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Prospective evaluation of diagnostic tools for respiratory viruses in children and adults

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ABSTRACT

Aim: To compare the performances of molecular and non-molecular tests to diagnose respiratory viral infections and to evaluate the pros and contras of each technique.
Methods: Two hundred ninety-nine respiratory samples were prospectively explored using multiplex molecular techniques (FilmArray Respiratory Panel, Clart Pneumovir), immunological techniques (direct fluorescent assay, lateral flow chromatography) and cell cultures.
Findings: Molecular techniques permitted the recovery of up to 50% more respiratory pathogens in comparison to non-molecular methods. FilmArray detected at least 30% more pathogens than Clart Pneumovir which could be explained by the differences in their technical designs. The turnaround time under 2 hours for the FilmArray permitted delivery of results when patients were still in the emergency room.

1. Introduction

Since the discovery of viruses in the twentieth century, considerable efforts have been made to improve the technics to detect and identify them. Cell cultures were the first diagnostic tool to be used in the mid-1950s, and since then, new techniques have been developed to decrease the time to a result (immunofluorescence, lateral flow chromatography) or to boost the sensitivity (molecular techniques) (Levine, 1996; Ginocchio and Harris, 2011). Many improvements have been made, and techniques combining speed and sensitivity are currently available, such as fully automated 'sample-in, result-out' multiplexed syndromic molecular tools (Bluchan and Ledeboer, 2014). Aside from being effective in terms of sensitivity and specificity, these latter diagnostic tools are able to recover non-cultivable viruses. As a consequence, questions regarding the usefulness of 'older' diagnostic methods regularly arise (Leland and Ginocchio, 2007; Hodinka and Kaiser, 2013). Meanwhile, important questions concerning these 'new' expensive rapid molecular techniques remain unanswered, such as their cost-effectiveness in terms of patient's management, or the clinical significance of detecting nucleic acids of micro-organisms that could be non-infectious at the time the sample is collected.

The objective of this work was to compare the performances of antigen detection and cell cultures techniques routinely used since years for the diagnosis of respiratory viral infections in the setting of a tertiary care hospital to those of newer molecular techniques (Clart Pneumovir, Genomica, Coslada, Spain and FilmArray Respiratory Panel, Biofire, Biomérieux, Marcy L'Etoile, France).

2. Materials and methods

2.1. Population and inclusion criteria

The study was initiated on the 1st of February (week 5) and ended on the 15th of March 2016 (week 11) in the Saint-Pierre University Hospital, a tertiary general hospital with 626 beds located in downtown Brussels. This was during the peak of the 2015–2016 influenza season which was moderate in Belgium and lasted from week 4 to week 13. More than 90% of influenza A isolates collected in Belgium were

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A(H1N1)pdm2009. Regarding influenza B, circulating strains were almost exclusively from the Victoria lineage according to the Belgian Scientific Institute of Public Health (Belgian Public Health Institute, 2019). The enrolment period was chosen in order to be sure to gather positive samples for influenza as it is intended to evaluate, in another article, the impact of the results on antiviral prescription. This choice obviously affects the prevalence of other viruses. Adults and children attending the emergency room (ER) and presenting with upper or lower respiratory symptoms were prospectively included if either intended to be maintained in the hospital or had any of the following conditions known to expose to a higher rate of complications of viral respiratory infections: chronic respiratory diseases such as cystic fibrosis or asthma, sickle-cell disease, asplenia, neuromuscular diseases, severe neurological affections, hereditary metabolic disorders including diabetes, congenital or acquired immunosuppression, heart defects, chronic nephropathies, chronic liver diseases and pregnancy. Children under 3 months of age with a fever without focus of infection were also included.

2.2. Study workflow

Upon inclusion, a respiratory sample was collected. Nasopharyngeal aspirate (NPA) samples were typically collected from children under 2 years old, and nasopharyngeal swabs (NPS) (flocked swab + UTM 3 mL, Copan, Brescia, Italy) were collected from older children and adults. The samples were immediately sent to the microbiology laboratory for testing. Prior to testing, NPA were diluted with 3 mL of viral transport medium composed of veal infusion broth (Difco, Becton Dickinson, Sparks, MD, USA) supplemented with bovine albumin (Sigma Aldrich, St. Louis, MO, USA). Lateral flow chromatography (LFC) tests and the FilmArray Respiratory Panel were used to test samples 24/7, whereas direct fluorescent assays (DFA) and cell cultures were performed during working hours (8:00 am to 5:00 pm) from Monday to Saturday. The Clart Pneumovir test was performed once a week. Lab results as well as clinical data from patients' chart were recorded and analyzed. Antigen detection tests and cell cultures are routine tests performed for patients attending the emergency departments. FilmArray and Clart Pneumovir were performed for the study.

2.3. Antigen detection tests

Because only 3 tests per day are reimbursed by the social welfare, the combination of LFC and DFA tests performed varied during the evaluation based on the most prevalent circulating viruses. From February 1st to the 10th, influenza (Sofia influenza A + B, Quidel, San Diego, CA, USA), RSV (BinaxNOW RSV, Alere, Waltham, MA, USA) and human metapneumovirus (hMPV) DFA (Argene, Biomérieux, Marcy L'Etoile, France) tests were performed. Fifty-nine samples were analyzed with this combination. From February 10th to March 15th 2016, metapneumovirus detection was replaced by an adenovirus detection test (Adenorespi K-set, Coris Bioconcept, Gembloux, Belgium).

2.4. Cell cultures

Cell cultures were performed as follows: an aliquot of the sample was inoculated on confluent Vero (African green monkey kidney), MRC5 (human lung) and LLC-MK2 (rhesus monkey kidney) cell cultures (Vircell, Santa-Fé, Spain) in 24-well or 6-well tissue culture plates (Greiner-Bio One, Frickenhausen, Germany). Cultures were incubated at 36 °C in a 5% CO_2 atmosphere for 2 weeks for the Vero cultures plates and LLC-MK2 cells and 3 weeks for the MRC5 cells. The culture media were replaced weekly. Cultures were examined every two to three days using an inverted microscope. Hemadsorption was performed on the LLC-MK2 cells at the end of the second week of incubation.

2.5. Molecular techniques

The FilmArray Respiratory Panel 1.7 is a closed 'sample-in, result-out' multiplex PCR system that integrates sample preparation, amplification, detection and analysis of results in approximately an hour. The panel detects the most common respiratory viruses: adenovirus, coronavirus (229E, HKU1, NL63 and OC43), human metapneumovirus, human rhinovirus/enterovirus (without distinction between the two), influenza A (with differentiations of H1, H1-pdm2009 and H3 strains), influenza B, parainfluenza 1–4 and respiratory syncytial virus (RSV). The panel also detects 3 bacteria; *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae* and *Bordetella pertussis* which were not evaluated in this study. Tests were performed according to the manufacturer's instructions.

The Clart Pneumovir test is a microarray technique that targets the same pathogens as the FilmArray Respiratory Panel, with the exception of coronaviruses HKU1, NL63 and OC43 and the 3 aforementioned bacteria. Among enteroviruses, this technique only detects echoviruses, but it can differentiate them from rhinoviruses. The extraction of nucleic acids was carried out with the QiaSymphony system SP (Qiagen) using the QiaSymphony Virus/Bacteria Midi kit (input volume 1500 µL, output volume 110 µL). The Pneumovir assay was performed according to manufacturer's instructions, and detection and interpretation of the results were conducted by a CARreader (Genomica).

2.6. Establishment of "composite" reference standard

As the molecular tests used were presumably more sensitive than the reference standard (viral culture), we constructed a "composite" reference standard to avoid bias in establishing the specificity of the evaluated tests. This "composite" reference standard was constructed as follows: when discrepant results between the 2 molecular techniques were observed, a third molecular technique was performed (Argene, Biomérieux, Marcy L'Etoile, France). A sample was considered truly positive for a pathogen if at least 2 molecular techniques were positive for this pathogen. For the targets included in the FilmArray test but not in the Clart Pneumovir panel (coronaviruses HKU1, NL63 and OC43), only the specificity of the positive results were evaluated using the Argene or laboratory developed tests.

2.7. Statistical analyses

Data were analyzed using two sided exact chi-square Fisher's tests followed in case of statistical significance by chi-square trend tests. The statistical software used were Medcalc V14.12.0 and IBM SPSS V24.0.

3. Results

A total of 299 samples (93 NPA and 206 NPS) from 291 patients were analyzed: 149 samples were obtained from 142 children, of whom 62 were female and 80 were male (mean age: 1 years and 10 months old; median: 7 months old); and 150 samples were obtained from 149 adults, of whom 73 were female and 76 were male (mean age: 53 years old; median: 52). For children, 80% (119/149) of samples were positive for at least one pathogen. The pathogen detected, ranging from most to least, was rhino/enteroviruses (49), influenza B (32), influenza A (25), coronaviruses (22), adenovirus (18), metapneumovirus (10), RSV (7) and parainfluenza (2). For adults, 63% (94/150) of samples were positive, and the detected viruses were influenza A (32), influenza B (26), rhino/enteroviruses (13), coronavirus (12), adenovirus (8), metapneumovirus (6) and RSV (4). No parainfluenza viruses were detected in the adult patients. All influenza A isolates were A(H1N1)pdm09 with the exception of one A(H3N2) from one adult patient. Influenza B strains were not subtyped. Co-detection was more common in children than in adults (35.3% vs 8.5%; $p < 0.001$).

Table 1 details the sensitivity and specificity observed for the

Table 1
Sensitivity and specificity of the techniques for the different pathogens.

	n° positive (n° co-detection)	Antigen detection		Cell cultures		Clart Pneumovir		FilmArray	
		Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Adenovirus	26 (20)	2/22 (9.1%)	100%	4 (15.4%)	100%	13 (50%)	100%	24 (92.3%)	98.5%
Influenza A	56 (10)	43 (76.8%)	98.3%	49 (87.5%)	99.6%	55 (98.2%)	99.6%	54 (96.4%)	99.6%
Influenza B	58 (23)	28 (48.3%)	98.3%	43 (73.1%)	99.3%	56 (96.5%)	98.7%	53 (91.4%)	100%
Metapneumovirus	16 (4)	2/3 (66.6%)	100%	0 (0%)	100%	9 (56.2%)	98.9%	14 (87.5%)	100%
RSV	11 (3)	7 (63.6%)	100%	6 (54.5%)	100%	8 (72.7%)	98.6%	11 (100%)	98.6%
Parainfluenza	2 (2)	NA	NA	1 (50%)	100%	2 (100%)	99.7%	2 (100%)	98%
Rhino/enterovirus	62 (30)	NA	NA	16 (25.8%)	100%	32 (51.6%)	99.6%	61 (98.4%)	96.6%
Coronavirus 229E	1 (1)	NA	NA	NA	NA	1 (100%)	100%	1 (100%)	100%
Coronavirus HKU1	20 (10)	NA	NA	NA	NA	NA	NA	20 (NA)	98.7%
Coronavirus NL63	6 (1)	NA	NA	NA	NA	NA	NA	6 (NA)	99%
Coronavirus OC43	7 (1)	NA	NA	NA	NA	NA	NA	7 (NA)	98.7%

Table 2
Rate of false negative (FN), partial agreement (PA) and total agreement (TA) of the different techniques for the positive samples in comparison to the composite standard.

Non-molecular techniques			Clart Pneumovir			FilmArray		
FN	PA	TA	FN	PA	TA	FN	PA	TA
88	31	94	50	33	130	6	6	201
41.3%	14.5%	44.1%	23.5%	15.5%	61%	2.8%	2.8%	94.4%

different techniques, depending on the pathogens. Table 2 provides the rate of false negative results, partial agreement (meaning at least one but not all the expected pathogens were detected) and complete agreement (meaning all the expected pathogens were detected) of the non-molecular techniques and molecular techniques compared to the established standard. The FilmArray test produced fewer false negative results and partial results compared to non-molecular techniques and the Clart Pneumovir test ($p < 0.001$). False negative results with molecular techniques were significantly more frequent in samples with co-detections compared to those with only one pathogen: 12% vs 3% for the FilmArray test ($p = 0.034$) and 76% vs 11% for the Clart Pneumovir test ($p < 0.001$). Similar result was also observed for cell cultures when limiting the analysis to culturable viruses (100% Vs 37%; $p < 0.001$).

Table 3 reports the mean turnaround time (TAT) of the techniques from the reception of the sample in the laboratory to the introduction of the results into the laboratory information system. The LFC and FilmArray techniques have the shortest TAT, while cell cultures and the Clart Pneumovir test have the longest TAT. The TAT for negative cell cultures is about 3 weeks as they are discarded after this delay.

Tables 4 and 5 provide the detection rates for different techniques for influenza A and B, depending on the duration of the cough or the age of the patients. There is an effect of cough duration for influenza A with the antigen detection test ($p = 0.032$) and cell cultures ($p = 0.010$), due to a decrease in diagnostic sensitivity with cough duration ($p = 0.017$ and $p = 0.010$, respectively). The decrease is observed as soon as the cough duration exceeds 5 days. An age effect was also observed for influenza A with the antigen detection test ($p = 0.006$) and cell cultures ($p = 0.023$), due to a decrease in sensitivity with age ($p = 0.002$ and $p = 0.004$, respectively). The decrease is observed from the first age category with the antigen detection and from 15 years for the cell cultures. No effect of cough duration or age was observed with the detection of influenza A for the FilmArray and Clart Pneumovir tests or on the detection of influenza B with any method.

Table 4
Rate of positive influenza tests depending on the duration of the cough (when reported).

		≤ 1 day	> 1–3 days	> 3–5 days	> 5 days
Influenza A	Antigen detection	11/14	14/16	4/6	2/7
	Cell cultures	13/14	15/16	6/6	3/7
	Clart Pneumovir	14/14	16/16	6/6	6/7
	FilmArray	14/14	16/16	6/6	6/7
Influenza B	Antigen detection	5/15	6/13	4/6	1/3
	Cell cultures	11/15	9/13	6/6	2/3
	Clart Pneumovir	14/15	12/13	6/6	3/3
	FilmArray	14/15	13/13	6/6	3/3

4. Discussion

4.1. Analytical performance and factors influencing molecular tests

As expected, molecular techniques were observed to be the most sensitive.

However, the performances of the two evaluated techniques differed; lower sensitivity for the detection of several targets was observed for the Clart Pneumovir test, notably this technique only detects echoviruses among all enteroviruses. For adenoviruses, metapneumoviruses and RSV, the explanation probably relies more on technical considerations. Indeed, false negative results with the FilmArray and the Clart Pneumovir tests were not always for samples with a low viral

Table 3
Mean turnaround time of the different techniques from the reception of the sample in the laboratory to the introduction of the result in the laboratory information system. *Samples received during working hours and analyzed the same day. **Samples received outside working hours. d: day(s); h: hour(s); hMPV: human metapneumovirus; min: minute(s); RSV: respiratory syncytial virus.

	Antigen detection		Cell cultures						Clart Pneumovir	FilmArray
	Influenza A + B	hMPV	Influenza A	Influenza B	Adenovirus	Enterovirus	Rhinovirus	RSV		
Turnaround time	1h01min	3h22min* 18h17min**	4d22h41min	4d15h27min	5d13h14min	4d21h43min	6d22h01min	7d18h14min	4d16h46min	1h49min

Table 5
Rate of positive influenza tests depending on the age of the patients.

		≤ 2 years	> 2–15 years	> 15–50 years	> 50 years
Influenza A	Antigen detection	18/18	6/7	15/24	4/7
	Cell cultures	18/18	7/7	20/24	4/7
	Clart Pneumovir	18/18	7/7	24/24	6/7
	FilmArray	18/18	7/7	22/24	7/7
Influenza B	Antigen detection	8/19	8/13	9/16	3/10
	Cell cultures	12/19	11/13	13/16	7/10
	Clart Pneumovir	18/19	13/13	15/16	10/10
	FilmArray	17/19	13/13	16/16	8/10

load, as indicated by the cycle threshold (which correlates with the viral load) of the third molecular technique used to elucidate discrepant results between the first two techniques. As an example, the mean Ct value and standard deviation of negative and positive samples for adenoviruses with Clart Pneumovir were 32.7 (1.6) and 31.8 (2.2) respectively ($p = 0.402$). The choice of primers and probes used to design tests can determine whether a certain strain of virus will be detected. Although the FilmArray test had a better sensitivity than the Clart Pneumovir test for the detection of adenoviruses, it may lack sensitivity in detecting certain strains, which could impact the management of immunocompromised patients (Song et al., 2016).

A huge advantage of molecular techniques is that they allow the recovery of those pathogens that are difficult to culture, that will not grow in culture or for which no antigen detection tests are available. In the present study, the rate of complete detection of the pathogens present in the samples was far more important with molecular techniques compared to non-molecular ones. This has already been reported. (Weinberg et al., 2004) Viruses with poor detection using non-molecular techniques primarily include adenoviruses, metapneumoviruses and rhino/enteroviruses. There were also positive samples for coronaviruses (Mahony, 2008) which, in our experience, do not commonly grow with routine cell lines, even if coronavirus NL63 was originally described in LLC-MK2 (van der Hoek et al., 2004).

The rate of co-detection was more important for children, which is a common finding and could be explained by notably increased, longer-lasting viral shedding in children under 3 months of age due to less developed mucosal immunity (Sharma et al., 2012). The co-detection of viruses in children is apparently not linked with a more severe outcome (Comerlato Scotta et al., 2016), and the molecular detection of a virus in a respiratory sample is not always associated with symptoms. The type of detected virus and the age of the patient can be helpful to decide on a care plan (Self et al., 2016).

As previously stated, false negative results with molecular techniques were significantly more frequent in samples with multiple pathogens compared to those with only one pathogen; this finding could be due to possible competition for the reagents when multiple targets are to be detected (Bezerra et al., 2011).

The specificity of all molecular techniques was over 98%, except that for rhino/enteroviruses with the FilmArray test (96.6%). The differences observed between molecular techniques could also be due to primer and probe choices.

Overall, the Clart Pneumovir is less sensitive than the FilmArray except for influenza B and its hands-on time and turnaround time are longer.

4.2. Analytical performances and influencing factors of non-molecular techniques

Only 3 positive samples were analyzed for metapneumovirus using an antigen detection test, as it was substituted for adenovirus test during the evaluation. This test nonetheless appeared more sensitive than the cell culture tests, as no metapneumovirus was recovered from cell cultures. This finding was under the expected sensitivity, which is approximately 50% according to the literature (Tang and Crowe, 2011).

The apparent low sensitivity of non-molecular techniques observed for adenoviruses deserves comment. Indeed, prolonged shedding after the primary infection is classically described for adenovirus. Adenoviruses can also be detected in tonsillar tissue or isolated from a throat sample of up to 11% of healthy children (Song et al., 2016; Kalu et al., 2010). These states of prolonged shedding or “latency” are more likely to be detected with molecular techniques. It is also noteworthy that 85% of adenoviruses detected in this evaluation were associated with one or more other pathogens, supporting the hypothesis that they could be bystanders in some cases.

The sensitivity of cell cultures for rhino/enterovirus was also low and could be partially attributed to the fact that group C rhinoviruses do not grow on standard cell cultures (Jacobs et al., 2013). Enteroviruses grow inconsistently on cell cultures, depending on their type, and no cell line enables the detection of all strains (Stellrecht et al., 2011). Unfortunately, rhinoviruses and enteroviruses in this study were not typed.

The presence of multiple viruses in a sample also influences cultures because the growth of the fitter or more abundant virus can mask the growth of others (George et al., 2002). For example, on the 46 false negative cultures for rhino/enteroviruses, 16 were positive for another virus. Likewise, for adenoviruses, for the 22 false negative cell cultures, 8 recovered another virus. Molecular techniques supposedly do not suffer from this drawback, although as previously discussed, a higher rate of false negatives was observed when multiple pathogens were present in the sample.

Cell cultures, however, can enable the detection of unsuspected viruses. This detection was the case in this evaluation for one measles virus, which was not originally suspected, and 2 herpes simplex viruses and 5 cytomegaloviruses (CMV). In one case, CMV could explain the symptoms exhibited by a 3-month-old patient with a fever without focus of infection. The other cases were more likely to be recurrences or prolonged shedding.

Regarding the sensitivity of antigen detection tests for influenza, influenza A is more easily detected than influenza B, which has already been reported (Busson et al., 2014). The decreased sensitivity for influenza A with antigen detection and cell cultures, which depends on the age of patients, can be explained by the higher viral shedding in children, and also because for younger children, NPA were preferred to NPS. Aspirates usually increase the detection rate for viruses over swabs (Loens et al., 2009). The decreased sensitivity of antigen detection and cell cultures when the duration of the cough increases is expected, because viral shedding is more important in the first days of the disease (Aoki and Boivin, 2009). The reason for the lack of similar findings for influenza B is unclear. The absence of sensitivity loss for molecular techniques, depending on the age of the patients and the duration of the cough, can be attributed to the high sensitivity of the techniques.

Usually, antigen detection tests have a faster turnaround time than cell cultures but a lower sensitivity except for viruses growing poorly, especially metapneumovirus and RSV, for which the sensitivity of antigen detection tests can be better than the one of cell cultures.

The specificity of non-molecular tests was above 98%. False positive

results in cell cultures can occur due to cross-contamination between wells on culture plates.

4.3. Turnaround time

Antigen detection tests based on lateral flow chromatography are usually the easiest and fastest to perform. Techniques based on immunofluorescence are slightly more time-consuming. Currently, totally automated techniques with a short hands-on time, such as FilmArray, can be realized in a time frame comparable to that of LFC tests. However, only one can be performed at one time, and the analyzer remains occupied for slightly more than an hour. The number of required instruments is based on the test volume at each facility and the desired TAT. Cell cultures remain the most time-consuming techniques, and results often arrive too late to impact patient management (Ginocchio, 2007). This was also the case for Clart Pneumovir in our setting, as it was only performed once a week due to the complexity of the corresponding analytical process.

4.4. Cost

In Belgium, non-molecular techniques for the diagnosis of respiratory viruses are reimbursed by the social welfare program, which is not the case for molecular techniques which are charged to the patients. During this evaluation, molecular techniques were free of charge for patients, but they cost approximately 135 euros per patient. The necessity of molecular tests must be seriously considered before prescription. These tests should probably be reserved for the most severely ill patients, for whom rapid and comprehensive microbiological evaluation is most likely to impact patient management. As a comparison, antigen detection tests cost, including workforce, can range between 5.5 and 14 euros depending on the test and cell cultures cost about 15 euros. However, non-molecular tests are reimbursed by the social welfare in Belgium permitting a broader prescription. The possibilities to implement molecular tests can vary between facilities and countries depending on local policies. Using molecular rather than non-molecular techniques could impact patients' isolation strategies (Richardson et al., 2016), antibiotic/antiviral use or reduce of the length of stay (Brendish et al., 2017; Rogers et al., 2015), but cost-benefit analyses are difficult to appraise because of many intertwined elements.

5. Conclusions

Molecular techniques have considerably increased our capacity to detect respiratory viruses in a timely manner. The main factors limiting a wide utilization of these techniques are cost and the difficulty to absorb the workload in large facilities with numerous samples to analyze per day. Another drawback is that latent viruses or traces of genetic material may be detected. The interpretation of a positive result might be difficult, and it must be carefully correlated to the clinical history of the patient as the presence of a virus in the respiratory tract is a factor exposing to bacterial superinfection (Vareille et al., 2011). It is possible to quantify the viral load in a respiratory sample, but there are conflicting reports regarding correlations between viral load and outcome (Granados et al., 2017; Wishaupt et al., 2017). Technical improvements should be made to prevent variation in quantification caused by sample dilution with saline instilled during aspirates or by the variable quantity of sampled material with swabs. These improvements could render comparisons between studies more reliable and permit the establishment of a threshold, above which detected viruses are indeed involved in an ongoing infectious process. In addition, a positive antigen detection test usually correlates with a high viral load in a sample, and a positive cell culture can only be achieved with infective viral particles. Whatever technique is used, fully understanding the benefits and limitations of each is crucial for interpretation. The choice of technique should depend, besides financial considerations, on the necessary sensitivity and speed based on symptoms, comorbidity and risk of a detrimental outcome for each patient. A tertiary care hospital should be able to offer diagnostic tools suitable for each individual case; if testing all patients with molecular techniques is not possible, the use of non-molecular techniques is preferable over nothing at all.

Conflict of interests

None declared.

Ethical approval

This study was approved by the ethics committee of University Hospital Saint Pierre (Brussels).

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