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Development of a Customized TaqMan® Array Card for Simultaneous Detection of 32 Respiratory Pathogens

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Abstract

Background: Syndromic testing with a rapid and complete diagnostic panel would be extremely useful for routine respiratory testing.

Objective: We developed and validated a real-time PCR-based Taqman array card (TAC) including several pathogens specific for immuno-compromised patients, using sequences designed and supported by the manufacturer.

Study design: Analytical validation was performed in 2 phases. Phase 1 testing was performed in 96-well plates and included efficiency, reactivity and sensitivity testing. The assays selected after phase 1 were validated in TAC format in phase 2 which included analytical sensitivity (LoD), reactivity, specificity and reproducibility testing. Clinical validation was performed in phase 3 by testing respiratory samples that were sent to the lab between December 2016 and May 2017.

Results: In total, 90 assays were tested in phase 1 of which 43 were retained for further validation in TAC format. Seven of the 43 assays did not meet the predefined criterion of efficiency ≥ 80%, but were accepted for further testing in phase 2 and 3 due to lack of alternatives. All 43 targets together detected 89% (481/539) of positives included in the reactivity testing in phase 1 and 98% (246/252) of positives in phase 2. All tested assays showed excellent analytical sensitivity, with LoD ranging from 1 to 100 copies/µL. An overall specificity of 99.96% was found. Reproducibility ranged from 76%-100%, with a mean of 91%. For the clinical validation, a total number of 428 samples were tested, with an overall positivity rate of 56.3% and a co-infection rate of 15.9%. 15 results (4% of total number of positives) were not confirmed and considered false positives. One result (0.2%) was considered false negative.

Conclusion: This syndromic panel was analytically and clinically validated and will be implemented for routine respiratory testing in the Erasme University Hospital in high risk patient populations.

Keywords: TaqMan® array card; Real-time polymerase chain reaction/methods; Respiratory tract Infections/microbiology syndromic testing; Development and validation; Sensitivity; Specificity; Reproducibility

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Background

Acute respiratory tract infections (RTI) are responsible for 75% of all consults for acute complaints in the world, resulting in a spectrum of clinical pictures going from common cold to life threatening pneumonia. Accurate and rapid diagnosis of etiologic agents can be crucial for high risk populations such as immunocompromised patients, patients in ICU, neonates etc. However, today's targeted infectious disease diagnostics limit testing to only the most common pathogens and include only a

limited number of respiratory viruses. This leaves most infections undiagnosed and leads to additional downstream testing and compromised patient care. Introducing symptom-driven testing (aka syndromic testing), targeting a large panel of pathogens in one step, can impact all areas of patient care. For healthcare providers, this implies faster diagnoses, improved antibiotic stewardship and avoiding unnecessary examinations and/or hospitalizations. Advantages for labs are improved efficiency, reduced downstream testing and costs. Several commercial platforms and assays exist for the simultaneous detection of multiple respiratory pathogens in a specimen. One common theme among all the commercial platforms is that the end user does not have control over the menu of assays included in the panel or the ability to customize individual assays. An exception is the TaqMan® Array Card (TAC; Thermo Fisher Scientific, Life Technologies, South San Francisco, CA) technology, a low density microfluidic card arranged in an 8 specimen × 48 assay array for a total of 384 individual real-time PCR reactions. This technology was originally intended for cancer research. For infectious diseases diagnostics, only in-house developed custom-made TAC assays exist to date. The CDC pioneered the use of TAC for detection of respiratory pathogens [1]. Since then, other TACs have been designed for respiratory diseases [2,3], hepatitis [4], enteric diseases [5-7], etiologies of neonatal sepsis [8], central nervous system infections [9], sexually transmitted infections [10] and bioterrorism agents [11,12]. In addition, more specialized cards were also developed for testing of malaria [13], TB drug resistance [14], pneumococcal serotyping [15] and acute febrile illness outbreak investigation [16].

Objective

Our goal was to develop and validate a respiratory TAC, using sequences designed and supported by the manufacturer and to include several pathogens specific for immunocompromised patients.

Study Design

TAC content

The pathogens to be included on the card were chosen in collaboration with the infectious diseases team and other key specialization teams of the Erasme University hospital, with a focus on immunocompromised patients. All the assays and their respective target genes are listed in **Table 1**. The cards also contain three control assays, including an internal positive control (Phocine Distemper Virus – PDV), one manufacturer control (18S) and one specimen quality control (hDNA). For the most important respiratory pathogens, at least two different gene targets were included to optimize sensitivity, and in case of RSV and influenza A viruses, additionally for subtyping. More than one assay was also needed to cover viruses that are part of a large and diverse group such as adenoviruses, enteroviruses and rhinoviruses.

Assay design

TaqMan™ MGB (minor groove binding probe) assays were designed using proprietary signature sequence finding algorithms and proprietary assay design pipelines (Thermo Fisher Scientific, South San Francisco, CA). For the identification of signature sequences, for each species in the panel inclusivity sets were comprised with complete genome sequences of strains of the organism to be targeted and exclusivity sets with complete genome sequences of strains of the organism's near neighbors. All sequences were downloaded from the National Center for Biotechnology Information (NCBI). The designed assays were mapped against all contents of NCBI in assembled and unassembled genomes and were selected based on 4 criteria: specificity, sensitivity, strain coverage and primer-dimer formation probability score. The assays targeting bacterial organisms were designed by identifying unique genome regions that *in silico* exhibited 98% or higher strain coverage and absolute specificity. The same criteria were followed for assays targeting viral organisms, however due to high viral mutation rates, especially in RNA viruses, pools of 3 or 4 assays were comprised in order to achieve *in silico* strain coverage of 95% and higher for complete genome sequences or, in the case of Influenza viruses, protein segments, submitted in NCBI since 2011 and onward.

Specimens

For phase 1 and 2, clinical samples (nose-throat swabs, BAL samples, nasopharyngeal aspirates), viral and bacterial isolates and/or their nucleic acid extract originated from the microbiology biobank at our hospital. These samples were previously tested and found positive by conventional techniques and/or other molecular assays. For pathogens that were not present in the biobank or pathogens with low prevalence, positive samples were obtained from other labs. When available, external quality control samples were purchased from different suppliers (QCMD, INSTAND, VIRCELL, Zeptometrix). Controls were treated as specimens from sample preparation to result, except for controls which contained the purified complete genome of the infectious agent with absolute quantification (VIRCELL). For phase 3 testing, respiratory samples that were sent to the lab for routine testing between December 2016 and May 2017 were included.

Nucleic acid extraction

Nucleic acids were extracted from 800 µL input sample on the QiaSymphony (Qiagen, Valencia, CA) using the DSP viral pathogen midi kit in combination with the "Pathogen/Complex 800 V5 DSP" generic DNA-RNA extraction protocol and eluted in 130 µL. A dilution of Phocine Distemper Virus (PDV) was added during extraction as internal extraction and inhibition control.

Phase 1: Analytical validation primer/probe sets

This phase consisted of preliminary testing to select only those assays that met predefined criteria and when several options, to select the best assay for each target.

PCR protocol: Singleplex qPCR was performed in 96-well plates on QuantStudio7 with TaqMan® Fast Virus 1-Step MMx and 900 nM primer/250 nM probe in an end volume of 20 µL. Following thermocycling profile was used: 50°C for 5 mins, 95°C for 20 s and 45 cycles of 95°C for 3 s followed by 60°C for 30 s.

Table 1 Assay overview with information on subtyping and respective target genes, limit of detection and reproducibility data.

Assays	Sub typing	Gene target(s)	LoD	Reproducibility (b)			
				St dev	Count	Expected number	% Concordance
Adenoviruses	-	Hexon/Protein VI analogs/L3 protein analogs	10 copies/µL	0.5117	27	27	100%
Bocavirus	-	NP-1	1 copy/µL	0.2499	15	18	83%
CMV	-	UL13	NP	0.5453	32	36	89%
Coronaviruses	CoV NL 63	N	1 copy/µL	0.1574	21	21	100%
Coronaviruses	CoV OC43	N	1 copy/µL	0.2737	22	24	92%
Coronaviruses	CoV 229E	Replicase polyprotein 1ab	10 copies/µL	0.0833	12	15	80%
Coronaviruses	CoV HKU1	5' UTR	10 copies/µL	0.3472	38	42	90%
HSV-1	-	UL41/Virion host shutoff protein	1 copy/µL	0.2595	27	33	82%
HSV-2	-	UL41-UL42 intergenic spacer	10 copies/µL	0.4310	9	9	100%
HHV-6	-	U77	NP	0.1072	6	6(c)	100%
Influenza A	H3	HA	10 copies/µL	0.2336	26	27	96%
Influenza A	H1-2009	HA	10 copies/µL	0.2138	18	21	86%
Influenza A	Pan-assay	Matrix protein	10 copies/µL	0.2568	52	57	91%
Influenza B	-	Matrix protein/polymerase	10 copies/µL	0.3953	24	24	100%
hMPV	-	Fusion glycoprotein precursor/ nucleoprotein	10 copies/µL	0.4099	14	18	78%
Parainfluenza viruses	PIV 1	RNA Polymerase	1 copy/µL	0.2664	18	18	100%
Parainfluenza viruses	PIV 2	Large protein	1 copy/µL	0.2168	21	21	100%
Parainfluenza viruses	PIV 3	Phosphoratein-D-protein	10 copies/µL	0.7455	21	21	100%
Parainfluenza viruses	PIV 4	Matrix protein/large protein N696_gp7/nucleocapsid protein	10 copies/µL	0.1840	12	15	80%
RSV	RSV A	Attachment glycoprotein	NP	0.1859	15	18	83%
RSV	RSV B	Glycoprotein	NP	0.2152	14	18	78%
RSV	Pan-assay	Attachment glycoprotein/ glycoprotein	NP	0.3435	29	33	88%
Rhinoviruses	-	5' UTR	10 copies/µL	0.6050	24	27	89%
VZV	-	ORF33.5 capsid maturation protease	NP	0.1362	12	15	80%
Bordetella pertussis	-	Anti-ECFsigma factor/ChrR	10 copies/µL	0.5052	12	12	100%
Bordetella "pan": *B. pertussis*+ *B. parapertussis*+*B. bronchiseptica*	-	Putative ferrodoxin	10 copies/µL	0.2499	16	21	76%
C. psitacii	-	Iron-regulated ABC transporter permease protein SufD	NP	0.0664	6	6	100%
C. pneumoniae	-	Putative mazG protein	1 copy/µL	0.1802	15	15	100%
C. burnetii	-	Rare lipoprotein A	10 copies/µL	0.2548	12	12	100%
L. pneumophila	-	Putative protein-spore maturation protein	10 copies/µL	0.3203	17	18	94%
M. pneumoniae	-	hmw1.cytaherence-accessory protein	NP	0.2383	12	15	80%
P. jirovecii	-	Mitochondrial gene	NP	0.2259	12	15	80%
Phocine Distemper Virus (PDV)	-	HA	NA	0.1697	158	165	96%
Human DNA Control	-	LOC102724344	NA	0.2408	146	165	88%

NA: Not Applicable; NP: Not Performed

(a)Assay detects both CoV HKU-1 and Cov OC43. When this assay and specific assay for CoV OC43 are positive: positive for CoV OC43. When only CoV HKU-1 assay positive positive for CoV HKU-1

(b)When more than 1 gene target results for combination of targets

(c)6 replicates were removed due to too low viral load (below the limit of detection)

PCR efficiency testing: Efficiency was defined from a standard curve based on tenfold serial dilution (in elution buffer) of the DNA or RNA (reference material). Each dilution was analyzed in triplicate. Assays with efficiency between 80 and 110% were eligible for phase 2 testing. In addition, the R^2 value of the standard curve should be >0.99.

Reactivity testing: A maximum of external quality control samples and known positive clinical samples were tested to cover as much subtypes as possible for each target.

Sensitivity testing: Since the sensitivity in TAC format is theoretically lower (1 µL reaction volume), LOD experiments were not performed in phase 1. Assays were eligible for phase 2 testing if all core samples of the EQC panels tested were detected.

Phase 2: Analytical validation TAC

The assays selected after phase 1 were validated in TAC format. The primers and probe for each assay were preloaded and dried onto the designated wells.

PCR protocol: Taqman® Array Cards were run with fast TAC block on QuantStudio7 with TaqMan® Fast Virus 1-Step MMx according manufacturer guidelines. Following thermocycling profile was used: 50°C for 20 min, 95°C for 2 min and 45 cycles of 95°C for 3 s followed by 60°C for 30 s.

Reactivity testing: For each target, at least 5 different strains/clinical samples were tested to cover different strains and/or subtypes. At least one strongly positive sample for each target and one weakly positive sample were included. To reduce costs, the extracts of external quality control panels and known positive clinical samples were pooled as indicated in **Table S1** in the supplemental material. Each pool (n=55) was analyzed in triplicate. Where needed, verification testing was done by an independent molecular microbiology laboratory (OLV hospital Aalst).

Sensitivity testing: Four pools of synthetic nucleic acid controls for TaqMan assay target organisms **(Table 2)** at 10^5 copies/µl each were created (plasmid DNA for DNA viral and bacterial targets and RNA transcribed from cloned target sequences for RNA viral targets). A 1:10 dilution series down to 10^2 copies/µl for each pool was then generated. Duplicate 10^5 µL reaction mixtures were made that contained each pool, TaqPath 1-Step RT-qPCR Master Mix and water; the final concentration of each synthetic control was 1, 10, 100 or 1000 copies/µL. Reaction mixes were loaded into the well of a TAC and run on the Applied Biosystems QuantStudio 12k real time PCR instrument using the thermocycling profile: 50°C for 20 min, 95°C for 2 min and 45 cycles of 95°C for 3 s followed by 60°C for 30 s.

Specificity testing:

Cross-reactivity: The specificity was evaluated by testing the ability to exclusively identify the pathogen targeted by the assay with no cross reactivity to organisms that are closely related, or cause similar clinical symptoms, or to organisms that are present as normal flora in the specimen types of interest. Strongly positive DNA/RNA extracts of each of the pathogens included in the card were tested to exclude cross-reactivity. These data were available by testing the reactivity panel on TAC format. In addition, a near-neighbor panel containing closely related strains and other pathogens that might be present in a respiratory sample was tested. This list **(Table 3)** was based on expert opinion, literature and other IVD panels.

False positives: Negative specimens (no-template control: contained all reaction components except the DNA or RNA sample, in human matrix) were tested (n=6) to determine the false-positive rate. These samples were tested next to highly positive samples to exclude user cross contamination.

Reproducibility testing: Results from positive pooled samples were filtered for outliers. These were defined as data points with amplification curves, which yielded a high Crt value (>34) for only one of the three replicates. Concordance was calculated by target for all positive replicate results against the total expected replicates. qPCR data for reproducibility were analyzed using the relative threshold (Crt) method. The Crt method is preferable to the baseline threshold (Ct) method when working with dried down assays. This is because dried down assays may reconstitute at different rates and cause baseline variability; the Crt method corrects for baseline variability.

Phase 3: Clinical validation TAC

The PCR protocol used was identical to that of phase 2. An entry validation was done for the batch of phase 3 cards, using 9 pools covering all targets. Between December 2016 and May 2017, respiratory samples that were sent to the lab for routine testing were also tested with the TAC **(Figure 1)**. A TAC result was

Table 2 Target organisms in each pool used in LoD studies.

Pool	Nucleic Acid Type	Assay Targets
1	DNA	Bordetella pan, Adenovirus, *Bordetella pertussis, Streptococcus pyogenes*
	RNA	Coronavirus 229E, Flu A H1 2009, Flu A H3, Flu A pan, Rhinovirus A+B+C, hMPV, Measles, RSV A, Coronavirus 229E
2	DNA	Adenovirus, *Bordetella holmesii, Coxiella burnetii, Legionella pneumophila*, Herpes simplex virus 1
	RNA	Flu A H3, Flu B, Parainfluenza 1, Parechovirus, Coronavirus HKU1, Rhinovirus A+B+C
3	DNA	Adenovirus, *Haemophilus influenzae*, Herpes simplex virus 2, Bocavirus 2, HHV6
	RNA	Coronavirus NL63, Flu A H1 2009, Flu B, Parainfluenza 2, Parainfluenza 4, Mumps, RSV B, Enterovirus A+B, Internal control PDV
4	DNA	*Chlamydophila pneumoniae*, Adenovirus, *Streptococcus pneumoniae*
	RNA	Coronavirus OC43, Parainfluenza 3, Flu A pan, Flu A H1 2009, hMPV, RSV A, RSV B, Enterovirus D

considered positive if it passed internal positive controls and had an exponential amplification curve. A fixed cycle threshold (C_T) of 0.1 was applied to all assays. Since the assays were extensively validated in phase 1 and 2, results were verified in following situations: (i) weakly positive results with flat amplification curve and (ii) discordant result between TAC and routine testing. Verification testing was done by the national reference center for respiratory pathogens (UZ Leuven). These confirmatory PCRs were chosen as an independent molecular test which targets different genes from those targeted by the TAC, or different regions in case of identical gene target.

Results

Phase 1: Analytical validation primer/probe sets

In total, 90 assays were tested of which 43 were retained for further validation in TAC format **(Table 1)**. The most frequent reasons for rejection of an assay were insufficient PCR efficiency and/or reactivity. For some targets, several assays were acceptable. In this case, the assay with best efficiency was chosen. The remaining 5 positions on TAC were (i) manufacturer control assay (n=1) and in-house assays for (ii) enteroviruses (n=1), (iii) rhinoviruses (n=2) and (iv) *P. jirovecii* (n=1). Seven of the 43 assays that were retained showed efficiency between 70 and 80% and thus did not meet the predefined criterion of efficiency ≥ 80%, but were accepted for further testing in phase 2 and 3 due to lack of alternatives. For all 43 targets together, 89% (481/539) of positives included in the reactivity testing were detected.

All initial designed assays for fungal pathogens showed false positive results (positive result for no-template control), however no wet lab root cause analysis for this observation was carried out as part of this study. *In silico* analysis did reveal that these assays exhibited low specificity, as their off-targets were in the order of thousands of organisms. For this reason, assays for Aspergillus species and Rhizopus species were rejected and were excluded from further validation testing. Subsequently, gene-specific assays for various Aspergillus species were designed and pooled together to achieve complete coverage for all species of interest (data not included in this study). A new design for *P. jirovecii* was included in Phase 2 validation.

Phase 2: Analytical validation TAC

For all 43 targets together, 98% (246/252) of positives included in the reactivity testing were detected. In case of multiple gene targets for one pathogen, the different assays confirmed each other. For some positives however, not all gene targets were positive. This could be due to the large number of different genotypes (adenoviruses, rhinoviruses, enteroviruses), or a difference in sensitivity of the assays (in case of a very low viral load).

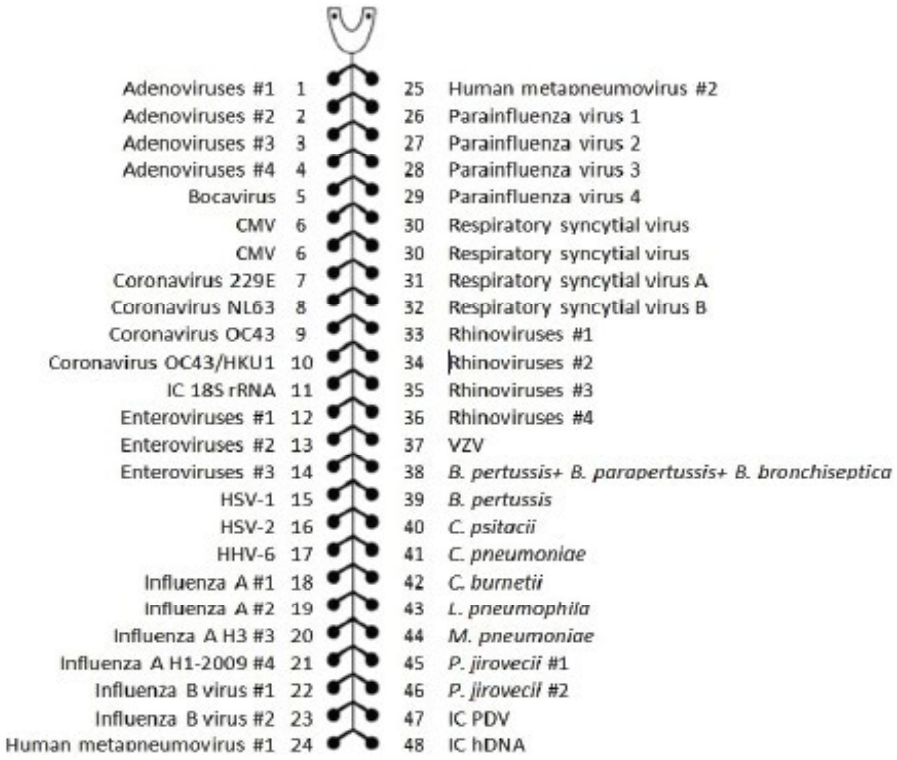

Figure 1 TaqMan Array Card layout (phase 3) for one sample with composition of the respiratory pathogens included. These microfluidic cards contain 384 individual wells separated into eight loading ports with 48 wells each. For each sample, one internal positive control (IC) with Phocine Distemper Virus (PDV) and one human DNA/RNA control are included. For several pathogens, more than one genetic target is included (indicated with #).

For each assay that was selected for Phase 2 testing, results for analytical sensitivity (LoD) and reproducibility are shown in **Table 1**. For some assays, LoD data are not available since a different target was tested compared to the ones included in our Phase 2 cards. For assays with multiple gene targets, the most conservative LoD result is shown. For 96% (23/24) of tested assays, a LoD of 1 or 10 copies/µL was found. One assay showed a LoD of 100 copies/µL.

Cross-reactivity was seen for the HSV-2 assay, that gave a weak positive amplification (Ct 32) of a positive HSV-1 sample. This was only seen in 1 out of 33 positive HSV-1 samples (11 pools in triplicate) in total. The RSV-B assay weakly amplified two samples positive for RSV-A (n=2) (out of 18 positive RSV-A samples in total). One off-target sample was weakly positive in the bocavirus assay (Ct>30, 3/3 replicates) but was not confirmed by verification testing. These 4 off-targets represent 0.06% of all results (55 pools each tested in triplicate, against 43 assays=7095 results).

None of the assays showed cross-reactivity with the near-neighbor panel **(Table 3)** or false positive results. However, low grade non-specific amplification was identified for following assays: adenoviruses (n=4), CoV 229E, rhinoviruses (n=2) and *M. pneumoniae*.

Concordance with orthogonal method showed reproducibility results ranging from 76%-100%, with a mean of 91%.

Phase 3: Clinical validation TAC

Based on the results of Phase 2, the content of Phase 3 cards differed slightly. The entry validation cleared the batch of cards for testing on clinical samples.

Patient and sample characteristics are shown in **Table 4**. A total number of 428 samples were tested, with an overall positivity rate of 56.3% and a co-infection rate of 15.9%. Detailed TAC results are shown in **Figure 2**. Non-specific amplification results were manually omitted when interpreting the plots, but could also be eliminated by automatic Ct thresholding. The nucleic acid extracts of 45 samples were send to the reference lab for confirmation of 50 results. Most of these results were weakly positive and/or positive in only one of multiple targets when present. 34 results were confirmed by the reference lab, 15 results (4% of total number of positives) were not confirmed and considered false positives. One result was considered false negative since the TAC result was negative, but routine and verification testing were (weakly) positive for CMV.

Discussion

Relationships between industry and the clinical microbiology laboratory are exceedingly valuable and serve as the basis for technological advancements [17]. Several reasons drove us to collaborate with Thermo Fisher Scientific for assay design. First, they can assure continuous performance monitoring of assays, which is of great importance. As new sequence data and other information on known pathogens become available, specific qPCR primers and probes may need to be altered or added.

Table 3 Near neighbor panel used to assess the analytical specificity.

Bacteria	Viruses
Legionella micdadei	Epstein-Barr virus
Legionella bozemana	Rubella virus
Legionella longbeachae	Parvovirus B19
Bordetella holmesii	
Bordetella hinzii	**Fungi**
Escherichia coli	*Penicillium chrysogenum*
Klebsiella pneumoniae	*Scedosporium boydii*
Pseudomonas aeruginosa	*Ransamsonia argiracea*
Staphylococcus aureus	*Exophiala dermatidis*
Staphylococcus pyogenes	*Candida albicans*
Staphylococcus pneumoniae	*Candida glabrata*
Staphylococcus oralis	*Candida tropicalis*

Table 4 Patient and sample characteristics (phase 3 testing).

Patient Characteristics	Value
Age: Median (range), Years	57 (1 day, 92)
Gender: Male, No. (%)	231 (54)
Type of sample	**No. (%) of samples**
Nose-throat swab	194 (45)
Bronchoalveolar lavage	125 (29)
Nasopharyngeal aspiration	76 (18)
Unknown type/other type	33 (8)

Second, as for gene expression assays (>1,000,000 inventoried assays exist), a library of primer and probe sets for a large number of pathogens could be created, ideally not only respiratory but also including pathogens involved in other infectious syndromes. These assays should be analytically and clinically validated and updated on a regular basis. Each lab would be able to put together their unique cards without having to validate extensively as was done in this study. Significant amounts of time and investment are required before medically useful products or developments make it from the research sector into the clinical laboratory. This panel is the product of a very intensive collaboration during a period of almost 2 years with weekly calls discussing raw data, troubleshooting, assay optimization, etc.

When relying on conventional diagnostic methods for respiratory infections, diagnostic reflexes are underdeveloped and/or underused because the results of these techniques are too slow, sensitive to sampling problems and have sensitivity problems. Rapid and complete diagnostic panels as described in this study would be extremely useful for routine respiratory testing. The implementation however involves a steep learning curve for microbiologists as well as for clinicians since the exact clinical interpretation of these multi-pathogen screening results is not always easy [18]. A prompt and comprehensive communication of the results is necessary. For some of the targets that can be categorized as opportunistic pathogens such as CMV, HSV-1, HSV-2, VZV, HHV-6 and *P. jirovecii*, a positive result in an upper respiratory sample (nasopharyngeal swabs, aspirations etc.) was not shown on the lab report during phase 3 testing. They were however communicated to the infectious diseases specialists who decided to consider the positive result as clinically relevant or not.

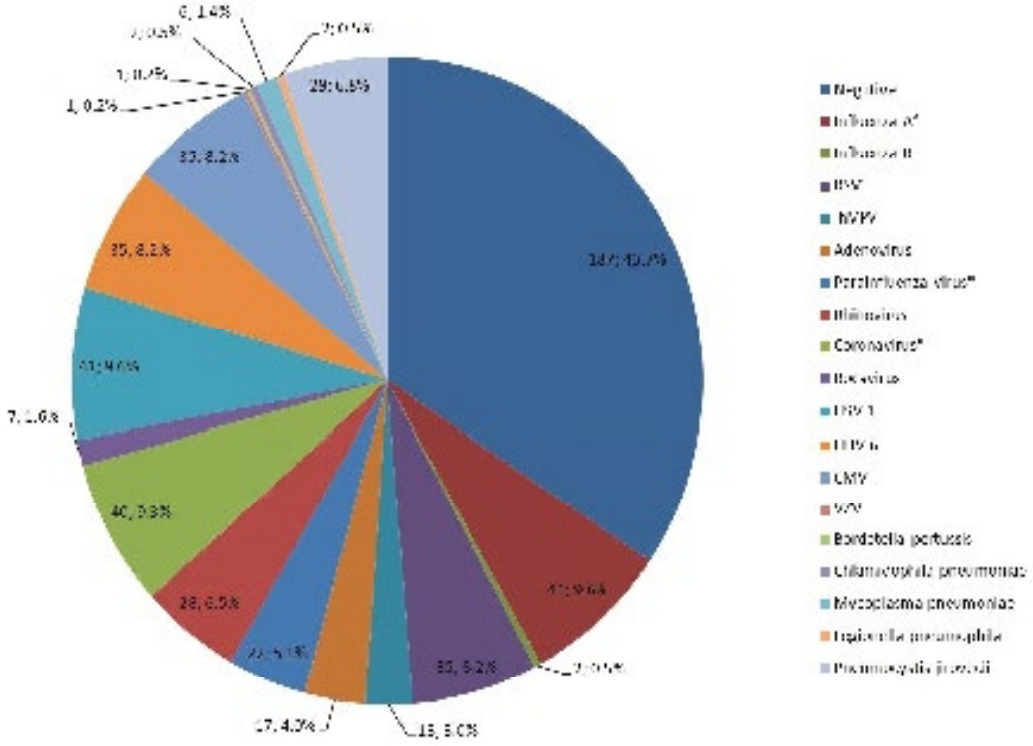

Figure 2 Results for TAC testing, including the numbers of pathogens and percentages of the total all H3 seasonal; 'RSV A (n=11), RSV B (n=24); *hPIV1 (n=1), hPIV2 (n=3), hPIV3 (n=15), hPIV4 (n=3); "CoV 229E (n=4), CoV OC43 (n=19), CoV NL63 (n=10), CoV HKU-1 (n=7).

In this TAC, more than one genetic target was included for several pathogens because the use of confirmatory targets allows to achieve a high as confidence result as possible. In addition, multi-target testing is important to limit false-negative results due to genome variability [19]. It also allowed strain subtyping for RSV and influenza A viruses.

The false negative results for reference and known positive clinical samples in reactivity testing of phase 1 do not necessarily imply a lack of sensitivity of the included assays. This could be explained by a very low DNA/RNA load or a deterioration of the sample quality and/or nucleic acid extract during conservation in the biobank. The reactivity testing in phase 2 showed excellent results and analytical sensitivity performed by LoD testing and reproducibility were very satisfactory for all tested assays.

The cross-reactive results that were noted were easily recognized since the true positive result was identified with a lower Ct-value. Even if this had not been the case, this cross-reactivity would not have impacted clinical decision making (HSV-2 versus HSV-1 or RSV-B versus RSV-A).

Since the assays for detection of Aspergillus species and Rhizopus species gave false positive results, these targets were left out of the study. Highly specific assays are needed for these targets since even a very low signal could be clinically significant in a lower respiratory tract sample. For these pathogens, gene-specific assays will be designed, pooled and included in the next version of the panel. New optimized assays are also being designed for RSV, hMPV, enteroviruses and rhinoviruses.

When comparing the results from phase 3 testing with those from previous winter seasons with an existing respiratory TAC [3,20], the overall positivity rate was comparable (56 versus 62 and 66%, respectively). The existing TAC (developed and validated by the microbiology departments of Addenbrooke's Hospital, Cambridge, UK and AZ St-Jan Brugge-Oostende Hospital, Brugge, Belgium) is very complete, but our goal was to use sequences designed and supported by the manufacturer and to add several pathogens specific for immunocompromised patients.

There are some limitations to our study. The number of reference strains and clinical samples was limited for some pathogens. The study was also limited by the lack of verification testing on all samples. Important to note is that this panel is a work in process, assays are updated on a regular basis and assay validation is continued. Also, the content of the card will be constantly evolving over time as new respiratory pathogens could be discovered and more data will become available on the clinical impact of known pathogens.

In conclusion, we developed and validated a customized respiratory TAC, using sequences designed and supported by the manufacturer. This TAC can test up to eight samples within 3 h from sample to results and can simultaneously detect 32 respiratory pathogens, including 23 viruses, 8 bacteria and 1 fungus. In a restricted patient population (immunocompromised, ICU, neonates), this syndromic panel will be implemented for routine respiratory testing in the Erasme University Hospital.

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References

1 Kodani M, Yang G, Conklin LM, Travis TC, Whitney CG, et al. (2011) Application of TaqMan low-density arrays for simultaneous detection of multiple respiratory pathogens. J Clin Microbiol 49: 2175-2182.

2 Waller JL, Diaz MH, Petrone BL, Benitez AJ, Wolff BJ, et al. (2014) Detection and characterization of *Mycoplasma pneumoniae* during an outbreak of respiratory illness at a university. J Clin Microbiol 52: 849-853.

3 Steensels D, Reynders M, Descheemaeker P, Curran MD, Jacobs F, et al. (2015) Clinical evaluation of a multi-parameter customized respiratory TaqMan® array card compared to conventional methods in immunocompromised patients. J Clin Virol 72: 36-44.

4 Kodani M, Mixson-Hayden T, Drobeniuc J, Kamili S (2014) Rapid and sensitive approach to simultaneous detection of genomes of hepatitis A, B, C, D and E viruses. J Clin Virol 61: 260-264.

5 Liu J, Gratz J, Amour C, Kibiki G, Becker S, et al. (2013) A laboratory-developed TaqMan array card for simultaneous detection of 19 enteropathogens. J Clin Microbiol 51: 472-480.

6 Liu J, Kabir F, Manneh J, Lertsethtakarn P, Begum S, et al. (2014) Development and assessment of molecular diagnostic tests for 15 enteropathogens causing childhood diarrhoea: A multicentre study. Lancet Infect Dis 14L: 716-724.

7 Platts-Mills JA, Gratz J, Mduma E, Svensen E, Amour C, et al. (2014) Association between stool enteropathogen quantity and disease in Tanzanian children using TaqMan array cards: A nested case-control study. Am J Trop Med Hyg 90: 133-138.

8 Diaz MH, Waller JL, Napoliello RA, Islam MS, Wolff BJ, et al. (2013) Optimization of multiple pathogen detection using the TaqMan array card: Application for a population-based study of neonatal infection. PLoS One 8: e66183.

9 Onyango CO, Loparev V, Lidechi S, Bhullar V, Schmid DS, et al. (2017) Evaluation of TaqMan array card (TAC) for the detection of central nervous system infections in Kenya. J Clin Microbiol 12: 02469-02416.

10 Patrick D, Steven De K, Ellen Van N, Sofie M, Charlotte V, et al. (2015) Clinical significance of multi parameter - Multi target screening of sexually transmitted infections by TaqMan® array card technology. ECCMID.

11 Weller SA, Cox V, Essex-Lopresti A, Hartley MG, Parsons TM, et al. (2012) Evaluation of two multiplex real-time PCR screening capabilities for the detection of *Bacillus anthracis, Francisella tularensis* and *Yersinia pestis* in blood samples generated from murine infection models. J Med Microbiol 61: 1546-1555.

12 Rachwal PA, Rose HL, Cox V, Lukaszewski RA, Murch AL, et al. (2012) The potential of TaqMan array cards for detection of multiple biological agents by real-time PCR. PLoS One 7: e35971.

13 Pholwat S, Liu J, Stroup S, Jacob ST, Banura P, et al. (2017) The malaria TaqMan array card includes 87 assays for *Plasmodium falciparum* drug resistance, identification of species and genotyping in a single reaction. Antimicrob Agents Chemother 24: e00110-17.

14 Banu S, Pholwat S, Foongladda S, Chinli R, Boonlert D, et al. (2017) Performance of TaqMan array card to detect TB drug resistance on direct specimens. PLoS One 4: e0177167.

15 Pholwat S, Sakai F, Turner P, Vidal JE, Houpt ER (2016) Development of a TaqMan array card for pneumococcal serotyping on isolates and nasopharyngeal samples. J Clin Microbiol 54: 1842-1850.

16 Liu J, Ochieng C, Wiersma S, Ströher U, Towner JS, et al. (2016) Development of a TaqMan array card for acute-febrile-illness outbreak investigation and surveillance of emerging pathogens, including Ebola virus. J Clin Microbiol 54: 49-58.

17 David TD, James JD (2011) Fostering partnerships between industry and the clinical microbiology laboratory. J Clin Microbiol 49: S78-S79.

18 Mahony JB (2008) Detection of respiratory viruses by molecular methods. Clin Microbiol Rev 21: 716-747.

19 Steensels D, Vankeerberghen A, De Beenhouwer H (2013) Towards multi target testing in molecular microbiology. Int J Microbiol 2013: 121057.

20 Steensels D, Reynders M, Descheemaeker P, Curran MD, Jacobs F, et al. (2017) Performance evaluation of direct fluorescent antibody, focus diagnostics simplexa™ Flu A/B & RSV and multi-parameter customized respiratory Taqman® array card in immunocompromised patients. J Virol Methods 245: 61-65.