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TWO-PHOTON EXCITATION FLUOROMETRY IN DETECTION OF INFECTIOUS DISEASES

by

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Preface

This work was carried out at the Laboratory of Biophysics, Institute of Biomedicine, University of Turku during the years 2002-2008; and at the Laboratory of Diagnostic Microbiology, Turku University of Applied Sciences during the years 2007-2008.

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Turku October 2008

Janne O. Koskinen

To my family

Abstract

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Because of the heavily overlapping symptoms, pathogen-specific diagnosis and treatment of infectious diseases is difficult based on clinical symptoms alone. Therefore, patients are often treated empirically. More efficient treatment and management of infectious diseases would require rapid point-of-care compatible *in vitro* diagnostic methods. However, current point-of-care methods are unsatisfactory in performance and in cost structure. The lack of point-of-care methods results in unnecessary use of antibiotics, suboptimal use of virus-specific drugs, and compromised patient care.

In this thesis, the applicability of a two-photon excitation fluorometry is evaluated as a tool for rapid detection of infectious diseases. New separation-free immunoassay methodologies were developed and validated for the following application areas: general inflammation markers, pathogen-specific antibodies, pathogen-specific antigens, and antimicrobial susceptibility testing. In addition, dry-reagent methodology and nanoparticulate tracers are introduced in context to the technique.

The results show that the new assay technique is a versatile tool for rapid detection of infectious diseases in many different application areas. One particularly attractive area is rapid multianalyte testing of respiratory infections, where the technique was shown to allow simple assay protocols and comparable performance to the state-of-the-art laboratory methods. If implemented in clinical diagnostic use, the new methods could improve diagnostic testing routines, especially in rapid testing of respiratory tract infections.

Keywords: infectious diseases, nanoparticles, rapid point-of-care testing, separation-free immunoassay, two-photon excitation fluorometry

Tiivistelmä

Janne O. Koskinen

KAKSOISFOTONIVIRITTEINEN FLUORESENSSI-TEKNIIKKA INFEKTIOTAUTIEN DIAGNOSTIIKASSA

Biofysiikan laboratorio, Biolääketieteen laitos, Turun yliopisto

Diagnostisen mikrobiologian laboratorio, Turun ammattikorkeakoulu

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Eri infektiotautien aiheuttamat kliiniset oireet voivat olla hyvin samankaltaiset. Tästä syystä infektiotautien patogeeni-spesifinen diagnosointi ja hoito on vaikeaa pelkän oirekuvan perusteella. Potilaita joudutaan hoitamaan usein empiirisesti. Tehokkaampi infektiotautien hoito vaatisi parempia *in vitro* –pikatestejä, sillä nykyisten pikatestimenetelmien suorituskyky on epätyydyttävä. Potilasläheisen diagnostiikan ongelmat johtavat toisaalta antibioottien liikakäyttöön ja toisaalta virus-spesifisten lääkkeiden tehottomaan käyttöön. Näistä seurauksena on tingitty potilaan hoito.

Tässä väitöskirjassa tutkittiin kaksoisfotonivirittеisen fluoresenssi -tekniikan soveltuvuutta infektiotautien pikadiagnostiikkaan. Työssä kehitettiin erotusvapaat immunomääritysmetodologiat seuraaville sovellus-alueille: yleiset tulehdusmerkkiaineet, patogeeni-spesifiset vasta-aineet, patogeenien antigeeniosoitus ja antibioottiherkkyys-määritys. Lisäksi työssä esitellään kuivattujen reagenssien ja nanopartikkelikoettimien käyttö uuden tekniikan yhteydessä.

Tulosten mukaan uusi määritystekniikka on monikäyttöinen työkalu infektiotautien pikadiagnostiikassa. Erityisen houkutteleva sovellus-alue olisi hengitystieinfektioiden monianalyyttinen potilasläheinen pikatestaus. Uutta tekniikkaa voidaan pitää merkittävänä parannuksena perinteisiin pikatesteihin verrattuna. Kliiniseen diagnostiseen käyttöön sovellettuna uudet menetelmät voisivat merkittävästi parantaa diagnostisia testausrutiineja.

Avainsanat: erotusvapaa immunomääritys, infektiotaudit, kaksois-fotonivirittеinen fluorometria, nanopartikkelit, pikadiagnostiikka

Contents

List of original publications

This doctoral thesis is based on the following original publications, which will be referred to the Roman numerals **I-V**. In addition, some unpublished data are presented.

I. Janne O. Koskinen, Jonne Vaarno, Niko J. Meltola, Juhani T. Soini, Pekka E. Hänninen, Juhani Luotola, Matti E Waris, and Aleksi E. Soini. Fluorescent nanoparticles as labels for immunometric assay of C-reactive protein using two-photon excitation assay technology. *Analytical Biochemistry* (2004), 328(2):210-218.

II. Janne O. Koskinen, Niko J. Meltola, Erkki Soini, and Aleksi E. Soini. A lab-on-a-chip compatible bioaffinity assay method for human alpha-fetoprotein. *Lab on a Chip* (2005), 5(12):1408-1411.

III. Janne O. Koskinen, Jonne Vaarno, Raija Vainionpää, Niko J. Meltola, and Aleksi E. Soini. A novel separation-free assay technique for serum antibodies using antibody bridging assay principle and two-photon excitation fluorometry. *Journal of Immunological Methods* (2006), 309(1-2):11-24.

IV. Janne O. Koskinen, Raija Vainionpää, Niko J. Meltola, Jori Soukka, Pekka E. Hänninen, Aleksi E. Soini. Rapid method for detection of influenza A and B virus antigens by use of a two-photon excitation assay technique and dry-chemistry reagents. *Journal of Clinical Microbiolology* (2007), 45(11):3581-3588.

V. Janne O. Koskinen, Teppo Stenholm, Jonne Vaarno, Jori Soukka, Niko J. Meltola, and Aleksi E. Soini. Development of a rapid assay methodology for antimicrobial susceptibility testing of *Staphylococcus aureus*. *Diagnostic Microbiology and Infectious Diseases*, in press (2008).

Abbreviations

AB	Antibody bridging
AI	Avidity index
AOM	Acute otitis media
AST	Antimicrobial susceptibility testing
BSA	Bovine serum albumin
CRP	C-reactive protein
EBV	Epstein-Barr virus
EDAC	N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride
ELISA	Enzyme linked immunosorbent assay
HIV	Human immunodeficiency virus
HNL	Human neutrophil lipocalin
hTSH	Human thyroid stimulating hormone
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MES	2-(N-morpholino)ethanosulfonic acid
mM	Millimolar (concentration)
MRSA	Methicillin resistant *Staphylococcus aureus*
MT	Molecular tracer
NAA	Nucleic acid amplification
nM	Nanomolar (concentration)
NPT	Nanoparticulate tracer
OECD	Organisation for economic co-operation and development
PCR	Polymerase chain reaction
pM	Picomolar (concentration)
POC	Point-of-care
RSV	Respiratory syncytial virus
RTI	Respiratory tract infection
TPX	Bioaffinity assay technique based on two-photon excited fluorescence
TR-FIA	Time-resolved fluoroimmunoassay
URTI	Upper respiratory tract infection
WHO	World Health Organization

1 Introduction

Infectious diseases are, along with cardiovascular diseases, a leading cause of death worldwide. Infectious diseases are estimated to be directly related to more than 25 % (15 million) of annual deaths, while respiratory tract infections (RTI) alone cause 7 % (4 million) and HIV (human immunodeficiency virus) 5 % of all deaths (WHO 2004). The percentages of different causes of death vary significantly between developed and developing countries (Guerrant 1999, WHO 2004, Lopez 2006), as well as by social status (Butler 2001). However, lower respiratory tract infections are a significant cause of death in all countries regardless of the average income level (Lopez 2006). The fact that infectious diseases often cause life-long disability and premature death at an early age increases their impact (Fauci 2001, WHO 2004, Lopez 2006). A significant percentage of the deaths and disabilities (a few million annual deaths) could be avoided by vaccination (GAVI Alliance 2004). Interestingly, the estimated number of infection-attributable cancers in the year 2002 was two million cases, 18 % of the global cancer burden (Parkin 2006).

The terms emerging and re-emerging infectious diseases are frequently used to describe infectious diseases. The emerging include new pathogens such as acquired immunodeficiency syndrome (AIDS) and severe acute respiratory syndrome (Peiris 2003). The re-emerging include old diseases such as malaria (Wellems 2003), tuberculosis (Espinal 2003), influenza A (avian influenzas, Olsen 2006), and drug-resistant microbes (WHO 2005). Many public health experts believe that the emergence of a new influenza pandemic is only a matter of time, and that it will occur during this century (Fauci 2001).

The threats that infectious diseases pose to public health today have been realized by the health care authorities and by the specialist (Fauci 2001, WHO 2004, Morens 2004) as well as by the general public. Infectious diseases are among the most significant threats (along with oil, water, and food) that endanger economic development and political stability. Therefore, massive resources will be directed to infectious diseases research; to the development of new vaccines, antimicrobial drugs, and diagnostics. (Fauci 2001, OECD 2003) In contrast to many other market fields of laboratory testing, infectious diseases testing is not highly saturated and the market potential is high.

Management of infectious diseases is complicated by the overlapping symptoms and the lack of efficient point-of-care diagnostic methods (Perkins 2006). From the physician’s point of view, efficient testing

would help to treat patients according to the cause of the symptoms (etiology) in contrast to empirical treatment. From the patient's point of view, the benefits are in minimizing human suffering and saving life. On a more general level, testing for infectious diseases allows epidemiological follow up, aids in directing resources, and improves public health.

The literature review of this thesis is focused on aspects related to *in vitro* diagnostic testing of infectious diseases present in every day clinical practice. In particular, the review aims to pinpoint common bottlenecks in the diagnostic practice, and to evaluate what kind of diagnostic tools would be needed to resolve these. RTIs are given most weight due to their frequency and economic impact.

1.1 Respiratory tract infections

Diagnosis of patients with RTI symptoms is usually based on clinical findings and anamnesis, while less frequently diagnosis is confirmed by laboratory analysis. Unfortunately, symptoms and clinical findings of different pathogens are variable and heavily overlap, even between viral and bacterial infections (Perkins 2006). Therefore, it is difficult to obtain a reliable etiological diagnosis in RTIs based on the clinical picture alone. Hence, usually the diagnosis is more descriptive of the symptoms (syndrome) than the causative agent. Rapid pathogen detection tests are available for several pathogens but their use is limited for several reasons. Tests for general inflammation markers such as C-reactive protein (CRP) are done more often to help in differential diagnosis, although the results have poor correlation with the etiological diagnosis.

1.1.1 Health and Economic impact

Respiratory tract infections are among the most common infectious diseases. On average, children suffer from up to dozen and adults from a few RTIs annually. RTIs cause 25 % of all infectious diseases deaths (WHO 2004), and their severity is pronounced in children, the elderly, the chronically ill, and in areas of low hygiene and poor nutrition. Globally, two million children die annually from acute RTIs, accounting for 10 to 20 % of all childhood deaths (Williams 2002). Influenza and respiratory syncytial viruses markedly increase mortality in infants and in the elderly. Generally, respiratory syncytial virus (RSV) is more severe in infants, whereas influenza is more severe in elderly people. (Willams 2002, Thompson 2003) The viruses are responsible for most of the RTI deaths in the developed countries

(Thompson 2003, Fleming 2005). Mortality is not usually a direct consequence of influenza or RSV infection but a result of complications, such as bacterial pneumonia (Beadling 2004).

Virus infections seem to predispose to secondary bacterial infections which may lead to lethal synergism (Beadling 2004). Because of this, the health of the most vulnerable should be monitored carefully if they are found to be positive for viruses. Importantly, treatments that diminish the magnitude of initial viral infection seem to protect from subsequent bacterial infection (Linde 2001, Beadling 2004, Peltola 2005a).

In Finland, adults have 0.5 RTI-related medical consultations per annum (Pirhonen 1994), and 40 % of persons having RTI seek medical attention (Aromaa 2002). RTIs are responsible for 25 % of outpatient doctor visits and 40 % of sick leaves in Finland (Jussila 2005). RTIs constitute more than three quarters of all infections, the most common diagnoses being common cold, acute otitis media (AOM), throat infection, and sinusitis (Rautakorpi 2006a). Similar statistics were found in the USA where every person had, on average, 0.8 respiratory infections per year. Patients with influenza, common cold, other acute upper respiratory tract infection (URTI), acute bronchitis, pneumonia, and acute ear infections sought medical attention in 36 %, 43 %, 87 %, 90 %, 91 %, and 99 % of cases, respectively. (Adams 1999)

The economic impact of all RTIs is significant. Most of the costs are due to sick leave and medical consultations, while medication is responsible for only some ten per cent (Birnbaum 2002, Fendrick 2003, Jussila 2005). Many of the consultations are unnecessary in terms of recovery and treatment. In the USA, 25 million people visit their family doctors because of uncomplicated RTI (Gonzales 2001). The common cold alone accounts for 20 million out-of-work days and a similar number of absent days from school annually (Adams 1999). A substantial number of out-of-work days of the parents are a result of their child's disease (Heikkinen 2004). The annual costs of viral RTIs in the USA have been estimated to amount to $40 billion (Fendrick 2003). Extrapolation to Finland gives costs amounting to €1 000 million.

Birnbaum *et al.* (2002) analysed the economic burden of RTIs in the USA from the employer perspective. Annual per capita employer expenditures totalled $4 400 and the overall costs, including treatment costs and time lost from work, were around $110 billion in 1997. The figures do not include indirect costs due to workforce distribution. The

huge health and economic impact of RTIs suggests that significant cost savings could be achieved with more efficient disease management.

1.1.2 Etiology and difficulty of diagnosis

Etiology of RTIs is extremely diverse. Symptoms are sometimes atypical, heavily overlap, and differ from patient to patient. Knowing the epidemiological status helps the clinician in making diagnoses. Still, pathogen-specific diagnosis is virtually impossible on clinical grounds alone. **Table 1** presents a summary of the most common causative agents of RTIs.

Rhinoviruses are responsible for up to half of all common cold cases (Mäkelä 1998, Pitkäranta 1998, Monto 2002 and 2004), while rapid diagnostic testing of rhinoviruses is complicated by the high number of serotypes (Heikkinen 2003a). This makes development of an immunoassay challenging; despite the high incidence and clinical significance of the virus, immunoassay tests are not commercially available. Rhinoviruses have also been associated with lower RTIs (Juven 2000, Savolainen 2003). The common cold is sometimes difficult to differentiate from bacterial infections (Puhakka 1998b, Heikkinen 2003a). However, only a small proportion of common colds are complicated by bacterial infections (Mäkelä 1998). Antibiotic treatment of patients with bacterial indication might shorten the duration of the symptoms, but prescription would require *in vitro* evidence of bacteria (Kaiser 1996).

Pharyngitis and tonsillitis cases are mostly (40 %) caused by viruses, especially in children under 3 years (Putto 1987). The most common etiological viruses are adenoviruses (20 %) and EBV (10 %) (Putto 1987). Bacterial pharyngitis is most common in children over 6 years. The clinical findings of group A streptococcal and viral pharyngitis broadly overlap, and accurate diagnosis by clinical signs alone is difficult. Streptococcus A is the most common bacterial etiological agent of acute pharyngitis. Still it causes only 5−10 % and 15−30 % of the total pharyngitis cases in adults and in older pediatric patients, respectively (Bisno 2002).

Table 1. Causative agents of common respiratory tract infections (see ***Appendix 1*** for reference information)

Diagnosis (syndrome)	**Etiology (pathogen found)**	**Reference**
Common cold	Rhinoviruses 50 %	9, 18
	Coronaviruses 5−15 %	5, 18
	Influenza viruses 5−30 %	5, 18
	Parainfluenza viruses 5 %	5, 18
	Adenovirus less than 5 %	5, 18
	Metapneumovirus 2−5 %	11, 14
	Bacterial 5−10 %	2, 10
Throat infection	Bacterial 20−40 %	2, 6
	Streptococcus pyogenes 15−30 % in youths, 5−10 % in adults	6
	Adenovirus 20 %	6
	Epstein-Barr virus 10 %	6
	All viruses together 40 % in children	6
	Unknown 30 %	6
Acute otitis media	Bacterial 65−80 %	2, 5, 16
	Streptococcus pneumoniae 25−50 %	16, 17
	Haemofilus influenzae 15−50 %	16, 17
	Moraxella catarrhalis 2−55 %	16, 17
	Sole viral 1-5 %, virus found in 20−70 %	5, 16, 17
	Co-infection of bacterium and virus 60−70 %	7, 8, 17
	Unknown less than 25 %	5, 17
Acute bronchitis	Viruses (influenza and others) 80−90 %	
	Bacterial 10 %	2
Pneumonia	*Streptococcus pneumoniae* 40 % in children (sole agent in 15 %), 20−30 % in adults	1, 4, 15
	Haemofilus influenzae 10 % in viral co-infections in children, 4 % in adults	1, 4
	Mycoplasma pneumoniae 5 % in children, 10 % in adults	1, 4
	Chlamydia pneumonia 3 % in children, 7 % in adults	1, 4
	Sole viral in children 30 %, virus found in children 60 %, sole viral in adults 10−30 %	1, 4, 12, 15
	Co-infection of bacterium and virus 30 % in children, 10−30 % in adults	1, 3, 12, 15
Acute Sinusitis	Bacterial 20−40 %	2, 10, 12
	Streptococcus pneumoniae 20−35 %	
	Haemofilus influenzae 5−25 %	
	Moraxella catarhalis 2−10 %	10
	Viruses 80 % in context of common cold	13

Acute bronchitis is usually caused by influenza, parainfluenza, RS-, or metapneumovirus (Hamelin 2005). However, chronic viral bronchitis can predispose to bacterial infection. Symptoms and causative agents closely resemble those of common cold and pneumonia. The differentiation between bronchitis (antibiotic treatment not recommended) and pneumonia (antibiotic treatment recommended) is difficult based on clinical findings. For instance, the positive predictive value, sensitivity, and specificity of clinical signs for influenza are very low, ranging from 20 % to 80 % at optimum conditions (Carrat 1999, Monto 2000, Peltola 2005b).

In bronchiolitis, RSV is the most prominent etiological agent in small children. Acute wheezing is caused by RSV or picornaviruses in three out of four cases (Jartti 2004, Lehtinen 2007).

In pneumonia, the diagnostic problem is the same as in bronchitis. Clinical signs are not sensitive and specific enough for differential diagnosis. Rapid tests that would give reliable information about the etiology are not available (File 2003). In adults, the etiological agent is usually bacterial but also viruses are a common cause (Angeles Marcos 2006, Hohenthal 2008). Bacterial pneumonia is most often caused by *S. pneumoniae*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, or *Haemophilus influenzae*. The etiological agent in children is usually viral but also co-infections with bacteria are found (Table 1) (Heiskanen-Kosma 1998, Juven 2000). During the first years of life, RSV, rhinoviruses, and *S. pneumoniae* are involved in the majority of child pneumonias (Juven 2000), while also metapneumovirus is found in older children (Hamelin 2005). Viral lower RTIs in children are often associated with bacterial upper RTIs, whereas co-existence with bacterial lower RTI is rare (10-20 %) (Lehtinen 2006). Development of bacterial pneumonia is found even less often (in a few percent of cases, Lehtinen 2006). According to this, a positive virus test result can be used to exclude bacterial complications with high probability. Moreover, it has been demonstrated that treatments that diminish the magnitude of initial viral infection help to protect from subsequent bacterial infection (Linde 2001, Beadling 2004, Peltola 2005a). This supports the need for effective virus drugs and rapid virus testing methods (Angeles Marcos 2006).

In acute otitis media, respiratory viruses can be detected in 50−70 % of the patients. The most common findings are rhino, entero, metapneumo, and RS-viruses (Arola 1990, Heikkinen 1999, Ruohola 2006, Heikkinen 2008) and they are major risk factors for AOM. The viruses seem to have an etiological role since they are often found in

the nasopharynx and in the middle ear fluid (Arola 1990, Heikkinen 1999, Heikkinen 2003b, Lehtinen 2006). Pathogenic bacteria are found in the middle ear fluid in 9 out of 10 cases (Heikkinen 1999, Ruohola 2006), but often also in the nasopharynx (Arola 1990). In 60−70 % of patients both bacteria and viruses are found (Heikkinen 2003b, Ruohola 2006), and they are thought to evoke a synergistic effect (Heikkinen 2003b).

Sinusitis and AOM caused by a virus may be accompanied with bacterial infection. The diagnostic problem is how to identify those who would recover well without an antibiotic from those who would benefit from it. Early treatment of influenza with virus-specific drug has been shown to reduce the development of AOM by more than 40 % (Whitley 2001). The few days time window between viral infection and the onset of AOM allows the opportunity for intervention, provided that rapid diagnostic methods are available (Heikkinen 2003b).

Approximately half of bacterial sepsis cases are preceded by acute RTIs (Beadling 2004, Peltola 2006a). Outbreaks of respiratory viral infections, especially RSV and influenza viruses, are associated with an increased incidence and/or severity of bacterial co-infections. The clinical symptoms associated with co-infections are more severe and last for a longer time compared to the respective viral or bacterial single infections. In co-infections, non-dangerous infections may become fatal. For effective and correct therapy, the causative agent should be determined. Monitoring of the pathogen repertoire of the patient would also help to predict the probability of sepsis. In some cases the antimicrobial response of the infectious agent should also be studied. (Beadling 2004, Angeles Marcos 2006)

So far, influenza is the only RTI virus disease for which specific anti-viral drugs are available. At present, the use of these drugs is still relatively uncommon. The situation may be considered unethical as influenza is a severe disease for which an effective medication exists (Personal communication, Docent Terho Heikkinen, May 16th 2008, ESPID, Graz, Austria). The lack of drugs for the other viruses has been used as an argument against wide testing of virus RTIs. Currently, specific drugs for RSV are in the pipeline (Datamonitor 2006). Nonetheless, testing of the other viruses is clinically useful, for example, as an exclusionary factor, in disease monitoring, and in reducing the use of antibiotics. Moreover, early identification of acute RTIs might enable the lowering of crib deaths (Samuels 2003). The

development of new methodologies for RTI pathogen testing, along with the development of anti-viral drugs, would be important.

1.1.3 Unnecessary use of antibiotics

Eighty per cent (80 %) of the total antibiotic burden is determined by outpatient use, with most antibiotics being prescribed for RTIs (Wise 1998). A significant fraction (up to 60−70 %) of the antibiotic use is questionable if not totally unnecessary (Wise 1998). In acute RTIs in the USA this fraction is about 55 % (Gonzales 2001). Most of the unnecessary antibiotics are used to treat bronchitis and pharyngitis, although both of the diseases are most often caused by viruses. No scientific evidence exists to support high antibiotic prescription rates in bronchitis (60−85 % of patients, Gonzales 1997, Rautakorpi 2006b) and in pharyngitis (60−95 % of patients, Nyquist 1998, Bisno 2002, Rubin 2005).

Most of the patients with acute cough (Fahey 1998), common cold (Mäkelä 1998), AOM (Glasziou 2004) or viral sinusitis (Puhakka 1998b) do not benefit from antibiotic treatment. Yet, most patients (80−95 %) with AOM and sinusitis are currently prescribed with antibiotics in Finland (Rautakorpi 2006b). The common cold, on the other hand, is less often treated with antibiotics in Finland (Rautakorpi 2006b). In the USA, the use of antibiotics for common colds is markedly higher; up to 50 % (Gonzales 1997, Nyquist 1998, Rubin 2005). When bacterial complications are considered probable, or the consequences of a secondary bacterial infection are severe, the use of antibiotics may still be justified (Beadling 2004).

Antibiotic treatment of acute bronchitis has little or no effect on the course of the illness (Orr 1993, Fahey 1998, Fahey 2004). Thus, it is not recommended for otherwise healthy patients unless there is evidence of bacterial etiology (WHO 2001). However, patients at risk of or having pneumonia should be distinguished. Even the concept of acute bronchitis is ambiguous since it is sometimes used as an excuse to justify the prescription of antibiotics. This has led to over-diagnosis of bronchitis (Gonzales 1998, Hueston 2000). A conservative estimate suggests that one third of patients with acute bronchitis or unspecified URTI could avoid antibiotic treatment with accurate and rapid *in vitro* diagnostic testing (Rautakorpi 2006b).

In pharyngitis, the patient group receiving antibiotics is significantly higher compared to patients actually having bacterial disease (Bisno 2002). Since *S. pyogenes* can not be reliably diagnosed based on clinical findings, an *in vitro* diagnostic test is required. This is usually

done with rapid strip tests. Due to the poor sensitivity of the tests (clinical sensitivity 80−90 %), negative test results need to be confirmed with more sensitive methods like culture (Gerber 2004). Sometimes patients are prescribed with antibiotics, despite the negative rapid test result. If the following laboratory result turns out negative, the therapy should be discontinued. Even this short antibiotic regimen and the use of back-up culturing could often be avoided if a reliable and sensitive rapid test method were available at the point-of-care. According to new guidelines, a negative rapid test result is accepted for exclusion of acute streptococcal pharyngitis, provided that the rapid test has comparable sensitivity to culture. (Bisno 2002) Indeed, implementation of high sensitivity rapid tests has been shown to improve therapeutic accuracy and patient outcome (Needham 1998). Positive adenovirus antigen detection test results strongly indicate non-group A streptococcal etiology (Putto 1987).

S. pneumoniae which is the most common pathogen in pneumonia and in AOM, is also found in healthy individuals. These individuals act as carriers of the bacteria, and this leads to low positive predictive values in *in vitro* diagnostic tests. However, negative bacterial test results from the nasopharynx and oropharynx have very high (90−100 %) negative predictive values in AOM (Eldan 2000, Syrjänen 2006) and in pneumonia (Boersma 1993b), respectively. Bacterial count (Syrjänen 2006) and detection of pneumococcal antigen (Boersma 1993a) correlate with the disease etiology. These observations enable the use of such tests, especially if they provide quantitative results, in excluding *S. pneumoniae* etiology and reducing the use of antibiotics.

Because broad spectrum antibiotics cause long-term changes in the composition of normal microbial flora of a patient, antibiotic treatment may predispose to new infections by colonization of pathogenic yeasts or drug-resistant microbial strains (Sullivan 2001). A fairly common direct side effect is diarrhoea (Beaugerie 2004), but antibiotic uptake may also predispose to cancer (Velicer 2004). General awareness of the nature of efficasy of antibiotics and about the drawbacks of antibiotics is growing. In the past, patients primarily sought antibiotic medication for RTIs, whereas currently, increasing numbers of patients are also interested in the cause of the illness (Butler 1998, Rautakorpi 2006b, Global Industry Analysts).

A rough estimate (based on the data by Gonzalez *et al.* 2001 and Ferech *et al.* 2006) suggests that, in Finland, annual savings of about ten million euro could be reached if antibiotic uptake were reduced by one third (estimated fraction of unnecessary courses). The savings

would cover the costs of significant numbers of rapid tests which could be done to obtain more accurate pathogen-specific diagnoses. However, more optimal rapid tests are needed to realize this reasoning.

The superfluous use of antibiotics has led to the emergence of bacterial strains which are resistant to the antibiotics (Steinke 2001, Goossens 2005). The spread of the resistant strains in the population is recognized as a major threat to public health (WHO 2000, Wise 1998). Increasing antibiotic resistance is often seen and felt as a community issue, whereas the general practitioners are mainly interested in the recovery of the individual patient (Butler 1998). The strategy of developing new antibiotic drugs has become rather inefficient and expensive, and therefore resources must be redirected to alternative disease management strategies. The formation of new resistant strains could be essentially slowed down by applying a more strict antibiotic administration policy (Rautakorpi 2006b, Peleg 2006). Strategies to reduce the unnecessary use have included promotion of public discussion and education of physicians, while success has varied from moderate to satisfactory (Rubin 2005, Peleg 2006). Better results could be achieved if the antibiotic prescription were directed by hard evidence, i.e. rapid identification of the pathogen and/or rapid *in vitro* susceptibility testing (Gonzales 2001).

1.2 Bioaffinity techniques for pathogen detection

The laboratory bioaffinity techniques most often used for detection of infectious diseases include enzyme linked immunosorbent assay (ELISA), chemiluminescence, electrochemiluminescence, time-resolved fluorescence, flow cytometry in its various modifications, immunofluorescence, turbidimetry, nephelopmetry and fluorescence polarization. The detection techniques are implemented in various instrumental settings including fluorescence microscopes, plate readers, plate readers with automatic dispensing units, and single well readers capable of random-access operation. Rapid point-of-care techniques include lateral flow and agglutination.

Current detection techniques fulfil the needs of a modern centralized clinical laboratory, whereas the most urgent need for new techniques lies in point-of-care (POC) testing. The use of the terms POC testing, decentralized testing, and near-patient testing in the literature is ambiguous. In this thesis, the term POC testing is used to denote testing that is carried out in a decentralized mode, regardless of whether the analysis is carried out next to the patient or in a laboratory of the same health care unit.

1.2.1 Diagnostic tests

The need for new efficient tools for POC testing of influenza and other infectious diseases has recently been emphasized by the National Institute of Health (www.nih.gov), the Organisation for Economic Co-operation and Development (OECD 2003), and the European Commission (http://cordis.europa.eu/fp7/home_en.html). Still, even the current methods are often notably underused in RTIs (Honkanen 2002). This contradiction reflects the unsatisfactory performance and usefulness of the current methods. New diagnostic methods are needed both to allow immediate and correct patient care, and to allow epidemiological surveillance (Linde 2001, OECD 2003, Peiris 2003, WHO 2005). In the case of a pandemic RTI outbreak, the upper limit of current laboratory capacity would be rapidly reached (Linde 2001). This halts both the diagnostic testing and the operation of the clinics as huge amounts of resources are bound to exclude patients with non-pandemic infections or psychological symptoms. In practice, specific diagnostic testing could currently be done only in the beginning of the epidemic. The threats warrant revision of the current testing capacity and capability.

Testing of RTIs is mostly carried out in centralized laboratories, which may lead to a long result turn-around-time. For efficient disease management, however, the results should be available within a few hours' time scale. Most preferably, the result should be available during the same patient visit. This warrants rapid POC methods. To ensure correct medication and palliative treatment, the pathogen should be recognized to a species level. From the test developer's and manufacturer's perspective, lack of access to clinical samples is often seen as a major limitation and risk for the test development (Perkins 2006).

Ideally, a test method should use specimens which are easy and painless to collect. The easiest specimen type can not always be applied as different pathogens replicate and accumulate in different locations of the respiratory tract. Nasopharyngeal aspirate is considered the most reliable sample type, but general practitioners regard sample collection cumbersome and painful, and consider it a major obstacle to carrying out pathogen detection (Linde 2001, Ipp 2002, Macfarlane 2005). Nasopharyngeal and nasal swabs have been studied as alternatives to aspirates. Aspirate samples are somewhat more sensitive compared to swab samples (Covalciuc 1999, Ipp 2002, Heikkinen 2001, Heikkinen 2002), while the difference is rather small when appropriate swab material and collection procedures are used. It

seems that swab samples could be used for all the commonly tested viruses, RSV being the most problematic (Heikkinen 2002, Waris 2007). These studies also encourage the use of nasal swabs, as the use of nasopharyngeal swabs is comparable to aspirate in discomfort (Ahluwalia 1987).

1.2.1.1 Differential diagnosis of viruses and bacteria

The main question in the treatment of RTIs is whether the infection is treatable with antibiotics or not. A simple and rapid test that would differentiate between bacterial and viral etiology (differential diagnosis) would be very useful. Several markers have been introduced for this purpose, such as C-reactive protein (CRP), procalcitonin, human neutrophil lipocalin (HNL), erythrocyte sedimentation rate, and white blood cell count. It is worth noting that any *in vitro* indication of viral etiology is often enough to discourage physicians from prescribing antibiotics.

The problem with the general systemic inflammation markers is that their levels depend on several factors such as the pathogen, patient, and infection phase. Certain levels can only depict the probability of bacterial or viral disease. Accordingly, sensitivity and specificity of the method is always a compromise between the two. Moreover, many RTIs are not systemic in nature.

Low CRP concentrations are regarded as justifying viral diagnosis, while high levels are used to justify a bacterial diagnosis. Recent results, however, show that CRP concentrations in bacterial and in viral infections heavily overlap (Hopstaken 2005, van der Meer 2005) and that results are often misinterpreted (Andre 2004). Overlapping is seen especially with adenoviruses (Peltola 2006b), in viral wheezing (Lehtinen 2006), and in pneumonia (Angeles Marcos 2006, Flood 2008). Thus, the sensitivity and specificity of CRP (the discriminatory power) can be as low as 50−60 % (Flood 2008).

Procalcitonin seems to differentiate viral and bacterial infections better than CRP, at least in certain systemic infections, like in bacterial pneumonia (Simon 2004). Another marker suggested for differential diagnosis is human neutrophil lipocalin (HNL). HNL is slightly better than CRP, but HNL concentrations still heavily overlap in bacterial and in viral infections (Xu 1995, Fjaertoft 2005).

All the markers lack sensitivity and specificity compared to pathogen detection methods. A combination of several markers into a diagnostic algorithm has also been studied, to increase the sensitivity and specificity of differential diagnosis. Still, no combination of clinical

signs and markers (CRP, HNL, erythrocyte sedimentation rate, and white blood cell count) was found to reliably differentiate between bacterial and viral acute lower RTIs (Nohynek 1995, Korppi 2004).

More recently, the expression of complement receptors on neutrophils has been shown to correlate with bacterial and viral etiologies, but a combination with several other laboratory test results is needed to obtain a high accuracy (Nuutila 2007). Moreover, the current instrumentation used for complement receptor testing (Nuutila 2006) is hardly implementable for POC testing.

1.2.1.2 Specific laboratory techniques

Diagnostic testing of infectious diseases is usually characterized by low frequency of positive test results. The most common bacterial etiological agent of acute pharyngitis, *S. pyogenes*, causes only 5−30 % of the total pharyngitis cases (Bisno 2002). In the USA, only 12 and 16 % of the tested samples (with clinical suspicions) were positive for influenza viruses and RSV, respectively (Thompson 2003). Common flu viruses can sometimes be found more often (30−50 %), but virus-specific drugs or rapid POC tests are not available for these viruses. As a result, current single analyte rapid tests need to be ordered and evaluated with care in order to obtain clinically meaningful information at a reasonable cost (Linde 2001).

In recent years, testing for RTI etiology has become more important, useful and cost-effective. The main reasons being the development of virus-specific drugs (Gubareva 2000), and the increasing opposition to the loose antimicrobial administration policy. Other reasons for carrying out pathogen testing are, for instance,

i) epidemiology
ii) identification of pathogen-specific clinical pictures
iii) shortening treatment and hospitalization times
iv) differential diagnostics
v) treatment design
vi) infection control, and
vii) avoiding unnecessary examinations.

It is also expected that the increasing general consciousness about the availability of virus drugs and about the shortcomings of antibiotics will affect the testing routines. In the future, physicians may refuse to prescribe antibiotics without *in vitro* evidence of bacteria. (Global

Industry Analysts) This will increase the use of and the demand for rapid tests.

Techniques that have been used for laboratory testing of RTIs include culture, serology, antigen detection, and nucleic acid based techniques. Conventional culture has strengths such as its broad spectrum, but also drawbacks such as price and long turn-around-time. The sensitivity of culture methods is sometimes lower compared to other methods. In addition, isolated viruses often need to be identified with antibody reagents.

Antigen detection

The most important property of viral antigen detection is specificity. In general, virus pathogen detection has clinical specificity close to 100 %. A false negative viral test result seldom leads to fatal consequences in RTIs. A false positive viral test result, in contrast, can lead to complications by another, undetected, disease-causing microorganism (Linde 2001). Results of bacterial antigen detection methods are sometimes complicated by healthy colonized carriers. Still, the bacterial antigen detection methods should have high sensitivity in order to avoid bacterial infections going untreated. Viruses can also be found from healthy carriers (van Gageldonk-Lafeber 2005), but healthy subjects are not tested in clinical practice. Hence, they do not pose a diagnostic problem if the testing coverage for other pathogens is sufficient.

Most of the rapid antigen detection methods have so far relied on the use of lateral flow or agglutination techniques. The tests are in routine use even in central hospitals, although facilities for culture or ELISA tests are also available nearby. Technically, lateral flow and agglutination tests can be brought to the market quickly for a moderate investment, and they can be used in a broad range of applications (O'Farrell June 2006, Yager 2006). Development and production costs markedly increase when high performance is needed. Most of the market niches are saturated by a few large companies (Global Industry Analysts).

In general, current rapid test methods are usually specific (over 95 %), but suffer from low sensitivity (80−90 %, Andreotti 2003, Linde 2001, Wild 2005, WHO 2005, Datamonitor 2006, Madeley 2007). Another major limitation of the current rapid tests is that they detect only one or two pathogens at a time (Madeley 2007). Neither of the techniques allows efficient testing of several analytes per patient or sample (multianalyte testing) (Yager 2006). Accordingly, reliable diagnosis

can be made only on the basis of a positive test result (Bisno 2002, Madeley 2007). Therefore, the current techniques are not well suited for RTIs where the positive sample rate is low.

Current rapid tests are characterised by long hands-on-times which means high overall cost per test. Moreover, most of the tests do not allow easy connections to external quality control or laboratory information management systems. The tests usually rely on detection by the human eye and are thus qualitative or semi-quantitative in nature. Such tests are prone to readout errors. These errors can be reduced with automated result read-out, and an increasing number of automated rapid test readers are available (O'Farrel July 2006, e.g. www.reagena.fi). Also the label (O'Farrel July 2006) and membrane (O'Farrel June 2006) technologies of lateral flow tests have been paid attention to in pursuit of higher sensitivity and precision.

Immunofluorescence is a common technique used for identification of respiratory viruses in hospital settings. The strengths of the technique include suitability for multianalyte detection and inherent quality control of sample withdrawal. However, the method is rather labour intensive, it takes several hours to complete, and the analysis requires high level of expertise. Therefore, it is not well suited to POC testing in primary care. In addition, its sensitivity seems to be somewhat lower compared to coated-tube techniques (Ahluwalia 1987, Juven 2000).

Immunometric assay principle and detection based on coated-tube techniques (like ELISA and time-resolved fluoroimmunoassay, TR-FIA), or flow-cytometry have the advantage of being sensitive, as well as having automated and quantitative results read-out. They also allow rational multianalyte testing. However, these techniques are too complicated and slow for POC testing (OECD 2003). Despite fairly short turn-around-times (1−3 hours), the sending and handling of the specimens lengthens the total assay times so that a result reported on the same day can be considered an achievement (Linde 2001).

Nucleic acid amplification

Nucleic acid amplification (NAA) techniques like polymerase chain reaction (PCR) are in principle very sensitive and specific. In clinical use, however, their reliability is often compromised. Polymerase inhibitors are frequently present in sample or swab material and they can lead to false negative results. Because of the inhibitors, detailed quality control procedures and internal controls are needed in clinical use. (Ieven 1997, Niesters 2004, Vernet 2004, Yang 2004) For this reason, and also to increase sensitivity, nucleic acid extraction is

usually needed. Extraction is the major bottleneck in the implementation of these techniques for routine cost-effective and rapid diagnostics (Niesters 2004, Vernet 2004). On the other hand, NAA methods are prone to contamination, which leads to false positive results.

To construct a NAA method, the target genetic sequence has to be well known. This requires a lot of basic research. In addition, changes in genetic sequence take place more often than changes in the corresponding amino acid sequence. Hence, NAA methods can give false negative results because of new mutations, or because sample or amplification processing has failed (Vernet 2004).

NAA methods can give false positive viral test results because sometimes genomic material is detected from the patient long after recovery from symptoms (Johnston 1993, Nokso-Koivisto 2002, Allander 2008). Also latent infections can give clinically false positive results as is the case for cytomegalovirus.

Due to the complexity of NAA methods, reactions have to be carefully optimized with respect to all reaction parameters for every microbe strain (Vernet 2004, Yang 2004)). When clinical samples are analysed, more than one pathogen-specific probe sequence is usually required for a reliable diagnosis (Roth 2004). Multiplexed NAA methods have been developed to simplify the test protocols but multiplexing is known to be complicated. It also tends to decrease sensitivity and specificity. (Ieven 1997, Vernet 2004) Automated NAA methods have been developed to shorten the hands-on-times. This, however, leads to complicated instrumentation and reagent cartridges, and further increases the costs.

In the detection of analytes for which sensitive immunoassay methods exist, NAA techniques can give little added value in terms of sensitivity or rapidity. For some pathogens, NAA methods provide a superior performance due to the insensitiveness of the classical methods for these particular pathogens. (Ieven 1997)

Despite the limitations, several multianalyte or multiplexed assay systems for up to 20 respiratory tract pathogens have recently been introduced (e.g. www.viracor.com, www.lab-21.com). However, turn-around-times are still often too long and the reagents are too expensive for rapid POC testing. Patenting and licensing-related issues have also slowed down the development and commercialization of the NAA techniques (Batchelder 2006). For these reasons, it is expected that the multianalyte NAA methods will be mostly used for research while diagnostic testing will be limited to patients with severe symptoms.

Serology

Viruses and intracellular parasites cause strong immunoresponses, whereas bacterial infections usually induce a good response only against carbohydrate antigens, and the response involves only IgM. Accordingly, detection of specific antibodies is mainly used for virus and intracellular parasite infections. Serology is extremely useful in epidemiological research and in retrospective detection, in particular, when antigen detection methods are not applicable. On the other hand, the use of serology in rapid testing of acute RTIs is limited due to the natural delay in the immunological response and the already existing serum antibodies due to past infections (Allwinn 2002).

1.2.2 Trends in laboratory testing

Despite being overlooked in the past (Batchelder 2006), diagnostics is expected to be one of the most important disciplines during the 21st century (Fauci 2001, OECD 2003). Interestingly, only 2−5 % of overall health expenditures are currently used for laboratory based testing, despite the fact that they are the key to high-quality clinical care (EDMA 2003, The Lewin Group 2005). Infectious diseases markets are expected to grow significantly within the coming years in all market segments, including antimicrobial and antiviral drugs, vaccines, and testing (Clinica Reports 2004, Pray 2006).

During recent years, the trend in laboratory testing of infectious diseases has moved from batch analysis towards random-access testing. The centralized approach has the drawback of a long lag time between the sampling and the result, and the risk of sample deterioration during transportation. On the other hand, centralised testing has the advantage of unifying and standardising the testing procedures. In addition, assay performance is usually superior to POC tests. In centralised testing, only a small proportion of overall expenses is usually accounted for by test reagent price, while logistics, labour and other indirect costs are responsible for most of the expenses.

At the same time, the use of POC tests has increased in general. The trend towards POC testing can be explained by the aims of health care to improve patient care and cost-effectiveness by shortening turn-around-times and by evaluating the overall cost effects (von Lode 2005). The development of suitable technique platforms for POC is reinforcing this trend.

It is likely that a balance between the two trends will be reached, so that clinical need and the nature of the tested analyte will determine the applied testing scheme (von Lode 2005). Analytes for which rapid

results are essential and/or for which cost-effectiveness can be shown, will be tested at the point-of-care.

When rapid diagnostic methods are implemented for POC testing of infectious diseases an important question is whether the method(s) has a positive impact on the overall management of infections. The test should lower the economic burden caused by the disease for the patient, employee, or for the health care system. This is realized by reduced hospitalization, mortality, number of doctor visits, prescription of antibiotics, and spreading of infections.

Despite the limitations of the current rapid tests methods, cost-effectiveness and the positive impact on patient management has been shown (Woo 1997, Barenfanger 2000, Noyola 2000, Sintchenko 2002, Gerber 2004).

Ideal test for diagnosing respiratory tract infections at the POC

Because of the multidimensional nature of RTIs, any test that concentrates on only one RTI virus to the exclusion of others must give a biased perspective on virus diseases in general (Madeley 2007). Hence, such tests have very limited diagnostic impact. In addition, identification of one RTI pathogen should not impede testing for other pathogens (File 2003). Accordingly, a clear trend towards multianalyte methodologies can be observed in the markets. The trend is dictated by clinical needs as multianalyte testing is needed to allow accurate pathogen-specific diagnoses. A multianalyte pathogen test method for *S. pyogenes*, *S. pneumoniae*, and for influenza A and B, RS-, metapneumo, adeno, and parainfluenza viruses would be able to detect most of the significant respiratory infection cases. Rapid lateral flow tests (one or two parameter tests) for most of these pathogens are available. Multianalyte reagents for immunofluorescence testing that cover many of the viruses are also available. Neither of these techniques, however, is well applicable for routine multianalyte POC testing.

An ideal diagnostic test for POC testing of RTIs should allow multianalyte pathogen testing from easily obtained samples. Most importantly, the product should allow accurate differentiation between bacterial and viral infections (Linde 2001). The test should also identify influenza viruses and RSV to allow targeted medication with specific antivirals. It would be valuable if the test also provided results for the other frequent viral pathogens. The clinical value of these tests would mainly be in ruling out more severe bacterial infections and to help the disease management. From the patient point of view, these

tests provide added value as an exact diagnosis for the symptoms can be obtained. Antiviral drugs for respiratory viruses other than influenza are expected to become available in the future. This would further increase the clinical value of such a multianalyte test. On the other hand, the lack of rapid and sensitive POC methods for many of the RTI pathogens constitutes a challenge for the commercialisation of virus-specific drugs (Abed 2006). Hence, the development of antiviral drugs and rapid assay methods should go hand-in-hand, as a specific assay result is needed to justify the use of virus-specific drugs.

Technical requirements of an ideal product for POC testing of RTIs include (i) ease of use, (ii) short turn-around-times, and (iii) robustness. The product should be (iv) suitable and designed for multianalyte detection, and it should provide (v) high sensitivity and (vi) specificity, as well as (vii) sufficient dynamic range. Results (viii) read-out should be automated in order to obtain unambiguous results and to enable (ix) connectivity to a health-care data network. The methodology should allow (x) quantitative analysis to better enable differentiation of healthy bacterial carriers from those with the disease. The reagents should have (xi) long shelf-life. As the success of sampling sets the limits for the quality of the test results, (xii) a general marker assessing the specimen quality would be of great value (Madeley 2007). So far, these objectives have not been reached by a single assay technique (Yager 2006).

1.2.3 New techniques proposed for rapid point-of-care testing

Commercial point-of-care compatible techniques include the ***Afinion system*** from Axis Shields PoC (Scotland). The Afinion concept consists of a bench top analyser and multi-compartment dry-chemistry testing cartridges. The detection system registers the transmitted or reflected light of the reactions, and converts it to a test result (www.axis-shield.com). Only modest sensitivity can be achieved using this technique because of the detection principle. So far, the company has launched tests for three blood analytes, while their clinical reference concentration ranges are high. Implementation of the test system for many of the POC analytes is limited due to insufficient sensitivity. The system is also characterised by high assay costs due to the complex cartridge design. Although the instrument can be used for several applications, multianalyte testing is probably not feasible.

The Triage® system by Biosite Inc. (USA) is aimed at near-patient testing of drugs and proteins. This platform applies dried reagents,

micro capillaries, and fluorescence energy transfer assay technique (www.biosite.com/products/triage.aspx). The company has launched multianalyte tests for parasites and for profiling of shortness of breath. The analytical sensitivities are significantly lower compared to standard laboratory methods (Clark 2002).

i-STAT by Abbott Laboratories (USA) is based on single-use test cartridges (www.abbottpointofcare.com/istat/index.asp). The test system applies enzyme conjugates and electrochemical sensors fabricated on a silicon chips. The cartridge wash fluid contains substrate for the enzyme. The product is detected electrochemically (amperometrically). The sensitivity of the technique reaches that of ELISA (picomolar level, Apple 2004). However, the complicated test cartridge makes the test expensive and demarcates the applications to those where multianalayte testing is not needed and high assay cost is acceptable (e.g. acute myocardial infarction testing).

Gyrolab Bioaffy® CD microlaboratory by Gyros (Sweden) applies a disc-shaped microfluidic device, where fluidics is realized by centrifugal force, and detection is based on fluorescence (www.gyros.com). This technique has been shown to provide multianalyte detection and sensitivity comparable to ELISA, whereas implementation in routine use has been hindered by problems in sample dispensing, transfer and mixing of fluids, signal intensity, large size of the instrumentation, complicated assay procedures, miniaturised disc design, and high assay costs.

Response Biomedical Corp (USA) has developed a ***RAMP^{TM} technique*** for quantitative immunochromatography that is based on the use of two different antibody-coated particles with two different fluorophores doped inside (www.responsebio.com). One particle type works as an internal control and the other as a specific tracer. The fluorescence from both chromatography lanes is measured. The signal ratios are used for reducing the variation arising from membrane properties, environmental changes, and internal inaccuracies. The sensitivities are close to standard ELISA (picomolar level, Wu 2004). Multiplexing is propably difficult to achieve with similar performance.

The ***OIA®*** Optical ImmunoAssay sold by Thermo Electron Corporation utilizes dependence of reflective properties of light from the mass accumulated on coated surfaces due to immunoreaction. The assay procedures are often multistep and, despite this, the assays are at best semi-quantitative in nature and suffer from inadequate sensitivity (Schultze 2001).

Åmic (Sweden) has developed a ***4Castchip*** that uses redesigned lateral flow strips. The liquid flow is driven by micro pillar capillaries instead of the lateral flow of chromatography paper. The detection is done by fluorescence. Comparable sensitivity to ELISA has been demonstrated (www.amic.se). Compared to nitrocellulose strips, the production cost of the 4Castchip is expected to be higher since it can not be processed in-line (O'Farrel July 2006). This arises from the fundamental differences between the manufacturing processes. The applicability of the technique for multianalyte detection remains to be shown. The product has not yet been launched on the markets.

Immunobiological sensors are characterized by being label free. The signal measurement is based on changes in physical properties of the test system components. Such sensors have been developed by several groups. Sensors are known to have problems associated with antibody attachment to solid-phase, sample matrix components, reagent stability, sensitivity of the used enzymes for environmental changes, vulnerability to extrinsic factors, repeatability, non-specific binding, and multistep assay protocols. Current assay sensitivities seem adequate only for drug screening and clinical chemistry, and the price of the chips tends to be high. (Wild 2005) Two sensor techniques have evoked especially high interest within the diagnostic field.

The SensiDX^{TM} biosensor by Ambri Ltd sensor systems for POC testing is based on changes in ionic conductivity of ion channels fused with analyte-specific antibodies on membranes. Currently, the development and the commercialisation have come to a halt because of several drawbacks encountered (www.ambri.com). Also surface plasmon resonance-based biosensor techniques developed by several groups suffer from low throughput, poor sensitivity, high assay cost, and need for a washing/separation system. Thus, they are generally more useful in research than in clinical POC testing.

Lab on a Chip is a term used to denote miniaturized microfluidic assay systems. The miniaturizing of reaction cartridges may bring several advantages such as faster reaction kinetics, better separation, and more rapid temperature changes, while the total assay system including the detection device may still be large (like a mass-spectrometer). Within recent years, lab-on-a-chip applications have been of great interest and have been actively developed. So far, the chips have been too expensive for POC applications because of the complicated chip designs needed to carry out the analytical tests, especially liquid handling and the separation of unbound reaction components (Kartalov 2006). Sensitivities of the current techniques are sufficient only for a

limited number of applications. Despite the huge resources put into the development of these techniques, they have not yet made significant market breakthroughs in the *in vitro* diagnostic field. Instead, most of the microfluidistics companies are in the field of pharmaceutical and biotech research. Genomics are overrepresented compared to proteomics and clinical diagnostics (Haber 2006).

1.3 Antimicrobial susceptibility testing

During the last decade, antimicrobial resistance of the clinically relevant bacteria has increased at an alarming rate (WHO 2005). The most significant resistance bacterium in terms of overall economic impact, treatment failures, and workload is methicillin-resistant *Staphylococcus aureus* (MRSA) (Gould 2005). *S. aureus* is often found in the human flora. However, as a result of trauma and the subsequent failure of the immune system to control the infection, the bacterium may cause clinical infections. For effective infection management in hospital, all patients at increased risk of MRSA carriership should preferably be contact isolated until tested negative (Kotilainen 2003).

MRSA strains are not necessarily more pathogenic than susceptible strains, but the treatment of MRSA infections is more demanding and expensive. It seems that MRSA infections have double the mortality of susceptible strain infections, at least partly explained by the inappropriate empirical treatment. The economic burden of resistant microbes arises mainly from indirect costs of morbidity and diminished quality of life; and from surveillance and control activities, including disinfection, patient contact isolation, and decontamination. Additional costs of MRSA infections amount to thousands to tens of thousands of dollars per hospitalized patient. (Gould 2005)

1.3.1 Methods based on culture

Conventional methods for antimicrobial susceptibility testing (AST) rely on microbial culture. These phenotypic methods usually have good correlation with the *in vivo* response of the drug. Culture methods are flexible and can be applied with minor modifications to new antimicrobial resistance strains. Standard disk-diffusion tests, Etests and broth dilution methods are, however, labour-intensive and prone to subjective observation. They also have long turn-around-times. Instrument development has aimed at shortening hands-on-times and increasing the reliability of the tests by automation (Felmingham 2001). Despite these efforts, turn-around-times of the assays have not

essentially decreased, since the methods still require the samples to be pure cultured isolates. New methods have been introduced in recent years but most of them still use culture at some point of the work flow.

1.3.2 Methods based on immunoassay

Immunoassay AST methods can apply different methodologies. Some of the methods are indirect and based on the correlation between certain antigens and phenotypic resistance. Because these antigens are usually not species-specific, the methods require pure culture of the samples. Rapid tests for MRSA usually detect protein PBP2a (alternative penicillin binding protein) which is involved in the cell wall synthesis (Hartman 1984). The protein has lower affinity for most β-lactam antibiotics compared to native PBP proteins, and this inhibits the antibiotic activity. Thus, detection of this protein is indicative of a resistant strain. Unfortunately, coagulase negative bacteria show the same resistance mechanism, and therefore pure culturing is needed prior to detection (van Leeuwen 1999).

Another principle relies on rapid identification of the bacterium after the culturing of pure cultured strains in the prescence of antibiotics. The tests for MRSA detect one or several target antigens simultaneously, such as clumping factor, protein A, group-specific antigens, and capsular polysaccharides (Wichelhaus 1999). Most of the tests rely on latex agglutination.

Very recently, a new test for rapid screening of MRSA, the BacLite™ Rapid MRSA Test, was introduced by 3M (www.3m.com). The test allows analysis directly from clinical samples within 5−24 hours. In the test, samples are first cultured in microtitre wells using a selective liquid growth medium. Then *S. aureus* cells are extracted with antibody-coated particles and further cultured in the presence of the antimicrobial. Finally, the cells are lysed and adenylate kinase activity is measured using bioluminescence. In contrast to conventional methods, this approach allows MRSA screening directly from clinical samples due to the extraction with antibody reagents. Similar AST approaches based on ELISA are known to be in in-house use, although the number of scientific reports and commercial tests applying the principle has so far been low. A major reason for this might be the need for washes and multistep assay protocols (including the test by 3M). In consequence, such tests do not provide significant improvements compared to conventional culture in terms of price and labour intensiveness.

1.3.3 Methods based on nucleic acid amplification

Genotypic methods are based on detection of determinants of the antimicrobial resistance at the genetic level and apply nucleic acid amplification (NAA). This approach is based on the correlation between genetic sequences and phenotypic antimicrobial resistance. Development of these methods has been driven both by the clinical need for rapid diagnostic tools and the increasing knowledge about genetic properties of the pathogens.

Most of the genetic methods for MRSA rely on testing for the *mecA* gene that encodes the PBP2a protein. Most coagulase negative bacteria have also the *mecA* gene, and they are abundant in clinical samples (Hyvärinen 1995). Thus, detection of genetic material of *S. aureus*, and the *mecA* gene directly from the screening specimen (not pure cultured) is not reliably indicative of MRSA infection (Becker 2006). Hence, pure culture is usually needed. Just recently, genetic methods that allow identification of MRSA directly from clinical samples have been developed. The detection of the *mecA* gene in these methods is linked with the detection of *S. aureus* specific genes from the same physical genome (Huletsky 2004).

Some authors believe that the NAA methods are about to replace the conventional phenotypic methods, and see them as alternatives or competitors with each other (Global Industry Analysts). However, the methodologies have different strengths and limitations, and they give answers to different analytical and clinical questions.

In principle, the NAA methods have very high analytical sensitivity and specificity in the detection of potential resistance genes. However, correlation to *in vivo* antimicrobial susceptibility can be poor. The presence of a resistance gene does not necessarily lead to treatment failure or *vice versa*. Poor correlation can arise from varying expression levels, or the resistance sequence can be from a silent or pseudogene (Sundsfjord 2004). On the other hand, the NAA methods may be superior compared to conventional culture in detecting heterogenically resistant strains.

The final steps of NAA and detection of the copy products can be seemingly rapid. This speed and the accuracy of finding certain resistance genes have been used to justify testing with NAA methods, which are more expensive than the phenotypic methods. Most of the NAA methods have so far required the sample to be pure cultured isolate, and the nucleic acid to be in extracted form. Hence, the sampling to result time has shortened only a little. In general, market entry of NAA methods has been markedly slower than was anticipated

some years ago (Global Industry Analysts). With the advent of new NAA methods that allow genetic testing directly from clinical samples, the applicability of the methods is expected to increase significantly.

Genetic testing is based on the testing of resistance determinants, whereas the antimicrobial therapy decision should be based on detection of susceptibility (Sundsfjord 2004). Genetic methods are searching for what is already known. Gathering genetic understanding about a pathogen to construct a method has so far required huge research efforts. For these reasons, genetic methods are best suited to epidemiology, screening, confirmatory purposes, and for research, rather than for patient-specific medication. Genetic methods are suitable for testing known strains, and the results should be analysed carefully when making treatment decisions. Thus, conventional phenotypic methods can not be expected to be supplanted by NAA methods (Sundsfjord 2004). Phenotypic methods are needed to detect emerging new resistance mechanisms, and to obtain clinically accurate data (Fluit 2001, Sundsfjord 2004).

1.4 Clinical phase of an infection

1.4.1 Systemic markers of acute infection

There are several systemic markers with concentrations correlating with the acute phase of inflammatory and immune systems, and they can be used in evaluation of the infection phase. They are also used in differential diagnosis of viral and bacterial infections (see 1.2.1.1). Positive test results should usually be followed by more specific clinical examination and laboratory tests.

1.4.2 Specific markers of infection

To enable high clinical accuracy of diagnoses, patients should be tested for disease-specific markers. When direct pathogen detection is not feasible, the most accurate results are provided by pathogen-specific antibodies (serodiagnostic testing). In screening applications, a qualitative test result is often sufficient. In other applications, more detailed quantitative information is needed in order to differentiate between primary, secondary and past infections.

Class-specific detection

In serodiagnostic testing, the infection phase and clinical status are usually determined by class-specific analysis of antibody responses. Although class-specific methods are widely used, they are known to

sometimes have poor correlation with the clinical phase of the infection. Methodological limitations include proneness to matrix interference caused by auto- and heterophilic antibodies (such as rheumatoid factor and inter-species antibodies), and by the complement. In addition, the signal response is contributed to by both the antibody concentration and the variable affinity distribution of the antibody population. For this reason, traditional coated-tube techniques do not allow quantitative measurements of specific antibody concentrations. (Lehtonen 1982)

Clinical diagnosis based on the relative amounts of IgM and IgG is not always reliable due to inter-individual variation. In some patients, the primary IgM response may remain below the detection limit of the method, while in other patients, the IgM response can persist for years (Hedman 1989, Andersson 1994). Moreover, false positive detection of IgM can occur due to non-specific polyclonal activation (Meurman 1983, Thomas 1999, Roque-Afonso 2004). In addition, the scheme of the response may depend on the pathogen. Sometimes IgG-class antibodies are detectable in primary infection already during onset of symptoms (Hedman 1991).

In order to allow more reliable clinical diagnosis, class-specific methods often require paired sample analysis, in which changes in the abundance of specific antibodies in two consecutive samples are compared. The long delay in diagnosis limits the clinical value of the technique. Obtaining a laboratory diagnosis when the symptoms and the infection have already resolved does not allow optimal treatment.

Antibody bridging assay principle

Getting rid of washing steps has been a long cherished goal in the development of bioaffinity assay techniques. A fundamental problem of the wash-free methods is that they do not usually allow class-specific serology. The reason for this is that only small fractions (in the order of one tenth of a percent) of the antibodies in a certain isotype class are specific for a particular antigen. The majority of the isotype represent specificities to other antigenic species. The non-specific fraction is usually in huge excess to the immunoassay reagent. Hence, in one-step assay methods the non-specific antibodies will, in practice, saturate all the anti-immunoglobulin reagent binding sites. For this reason, class-specific detection requires separation of the non-specific isotype fraction before addition of the second immunoassay probe.

An alternative assay scheme for class-specific detection is antibody-bridging (AB). In this binding scheme, specific antibody molecules

(analyte) bind with one arm to the antigen in the solid phase, and with another arm to a labelled antigen reagent. Thus, the antibody molecules form a bridge between the two separate antigen molecules. In the industry, the principle is known to provide excellent performance, and it is applied in several commercial immunoassay products such as Prism™, Elecsys™, and Centaur® platforms. However, the number of published scientific articles is very low compared to class-specific methods.

The AB assay principle has several advantages compared to traditional class-specific techniques, including resistance to interference caused by matrix effects (Siitari 1990, Bürgisser 1996, Ankelo 2003), superior specificity (Miyazawa 1988, Siitari 1990, Bürgisser 1996, Horimoto 1997, Miyazawa 1999), low non-specific binding (Chang 1984, Horimoto 1997, Miyazawa 1999, Ankelo 2003, Westerlund-Karlsson 2003), and freedom from bias caused by differences in the affinities of anti-immunoglobulin reagents. The assay principle may also provide high sensitivity for IgM-class antibodies, allowing earlier detection of an immune response (Chang 1984, Gallarda 1992, Bürgisser 1996).

The antibody-bridging principle *per se* does not allow differentiation between primary and secondary or past infections, and this has been considered the main drawback of the assay principle.

Measurement of antibody avidities

Avidity index (AI) assay methodology in its various modifications is based on determination of the avidity distribution of the antibody population in the sample. It is an alternative approach to class-specific assessment of the infection phase, i.e. to differentiate between primary, secondary and past infections (Pullen 1986, MacDonald 1988, Hedman 1993). In primary infection, the avidity distribution is wide, and the avidities are low, on average. Secondary and past infections, in turn, are characterised by a narrow avidity distribution, and high avidity. In the avidity index technique, the sample is exposed, in parallel, to a normal assay buffer and to a buffer containing a chaotropic agent. The ratio of the two results gives the avidity index. The higher the average avidity of the antibody population, the larger the proportion of solid-phase bound analyte antibodies able to remain bound with the antigen in the presence of the chaotrope. Alternatively, the chaotrope can be present already in the primary sample incubation buffer where it prevents the binding of low avidity antibodies. If the AI exceeds an analyte-dependent threshold, the infection can be deemed past or secondary. (Thomas 1991, Hedman 1993) When the AI information is combined with the signal levels of the serodiagnostic method

(proportional to concentration), reliable estimates of the nature and phase of the infection can be made from a single clinical sample. The AI method does not require analysis of paired serum samples.

The suitability of the AI technique for differentiation between primary and secondary phase immunoresponses has been comprehensively demonstrated in the literature for many pathogens (Hedman 1993, Granoff 1998, Korhonen 1999). The AI technique provides good clinical performance compared with traditional class-specific methods. Despite the advantages of the AI technique, it is applied less widely than the class-specific methods at present. One explanation for this is that many of the methods must apply the so-called end-point titre AI determination, which is needed to obtain reliable AIs. In IgG AI techniques the obtained AI is often dependent on IgG concentration (used titre). Due to the high price of such tests in heteregenous assay platforms, the technique is mainly used in confirmatory tests.

1.5 Two-photon excitation assay technique

Recent developments in *in vitro* diagnostic testing have aimed at physical downsizing, simplified protocols, automated random-access operation, speed, and adaptability for point-of-care testing. An ideal technique would avoid physical separation steps and its performance would be independent of the reaction volumes. These should be achieved without compromises in accuracy, cost-efficiency, or reliability. Usually some goals are achieved at the expense of others.

The two-photon excitation (TPX) assay technique is a novel micro-analytical bioaffinity technique that potentially fulfils the above mentioned criteria. The technique applies polymer microspheres as solid phase carriers for bioaffinity binding reactions (**Figure 1**). The microparticles (3 000 per µl) act as local concentrators for the fluorescent tracer molecules (present in 1 nM concentration). The tracer concentrates on the solid surface according to the immunometric principle. The detection of the formed complexes is based on two-photon excited fluorescence measurement of individual microparticles. (Soini 2002a) Fluorescence is excitated with a pulsed near infra-red laser (1064 nm), while the fluorescence from individual microparticles is measured at visible wavelengths (Hänninen 2000, Soini 2000).

Most luminescence techniques use single-photon excitation where the wavelength of the illumination (excitation) is shorter (higher in energy) than the fluorescence emission. Such techniques usually have relatively large excitation volumes, and their signal is directly

proportional to the measurement reaction volume. Excitation with two simultaneous photons, in contrast, takes place only when the density of photons per unit volume and per unit time is high enough for two photons to be simultaneously absorbed by the same fluorophore. The absorbed energy is the sum of the energies of the two photons. This leads to a situation where two-photon excitation is achieved only in a volume that corresponds approximately to the size of a microsphere (~1 fl). The excited state is relaxed by emission of a photon of higher energy (shorter wavelength) compared to the illumination (**Figure 2**).

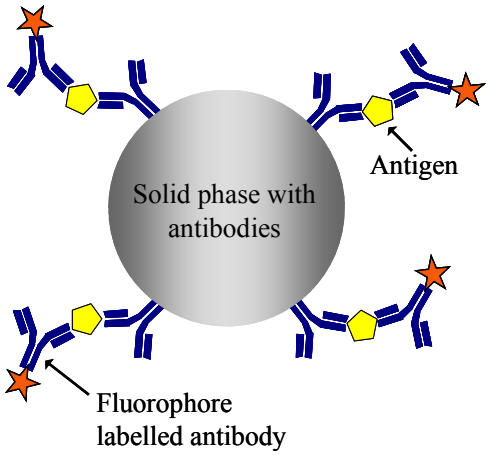

Figure 1. Schematic presentation of the immunocomplex formation between the solid phase and the tracer reagents with the analyte.

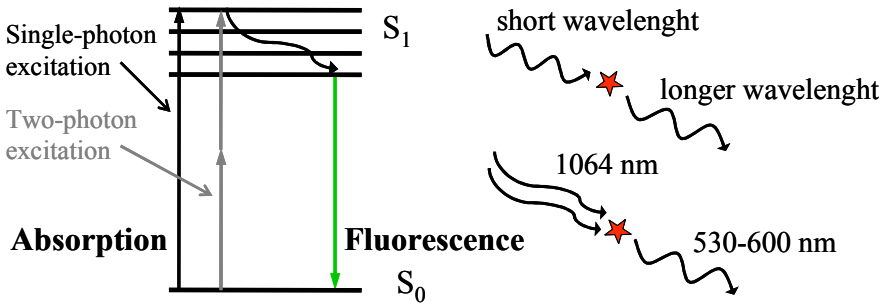

Figure 2. Left side shows a simplified Jablonski energy diagram for single- and two-photon excitations, and for emission (quantum nature of light). Right side presents schematically the wave nature of light in single- (up-right) and in two-photon (low-right) excitation.

In the TPX technique, discrimination of the fluorescence signal of the microsphere-bound tracer from the unbound tracer in solution takes place within the focal volume. The micro-fluorometer actively scans

the reaction mixture for microspheres, which are then trapped in the focus for less than 100 ms. The presence of a microsphere within the focal volume is indicated by the excitation light scattering signal in the infra-red region (Soini 2002a, Soini 2002b). When scattering is not detected, the signal is averaged for the solution background signal. When scattering is observed according to certain parameters and the microsphere is trapped in the focus, the signal is averaged for the microsphere signal. Accordingly, the optical system of the fluorometer separates the fluorescence of the bound tracer from the fluorescence originating from the unbound tracer free in solution. This enables the measurement of bioaffinity reactions without physical separation of the unbound tracer fraction. Hence the term, separation-free.

In two-photon excited fluorescence, excitation and emission take place in totally different wavelength ranges. For this reason, scattering of illumination light and fluorescence from optical components can be effectively eliminated by optical filtering. The measured signal is practically free of the scattering and the background fluorescence of the excitation. In practice, the background signals are governed by the concentration of the tracer free in solution, and not by the unspecific binding or instrument background as in coated-tube technologies.

Because of the extremely small excitation volume, the measured fluorescence signal, as well as the sensitivity and precision are independent of the reaction volume. Reagent consumption is very low because of low concentrations and small volumes. The separation-free assay format allows on-line monitoring of reaction kinetics (Hänninen 2000). Separation-free fluorescence measurement minimizes the need for automated liquid handling. A further advantage of the technique is that the microfluorometer provides information both from the microspheres and from the surrounding solution. This allows efficient flagging out and/or compensation of matrix effects.

When a molecular tracer is used, the sensitivities of immunometric assays are typically 1−10 pM, depending on the antibody affinities. The use of a nanoparticulate tracer enables sensitivities well within the femtomolar range (Koskinen 2004, Koskinen 2007). The TPX assay technique applies fluorescent labels which have been specially designed for two-photon excitation fluorescence (Meltola 2005c).

The TPX assay technique has been applied for a wide selection of bioaffinity assay types including antigens (Waris 2002), antibodies (Koskinen 2006), competitive binding assays (Vaarno 2003, Tirri 2005), recognition of nucleic acid sequences (Meltola 2005b), and single nucleotide polymorphism (Vaarno 2004).

2 Aims of the study

The continuous increase in drug-resistant microbe strains and the new virus-specific drugs will most likely lead to new more strict recommendations concerning the prescription of medication. The new recommendations are expected to call for *in vitro* identification of the pathogen before prescription. These will increase the need for new diagnostic tools which are adaptable for rapid point-of-care testing of infectious diseases. None of the mainstream rapid POC techniques allows efficient multianalyte detection, which is needed for efficient diagnostic testing of respiratory tract infections. The POC techniques used today no longer meet the requirements of today's healthcare delivery models (OECD 2003). Accordingly, the most urgent need for new rapid POC tests in the field of infectious diseases testing is in the segment of respiratory tract infections.

The purpose of this study was to evaluate the potential of two-photon excitation fluorometry as a diagnostic technique for rapid point-of-care testing of infectious diseases.

In more detail, the aims of this study were:

1. To study how the TPX technique is suited to high sensitivity detection of systemic markers of acute infections.

2. To study how sensitivity of the technique could be increased.

3. To study how the assay protocols of the technique could be further simplified.

4. To study the applicability of the technique for serodiagnostic testing.

5. To study the use of the technique for rapid testing of infectious diseases.

6. To study the suitability of the technique for real-time monitoring of microbial growth, and for antimicrobial susceptibility testing.

3 Materials and Methods

3.1 Materials

Most of the used chemical reagents, buffer compositions, antibody clones, antigens, and description of the clinical samples have been given in detail in Publications **I-V**. Those not described, are listed here. EDAC-sulfo-NHS-solution: 20 mM EDAC (N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride), 200 mM sulfo-NHS (N-hydroxysulfosuccinimide sodium salt) in 25 mM MES (2-(N-morpholino)ethanosulfonic acid) buffer. Glycine-BSA -solution: 100 mM glycine, 1 % BSA (bovine serum albumin), pH 8.5. BSA solution: 10 mM Tris-base, 0.01 % Tween 20; 20 % BSA, and 10 mM NaN_3, pH 8.0. Nanoparticle gel filtration -buffer: 10 mM Tris-base, 10 mM NaN_3, and 0.01 % Tween 20, pH 8.0. Nanoparticles (co-polymer of styrene and acrylic acid, diameter 55 nm, solid content 5 %) were a generous gift from Dr. Harri Härmä (University of Turku, Finland), and were prepared as described earlier (Huhtinen 2005). Dialysis tubes (Spectrapor Dispodialyzer cut off 10 kilodalton) came from Spectrum Laboratories Inc. NAP-5 and Superdex 200 (preparative grade) gel-filtration columns came from Amersham Biosciences Inc. The Nanosep centrifugal device (Omega 300 kilodalton) was from the Pall Corporation (NY, USA).

3.2 Methods

Coating of microspheres

Coating protocols for the microsphere reagents have been described in Publications **I-V**. Typically, antibody (0.2 mg/ml in 50 mM MES buffer pH 5.5) coating was done by passive adsorption to the microsphere surface (1.5 µg of protein / cm^2 of diameter 3.2 µm microsphere surface) followed by fixation with EDAC in 50 mM MES buffer pH 5.5 (0.5 mg/ml). EDAC cross-links free amine groups of the proteins to carboxylic acid groups of the proteins and the microsphere surface. This makes the microsphere reagents less susceptible to protein leaking. It also improves their binding capacity. Antigens were coated either using a similar method or via a biotin-avidin linkage (**III**).

Labelling of antibodies and antigens

Antibodies and antigens (2 mg/ml in 100 mM $NaHCO_3$, pH 8.4) were labelled with succinimidyl ester of the fluorescent labelling reagent BF 530 as described by Meltola *et al*. (2005a). The reagent binds covalently to free amine groups of the target protein molecules. After three hours of incubation (at room temperature, in dark) the protein-fluorophore conjugate was separated from the unreacted fluorophore by using size exclusion chromatography (NAP-5 column, phosphate buffered saline). The protein specific parameters (concentration of the labelling reagent) and the resulting conjugation degrees have been described in Publications **I-V**. For IgG, the optimal conjugation degree (fluorophores per protein molecule) as evaluated by immunoassay performance (sensitivity) was four fluorophores per molecule, and for other proteins, it was scaled in relation to the molecular weight.

Preparation of nanoparticle tracers

A solution of methyl ester derivative of the fluorescent dye ArcDia^{TM} BF 530 (500 µl, 2 mg/ml in dimethylformamide) was slowly added to a suspension of nanoparticles (500 µl of the stock supernatant) while vortexing. The mixture was incubated at +6°C for one day, and water (1 ml) was added. The mixture was transferred to a dialysis tube, and dialyzed against 1 litre of 50 % ethanol and water. The nanoparticle solution was transferred to a glass tube for storage, and NaN_3 was added to the final concentration of 10 mM. The solid content of the resulting solution was 0.5 % (7.3 x 10^{16} particles/l). The nanoparticle solution was analysed with a photon correlation spectrometer (Coulter N4 Plus submicron analyser, Beckman Coulter Inc., Fullerton, CA, USA). This indicated a mean nanoparticle diameter of 55 nm.

Equal volumes (240 µl) of fluorescent nanoparticles (1200 µg solid) and MES buffer (50 mM) were combined. To this solution, a 1/10 volume unit of freshly prepared EDAC-sulfo-NHS-solution was added while vortexing, and the reaction was bath-sonicated for 30 seconds. The reaction mixture was stirred in the dark at room temperature (Eppendorf ThermoShaker, 1400 rpm) for 10 minutes, sonicated again for 30 seconds, and stirred for another 10 minutes. The reaction mixture was eluted through a NAP-5 column with MES buffer (25 mM). The solution was concentrated to a volume of 240 µl by centrifugation with Nanosep (300 kilodalton). An aliquot (40 µl, 200 µg solid) of this solution was added drop by drop to monoclonal antibody solution in carbonate buffer (120 µl, 2.5 mg/ml) while vortexing. The solution was bath-sonicated for 30 seconds, and stirred

at room temperature for 6 hours. One volume of glycine-BSA solution (160 µl) was added to the solution while vortexing, and stirring was continued overnight at 10°C. The solutions were gel filtrated (1 ml/min, Superdex 200 preparative grade) with Merck Hitachi HPLC (High performance liquid chromatography) using gel-filtration buffer. Prior to the gel-filtration, the solutions were bath-sonicated for 15 seconds. Fractions (á 330 µl) containing the antibody-coated nanoparticles were combined. BSA solution was added to the final concentration of 0.25 %. The concentration of the solution was determined by fluorometry in relation to the original stock. The size distribution of the nanoparticles was determined with Coulter N4 Plus.

Preparation of dry-chemistry reagent assay plates

Method specific assay protocols, preparation of assay reagent mixtures (**I-V**) and dry-chemistry assay wells (**II, IV**, and **V**) have been described in detail in the original Publications. Typically, equal volumes of microsphere and tracer predilutions (2−4 x 10^7 pieces / ml, and 2−4 nM for molecular and 10−50 pM for nanoparticle tracers, respectively) in dry-chemistry assay buffer were combined, and dispensed (5 µl) with an automate dispenser onto 384-well plates, followed by drying over silica gel in a desiccator with a fan (at room temperature, in dark, over-night) (**II**, **IV**, and **V**). The plates containing the reagents in the dry form were stored in laminated foil bags with silica gel until use.

Typical assay protocol

For an assay, a dry-chemistry reagent plate is removed from the storage bag, an appropriately diluted sample in assay buffer is dispensed (20 µl) onto the wells, and the wells are sealed with a plate-sealing film. The plate is inserted into an ArcDia^{TM} TPX Plate Reader for shaking, incubation, and separation-free fluorescence measurement.

4 Results

4.1 Preparation and optimization of immunoassay reagents

The protocols for antibody coating (Waris 2002) and protein labelling (Meltola 2005a) used in this work usually provided excellent assay reagents (**I-V**). Further optimization of coating conditions (such as pH) rarely improved assay performance (**unpublished results**). This indicates the robustness and wide applicability of the coating methodology. For optimal coating of antigens, however, it turned out that biotin-(strept)avidin coating is often needed to maintain high immunological activity (**III**). For IgG-molecules, the optimal labelling degree turned out to be four fluorophores per antibody molecule (**IV**) which is in line with previous results (Meltola 2005a). The protein−fluorophore conjugates (ArcDia^{TM} BF530, Meltola 2005c) demonstrated stability and good performance.

Optimization of assay microsphere and tracer concentrations was done by cross-testing different concentrations of the reagents (in reaction $1.5{-}5{\times}10^6$ microspheres/ml and 0.25−4 nM tracer) (**I−V**). The theoretical and practical aspects of optimization are discussed in Publication **V**. Most often close-to-optimal assay conditions can be selected on the basis of previous experience. Usually, only a modest improvement (a factor of two) can be achieved by further optimization of the concentrations. Nevertheless, cross-testing of different reagent concentrations on the TPX platform is easy due to simple assay protocols. The on-line measurement option provides an easy means to optimize the concentrations also with regard to assay kinetics.

During the method development work, reagent antibody clones were typically optimized by testing a number of different antibody clones in all feasible combinations. For the influenza B virus method, clone optimization was needed because the antibodies applied in the reference method did not provide good sensitivity in the TPX platform. Despite the testing of seven different antibody clones, the method failed to provide sufficient clinical sensitivity with the molecular tracer. Hence, we had to apply a nanoparticulate tracer approach which was also optimized with respect to antibody clones (**IV**). According to our experience, the performance of antibody clones can vary significantly by detection platform (**IV**) and by tracer type (molecular versus nanoparticulate tracer, **Appendix 2**).

4.2 Dry-Chemistry reagents

The development and use of dry-chemistry reagents is described in Publications **II**, **IV**, and **V**. The common drying protectants, sorbitol and trehalose turned out to be compatible with the reagents of the TPX technique. Rapid and thorough dissolving of the dry-chemistry reagents, especially the microspheres, required efficient shaking of the assay plate. Once well shaken, the dried reagents were rapidly suspensed (**II**). Inclusion of the drying protectants in the wet-chemistry reagents did not affect the immunoassay kinetics (**unpublished results**). Based on these observations, it is safe to assume that in clinical point-of-care testing, the dry-chemistry reagents give as rapid and as sensitive results as the wet-chemistry reagents. The use of dried reagents allowed markedly simplified assay protocols (one-step).

In Publication **IV**, the dry-chemistry reagent approach was applied successfully also for a nanoparticulate tracer. This was expected to be challenging because of potential problems in colloidal stability and in unspecific binding, which tend to increase in conventional coated-tube techniques. The dry-chemistry reagents provided the same clinical results as the original wet-chemistry method. The wet- and the dry-chemistry methods also showed excellent signal correlation between each other for the clinical samples. Pearson correlation coefficients for influenza A and B virus methods were 0.97 with $P<0.0001$, and 0.96 with $P<0.0001$, respectively.

In Publication **V**, the dry-chemistry reagents were applied in a method for antimicrobial susceptibility testing of *S. aureus*. Both the anti-microbial agent and the immunoassay reagents were dried onto the reaction wells. Also in this application, the dry-chemistry reagent method gave the same results as the corresponding wet-chemistry reagent method. The dry-chemistry reagents retained their functionality during a stability testing period of three months (**II**, **IV**).

Unexpectedly, unspecific binding was not observed with the dried reagents despite the solid phase microspheres, and the tracer were stored in the same physical protective medium (**II**, **IV**, **V**). When dried reagents are applied in heterogeneous assays formats, such as TR-FIA, the tracer must be physically separated from the solid phase in order to minimize unspecific binding.

4.3 Nanoparticulate tracers

The detection sensitivity (lowest limit of detection) achievable with conventional molecular tracers in the TPX detection technique is 1-5

pM. This sensitivity, however, is not sufficient for all the clinically important analytes. For example, human thyroid stimulating hormone (hTSH, **Appendix 2**) and cardiac markers require subpicomolar detection sensitivity. In Publication **I**, we described a sensitive assay method for human C-reactive protein (CRP) that applied a nanoparticulate tracer (NPT). These NPTs were custom-made by a collaborator. The objective was to develop a method for the detection and monitoring of systemic inflammation/infection markers. The nanoparticulate tracer improved the detection sensitivity compared to the molecular tracer method by two orders of magnitude. The assay showed a dynamic assay range of 0.01−30 mg/L. The sensitivity was significantly better compared to most commercial methods (Roberts 2000). The method applied a total serum sample dilution factor of 1 000; thus the detection limit in the reaction mixture was 10 pg/ml (90 femtomolar). (**I**) If higher CRP levels are to be measured, more diluted samples must be used.

Subsequently, the CRP method has been used for studies of diabetes (Waris 2005), multiple sclerosis (Soilu-Hänninen 2005), photochemical photobiology (Laihia 2005), and general immunology (Koskinen 2003). A major clinical finding in these studies was that the serum CRP levels of healthy individuals were markedly lower (0.1-1 mg/L) compared to previous understanding (**I**, Koskinen 2003). Another interesting finding was that the serum CRP levels were sensitive even to the slightest inflammation conditions, such as sunburns and common flu (Koskinen 2003, Laihia 2005). To allow reliable assessment of general inflammation marker baseline levels, several consecutive samples, separated by at least a few weeks in time, should be analysed.

Despite extensive optimization of influenza B virus methods using a molecular tracer, this method failed to provide sufficient clinical sensitivity (**IV**). It is well known that assay methods for influenza B virus antigen detection are generally characterized by lower clinical sensitivity compared to methods for influenza A virus. In order to improve the performance of the influenza B virus method we chose to apply NPTs. Since the performance of a NPT is heavily dependent on the antibody properties we studied five alternative monoclonal antibody clones, and tested them against seven antibody clones on the microspheres. The most promising combinations were further optimized with respect to NPT concentrations.

For the preparation of nanoparticulate tracers, polystyrene nanoparticles were first impregnated with two-photon excitable

fluorophores. Based on fluorescence intensity measurements, each nanoparticle contained 250 fluorophores, while the effective fluorescence yield corresponded to the fluorescence of 150 molecules (**unpublished data**). For coupling with anti-influenza B virus antibodies of different clonalities, carboxylic acid groups on the fluorescent nanoparticle surface were activated using two-step EDAC - sulfo-NHS activation. After the coating with the antibodies, glycine and bovine serum albumin were added to deactivate the unreacted carboxyl groups and to block free sites on the polystyrene surface, respectively. Unconjugated antibodies were separated from the nanoparticle antibody tracer by size exclusion chromatography.

Aggregation is a common problem with nanoparticles. Aggregation results in higher signal variation and tends to increase unspecific binding. These compromise assay sensitivity and impair precision. During the NPT development, all the physical steps were chosen so as to minimize the aggregation of the nanoparticles. For example, gel-filtration was used instead of centrifugal filter devices. Conjugates showed rather narrow size distribution in photon correlation spectrometry, whereas mean diameters between the clones showed more significant variation (see **IV**). Typically, the diameters of the NPTs were 100−200 nm (before coating 55 nm) which is in line with the results reported by others (Soukka 2003). Thus, the NPTs seemed to consist of small aggregates of few nanoparticles. Passive adsorption methodology applied in initial studies resulted in significantly larger aggregates. Those NPTs showed slower kinetics and lower sensitivity.

The NPT method showed markedly increased signal-to-background ratios (a factor of 3–10), compared to the molecular tracer method. This allowed rapid detection of influenza B virus antigens. We also studied the applicability of the dry-chemistry reagents with the NPT reagent, and found that they could be stored dried without compromising the assay performance. (**IV**)

Usually, the signal imprecision of assay methods based on the TPX detection technique is independent of the analyte concentration. The NPT methods, and especially the method for influenza B virus, made an exception by showing a clear trend. Low analyte concentrations had higher imprecision (**IV**). The trend might be explained by molecular level phenomena as with high analyte concentrations, MT and NPT signal imprecisions were similar. Influenza B virus antigens are most probably in the form of large antigen complexes composed of nucleoprotein and nucleic acid. The binding of antigen complexes and NPT reagent to microspheres thus follows the Poisson distribution.

Hence, when the amount of nucleoprotein complexes approaches the number of microspheres (about 5−50 per microsphere), the number of bound NPT per microsphere varies by tens of percents. Also, variation in the number of nanoparticles per reagent aggregate should manifest itself in this manner. This trend was not observed with the NPT for CRP (**I**), most probably because this reagent (and the CRP analyte) was uniform and had narrow size distribution. In contrast to other NTP reagents, CRP tracer was prepared by coating the nanoparticles with $F(ab)_2$-fragments (instead of IgG). In our experience, coating with Fab-fragments results in smaller nanoparticle tracer conjugates compared to intact IgG´s of the same antibody clone (**unpublished data**). This is probably due to the lower tendency for an unspecific interaction as the Fc-fragment is more hydrophobic than the Fab-fragments.

4.4 Antigen detection of respiratory tract pathogens

Publication **IV** describes the development and validation of methods for rapid detection of influenza A and B virus antigens from nasal specimens. The performance of the methods was compared to TR-FIA reference methods. The new methods were tested using both wet- and dry-chemistry assay reagents. The wet-chemistry reagent assays were carried out using the kinetic measurement option to evaluate the positive detection rate, defined as a fraction of the samples deemed positive at a certain time point.

The key results of Publication **IV** were (i-vi):

(i) A rapid method for detection of influenza A virus antigens was developed and validated. At the 20-minute incubation time point, 23 out of 24 positive samples were determined as positive by the new method, while two borderline samples remained negative at this time point (**Figure 4**). At 60 minutes, all of the positive and borderline samples were deemed positive (sensitivity 100 %). In addition, the new method found one extra positive sample compared to the reference method. This sample was positive also in PCR. Furthermore, one sample which was TR-FIA positive but PCR negative was found negative in TPX. No cross-reaction was found to influenza B virus positive samples, indicating a specificity close to 100 % (N = 33).

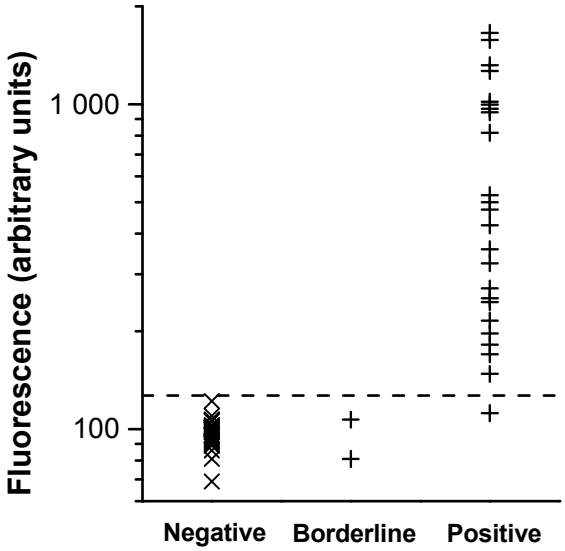

Figure 4. Performance of the method for influenza A virus detection at the 20-minute time point. The dashed line represents the cut-off value set for a positive test result.

(ii) A sensitive method for rapid testing of influenza B virus antigens was developed and validated. According to the results, at 25 minutes of incubation, 25 out of 25 positive samples were determined as positive, and four borderline samples were tested as negative (**Figure 5**). At the time point of 120 minutes, two of the four borderline samples barely cut the analytical detection threshold (intra-assay 3SD) set for positive detection in TPX, while these samples did not reach the method-specific cut-off (inter-assay 3SD). Of the two samples that remained negative in TPX one was positive by PCR and the other was negative by PCR (false positive in TR-FIA). No cross-reaction was found to influenza A virus positive samples, indicating a specificity close to 100 % (N = 36).

(iii) The results showed that the wet- and the dry-chemistry reagent methods provided the same results for each patient sample. Also application of NPT as dried reagent provided good performance without compromising sensitivity, kinetics, or precision.

(iv) The methods were validated by running a large number of clinical samples that included interfering matrix components. The results showed that the technique is robust and tolerates particulate material, mucus, and haemoglobin well. The TPX technique has

the unique property of giving separate information about the specific immunoreaction (microsphere signal), and about the matrix effects (solution background signal). This allows compensation and/or flagging out of the matrix effects.

(v) Due to the on-line measurement, most of the positive samples (about 90 %) can be detected rapidly within 20 minutes. This is possible without compromising in the overall assay sensitivity as the confirmation measurement is done after 2 hours of incubation (immunoreactions close to equilibrium).

(vi) The TPX technique would allow rapid respiratory pathogen testing at the POC with similar sensitivities and specificities as obtained with conventional techniques in laboratory conditions.

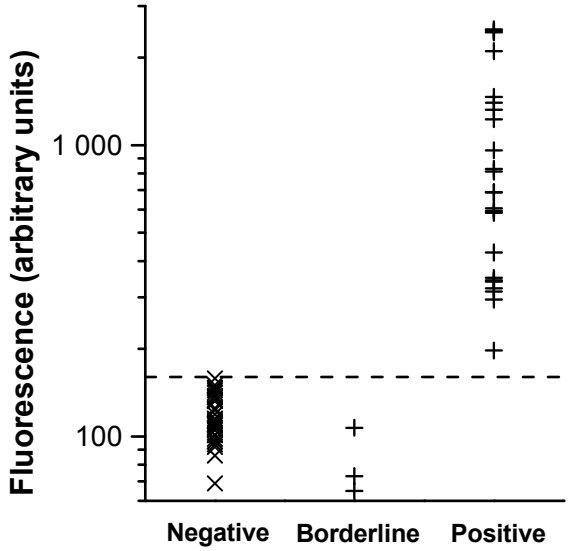

Figure 5. Performance of the method for influenza B virus detection at the 25-minute time point. The dashed line represents the cut-off value set for positive test result.

When considering the treatment of influenza virus positive patients, the subtype information (influenza A or B) is of minor importance because of the similar treatment. Keeping this in mind, we developed a duplexed assay for rapid detection of influenza A and B viruses. The method applies microspheres specific for both the viruses and a combination of two separate tracers. The concomitant assay method is described in **Appendix 3**.

4.5 Detection of specific antibodies

Applicability of the TPX technique for rapid serodiagnostic testing was studied and discussed in Publication **III**. Separation-free detection techniques do not allow conventional class-specific detection of specific antibodies. Instead, an alternative binding principle must be applied.

The antibody-bridging (AB) binding principle provides an alternative means for serodiagnostic testing. An assay method for adenovirus-specific antibodies was developed to prove the principle of this type of assay on the TPX platform (**III**). The response of the new AB method was compared to the response of a standard class-specific ELISA method. This study revealed that the new method allows detection of Immunoglobulin G and A class adenovirus (hexon antigen) -specific serum antibodies, whereas the method was unable to detect IgM-antibodies. IgM contributed to the signal response only when IgG was also present in high concentrations. The failure to detect IgM alone was accounted for by the inability of IgM antibodies to bind simultaneously solid-phase antigens and soluble tracer antigens (**III**).

An interesting kinetic phenomenon was observed in the new assay method (**III**). For IgG-molecules, at low analyte concentrations, the maximum signal was reached within 60 minutes, after which the signals started to decrease (**Figure 6**). At higher concentrations, normal immunoassay kinetics was observed. We explained this phenomenon by bivalent irreversible binding of the antibodies onto the solid-phase antigens. At low analyte concentrations (reagent excess) immuno complexes can rearrange: an antibody molecule bound by one arm to the antigen-coated surface, and by the other arm to a labelled antigen (signal-giving complex), can reorganize to bind on the solid phase using both of its Fab arms. This leads to a release of the labelled antigen from the solid phase, and a decrease in fluorescence signal levels. The phenomenon does not lead to false negative results as the detection will be finished before the signals start to decline.

If bivalent binding is to be avoided, then the surface antigen density has to be adjusted so that the average distance between two antigens on the surface is longer than the extent of the Fab arms. We did this by diluting the antigen density of the surface down to a 1/10 mass proportion using BSA as dilutant (**unpublished results)**. This resulted in lower signal dynamics and poorer signal statistics that hampered data analysis. Thus, conclusions could not be made with respect to bivalent binding. Nor did this study enable detection of IgM-class antibodies, supporting our suggestion for the mechanism.

Conformational change of IgM molecules from planar to staple-like conformation can happen even as a result of single Fab-arm binding (**III**). We have also demonstrated that after initial incubation with the tracer antigen, the IgM is able to bind to the antigen surface (**unpublished data** by Hanna Smolander) but it still does not give an antibody-bridging signal. Binding of the IgM to the antigen surface thus seems to lead to an instant dissociation of the labelled antigen. In the literature, IgM has been shown to give good signal response in antibody-bridging methods for other pathogens. This suggests that this phenomenon is antigen dependent and might have to do with the size and multivalency of the antigen.

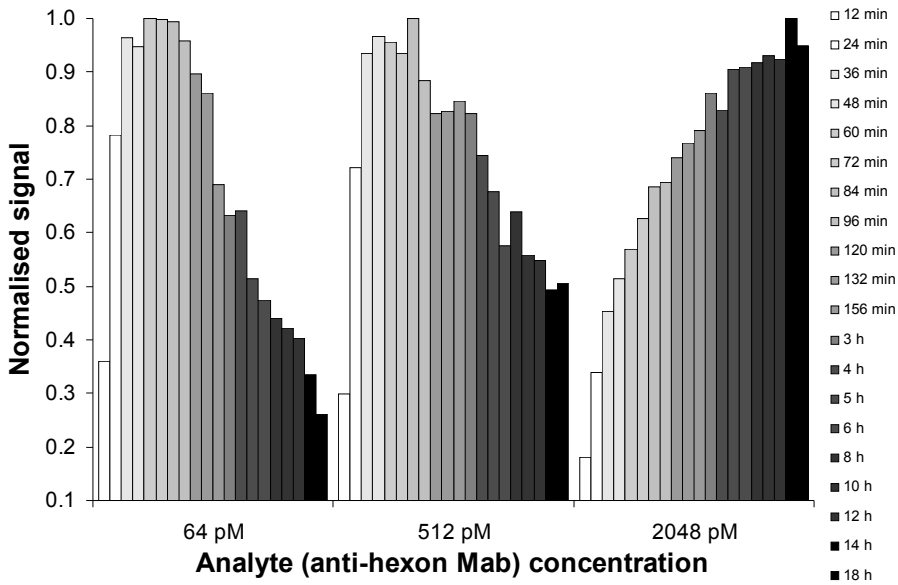

Figure 6. Signal kinetics of the antibody-bridging method at different analyte concentrations. Y-axis shows normalised signals (to the maximum signal of the respective reaction). The analyte concentration 2048 pM is at the hook of the dose-response curve. Measurement time points are shown on the right.

Because dilution of the antigen surface density results in lower signal levels and lower sensitivity, the dilution strategy should not be applied in one-step antibody-bridging methods. This is in line with the literature. It has been shown that in one-step incubation methods (in heterogeneous assay systems) the optimization of antigen density is not as crucial as it is in two-step incubation methods (Bourdage 2005).

In Publication **III** we reported that passive coating of the hexon antigen resulted in partial loss of functionality. This was seen as lower signals compared to the biotin-avidin strategy. This finding was

prominent, especially when monoclonal antibodies were used as analytes. In the assay of clinical samples, however, the passive coating strategy provided good results. This is most probably connected with the polyclonal nature of serum antibodies.

The antibody-bridging principle does not allow determination of the infection status (primary, secondary, or past) as the class-specific methods do. To overcome this limitation, we proposed combining the antibody-bridging technique with the avidity index technique (**III**). This combination should enable separation-free serodiagnostic testing, which would also provide information about infection status.

4.6 On-line detection of microbial growth

The possibility to apply the TPX technique to on-line monitoring of bacterial growth was studied in Publication **V**. The new methodology for monitoring bacterial growth is based on a combination of an immunometric assay principle and microbial culturing in closed assay reaction wells. We used *Staphylococcus aureus* as the model organism. When growth medium was used in place of a regular assay buffer, sample bacteria were able to grow. This resulted in increasing numbers of antigens, which were then sandwiched on the microspheres and detected. According to the results, the TPX technique allows on-line monitoring of species-specific growth. Increasing turbidity of the reaction mixture interfered with signal measurement but did not impair analysis of the results.

To ensure that the immunoreagents do not affect the bacterial growth and distort the results, we studied the effect of the reagents on growth. No effect on growth was observed (**V**).

The detection limits of the new method for whole bacterial cells were studied in both growth-inhibiting and growth-sustaining (after 6 hours of culturing) conditions. The detection sensitivities were strain-dependent and were, on average, 5×10^5 and 3×10^4 colony forming units/ml, respectively. The new method recognized all seven tested *S. aureus* strains and cross-reacted (slightly) with only one out of seven coagulase negative staphylococcus strains, indicating good specificity.

The new methodology could be used for several applications in microbiological research such as studies of cell respiration, cytotoxicity, and growth-inhibiting or growth-promoting substances.

4.7 Antimicrobial susceptibility testing

In Publication **V**, we developed a new methodology for antimicrobial susceptibility testing. This took advantage of the recent discovery that the TPX technique allows on-line monitoring of species-specific growth. Again, *S. aureus* was chosen as proof-of-concept analyte. The method applied dried reagents. When the growth medium was supplemented with an antimicrobial agent the growth of the bacteria was inhibited proportionally to the susceptibility of the bacteria. Thus, the new method allowed determination of antimicrobial susceptibility by a single-step assay protocol.

Correlation between the AST results of the new method and the conventional broth dilution method was studied with four oxacillin-susceptible and three oxacillin-resistant strains. The new method correctly identified all susceptible and resistant strains, and the results correlated well with the minimum inhibitory concentration values of the reference method (**V**).

To evaluate the performance of the TPX technique for direct sample analysis (without pure culture) the effect of competing growth was studied by growing *S. aureus* and *S. epidermidis* simultaneously in the reaction wells. We found that the monitoring of *S. aureus* specific growth was possible even when *S. epidermidis* was present in 350-fold excess compared to *S. aureus* (**V**). According to this result, the technique seems to be rather tolerant of competing bacteria.

5 Discussion

The dry-chemistry reagent concept and the nanoparticulate tracers developed in this thesis enabled the use of the TPX assay technique for rapid testing of infectious diseases in different application areas. Compared to current laboratory methods, the TPX methods are simple to perform and still they provide high performance. Due to the dried reagents and the separation-free fluorescence detection, the assay protocols are one-step. This is in contrast to many of the proposed POC techniques which must apply complicated test cartridges and liquid handling.

The TPX assay technique uses coated microspheres as solid phase carriers for the immunoaffinity reactions. This makes quality control easy compared to the coated-tube techniques. The technique and the reagents showed robustness throughout the studies. Small reaction volumes, low reagent concentrations, and the microsphere solid-phase chemistry mean low manufacturing costs. Furthermore, compared to many of the POC techniques, the new technique provides higher sensitivity, and it gives quantitative test results in electronic format. In conclusion, the new technique seems to meet most of the desirable features of a POC immunoanalyser (von Lode 2005).

Limitations of the technique include limited applicability to whole blood samples. This property would be of value when analyzing samples in emergency care. Nevertheless, analytes with high reference range (nM) can be analysed from whole blood upon sufficient dilution.

Another limitation is the relatively long measurement time per reaction well (20−60 seconds). This is not a major limitation in POC testing but it may limit the applicability in centralized high-throughput testing. Unlike in ELISA, bioaffinity binding reagents in the TPX platform must be very pure and of high specific acitivity. This may pose challenges in the method development. Yet another drawback of the TPX technique is that the fluorescence reader is more complex in design, and thus more expensive, compared to the conventional photometrical detectors used in conventional ELISA methods.

5.1 Dry-chemistry reagents

Conventionally, immunoassay methods have used soluble assay reagents. Such reagents must be dispensed into the reaction chambers at the point of use, typically in a multistep mode with washes in between the steps. An alternative approach is to use dry-chemistry reagents – the analyte specific reagents are dispensed into the reaction

chambers or onto the test slide already during the manufacturing process of the test kits, followed by drying. Dried reagents markedly simplify test protocols, prolong the shelf-lives at room temperature, and make shipping of the test reagents easy. The use of dried reagents can be considered to be a prerequisite for POC testing and lab-on-a-chip applications (**II**). Pre-dispensed reagents also mean better accuracy in reagent concentrations due to automated dispensing at the manufacturing facilities.

Dried reagents have traditionally been applied in lateral flow tests but currently, also an increasing number of immunochemistry analysers use dried reagents. However, as long as the signal detection requires physical separation of the bound and unbound fractions, the test instruments must use complicated liquid handling. In other words, physical separation-steps mean complicated instrument design and high assay costs.

The use of dried reagents is sometimes associated with compromised performance in comparison to corresponding soluble reagents. For example, drying may result in reduced functionality of binding reagents, and increased imprecision and non-specific binding. Sensitivity and precision are thereby compromised. These problems are usually minimized by including buffer additives and by physically separating the solid phase reagent and the tracer. Such problems were not encountered with the methods developed in this study (**II**, **IV**, **V**). In general, unspecific binding is seldom met in the TPX technique. This is most probably due to i) low reagent concentrations, ii) the quality of the used reagents, as otherwise the obtained sensitivities would not be possible, and iii) the relatively high level of background signal, which originates from the unbound fraction of the tracer (in nM concentration). In the TPX technique, the background signal is governed by the free fraction of the fluorescent tracer. Therefore, it is rather tolerant of unspecific binding.

Surprisingly, also the influenza B virus method which applied nanoparticulate tracer showed the same performance with soluble and dried reagents. Non-specific binding was not observed either. Initial dry-chemistry reagent studies using the NPT method for CRP (**I**) suggested that the NPT and microsphere reagents sometimes aggregate in high analyte concentrations (**unpublished results**) requiring the use of physical separation. Because such problems were not encountered with the other methods, we concluded that the problems with the CRP reagents were related to the pentameric nature of the CRP molecule.

In conclusion, the developed dry-chemistry reagent methods showed good performance and robustness throughout the studies with different application types. The use of dried reagents with the separation-free TPX assay technique makes it well suited for POC applications.

5.2 Multianalyte point-of-care testing of respiratory pathogens

In the management of respiratory tract infections, physicians usually make the diagnosis on the basis of clinical findings, anamnesis, and epidemiologic picture. Sometimes, samples are taken after visiting the physician, and they are analysed for specific pathogens either with rapid POC tests or with laboratory methods. Thus, the patient may need to return to the physician for the final diagnosis. Rapid and cost-effective multianalyte POC tests would enable reversing this mode of operation. Nursing stuff could be trained to recognize symptoms according to a standardized protocol (strategy already in use, Rautakorpi 2006b), followed by collection of appropriate specimen(s), and analysis of the sample(s) at the point-of-care. The test result would then be available for the physician when meeting the patient for the first time. According to this vision, a specific microbiological diagnosis can be given for most patient cases within the same patient visit (Linde 2001). Such an operation mode would allow rapid initiation of correct treatment, including virus-specific drugs, and is expected to reduce unnecessary use of antibiotics. So far, implementation of this approach has been hampered by lack of suitable diagnostic methods, which would allow multianalyte POC testing.

Although authors of studies dealing with the over-prescription of antibiotics and antimicrobial resistant bacteria do not usually call for better *in vitro* diagnostic tests, they feel uncomfortable treating the patients empirically. Recently, the physicians and microbiologists have started asking suppliers about multianalyte antigen detection tests (Personal observation, ESPID 2008 congress, Graz, Austria).

In terms of disease burden (incidence, deaths, and economic loss) influenza is one of the most severe infectious diseases in the developed world. Secondary bacterial complications of influenza are major health risks, and they could be reduced by more efficient use of antiviral treatments. However, if the clinical diagnostic accuracy for influenza is poor (no *in vitro* pathogen detection), also the reputation of the antiviral medication will deteriorate. (Linde 2001) For this reason, virus drug companies should promote rapid *in vitro* testing.

Public awareness is expected to affect the testing routines of clinical *in vitro* diagnostics in the near future. It is expected that, in the future, patients will actively ask for a specific diagnosis in order to avoid unnecessary broad spectrum antibiotics, and to allow correct medication with specific antiviral drugs. If the test prices are reasonable, symptomatic patients will be eager to adopt the new diagnostics (Billings 2006).

Despite the widely acknowledged need for rapid multianalyte POC tests, no such methods are on the markets. There are several multianalyte test systems available, such as immunofluorescence, ELISA, and those based on nucleic acid amplification (NAA), but none of them is well suited for rapid POC testing due to their high cost structure and complicated assay protocols. Mere reagent cost, at present, of one multiplexed NAA test with 10–20 analytes is approximately 100 €, and typically a single analyte PCR test ordered from the lab costs 50–100 €. For these reasons, it seems probable that the NAA methods will remain as a research tool or as an alternative technique for those rare patient cases where high assay cost is acceptable.

On the basis of Publication **IV**, the TPX assay technique seems to be well suited for rapid POC testing of RTI diseases. Taking advantage of the kinetic measurement option of the TPX technique, we were able to determine the sensitivities of the methods at various time points. According to the results, sensitivities of the new methods at the 15–20 minute time point are comparable to lateral flow tests. Prolonged incubation (2 hours) and repeated measurement provide performance comparable to the state-of-the-art laboratory methods. The kinetic measurement option provides fast results without compromising the overall sensitivity. A limitation of the new technique is the need for sample pre-treatment with detergent buffer prior to analysis, that takes one minute. On the other hand, a similar step is also needed in any other current POC test. Because the TPX measurement technique relies on detection of individual microspheres by their scattering, clinical specimens should be obtained by using swabs made of artificial fibre (instead of cotton) that do not release particulate material.

The challenge associated with the development of a multianalyte RTI test relates to the specimen collection and pre-treatment. The optimal specimen type depends on the pathogen species. In the clinical practice of a multianalyte test with high coverage of both viruses and bacteria, probably two swab specimens should be taken, one from the nose and one from the throat. These swabs would then be combined with

appropriate extraction reagents, followed by dilution and dispensing into the test cartridge. However, the sample pre-treatment poses another challenge as different pathogens may reguire different pre-treatments for optimal sensitivity and specificity.

Following the development of influenza A and B virus antigen detection methods (**IV**), we have studied the applicability of the TPX assay technique for multinalyte testing of RTIs in the described format. We have developed new methods for common respiratory tract pathogens including *S. pyogenes*, *S. pneumoniae*, respiratory syncytial virus, metapneumovirus, adenovirus, and parainfluenza viruses. The new methods show excellent sensitivity (90-100 %) and specificity (close to 100 %) compared to current rapid tests based on lateral flow (bacteria), or laboratory tests based on TR-FIA or ELISA (viruses). (Koskinen 2008a, Koskinen 2008b, Koskinen 2008c) By running the new assays in multianalyte format at the POC it would be possible to obtain the test results within the same patient visit. This would allow rapid and accurate pathogen-specific diagnoses.

The manufacturing cost of the new multianalyte RTI test is expected to be close to those of the current single analyte rapid tests because of the simple assay procedures and low reagent consumption. A further advantage of the new technique is that the same instrument can be used for the measurement of systemic infection markers such as C-reactive protein (CRP) (**I**), thus complementing the analyte portfolio.

Currently, multianalyte detection methods are often developed by multiplexing several reactions into a one reaction vessel. Multiplexing is known to be difficult, and it results in compromised assay performance. We studied multiplexing by combining the influenza A and B virus antigen detection methods (**IV**) into one reaction (**Appendix 3**). This duplexed method showed good performance in relation to the reference methods on DELFIA^{TM}, but sensitivity was compromised in comparison to the corresponding single analyte methods. Most probably, further multiplexing would further decrease assay sensitivity. The multianalyte testing on the TPX platform would not be realized by multiplexing, but instead, by running separate tests in parallel assay wells. This multianalyte approach meets the goals of multiplexing, but returns an uncompromised detection performance.

Since the TPX detection technique relies on microscopic optical detection, the performance of the technique is independent of the assay volume. This property makes TPX a suitable detection technique for miniaturized assay formats, such as “Lab-on-a-Chip” formats (**II**). The combination of the TPX technique and miniaturized assay chips would

allow the design of disposable multianalyte testing cartridges for POC testing. Multianalyte assays could be brought about, as described above, by having several parallel reaction wells or by multiplexing several reactions to a single reaction cuvette. Multianalyte tests using the TPX technique can be designed for different disease types and screening applications, e.g. a cartridge for respiratory infections (**IV**) or for pathogen-specific antibodies (**III**). The exact composition of the reagents in the cartridge could be easily modified, for example, according to market region, prevailing infectious agents within a population, or the needs of a laboratory.

The multianalyte infectious diseases test could also be used for real-time epidemiological surveillance. In contrast to conventional rapid assays, the TPX technique is characterized by an automated results read-out. Thus, the analysers could be connected via the Internet to a global database and surveillance system allowing health-care authorities to monitor ongoing infections on-line. Such a surveillance system would support the control over infectious diseases and epidemics, and provide an "early warning" system for changes in pathogen activities (Linde 2001, OECD, WHO 2005). Such multianalyte testing would have a high impact, for both the individual and for society.

5.3 Antibody bridging − avidity index technique

For decades serodiagnostic testing has been based on the use of class-specific methodology and paired serum analysis. Limitations of the class-specific methods were discussed in Section 1.4.2. Because of the slowness of the immunoresponse and the time lag of paired serum analysis, rapid POC testing does not usually provide added value in comparison to conventional centralised laboratory serology. Furthermore, class-specific detection can not be realized by separation-free techniques that would be adaptable for POC testing.

In recent years, method specificity (Valdes 1992) and matrix interferences (Kricka 1999, Selby 1999, Levinson 2002) have gained wide publicity. This is highlighted, for example, by a recent Editorial (Bjerner 2005) and an Opinion (Ismail 2005) in the Clinical Chemistry journal. In order to improve the accuracy of clinical immuno-diagnostics, alternative methodologies should be developed. The field of serodiagnostic techniques, however, has shown relatively little research activity in the last few decades.

One strategy for reduction of sample interferences in class-specific methods is to remove the interfering substances. This, however, is

tedious and markedly increases the turn-around-time of sample analysis. Other strategies include modification of anti-immunoglobulin reagents, and the use of appropriate buffer additives. (Bjerner 2005) An ideal strategy would be the use of an immunoassay scheme less prone to interferences. Therefore, the serological methodology alternative to class-specific detection has been developed.

An alternative approach to the use of anti-immunoglobulins is the antibody-bridging (AB) assay scheme. In this approach, only antigens are used as reagents, both in the solid-phase and as tracer. Theoretically, this approach is the only one that allows separation-free detection of specific antibodies. The principle has several advantages:

(i) It has been demonstrated both theoretically and experimentally (Siitari 1990, Bürgisser 1996, Ankelo 2003, Westerlund-Karlsson 2003) that assay methods following the principle are free from interference caused by rheumatoid factors, heterophilic antibodies and the complement, which frequently cause problems in assays based on anti-immunoglobulin antibodies. This is because the assay principle does not employ anti-immunoglobulin reagents.

(ii) In contrast to conventional class-specific methods, AB methods are free from bias caused by differences in the affinities of anti-immunoglobulin reagents.

(iii) In the AB assay, the analyte has to bind to two or more antigens to contribute to the final signal output. This leads to superior specificity compared to class-specific methods (Miyazawa 1988, Siitari 1990, Bürgisser 1996, Horimoto 1997, Miyazawa 1999).

(iv) For the same reason, the principle effectively eliminates the signal originating from unspecific binding. This allows the use of low sample dilutions (Chang 1984, Horimoto 1997, Miyazawa 1999, Ankelo 2003, Westerlund-Karlsson 2003).

(v) The signal output of the assay principle is proportional to the number of labelled antigens bound on the analyte antibody. The assay principle may thus provide superior sensitivity for multivalent IgM-class antibodies. This leads to earlier detection of an immune response. This theory has also been demonstrated experimentally (Chang 1984, Gallarda 1992, Bürgisser 1996).

(vi) The principle allows more convenient determination of specific antibodies from different species compared to class-specific methods (Kristiansen 1997).

The advantages of the antibody-bridging principle are expected to provide improved diagnostic performance compared to classical

methods. Although the AB principle is widely applied in commercial serodiagnostic tests (e.g. on Prism™, Elecsys™, and Centaur® platforms), there are very few reports in the literature which describe methods based on this assay principle. None of the published reports describes a separation-free method; all of them rely on heterogeneous assay format with washing steps for specific signal generation.

The proof-of-principle AB method for adenovirus specific antibodies showed good performance in comparison to class-specific ELISA (**III**). Interestingly, IgM-class antibodies did not give a signal response either when using the AB method on a TPX platform (**III**), or when using the AB on a DELFIA™ platform (**unpublished data**). The same phenomenon has been reported in the literature (Aggerbeck 1996, Miyazawa 1988), although, in other studies AB has been shown to give a strong signal response for IgM-class antibodies (Chang 1984, Gallarda 1992, Bürgisser 1996). The aspects affecting the reactivity of IgM in AB methods need to be studied in more detail in the future.

The main drawback of the AB principle is that it does not provide information about the immunoglobulin subclasses. Thus, it can not differentiate between primary and secondary infections. This limitation could be overcome by combining the AB principle with the avidity index technique (see **III**). The antibody-bridging principle and the avidity index methodology, in combination, could provide superior diagnostic performance compared to conventional techniques. This claim is based on the above-mentioned facts (see 1.4.2, and 5.3). The proposed technique combination: i) is less susceptible to matrix interferences, ii) has higher specificity, iii) is less influenced by unspecific binding, iv) is highly sensitive, and v) provides results from a single serum sample.

If the hypothesis proves to be correct, the new methodology would provide added value to immunity testing and assessment of infection status and phase. Compared to the current practice (class-specific methods), the new methodology would speed up the diagnosis, as paired sample analysis would be avoided. A faster and more accurate diagnosis improves patient care and leads to faster clinical recovery.

5.4 Antimicrobial susceptibility testing

New approaches for antimicrobial susceptibility testing have been introduced in recent years. Although most of them are based on nucleic acid amplification, novel methodologies that rely on bacterial culturing are still required because of the constrains of the genetic methods. The new methods should be able to challenge the traditional phenotypical

methods. In Publication **V**, we provided proof-of-principle for the suitability of the TPX technique for on-line monitoring of bacterial growth and for AST. The new methodology could allow AST directly from clinical samples with significantly shorter turn-around-times (6−8 hours) compared to conventional methods.

The main drawback of the method presented in Publication **V** is the narrow dynamic range. Samples have to be appropriately diluted to bring the initial bacterial concentration below the detection limit. On the other hand, the higher the dilution, the longer time required for a positive result. In consequence, the methods must apply several sample dilutions. This lowers the throughput and increases the assay cost.

During the incubation, antibiotics disintegrate bacterial cells which results in an increase in the concentration of soluble antigens. This leads to a crawling up of the signal despite the fact that bacterial growth is inhibited. The phenomenon poses challenge for data analysis as pre-set cut-off values may not always give optimal results.

The most time-taking step in current AST methods is the pure-culturing step which is needed to eliminate other bacterial species. Susceptibility must be tested for one species at a time. Pure culture leads to loss of most of the diversity information of the population concerning antimicrobial resistance. In addition to acquisition of new infections, this might be one reason why several consecutive samples must sometimes be analysed before the patient is finally screened positive for MRSA.

The new methodology tolerates other sample microbes rather well. This suggests that it could be used to analyse clinical samples without pure culture, thus maintaining the population diversity information. This should be a major advantage over the present methods. Before implementation for clinical diagnostics, the microbe-specific method should be further optimized with respect to antibodies (higher affinity, better recognition spectrum), selective growth medium, and atmospheric conditions. The re-optimised method should then be tested with clinical samples to reveal its true performance.

The potential of the proposed new methodology is supported by the recent product launch by 3M (BacLite Rapid MRSA Test). The test takes advantage of a similar immunoassay approach. However, the two methodologies differ with respect to the detection technique; the method used by 3M applies a wash assay platform, whereas the TPX technique is wash-free. Other potential targets for rapid phenotypical AST would be *S. pneumonia* and *Escherichia coli*, for example.

6 Conclusions and prospective

This study shows that the two-photon excitation assay technique in combination with dry-chemistry reagents is a versatile tool for rapid infectious diseases testing. The technique was shown to allow assays in the following application fields: i) general inflammation markers, ii) antigen detection, iii) serology, and iv) antimicrobial susceptibility testing. The platform allows one-step assay protocols that are a prerequisite for cost-effective and user-friendly point-of-care testing. The new technique can be considered a significant improvement over current rapid test methods.

This study showed that the sensitivity of the TPX methods can be increased by using nanoparticulate tracers up to one to two orders of a magnitude. We also conclude that multianalyte detection is best realized by running the tests in parallel in separate assay wells. In those cases where relative amounts of two analytes in the sample are of clinical interest, multiplexing should be considered.

Serological and microbial (AST) applications must be further studied in order to reveal their potential as tools for *in vitro* testing. In the near future, we aim to combine the antibody-bridging principle with the avidity index technique and evaluate the significance of this combination in clinical serodiagnostic testing.

The most promising application field of the TPX assay technique is the rapid multianalyte testing of respiratory tract infections. Our future objective is to validate the multianalyte test in clinical settings. The described multianalyte test could be used in the “doctor’s office” as well as the in clinical laboratory, where it could replace current single analyte rapid tests and traditional laboratory methods.

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Appendix 2: Sensitive nanoparticulate tracer method for thyroid stimulating hormone

Introduction and Experimental

Some of the clinically important analytes, such as human thyroid stimulating hormone (hTSH) and cardiac markers, require very high detection sensitivities. To study the potential sensitivity of the TPX assay technique we chose to develop a nanoparticulate tracer (NPT) method for hTSH. This analyte has traditionally been used to reflect the sensitivity of new assay techniques. The preparation of NPT specific for hTSH was carried out as described in section 3.2. Antibody clone type optimization was needed for the method because the analyte requires extreme sensitivity and because of the chosen NPT approach. We also optimized the buffer composition for high sensitivity.

Results and Discussion

The optimized NPT method (50 000 microspheres per 20 µl reaction volume, NPT concentration 0.4 x 10^{13}/L, assay buffer supplemented with 3 % PEG 6000) for hTSH was compared to a corresponding method applying molecular tracer (**Figure 1**). It turned out that different antibody clone pairs and configurations (solid-phase versus tracer) gave optimal performance depending on whether the method used either NPT (clone 5409 solid phase, clone 5405 tracer) or molecular tracer (MT, clone 5405 solid phase, clone 5404 tracer). The NPT method had lowest limit of detection (zero control + 3SD) in the reaction mixture of 0.01 mIU/L (20 femtomolar). The functional sensitivity, defined as the concentration co-efficient of variation ≤ 20 %, was 0.05 mIU/L. Intra-assay imprecision at the concentration of 0.5 mIU/L was 8 %. The corresponding values for the MT method were 0.2 mIU/L, 0.5 mIU/L, and 20 %. The NPT method showed excellent linearity of the response ($R^2 \geq 0.996$). The NPT improved the detection sensitivity of the hTSH method by an order of magnitude. This sensitivity is expected to allow screening of thyroid dysfunctions in primary care.

The performance of the TPX technique is independent of the assay volume and hence allows analysis from 1 µL reaction volumes. In such a volume, the 20 femtomolar detection limit amounts to 10 000 analyte

molecules. The method is one of the most sensitive assays reported in the literature in terms of the number of analyte molecules. It can be argued that the method approaches the theoretical lowest limit of detection of the current instrumentation; the detection limit corresponds to an analyte concentration of four antigen molecules per solid phase microsphere. In such low analyte concentrations, imprecision is expected to be tens of percents as individual microspheres which are bound from zero to four nanoparticles (via zero to four antigens) are measured.

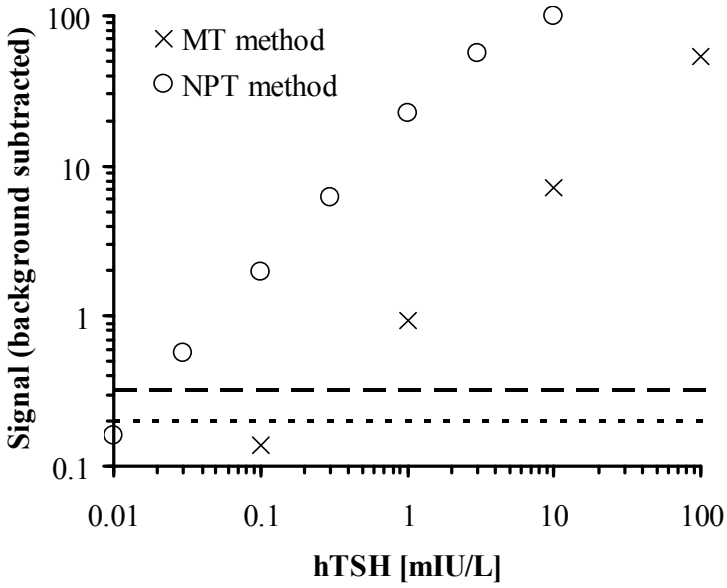

Figure 1. Dose-response curves for hTSH methods applying molecular tracer (MT, dashed line zero + 3SD) and nanoparticulate tracer (NPT, dotted line zero + 3SD). Standard sample was diluted into the assay reaction by a factor of two. Incubation time was two hours.

Appendix 3: Concomitant assay method for influenza A and B viruses

Introduction and Experimental

The clinical manifestations of, and the empirical treatment for, influenza A and B virus infections are the same. Moreover, new antiviral drugs are effective in both infection types. Therefore, differentiation between the two (A or B) is of minor importance, both for the physician and for the patient. On this basis, it would be justified to combine the two assay methods in one more general influenza virus method, which would detect both virus types.

To demonstrate this method option in the TPX platform, we prepared a combination reagent for influenza A and B viruses by coating microspheres with a 1:1 mixture of influenza virus antibodies against A (clone A3) and B (clone B2). These microspheres were used to capture influenza A and B virus nucleoprotein antigens from the samples. A mixture of fluorescently labelled antibodies (molecular for influenza A virus of clone A1, and nanoparticulate for influenza B virus of clone 2/3) was used as detection probe. Consequently, the method gives a signal response irrespective of the virus type.

Results and Discussion

The results of the concomitant assay method showed that at the 15-minute incubation time point 19 out of 20 influenza A virus positive samples and 11 out of 16 influenza B virus positive samples were deemed positive (**Figure 1a**), while at 35 minutes, all 36 samples were tested positive (**Figure 1b**). All negative samples (N=20) were below the cut-off level. **Figures 1a** and **1b** demonstrate how the detection rate (sensitivity) and the signals increase with incubation time.

Pearson correlations for the concomitant assay signals versus reference TR-FIA method signals, and concomitant assay signals versus individual TPX method signals were 0.8 ($p<0.0001$) and 0.9 ($p<0.0001$), respectively. Despite differences in the detection techniques and the methods, the signals obtained from the clinical samples with different methods correlated well, and each sample provided the same clinical result.

When compared to the individual assay methods, the signal-to-background ratios of the concomitant method for influenza A and B virus positive samples were lower by a factor of two (2) and three (3),

respectively. The absolute signal levels were, however, lower only by a factor of 1.3 for influenza A virus, and 1.7 for influenza B virus. The lower absolute signal levels can be explained by the lower capacity of the solid-phase per specific antibody, while lower signal-to-background ratios are also contributed to by increased solution background fluorescence. In the concomitant assay, the solution background fluorescence was increased by a factor of approximately two because the assay reaction contained two different tracers, one for influenza A and one for influenza B viruses. The decrease in solid-phase capacity could be compensated for by the use of high capacity streptavidin microspheres or similar approach.

In conclusion, the performance of the concomitant method was somewhat compromised compared to individual methods. The results indicate, however, that the method still provided sufficient clinical sensitivity. Using this approach, measurement time per sample was cut to half (from 50 seconds to 25 seconds per sample per measurement time point). This increases the throughput of the technique.

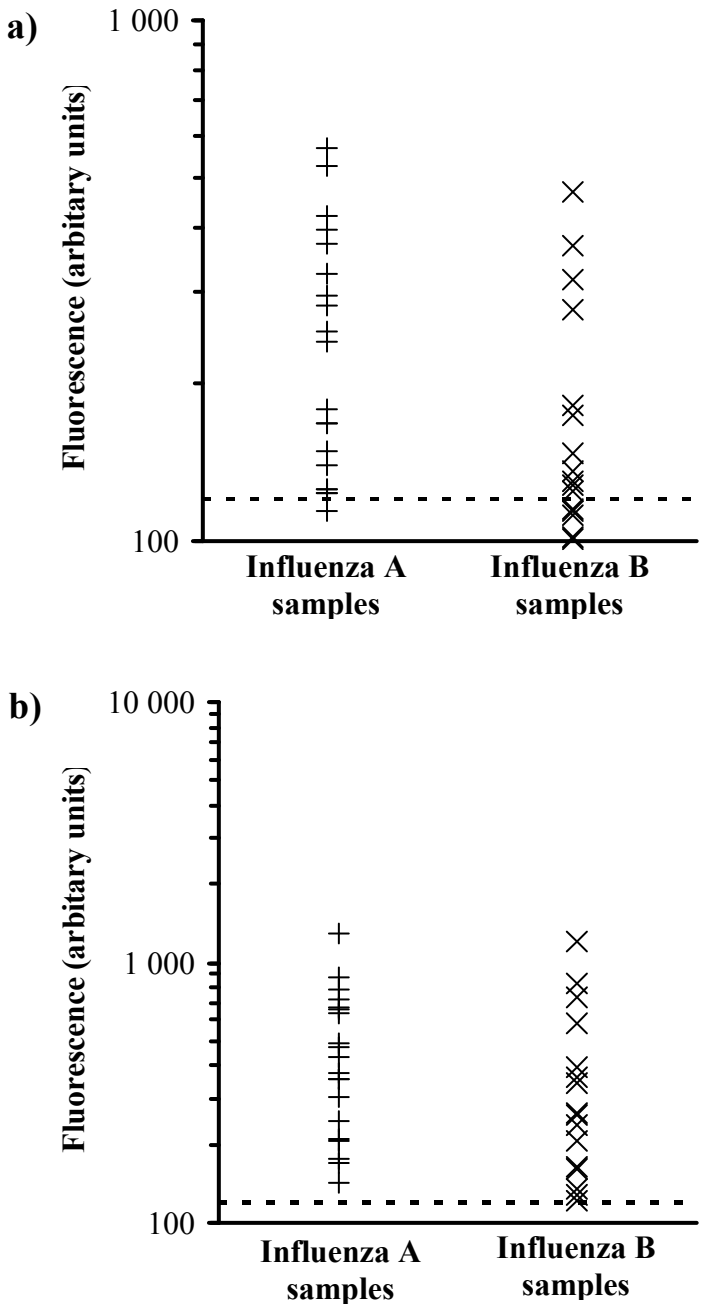

Figure 1. Figures demonstrating the performance of the concomitant TPX method for rapid detection of influenza A and B virus antigens (a) at the 15-minute and (b) at the 35-minute time points of incubation. (+) samples positive for influenza A virus by TR-FIA, (×) samples positive for influenza B virus by TR-FIA. Dashed line represents the cut-off level of the method. All influenza negative samples (N=20) remained below the cut-off.