasal and throat swabs with health care worker collected nasal and throat swabs or nasopharyngeal swabs for the detection of respiratory viruses including SARS-CoV-2. They found that self-collection (SC) was highly concordant with health care worker collection (HC) (κ = 0.890) for all viruses including SARS-CoV-2 and more concordant than HC to positive results by any method (κ = 0.959 vs 0.933). (57)

- In a not yet peer reviewed study Teo et al. evaluated the performance of self-collected of naso-oropharyngeal saliva and nasal swabs (SN) compared to nasopharyngeal (NP) swabs in 200 migrant workers, who were given an instruction video on how to self-sample (45 with acute respiratory infection, 104 asymptomatic close contacts, and 51 confirmed COVID-19 cases). Of 337 sets of tests (workers were tested repeatedly), there were 150 (44.5%) positive NP swabs, 127 (37.7%) 17 positive SN swabs, and 209 (62.0%) positive saliva. In COVID-19 confirmed patients, saliva performed better than nasal swab at all-time points after onset of symptoms. (58)

### 7.3. CONCLUSION SELF-COLLECTED SWABS

- The acceptability of self-collecting nasopharyngeal swabs for the diagnosis of SARS-CoV-2 infection has only been assessed in health care workers (95% willingness to repeat). There is very limited available evidence on the performance of self-collected nasopharyngeal swabs for the RT-PCR detection of SARS-CoV-2.
- There is limited available evidence on the performance and acceptability of self-collected throat swabs for the diagnosis of SARS-CoV-2.
- There is limited available evidence on the performance and acceptability of self-collected nasal swabs, though 2 studies suggest saliva is more sensitive than self-collected nasal-swabs.
- Sensitivity is higher if self-sampling is performed under medical supervision.

### 7.4. RECOMMENDATION SELF-COLLECTED SWABS

- Based on the very limited available evidence on the performance of self-swatting, it is currently not recommended to use this technique.
- More studies are urgently needed to assess acceptability and performance in order to determine if self-swatting of nasal and/or throat samples can be used as an alternative to saliva testing in screening settings (compare saliva results with results of self-collected nasal/throat swabs; comparing self-swatting with saliva and/or swabbing performed by healthcare workers).
- Self-swatting could be an interesting option in e.g. test villages (under medical supervision), but further studies are urgently needed before implementing it.

### 8. Multiplex

#### 8.1. BACKGROUND MULTIPLEX

In the coming months, it will become increasingly important to distinguish a SARS-CoV-2 infection from other respiratory infections such as influenza and RSV, both from a clinical as from a surveillance point of view. This will put an increased pressure on lab capacity and reagent supply. Moreover, in the context of respiratory disease surveillance, the collection of samples within the existing surveillance networks of health care providers may be very difficult because of the changes in testing facilities. Additional efforts are needed to make the collection of samples possible. Multiplex testing will therefore become increasingly important. At this moment, these multiplexes are not reimbursed by RIZIV/INAMI (unless for some categories of patients, eg transplants patients).
Currently, the necessary steps are being undertaken to include multiplexes with SARS-CoV-2 and other respiratory pathogens in RIZIV/INAMI nomenclature. The plan is to have this finalized in the first half of September.

At this moment, the proposal is to include two types of multiplex:

- one for a limited number (at least 3) of respiratory pathogens, with at least influenza (including mandatory typing: separate result for influenza A and influenza B) and RSV,
- one for a large set (initial proposal: 10) of respiratory micro-organisms, with freedom on the type of organisms to include (will depend on clinical context of patient). The cost of the necessary machine with acceptable turn-around-time, is significantly higher. This type of multiplex, also due to much higher pricing, will be restricted for use only in hospitalized critically ill patients.

At present, in Belgium, multiple laboratories have developed or are developing and validating multiplex assays to detect and differentiate RNA from SARS-CoV-2 from other pathogens in respiratory specimens. Some examples from the relevant NRC’s:

- At the NRC Influenza currently triplex qPCR used for influenza typing (detection of influenza A viruses, influenza B viruses and RNaseP housekeeping genes) is adapted to add a fourth target (E gene of SARS-CoV-2). Validation is almost finished. The NRC Influenza also collaborates with in the development of a quadruplex to detect SARS-CoV-2, influenza (A and B, but no typing), RSV (A and B, no typing), and RNaseP as housekeeping genes. A multiplex for 16 non influenza respiratory viruses for surveillance purposes is also available. This is based on primers/probes used by the NRC Influenza. For surveillance

- The NRC respiratory pathogens (SARS-CoV-2) performs a multiplex on a respiratory panel of 29 pathogens, including SARS-CoV-2.

At this stage, we do not have an exhaustive overview of the multiplexes currently used/developed/validated in Belgium.

### 8.2. CONCLUSION AND RECOMMENDATION MULTIPLEX

- Finalization of incorporation of multiplex in RIZIV nomenclature is urgent, and planned for the first half of September.
- Labs should aim at implementing/developing/validating multiplexes that are subject to reimbursement.
- When reimbursement is implemented, it is recommended to, during the season of respiratory infections (week 40 till week 20, cfr influenza surveillance):
  - Use multiplex (respiratory “minipanel”), including SARS-CoV-2, influenza (A and B) and RSV, in all patients presenting with influenza-like illness, including in primary care. The costs of the test in this context will be regulated in the same way as it is currently regulated for SARS-CoV-2 testing for patients in primary care and will be incorporated within the regular RIZIV/INAMI reimbursement for hospitalized patients.
  - The use of a respiratory “maxipanel” (at least 10 respiratory micro-organisms) will be restricted to hospitalized critically ill patients. The recommendations and reimbursements issues will be further discussed by the Microbiology Working Group of the Commission Clinical Biology (MWG CKB-CBC).
  - Do not use multiplex for testing of contacts, nor for SARS-CoV-2 screening of asymptomatic populations.
  - Prioritize SARS-CoV-2 testing in symptomatic persons (see case definition of possible case) in primary care, in situations when multiplex is not or limited available.

- An overview of the Belgian capacity regarding multiplex testing is urgently needed.
- Multiplex PCR will further be used for surveillance purposes (separate protocol/budget).
9. Elements for further discussion:

1. More studies are needed to compare nasal swabs, throat swabs and saliva (container versus swab) for self-sampling, including in combination. Published studies focus mainly on comparison to nasopharyngeal swabs.

2. It will be difficult for laboratories to use different tests for the symptomatic (ILI) patients and the asymptomatic persons, even if the information is available. How can this be solved (practically)? Moreover, reimbursement of the regular RT-PCR for SARS-CoV-2 falls under two different regulations.

3. The case definition for a possible case of COVID-19 is much wider than ILI. Should the case definition be restricted to ILI during the season of respiratory infections to avoid wasting multiplex PCR tests on persons with e.g. fever and diarrhea? More information is needed on the symptoms presented by the COVID-19 patients in ambulatory settings, to be able to evaluate this (Sciensano).

10. General remark

The testing approaches covered in this review, such as pooling of samples or saliva testing, offer a reduced sensitivity in comparison to nasopharyngeal RT-PCR. Nevertheless, they may represent valid tools for population-based screening. The rationale behind using tests with a suboptimal or lesser sensitivity for screening purposes is the fact that people with high Ct values are likely to be non-contagious (see also RAG advice on Interpretation of PCR results and infectivity). Moreover a not yet peer reviewed study by Larremore et al. modelled surveillance effectiveness considering test sensitivities, frequency, and sample-to-answer reporting time. They found that effective surveillance, including time to first detection and outbreak control, depends largely on frequency of testing and the speed of reporting, and is only marginally improved by high test sensitivity. The authors therefore conclude that surveillance should prioritize accessibility, frequency, and sample-to-answer time whereas analytical limits of detection should be secondary. (59)

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ANNEX 1: SCHEMATIC OVERVIEW OF POOLING METHODS

**Method 3**

This method uses two rounds of testing. In the second round, samples are tested in multiple overlapping groups, represented by rows and columns on a square. More people can be tested by adding dimensions (see the cube).

**Round 1:** 3 tests

- Negative
- Positive

**Round 2:** 6 tests

- Negative test
- Positive test

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**Method 4**

This method uses only one round of testing. Samples are distributed into a matrix of overlapping groups.

9 people

6 tests

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