Serum complement activity in differentiating between bacterial and viral infections

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Master's thesis

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The serum complement system is a humoral part of the innate immune system that takes part in killing pathogens via opsonization, induces inflammation or uses its proteins to form membrane-attack complex (MAC) on pathogens surface leading to its lysis. Complement cascade has three activation pathways: classical-, lectin- and alternative pathway which converge in terminal pathway leading to the formation of MAC. In this study we measured complement activity from patient serum samples provided by TYKS to determine if there is difference between serum samples from patients with bacterial infections and patients with viral infections. The method we used had luciferase-modified *Escherichia coli* (*E.coli*-lux), which emits measurable bioluminescence, exposed to the serum samples complement activity. As *E.coli*-lux emits bioluminescence signal while alive, we could measure the complement activity by measuring the signal produced while the cells die to the lysis. We found out that there were significant differences between the complement activity of bacterial samples and viral samples as well as to the healthy control samples. We did not find any differences comparing the subgroups divided by the diagnosis. The method used for this study is quite simple and fast to perform and could thus be a good addition to the diagnostic methods used in clinical setting to differentiate between bacterial and viral infections. It cannot however be used on its own as there was also overlap between the infection types.

Key words: complement, serum, bacteria, virus, infection, diagnostics

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1 Abbreviations

CP = classical pathway

LP = lectin pathway

AP = alternative pathway

MAC = membrane attack complex

C1-9 = complement components 1-9

E.coli = Escherichia coli

E.coli-lux = luciferase modified Escherichia coli

BL = Bioluminescence

C1 complex = C1qr2s2

MBL = mannose-binding lectin

PRM = pattern recognition molecule

MASP = MBL-associated serine protease

MACPF = MAC/Perforin

RCA = regulator of complement activation

CR1 = complement receptor type 1

MCP = membrane cofactor protein

C4bBp = C4b-binding protein

C1inh = C1 inhibitor

DAF = decay-accelerating factor

HRF = homologous restriction factor

MIRL = membrane inhibitor of reactive lysis

TYKS ER = TYKS emergency room

HSLA = High sensitivity luminescence assay

HBSS = Hank's Balanced Salt Solution

CPS = counts per second

rCPS = relative counts per second

CI = complement index

2 Introduction

The complement system is a humoral part of the immune system which consists of 50+ proteins that may help antibodies kill bacteria or lyse the pathogens on their own. It can be divided into classical-, lectin- and alternative-pathways (CP, LP and AP respectively) all of which can contribute to the formation of the membrane-attack complex (MAC) which is responsible for the lysing of the cells. The different pathways have different proteins on the stage of cascade activation, but they all meet when the different C3 convertases cleave C3 into C3a, an anaphylatoxin which causes inflammation and C3b which binds to the C3 convertase and forms C5 convertase. The previous phase of cleaving C3 is called major amplification step as in all three pathways the cascade activation multiplies in strength greatly as C3 convertases can cleave hundreds of C3s quickly. (Mastellos et al. 2024; Owen et al. 2013)

After forming the C5 convertase, C5 protein is then cleaved into C5a, another anaphylatoxin and C5b which starts forming the MAC by recruiting C6 and C7 which forms C5b67 complex at which point it anchors itself hydrophobically to the lipid layer of the target cell. When C8 is bound to the complex and also anchored to the lipid layer, it recruits 10-17 C9 proteins that form the actual MAC which makes a ring-shaped hole to the cell's lipid layer and leads to the lysing of the cell. The lysing effect of MAC has its flaws and thus doesn't work on all cells, but for the purpose of this research we use *Escherichia coli* (*E. coli*) which it does work on. (Mastellos et al. 2024; Owen et al. 2013)

As serum doesn't contain blood cells, we can use the serum's ability to lyse cells as a measurement of the serum's total complement activity. Luciferase modified *E. coli* (*E. coli*-lux) cells used in this research produce bioluminescence (BL) which can be measured as proof of life and as such when the cells divide, the BL levels rise and when they die, the levels start to drop (Atosuo et al. 2013, 2021; Virta et al. 1998).

2.1 Pathway differences

The three pathways have different criteria for activation (Figure 1). The CP is initiated by $C1qr_2s_2$ (C1 complex) binding its C1q subunit to two available Fc regions of immunoglobulin (Ig) -antigen complex. The Fc regions can be from one IgM on pathogen surface or two IgG molecules as they only have one available Fc region per molecule. The conformation changes on the C1 complex following the binding to the Ig activates C1r subunits serine protease function. Activated C1r then cleaves C1s which activates its serine protease function which then allows cleaving of C4 and C2 into C4a, C4b, C2a and C2b. C4a and C2b act as an anaphylatoxin and thus don't contribute to the further activation of the complement pathway directly. C4b and C2a then form C4b2a complex which is CPs C3 convertase. (Mastellos et al. 2024; Owen et al. 2013)

The LP shares steps with CP, but it is initially activated when mannose-binding lectin (MBL) or other pattern recognition molecules (PRM) belonging to the same molecular family, such as collectins and ficolins, bind to the cell wall of the microorganism (Ma et al. 2017). Collectins CL-L1 and CL-K1 (or CL-10 and CL-11) activate LP by forming soluble CL-LK complex that can bind to MASPs. (Hansen et al. 2016; Henriksen et al. 2013). Three ficolins: ficolin-1, ficolin-2 and ficolin-3 can also act instead of lectins to activate the pathway (Ma et al. 2017). The main difference from the CP is that there is no need for antibody-antigen binding to initiate complement cascade. In LP, MBL acts instead of the CPs C1q to activate C4 and C2 by forming a complex with MBL-associated serine proteases 1 and 2 (MASP) which in turn act instead of the C1r and C1s. Otherwise it proceeds the activation in the same way as described in CPs activation in previous chapter. (Mastellos et al. 2024; Owen et al. 2013)

The AP is mainly activated in fluid state by spontaneous C3 tick over which means that C3 hydrolyses continuously at a low-level. The cleaved C3b can then bind to cell-surface molecules foreign to the host which allows binding of factor B. Cell-surface bound C3b is inactivated by sialic acid which is found plentiful on the surface of most mammalian cells which disallows activation on host cells. The membrane bound complex is then cleaved by factor D to form active C3bBb complex, the C3 convertase of AP. The C3 convertase then binds another C3b after cleaving it to form C3bBb3b which acts as a C5 convertase thus leading to the same result of the joined terminal pathway of all three initial pathways. (Fearon et al. 1973; Mastellos et al. 2024; Owen et al. 2013) A novel activation of AP could also be

induced by collectin CL-P1 (or CL-12) via properdin binding of soluble form of CL-12. It is highly restricted as the more common cell membrane bound form of CL-12 is incapable of binding to properdin. (Ma et al. 2015).

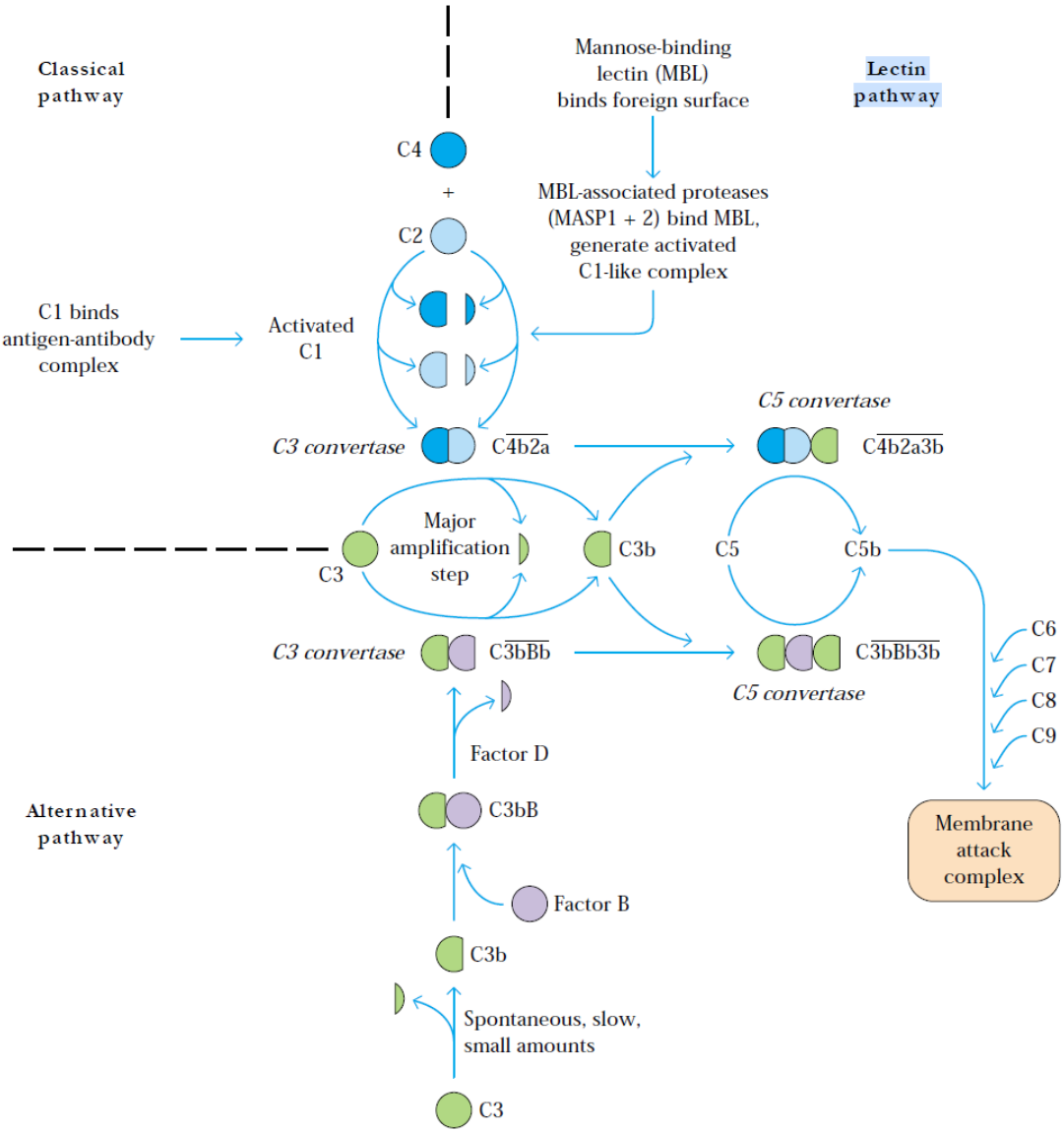

Figure 1. Overview of complement cascade. Complement system has three different activation pathways. Classical pathway which activates on C1 binding to Ig bound to its antigen. C1 then cleaves C2 and C4. The cleavage products then form classical pathways C3 convertase. Lectin pathway activates when mannose-binding lectin binds to the cell wall of the micro-organism and thus form C1-like complex which acts similarly to the C1 in cleaving the C2 and C4 and thus lead to the formation of C3 convertase. The alternative pathway mainly activates through slow spontaneous cleavage of C3 into C3b which together with Factors B and D form alternative pathways C3 convertase. From this point onward all three pathways function similarly cleaving C3 into C3b and C3a from which the former then assembles with the C3 convertase into C5 convertase. The C5 convertase then cleaves C5 into C5b and C5a from which the former starts recruiting C6, C7, C8 and C9 to form C5b-9 (MAC). (Mastellos et al. 2024; Owen et al. 2013)

2.2 Formation of MAC

The terminal pathway of the complement cascade is when all three initiating pathways have converged and leads to the formation of the MAC (Figure 2). It starts when C5 is cleaved into C5a and C5b, C6 binds quickly to the C5b on pathogen surface forming C5b6. Following C7 binding to the previous complex it becomes lipophilic which allows its insertion into the membrane during C5b67 complex binding the C8. C8 is a heterotrimeric protein with α-, β- and γ-subunits. C8α binds to the membrane and C8β is responsible for the binding to the C5b67 complex while C8γ is non-essential subunit that boosts the bacterial-killing property of the MAC, but exact functions are still unknown. However, C8γ might stabilize the MACPF-MACPF (MAC/Perforin) domain structure based on its orientation in the plasma soluble C5b9 complex (sC5b9) which is the result of regulatory proteins binding the hydrophobic heads of the complex and thus preventing it from attaching to the cell surface. In the end the C5b8-complex recruits 10-17 C9 proteins which form the pore on the cell membrane. (Hadders et al. 2012; Owen et al. 2013; Parker and Sodetz 2002)

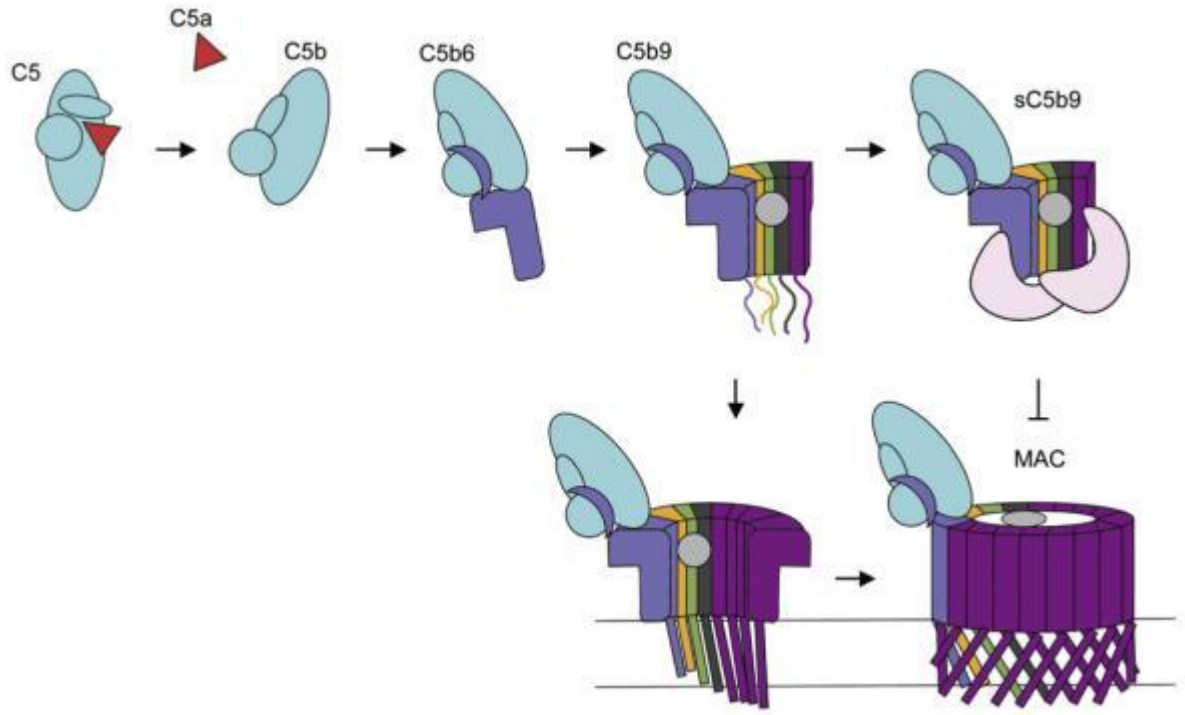

Figure 2. Terminal pathway. When C5 is cleaved into C5a and C5b, the latter binds with C6 forming C5b6. It then recruits C7 and C8 while binding to the target cells membrane. Bound C5b8 then binds 10-17 C9 which forms the pore. The formation of sC5b9 on the other hand inhibits the MAC formation as it is the plasma soluble bound version of the MAC that is prevented from attaching to the cell surface by regulatory proteins. (Hadders et al. 2012; Owen et al. 2013)

2.3 Regulation

Main regulatory mechanism used widely by the whole cascade are zymogens that are highly labile and thus inactivate easily if they are not reacting quickly after activation. For example, when C3 is cleaved into C3a and C3b, C3b is produced abundantly as C3 concentration is high and all three pathways produce C3 convertases. C3b being the active component formed in the cleaving, starts to opsonize cells in the vicinity and to prevent targeting host cells it hydrolyses when further than 40nm away from the C3 convertases that cleaved it. There are also number of other important regulatory proteins, most of which belong to the regulator of complement activation (RCA) gene family, which are either membrane-bound, that control all three pathways, or soluble that control more specific ones. (Owen et al. 2013; Zipfel and Skerka 2009)

Membrane-bound regulators complement receptor type 1 (CR1) and membrane cofactor protein (MCP) both act in the same way binding to the C4b in CP or LP or to the C3bBb in AP which are then cleaved by factor I and thus preventing formation of C3 convertase. Soluble C4b-binding protein (C4bBp) acts similarly stopping the formation of the convertase in CP and LP and a soluble factor H does so in AP. (Iida and Nussenzweig 1981; Lublin et al. 1988; Owen et al. 2013; Zipfel and Skerka 2009)

Another common regulator of CP and LP is soluble C1-inhibitor (C1Inh). It is a serine protease inhibitor, so it prevents the activated formation of C1 from assembling and thus preventing the cleavage of C4 and C2. It does so by dissociating $C1r_2s_2$ from C1q by binding to the former. Decay-accelerating factor (DAF) in accordance with its name accelerates the decay of C3 convertase by cleaving it into two parts from which factor I cleaves the membrane-bound portion permanently. (Owen et al. 2013; Zipfel and Skerka 2009)

One of the regulatory targets during the terminal cascade, from C5 to MAC formation, is the first formed complex C5b67. Soluble S protein binding to C5b67 can prevent its binding to host cells which may cause “innocent-bystander lysis”. Another two terminal cascade regulators are membrane-bound proteins homologous restriction factor (HRF) and membrane inhibitor of reactive lysis (MIRL) which prevent non-specific binding of C9 to C5b8 on host cell membrane. They block the binding sites by binding themselves to the C5b8 if it originates from same species as the target cell. (Owen et al. 2013)

2.4 Complement evasion

Different viruses employ different tactics on evading complement mediated attacks on the virus. Often the viruses use complement regulators against the complement system which makes it either harder for the system detect the virus or kill it. HIV, human T cell leukemia virus and human cytomegalovirus attach CD46, CD55 and CD59 respectively to the viruses envelope to avoid complement system. Large DNA-viruses can also attach host cells in the same vein. Herpesviruses can also use surface-bound regulators. Vaccinia, cowpox and variola viruses use structurally similar proteins to C4bBp that is C3 decay accelerator. HIV on the other hand recruits factor H indirectly by using glycoproteins and West-Nile virus appears to incorporate the factor H to itself for protection against the complement system. (Diamond 2009)

Yeasts may deploy the same tactics as viruses on evading the complement system. *C.albicans* for example uses factor H, FHL1 and C4bBp to control the activation of the complement system during invasion. It has a goal to inactivate C3 by using its very own complement regulators bound to the yeasts surface. As another example fungus *Aspergillus fumigatus* and bacteria *Streptococcus pneumoniae* and *Borrelia burgdorferi* use similar tactics to the *C.albicans*. (Gropp et al. 2009)

3 Aim of this thesis

This thesis aims to compare serum complement activity between bacterial and viral infections and their subgroups. Focus is to find significant differences in the time it takes for patients' serum to kill pathogens with the formation of MAC by perforating the target pathogen cell and thus lysing it. These differences are then evaluated if they can be used to differentiate bacterial and viral infections *in vitro* consistently and reliably to assist in diagnosing infected patients with yet undiagnosed diseases that by symptoms could be either bacterial or viral diseases.

The method used is quite fast and easy to replicate and thus could prove to be valuable asset in addition to other methods available. However, in this study the aim is not to optimize the method but to simply provide framework for it for further studying and to provide valuable information of the viability for clinical setting. Hypothesis is that there is a difference in the complement activity, but it remains to be seen if it can be easily identified.

4 Materials and methods

4.1 Serum samples

Patient samples (Figure 3) used for the high sensitivity luminescence assay were collected from TYKS ER and infectious disease ward from suspected infectious patients with fever. Etiological diagnosis was conducted by doctor specialized in infectious diseases with microbiological, serological and radiological methods. 40 healthy volunteers were measured as a control group. Samples were stored in -80°C.

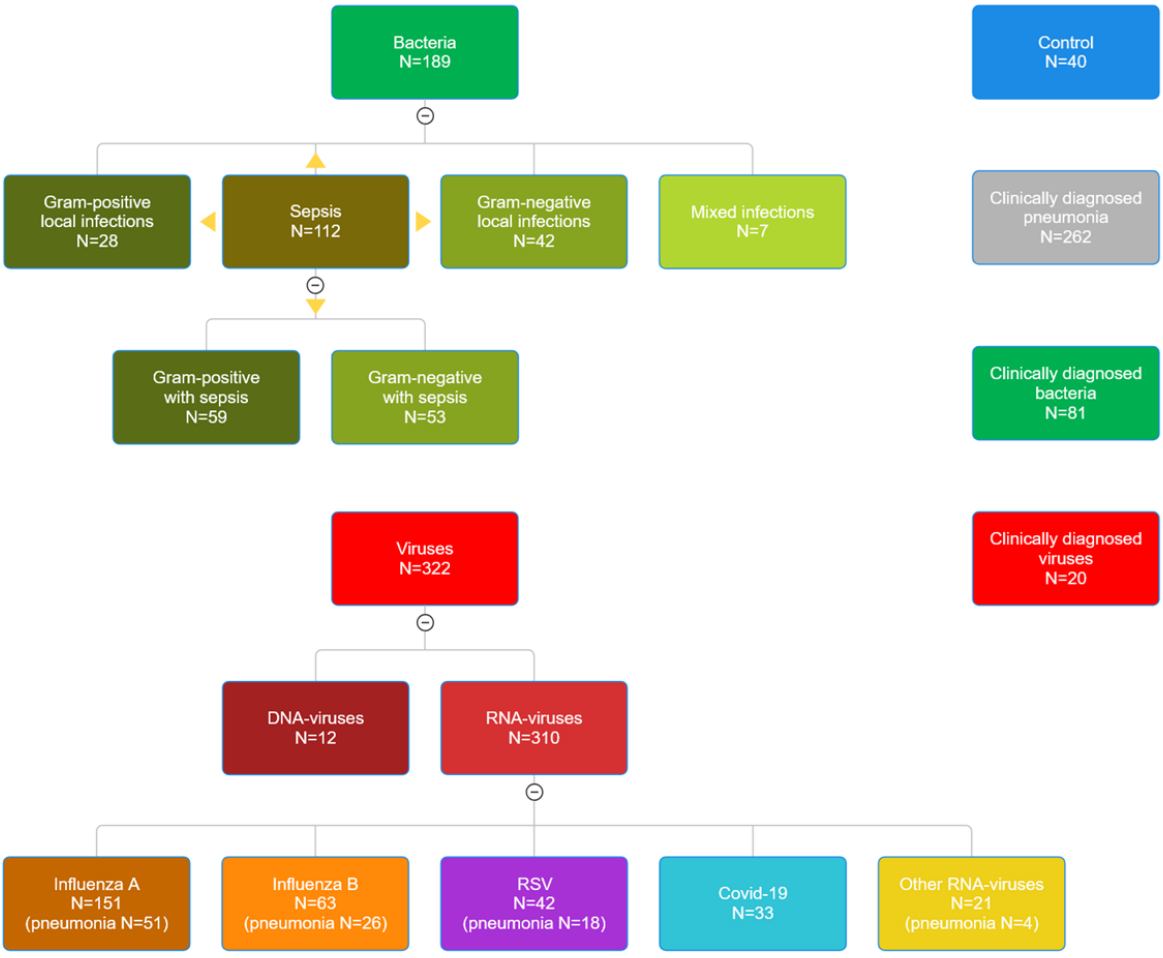

Figure 3. Patients diagnosis. Patient samples used in the study categorized by diagnosis done at TYKS.

4.2 Luciferase modified *Escherichia coli*

Cells used in this research were luciferase (plasmid pEGFPluxABCDEamp) modified K12 strain *E.coli* which emit BL as a result of operon expressing luciferase enzyme complex. Plasmid pEGFPluxABCDEamp was created by ligating plasmid pEGFP (Clontech, Saint-Germain-en-Laye, France) with luciferase genes (luxABCDE) modified from plasmid pSB2025. It was then transformed via electroporation using the Bio-Rad Gene Pulser system (Bio-Rad Laboratories, Richmond, CA, USA) into *E.coli* K-12 strain M72 (SmRlacZ(Am)ΔbiouvrBΔtrpE42[λn7(Am)N53(Am)ca857ΔH1]) (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and identification of positive transformants of the created *E.*coli-lux was conducted using ampicillin resistance and monitoring emission of BL and fluorescence signals. (Atosuo et al. 2013)

High sensitivity luminescence assay (HSLA) was used to determine the activity of complement system by measuring the BL levels produced by *E. coli*-lux cells after exposed to diluted human blood serum samples.

4.3 Complement activity assay

The reaction was carried out in plate wells containing *E. coli* (with $OD_{600\,nm}$ of 0.00175) and diluted serum (0.5%, 0.25%, 0.125% and 0% as a control) in a total volume of 100µl. 0.5% serum dilution was chosen because it has enough complement activity in most samples to kill most of the *E.coli* cells in the measured time of 210 minutes yet still show differences between bacterial and viral samples. In contrast 0.25% serum samples have enough complement activity only on some of the samples and 0.125% serum samples don't have enough complement activity in almost all samples to kill the *E.coli*. Statistics are shown on results. Hank's Balanced Salt Solution (HBSS) was used as a buffer solution. Unfrozen *E. coli* cells from glycerol stock were incubated in buffer solution for 20 minutes RT before adding to the wells. Assay was conducted in 37°C with 3 min intervals for 3 hours and 30 minutes on a Hidex Sense plate reader.

4.4 Data analysis

Raw data from the HSLA was analyzed and calculations regarding the data were done in Excel (Microsoft 365 Apps for enterprise). Graphs were done in Excel apart from the boxplots which were done in Origin (OriginLab). Box plots presented in this study always have lines

representing 95%, 75%, 50%, 25% and 5% of the values fall under the line from top to bottom. Significant differences were calculated using Mann-Whitney U-test in the case of two samples in comparing DNA-viruses and RNA-viruses and Kruskal-Wallis on the rest using SPSS (IBM SPSS Statistics). P-value used to determine whether null hypothesis is rejected, and results are statistically significant is $p<0.05$.

5 Results

5.1 Bioluminescence comparison

We measured the BL signal value (Figure 4), presented as counts per second (CPS), of the *E.coli*-lux over the measured 210 minutes while exposed to the sera of patients with different infections (Figure 3) according to the diagnosis done by TYKS ER and infectious ward. Compared to the *E.coli*-lux 0% serum baseline, the samples exposed to the sera have increased BL signals during the experiment as shown on Figure 4. There seems to be a slight acceleration of *E.coli*-lux metabolism while it is confronted by complement proteins. Figure 4 gives us a visual representation of the difference between bacterial, viral and healthy samples as a result of chosen gradient of dilutions.

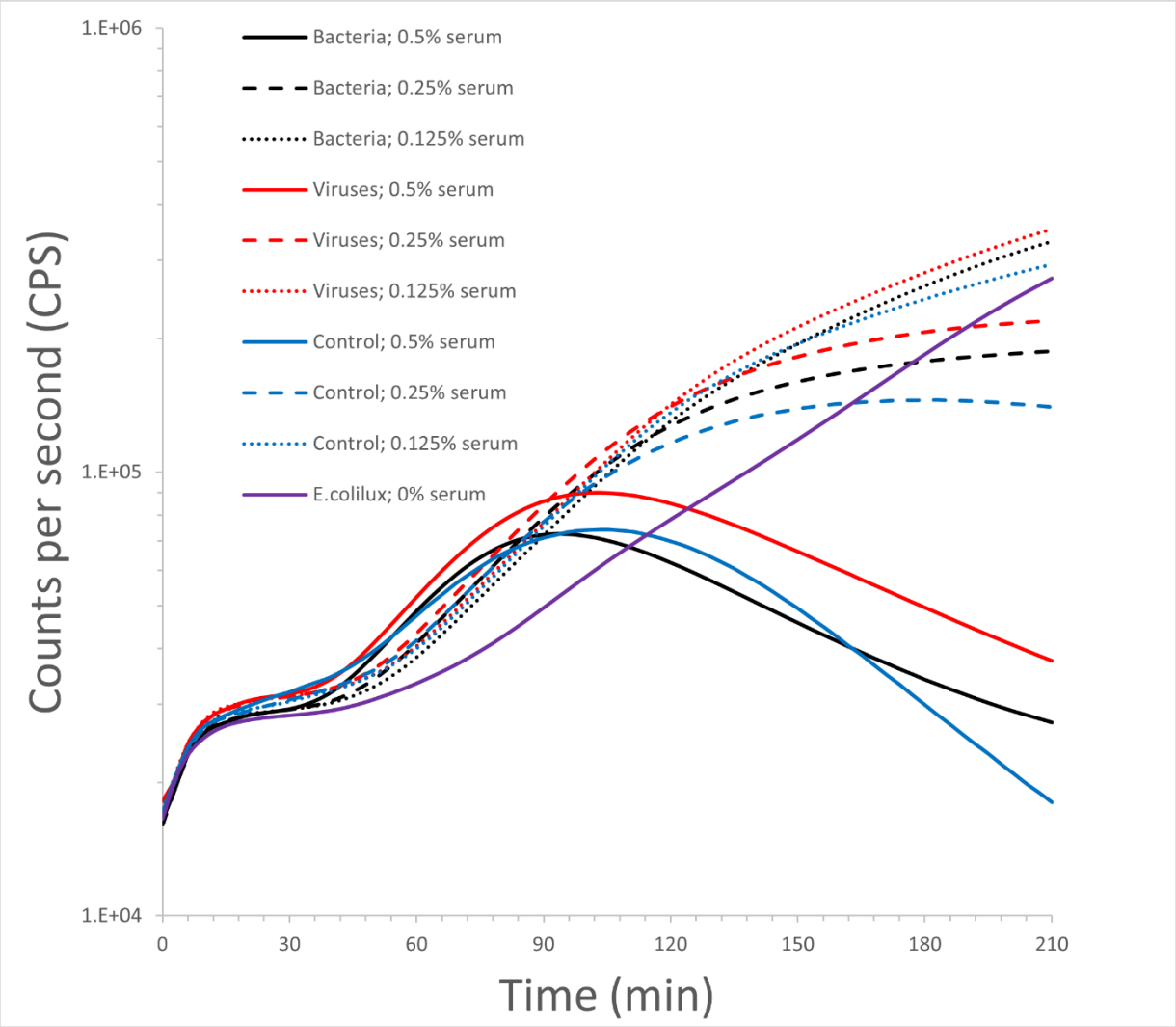

Figure 4. Average bioluminescence values. Bacterial, viral and healthy control samples presented as counts per second during the 210 min measurement.

We then compared the CPS values to the baseline of *E.coli*-lux with 0% serum (Figure 5).

$$relative\ CPS = \frac{CPS}{CPS(0\%\ serum)}$$

From Figure 5 we can see that there are differences in rCPS values and peak times between all three groups.

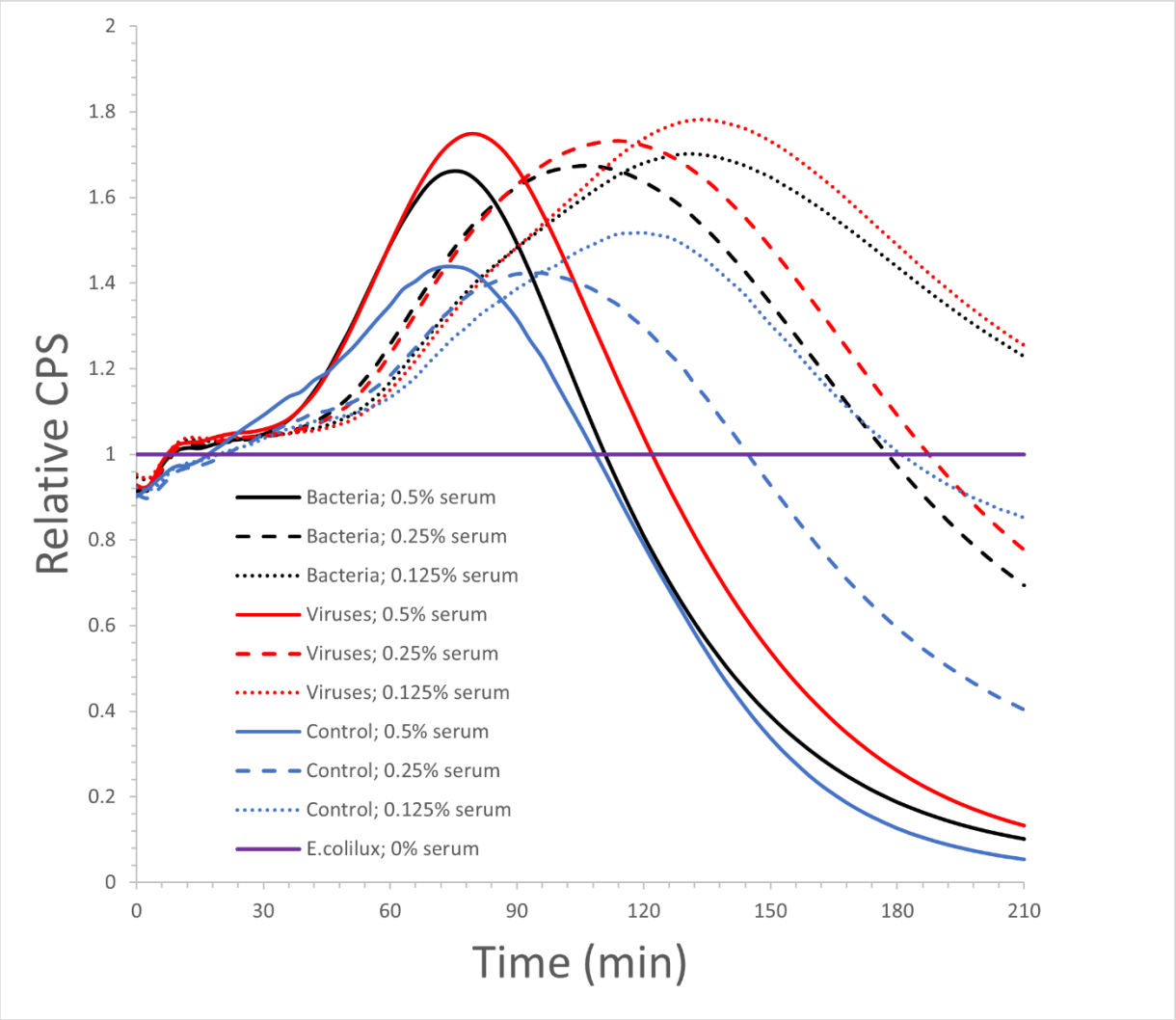

Figure 5. Average relative bioluminescence values. Bacterial, viral and healthy control samples presented as relative counts per second (rCPS) where rCPS = CPS/CPS(0% serum).

5.2 Complement total killing activity

Next, we compared how many of the samples rCPS values dropped at least below 1.00 at 210 min mark. It shows that the 0.50% serum has enough complement activity to kill enough of the *E.coli* to have lower population than the 0% serum background in over 95% of samples in all sample groups (Table 1) but still has differences in kill times as well as total BL activity of

the *E.coli* (Figure 4). 0.25% serum on the other hand has varying kill times and total BL activity within all three sample groups presented as well as 0.125% serum being too diluted to have enough complement activity for us to see differences at all. Interestingly in healthy control samples even the 0.125% diluted serum is enough to kill the *E.coli* population under the 0% serum levels (82.50%) (Table 1).

Table 1. Percentile presentation of the individual samples' rCPS values across all different verified groups with diagnosis at 210 min. It shows that in 0.50% sera, over 95% of all samples in all groups had enough complement activity to reach relative bioluminescence values of under 1.00.

rCPS under 1.00 at 210 min			
	0.5% serum	**0.25% serum**	**0.125% serum**
Bacteria (N=189)	97.38%	75.39%	14.66%
Gram+ (N=87)	96.55%	73.56%	13.79%
Gram- (N=95)	97.89%	75.79%	13.68%
Sepsis (N=112)	96.43%	67.86%	12.50%
Viruses (N=322)	98.76%	71.12%	4.04%
DNA-viruses (N=12)	100.00%	91.67%	8.33%
RNA-viruses (N=310)	98.71%	70.32%	3.87%
InfA (N=151)	98.68%	70.20%	4.64%
InfB (N=63)	100.00%	69.84%	4.76%
RSV (N=42)	100.00%	73.81%	2.38%
Covid-19 (N=33)	96.97%	66.67%	0.00%
Control (N=40)	100.00%	100.00%	82.50%
Clinical pneumonia (N=262)	98.85%	80.53%	11.45%
Clinical bacteria (N=81)	100.00%	86.42%	12.35%
Clinical viruses (N=20)	95.00%	95.00%	15.00%

5.3 Peak time comparison

As a result of clearly different peak times of average samples' relative BL values (Figure 5), we compared the peak times of bacterial, viral and healthy control samples on 0.50% serum (Figure 6), 0.25% serum (Figure 7) and 0.125% serum (Figure 8). On all three figures (Figure 6, Figure 7, Figure 8), we can see that viruses rCPS peak times are achieved significantly slower compared to the peak times of bacteria and healthy control samples as well as bacteria's peak times are achieved significantly slower than healthy control samples' peak times. In other words, bacterial peak times are higher than control peak times and viral peak times are higher than both bacterial and control peak times.

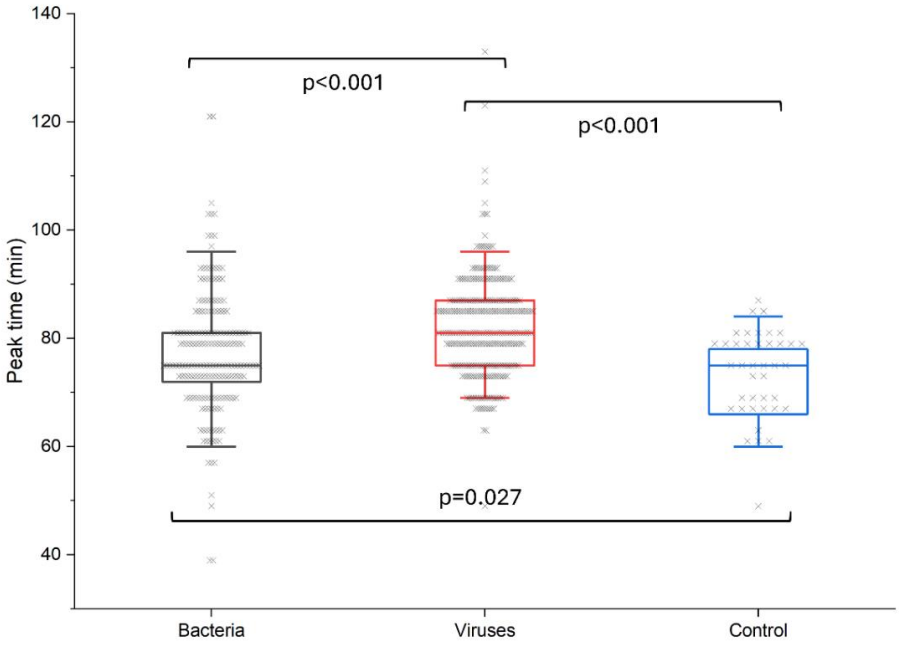

Figure 6. Peak time values of individual 0.50% serum samples rCPS values during the 210 min measurement in bacterially and virally infected patients and healthy controls. Viruses peak times are significantly higher than both bacteria's and controls peak times as well as bacteria's peak times are significantly higher than controls peak times. Significant differences were calculated using Kruskal-Wallis test.

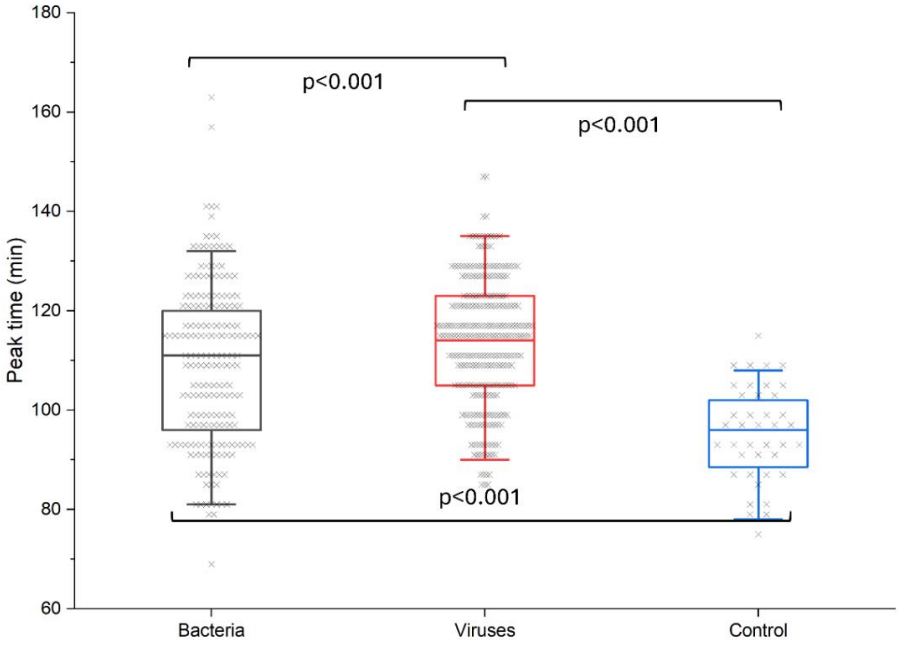

Figure 7. Peak time values of individual 0.25% serum samples rCPS values during the 210 min measurement in bacterially and virally infected patients and healthy controls. Viruses peak times are significantly higher than both bacteria's and controls peak times as well as bacteria's peak times are significantly higher than controls peak times. Significant differences were calculated using Kruskal-Wallis test.

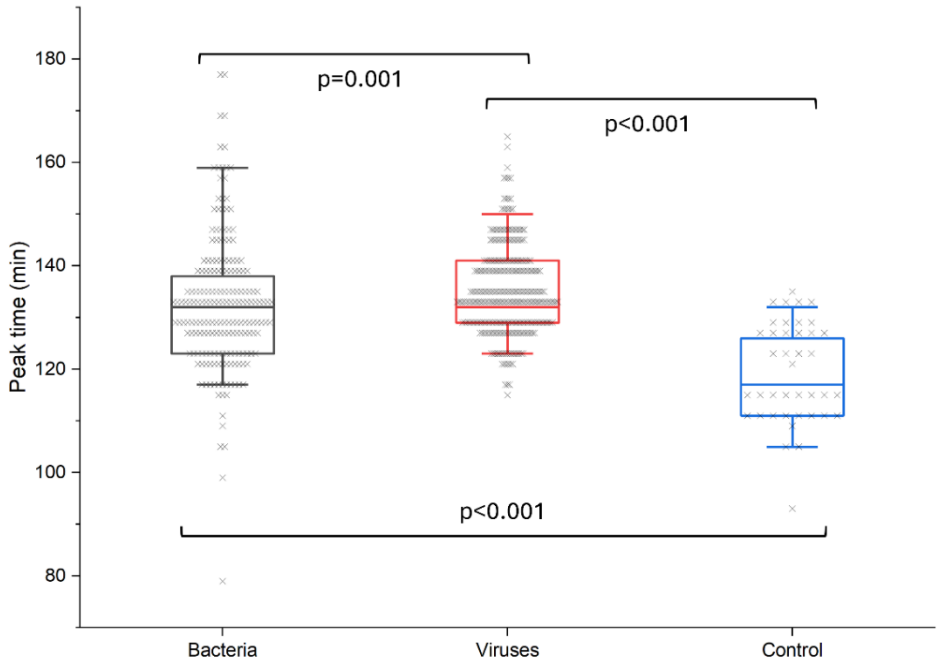

Figure 8. Peak time values of individual 0.125% serum samples rCPS values during the 210 min measurement in bacterially and virally infected patients and healthy controls. Viruses peak times are significantly higher than both bacteria's and controls peak times as well as bacteria's peak times are significantly higher than controls peak times. Significant differences were calculated using Kruskal-Wallis test.

Next, we compared the rCPS peak times together with the rCPS values at the 60-, 90-, 120-, 150-, 180- and 210min marks (Table 2). On all three dilutions the rCPS peak times differ significantly between bacterial, viral and healthy control samples as we saw in previous three figures. The 60min mark rCPS values do not have significant differences on any of the three dilutions as well as 90min mark rCPS values do not have any significant differences on 0.25% serum samples and 0.125% serum samples. At 210min mark on 0.125% serum samples the rCPS values also do not differ. We do see significant differences between the rCPS values at 120-, 150- and 180min marks at all three dilutions with viruses being always the highest values and controls being the lowest values, but more importantly there are significant differences on 0.50% serum samples from 90min mark onwards during the rest of the measurement with rCPS values from highest to lowest in order of viruses, bacteria, controls. P-values for the T-tests without significant differences are as follows: 0.5% 60min p=0.811; 0.25% 60min p=0.220; 0.25% 90min p=0.596; 0.125% 60min p=0.225; 0.125% 90min p=0.804; 0.125% 210min p=0.174.

Table 2. 0.50% serum, 0.25% serum and 0.125% serum samples' rCPS peak times and rCPS values, every 30min from 60min mark onwards, between bacterial- and viral infections compared to the control samples. There are significant differences between bacterial and viral infections on all three dilutions with viruses having always the highest rCPS values and control samples' having always the lowest. Significant differences were calculated using Kruskal-Wallis test.

0.5% serum sample								
	Bacteria		**Viruses**		**B vs V pairwise**	**Control**		**All 3 K-W**
	MEAN	SD	MEAN	SD	T-TEST	MEAN	SD	T-TEST
rCPS peak time (min)	77	11	81	9	p<0.001	73	8	p<0.001
relative (r) CPS (60 min)	1.49	0.26	1.48	0.23	nsd	1.35	0.14	p=0.001
relative (r) CPS (90 min)	1.49	0.47	1.65	0.39	p<0.001	1.32	0.24	p<0.001
relative (r) CPS (120 min)	0.81	0.53	1.02	0.49	p<0.001	0.79	0.22	p<0.001
relative (r) CPS (150 min)	0.39	0.42	0.53	0.41	p<0.001	0.34	0.14	p<0.001
relative (r) CPS (180 min)	0.19	0.30	0.26	0.28	p<0.001	0.13	0.07	p<0.001
relative (r) CPS (210 min)	0.10	0.22	0.13	0.18	p<0.001	0.05	0.03	p<0.001
0.25% serum sample								
	Bacteria		**Viruses**		**B vs V pairwise**	**Control**		**All 3 K-W**
	MEAN	SD	MEAN	SD	T-TEST	MEAN	SD	T-TEST
rCPS peak time (min)	109	16	113	13	p<0.001	95	9	p<0.001
relative (r) CPS (60 min)	1.26	0.17	1.23	0.15	nsd	1.18	0.15	nsd
relative (r) CPS (90 min)	1.62	0.28	1.63	0.23	nsd	1.42	0.24	p<0.001
relative (r) CPS (120 min)	1.64	0.38	1.71	0.29	p=0.030	1.30	0.29	p<0.001
relative (r) CPS (150 min)	1.35	0.47	1.47	0.37	p=0.008	0.93	0.37	p<0.001
relative (r) CPS (180 min)	0.97	0.48	1.09	0.38	p=0.007	0.59	0.38	p<0.001
relative (r) CPS (210 min)	0.69	0.43	0.78	0.35	p=0.012	0.40	0.34	p<0.001
0.125% serum sample								
	Bacteria		**Viruses**		**B vs V pairwise**	**Control**		**All 3 K-W**
	MEAN	SD	MEAN	SD	T-TEST	MEAN	SD	T-TEST
rCPS peak time (min)	135	13	135	10	nsd	119	10	p<0.001
relative (r) CPS (60 min)	1.17	0.14	1.15	0.11	nsd	1.13	0.10	nsd
relative (r) CPS (90 min)	1.48	0.23	1.49	0.19	nsd	1.39	0.20	p=0.009
relative (r) CPS (120 min)	1.68	0.27	1.73	0.22	p=0.006	1.52	0.20	p<0.001
relative (r) CPS (150 min)	1.65	0.24	1.73	0.20	p<0.001	1.30	0.17	p<0.001
relative (r) CPS (180 min)	1.44	0.23	1.50	0.18	p=0.010	1.01	0.14	p<0.001
relative (r) CPS (210 min)	1.23	0.21	1.27	0.16	nsd	0.85	0.13	p<0.001

5.4 Complement index value

Relative BL values (rCPS) taken at different times during the measurement period combined with peak times reduced by 30 minutes allows us to calculate complement index (CI) value using formula presented below. CI value is calculated to have as high values as possible for bacterial samples and as low values as possible for viral samples. rCPS values are BL values presented as counts per second relative to the baseline BL values of the *E.coli*-lux with 0% serum concentration. These are taken from 90-, 120-, 150-, 180- and 210-minute marks during the measurement. Peak time is the timing of the peak of the relative BL value.

$$CI = \frac{rCPS(90min) \times rCPS(120\text{min}) \times rCPS(150\text{min}) \times rCPS(180\text{min})}{[rCPS(210\text{min})]^4} \times \frac{1}{(peaktime - 30min)}$$

This CI value is used to better differentiate the different subgroups from each other. The main interest is between the calculated CI values of bacterial (n = 191) and viral infections (n = 288) with healthy control group (n = 40) that are compared on Figure 9. The CI values of bacteria were significantly higher compared to the CI values of viruses. Control groups CI values were also significantly higher than viruses. As the biggest differences were at 0.50% serum samples, we carry on with the CI value comparisons later with only the 0.50% serum samples.

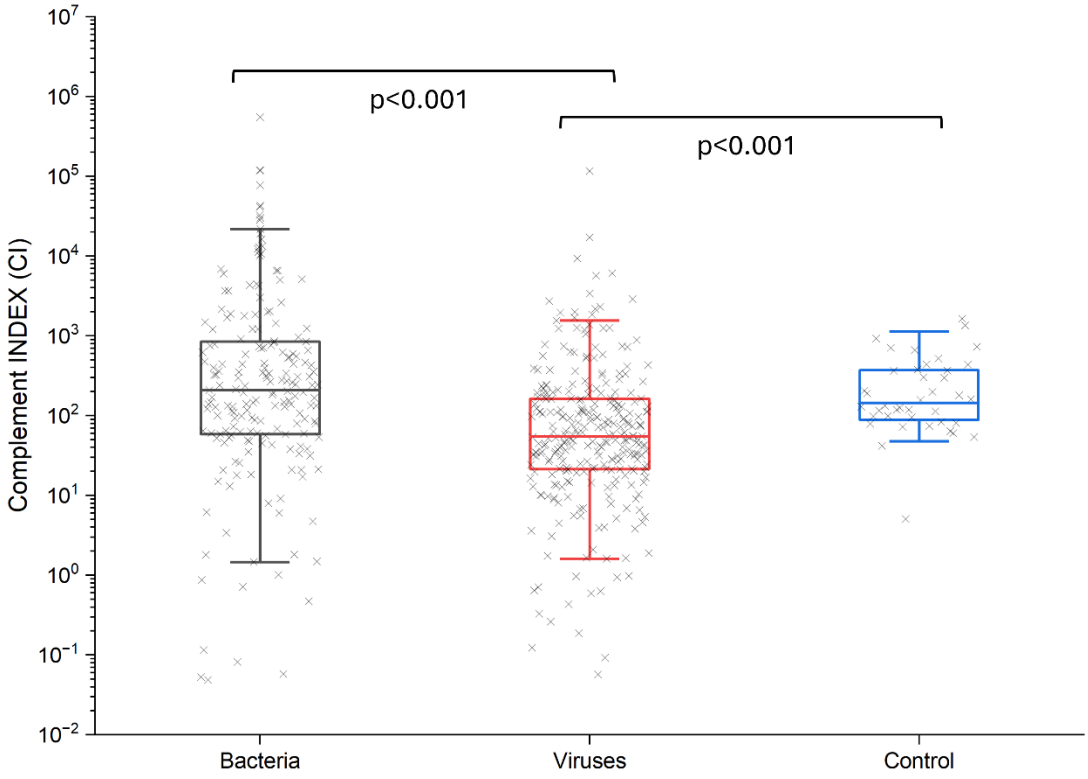

Figure 9. Comparison of complement index values (CI) between bacterial, viral and healthy control samples with 0.50% serum. CI value was calculated from the 0.50% samples' relative bioluminescence values (rCPS) and peak times of the 210min measurement of *E.coli*-lux exposed to serum samples. CI values of both bacteria and controls were significantly higher than CI values of viruses. P-value for the comparison between bacterial and the healthy control samples was p=0.965. Significant differences were calculated using Kruskal-Wallis test.

5.5 Bacterial subpopulations

Using the same procedure as previously we next compared gram-positive local infections (n = 88) and gram-negative local infections (n = 96) as well as samples with sepsis (n = 116) and clinically diagnosed pneumonia (n = 264). Starting with the peak time comparison of 0.50% serum samples (Figure 10), 0.25% serum samples (Figure 11) and 0.125% serum samples (Figure 12).

There were no significant differences between the peak times of the groups in 0.50% dilution (Figure 10). Kruskal-Wallis p=0.384 was for the 0.50% comparison with individual pairwise comparisons as follows: gram-negative and gram-positive p=0.667, sepsis and pneumonia p=0.091, gram-positive and sepsis p=0.250, gram-positive and pneumonia p=0.833, gram-negative and pneumonia p=0.453, gram-negative and sepsis p=0.473.

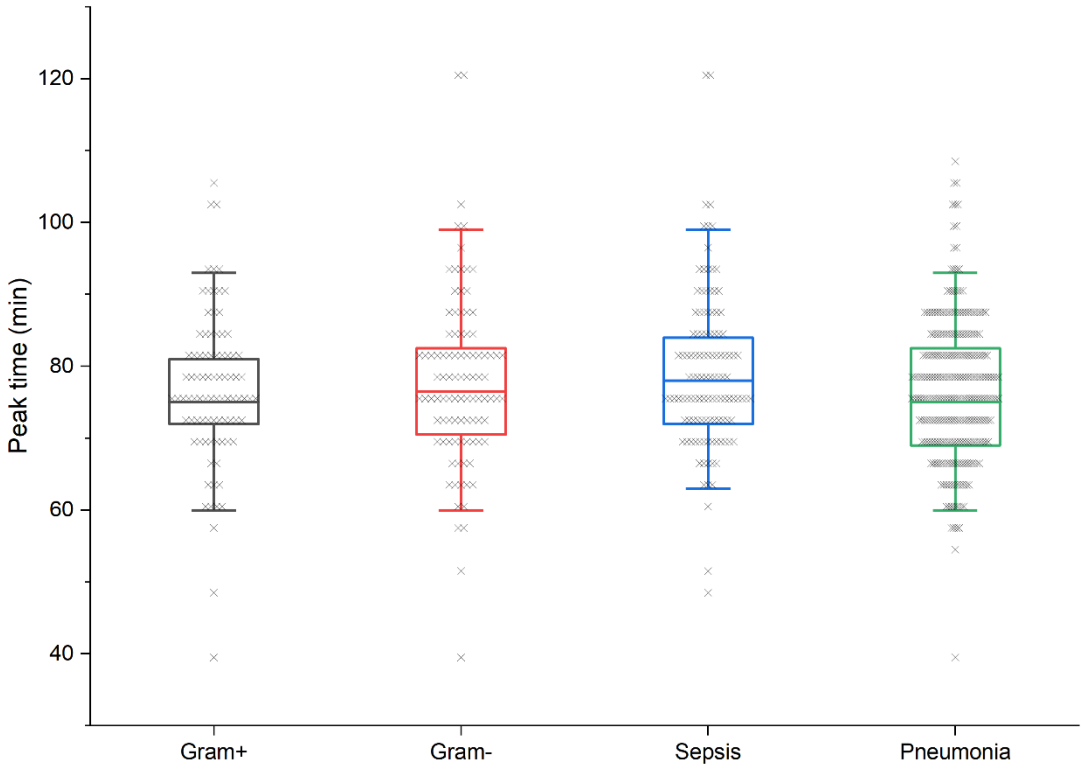

Figure 10. Peak time values of individual 0.50% serum samples rCPS values during the 210min measurement in gram-positive and gram-negative local infections as well as in samples with sepsis and clinically diagnosed pneumonia. There were no significant differences between the groups. Significant differences were calculated using Kruskal-Wallis test.

The peak times of sepsis samples were significantly higher than the peak times of pneumonia samples in 0.25% dilution (Figure 11). Kruskal-Wallis p=0.246 was for the 0.25% comparison with individual pairwise comparisons as follows: gram-negative and gram-positive p=0.950, sepsis and pneumonia p=0.042, gram-positive and sepsis p=0.261, gram-positive and pneumonia p=0.583, gram-negative and pneumonia p=0.625, gram-negative and sepsis p=0.223.

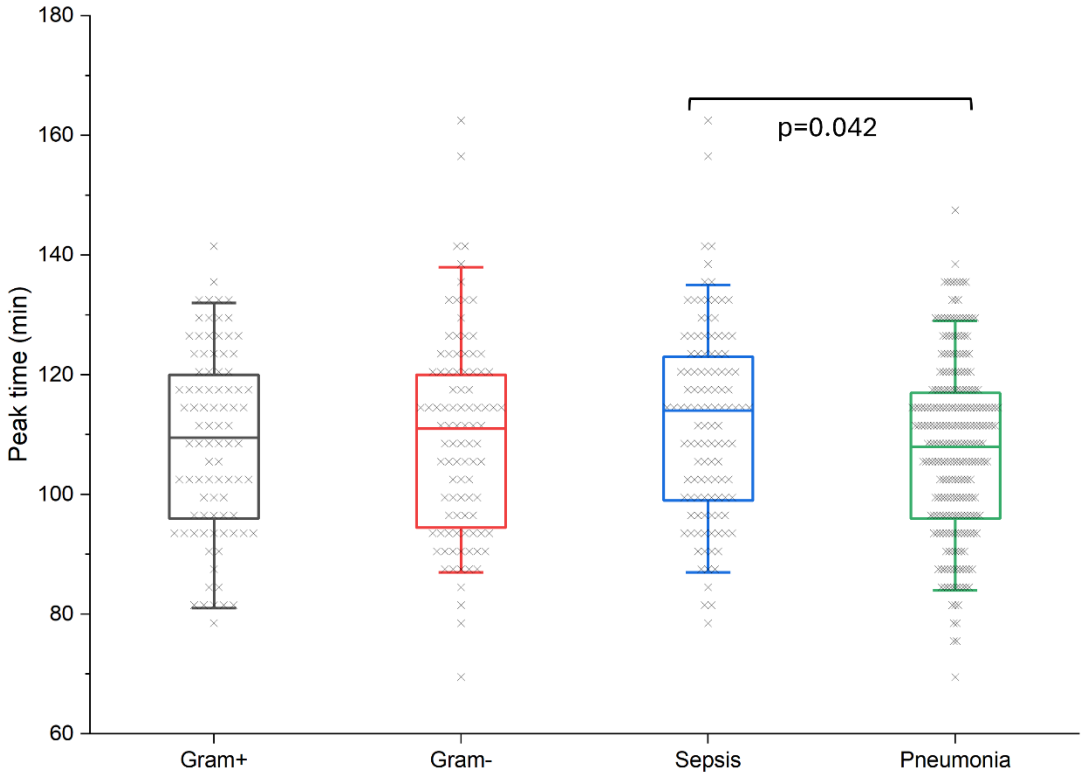

Figure 11. Peak time values of individual 0.25% serum samples rCPS values during the 210min measurement in gram-positive and gram-negative local infections as well as in samples with sepsis and clinically diagnosed pneumonia. There were significant differences between sepsis and pneumonia samples with sepsis peak times being higher. Significant differences were calculated using Kruskal-Wallis test.

The peak times of pneumonia samples were significantly higher than the peak times of both gram-positive and sepsis samples in 0.125% dilution (Figure 12). Kruskal-Wallis p=0.066 was for the 0.125% comparison with individual pairwise comparisons as follows: gram-negative and gram-positive p=0.086, sepsis and pneumonia p=0.047, gram-positive and sepsis p=0.862, gram-positive and pneumonia p=0.045, gram-negative and pneumonia p=0.951, gram-negative and sepsis p=0.097.

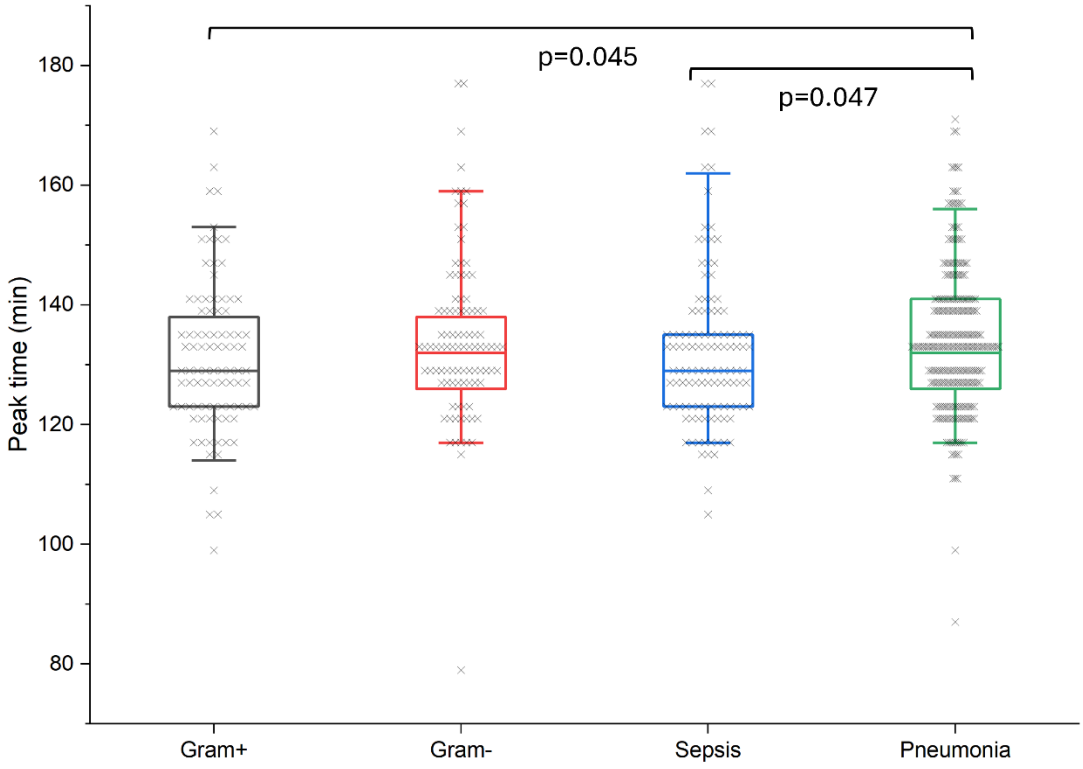

Figure 12. Peak time values of individual 0.125% serum samples rCPS values during the 210min measurement in gram-positive and gram-negative local infections as well as in samples with sepsis and clinically diagnosed pneumonia. There were significant differences between pneumonia samples and both gram-positive and sepsis samples with pneumonia peak times being higher. Significant differences were calculated using Kruskal-Wallis test.

Overall gram-positive and gram-negative bacteria have peak times very similar to total bacterial population as expected and sepsis samples sitting little higher than them. Pneumonia sits very close to total bacterial samples on all three dilutions, but just a little lower.

Then we proceeded to compare the rCPS peak times together with the rCPS values every 30min starting from 60min mark (Table 3). There are significant differences on 0.50% serum samples on 60min mark and 90min mark as well as on 0.125% serum samples with the addition of 210min mark rCPS values. Interestingly there are significant differences between all the measured rCPS values on 0.25% serum samples. Where there are differences, both sepsis- and gram-positive samples have always significantly higher rCPS values than both pneumonia- and gram-negative samples.

Table 3. 0.50% serum, 0.25% serum and 0.125% serum samples' rCPS peak times and rCPS values, every 30min from 60min mark onwards, between gram-positive, gram-negative, sepsis and pneumonia serum samples. There are significant differences between the groups in 0.25% serum samples but less differences on 0.50% serum samples compared to the differences between bacterial and viral samples at the same dilution level. Sepsis- and gram-negative samples always having higher rCPS values than both pneumonia- and gram-negative samples rCPS values where there are significant differences. Significant differences were calculated using Kruskal-Wallis test.

0.5% serum sample									
	Gram+		**Gram-**		**Sepsis**		**Pneumonia**		
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	T-TEST
peak time (min)	76	11	78	12	79	11	76	10	
relative (r) CPS (60 min)	1.54	0.28	1.44	0.24	1.55	0.26	1.46	0.25	p=0.002
relative (r) CPS (90 min)	1.52	0.49	1.46	0.46	1.59	0.46	1.44	0.45	p=0.018
relative (r) CPS (120 min)	0.79	0.52	0.83	0.55	0.90	0.57	0.76	0.47	nsd
relative (r) CPS (150 min)	0.37	0.40	0.41	0.45	0.44	0.46	0.36	0.35	nsd
relative (r) CPS (180 min)	0.18	0.30	0.20	0.31	0.22	0.33	0.17	0.24	nsd
relative (r) CPS (210 min)	0.10	0.24	0.11	0.22	0.12	0.24	0.08	0.16	nsd
0.25% serum sample									
	Gram+		**Gram-**		**Sepsis**		**Pneumonia**		
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	T-TEST
peak time (min)	109	15	109	17	112	16	108	14	
relative (r) CPS (60 min)	1.29	0.19	1.22	0.15	1.29	0.17	1.23	0.16	p=0.001
relative (r) CPS (90 min)	1.67	0.30	1.57	0.24	1.68	0.27	1.58	0.25	p=0.002
relative (r) CPS (120 min)	1.68	0.40	1.59	0.35	1.73	0.35	1.59	0.34	p<0.001
relative (r) CPS (150 min)	1.39	0.49	1.32	0.46	1.47	0.44	1.30	0.43	p=0.003
relative (r) CPS (180 min)	1.01	0.49	0.94	0.47	1.08	0.46	0.91	0.43	p=0.007
relative (r) CPS (210 min)	0.73	0.45	0.66	0.42	0.79	0.43	0.64	0.39	p=0.007
0.125% serum sample									
	Gram+		**Gram-**		**Sepsis**		**Pneumonia**		
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	T-TEST
peak time (min)	131	13	134	14	132	13	134	12	
relative (r) CPS (60 min)	1.20	0.15	1.14	0.11	1.19	0.14	1.14	0.12	p=0.002
relative (r) CPS (90 min)	1.52	0.26	1.44	0.19	1.51	0.25	1.45	0.21	p=0.025
relative (r) CPS (120 min)	1.71	0.30	1.64	0.23	1.71	0.29	1.65	0.25	nsd
relative (r) CPS (150 min)	1.67	0.28	1.62	0.20	1.68	0.25	1.63	0.24	nsd
relative (r) CPS (180 min)	1.47	0.27	1.41	0.19	1.47	0.24	1.41	0.21	nsd
relative (r) CPS (210 min)	1.26	0.24	1.20	0.18	1.26	0.22	1.20	0.18	p=0.032

rCPS values across the board are little higher than total bacterial serum samples' rCPS values for both gram-positive and sepsis samples and little lower for gram-negative and pneumonia samples. Gram-positive and sepsis samples go quite hand in hand as well as gram-negative and pneumonia across all dilutions.

Next, we calculated the CI values from the values presented above on table 3 as we calculated the CI values before on chapter 5.4. CI value comparison between the bacterial subpopulations showed that gram-positive samples' CI values are significantly higher than pneumonia samples' (Figure 13). Kruskal-Wallis p=0.028 was due to the pairwise difference

between gram-positive and pneumonia samples. All CI values were similar to the levels of total bacterial samples' CI values except gram-positive CI values sitting a little higher.

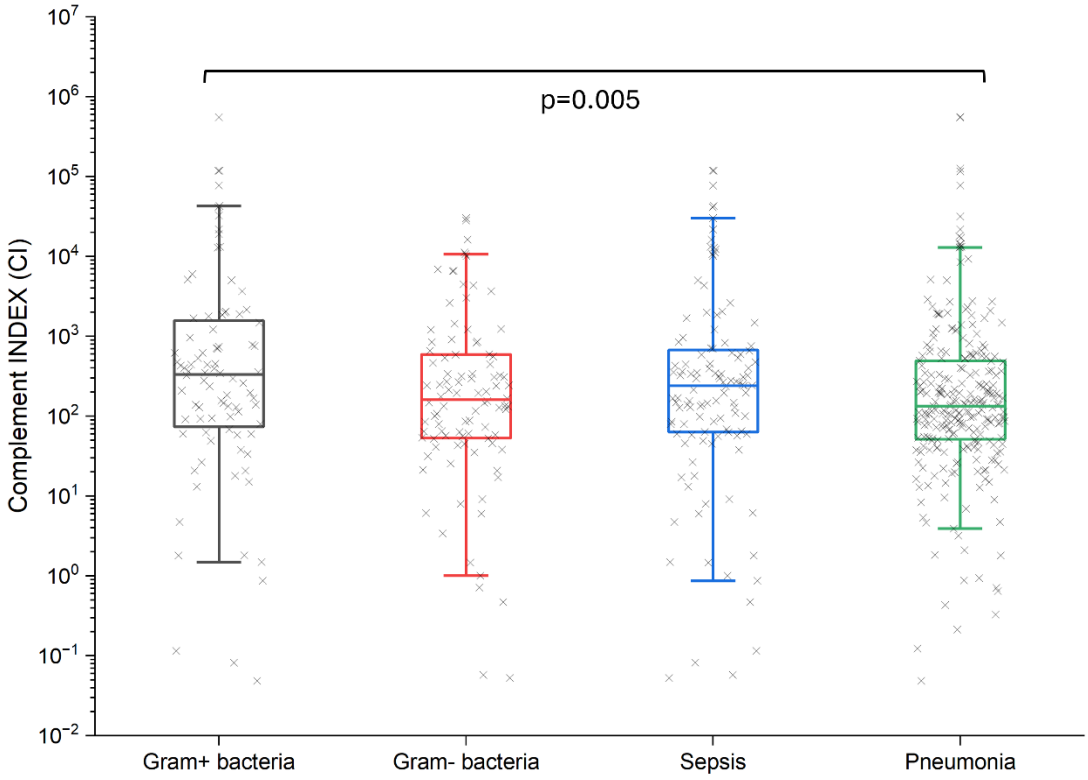

Figure 13. Comparison of complement index values (CI) between gram-positive, gram-negative, sepsis and pneumonia serum samples with 0.50% serum. CI value was calculated from the 0.50% samples' relative bioluminescence values (rCPS) and peak times of the 210min measurement of *E.coli*-lux exposed to serum samples. Only significant difference between groups was gram-positive samples' CI values being significantly higher than pneumonia samples' CI values. Significant differences were calculated using Kruskal-Wallis test.

5.6 RNA-virus subgroups

First as usual we start by comparing the peak times of samples with Influenza A (n = 153), Influenza B (n = 64), RSV (n = 43) and Covid-19 (n = 33). Figure 14 is for the 0.50% serum samples, Figure 15 for the 0.25% serum samples and Figure 16 for the 0.125% serum samples.

On 0.50% dilution influenza B serum samples' peak times were significantly higher than Covid-19 serum samples' peak times (Figure 14). There were no other differences between the groups. Other pairwise comparisons p-values were for influenza A and influenza B

p=0.087, influenza A and RSV p=0.549, influenza A and Covid-19 p=0.380, RSV and Covid-19 p=0.240 and with influenza B and RSV p=0.443. Kruskal-Wallis was p=0.190.

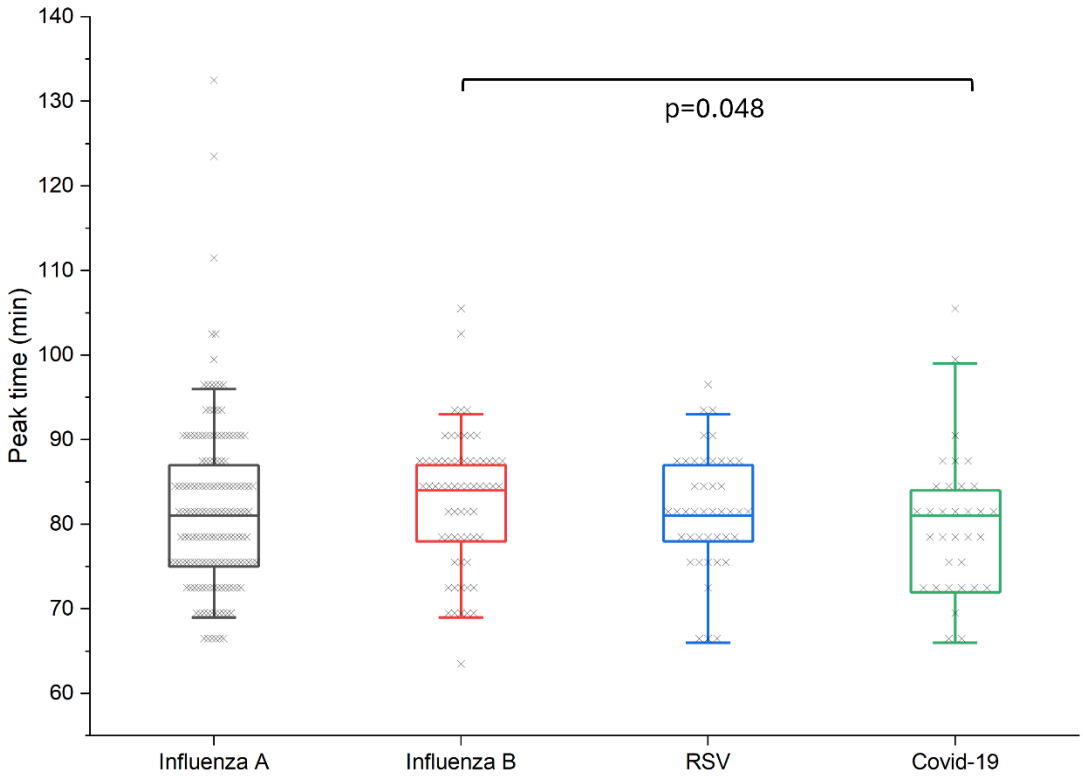

Figure 14. Peak time values of individual 0.50% serum samples rCPS values during the 210min measurement in Influenza A, influenza B, RSV and Covid-19 serum samples. There were significant differences between Influenza B and Covid-19 samples with influenza B serum samples' peak times being significantly higher. Significant differences were calculated using Kruskal-Wallis test.

On 0.25% dilution, the Covid-19 serum samples' peak times were significantly lower than all the other groups' peak times present in this comparison (Figure 15). The p-values with no differences were as follows: influenza A and influenza B p=0.868, influenza A and RSV p=0.871 and influenza B and RSV p=0.986. Kruskal-Wallis was p=0.062.

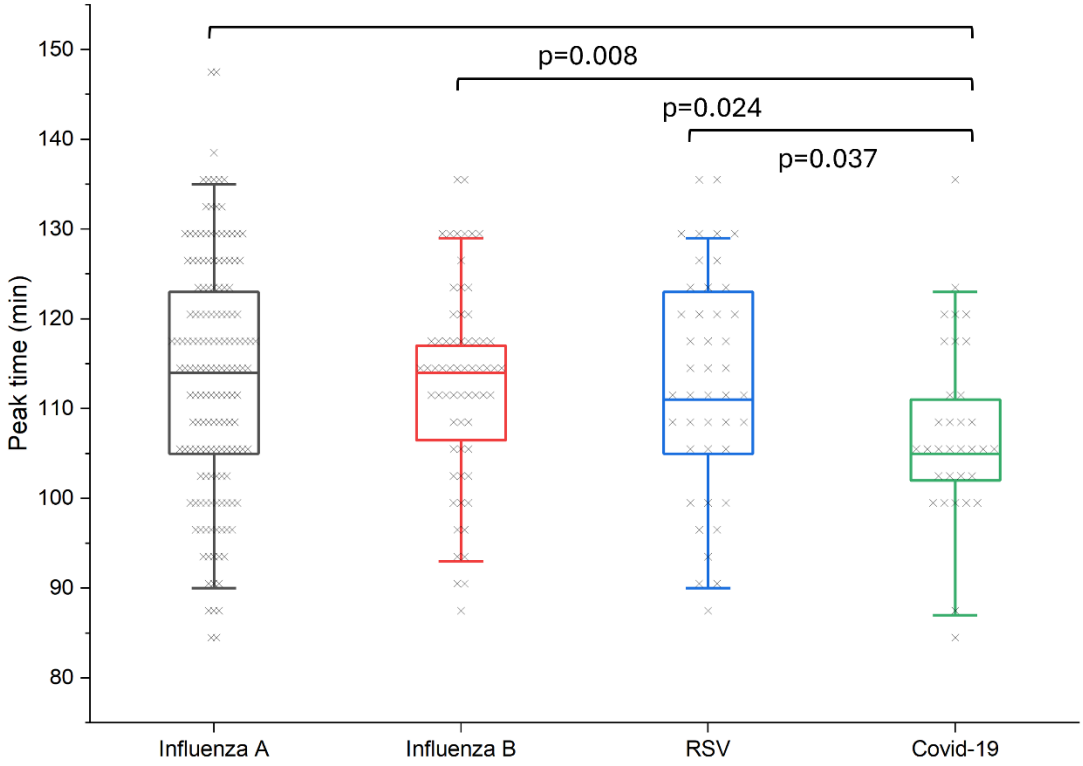

Figure 15. Peak time values of individual 0.25% serum samples rCPS values during the 210min measurement in Influenza A, influenza B, RSV and Covid-19 serum samples. There were significant differences between Covid-19 serum samples compared pairwise to all the other sample groups with Covid-19 serum samples' peak times being significantly lower. Significant differences were calculated using Kruskal-Wallis test.

On the 0.125% serum samples comparisons (Figure 16) there were no significant differences. Pairwise comparisons were as follows: influenza A and influenza B p=0.994, influenza A and RSV p=0.556, influenza A and Covid-19 p=0.855, influenza B and RSV p=0.603, influenza B and Covid-19 p=0.866 and for RSV and Covid-19 p=0.774. Kruskal-Wallis was p=0.944.

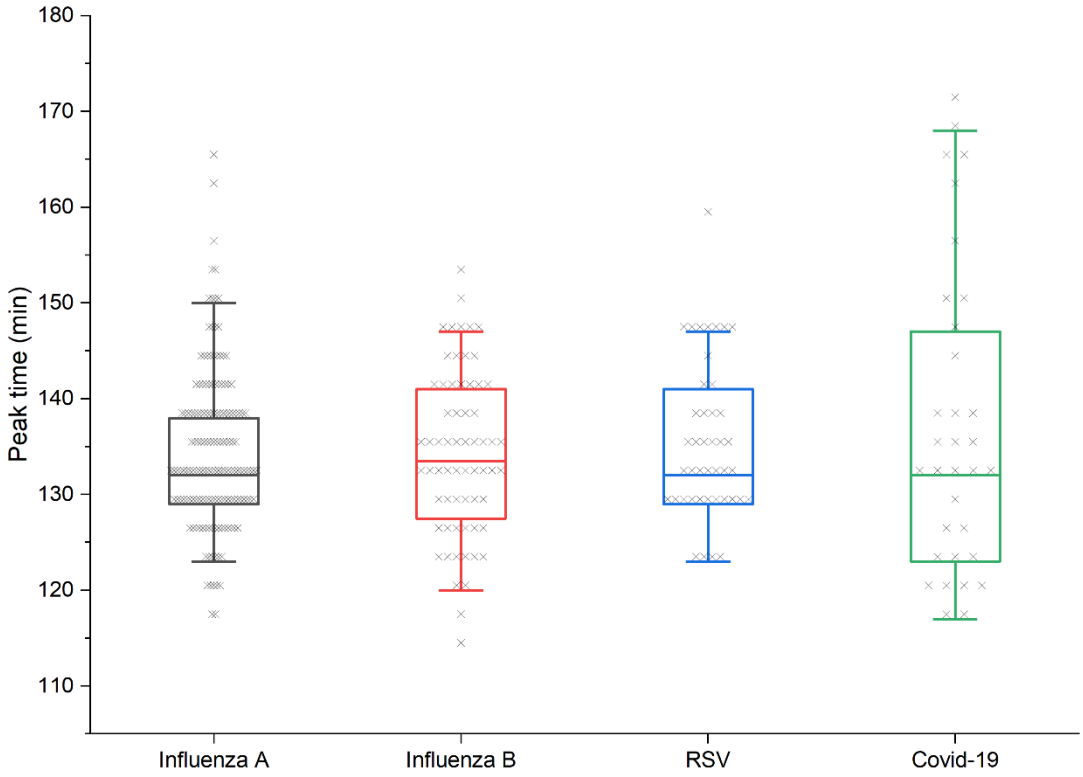

Figure 16. Peak time values of individual 0.125% serum samples rCPS values during the 210min measurement in Influenza A, influenza B, RSV and Covid-19 serum samples. There were no significant differences between the groups. Significant differences were calculated using Kruskal-Wallis test.

There is quite a lot of variances within the RNA-subgroups. Even though the peak times differ only slightly from the total viral peak times they do not differ consistently. On 0.50% serum samples peak times are higher for influenza B and lower for the rest compared to the total viral population. On 0.25% Covid-19 peak times are quite a bit lower yet all other groups are identical to the total viral population and on 0.125% all the groups have higher peak times compared to the total viral peak times.

Next as with previous comparisons we compared the peak values with the rCPS values on 60min mark and every 30min onwards (Table 4). There were only few differences between the rCPS values across the board with them being inconsistent as well. Covid-19 serum samples' rCPS values were significantly lower than rest of the groups rCPS values on 0.50% dilution during 60min mark and 90min mark measurements. On 0.25% dilution and on 0.125% dilution during 60min mark measurement Influenza B serum samples' rCPS values were significantly higher than rest of the groups and during 180min mark and 210min mark

measurements Covid-19 rCPS values were significantly higher than rest of the groups' rCPS values. Expectance for CI value differences is that there is none according to the Table 4.

Table 4. 0.50% serum, 0.25% serum and 0.125% serum samples' rCPS peak times and rCPS values, every 30min from 60min mark onwards, between influenza A, influenza B, RSV and Covid-19 serum samples. There were only significant differences between the 60min mark rCPS values across all dilutions, 90min mark rCPS on 0.50% serum samples and 180min mark and 210min mark on 0.125% serum samples, but way less compared to the comparison between bacterial and viral infections. Influenza A serum samples' rCPS values being significantly higher than the rest on all but the 0.125% 180min mark measurement and 210min mark measurement where Covid-19 had significantly higher values than the rest. Significant differences were calculated using Kruskal-Wallis test.

0.5% serum sample									
	Influenza A		**Influenza B**		**RSV**		**Covid-19**		
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	T-TEST
peak time (min)	81	10	83	8	81	7	80	8	
relative (r) CPS (60 min)	1.54	0.24	1.44	0.18	1.46	0.21	1.34	0.18	p<0.001
relative (r) CPS (90 min)	1.70	0.42	1.65	0.32	1.64	0.34	1.47	0.33	p=0.035
relative (r) CPS (120 min)	1.05	0.53	1.04	0.41	1.01	0.44	0.88	0.44	nsd
relative (r) CPS (150 min)	0.56	0.44	0.52	0.32	0.51	0.34	0.46	0.38	nsd
relative (r) CPS (180 min)	0.28	0.30	0.24	0.20	0.24	0.22	0.25	0.31	nsd
relative (r) CPS (210 min)	0.14	0.19	0.11	0.11	0.11	0.13	0.15	0.26	nsd
0.25% serum sample									
	Influenza A		**Influenza B**		**RSV**		**Covid-19**		
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	T-TEST
peak time (min)	113	13	113	11	113	12	107	10	
relative (r) CPS (60 min)	1.26	0.15	1.20	0.13	1.22	0.15	1.20	0.11	p=0.006
relative (r) CPS (90 min)	1.66	0.24	1.60	0.22	1.61	0.20	1.63	0.14	nsd
relative (r) CPS (120 min)	1.75	0.30	1.69	0.27	1.69	0.25	1.66	0.21	nsd
relative (r) CPS (150 min)	1.50	0.39	1.45	0.32	1.46	0.34	1.41	0.31	nsd
relative (r) CPS (180 min)	1.11	0.40	1.07	0.33	1.08	0.36	1.10	0.36	nsd
relative (r) CPS (210 min)	0.78	0.36	0.77	0.31	0.77	0.32	0.84	0.37	nsd
0.125% serum sample									
	Influenza A		**Influenza B**		**RSV**		**Covid-19**		
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	T-TEST
peak time (min)	134	8	134	9	135	8	137	16	
relative (r) CPS (60 min)	1.17	0.11	1.13	0.09	1.14	0.11	1.13	0.07	p=0.021
relative (r) CPS (90 min)	1.50	0.20	1.47	0.18	1.48	0.17	1.52	0.15	nsd
relative (r) CPS (120 min)	1.76	0.23	1.71	0.21	1.73	0.18	1.72	0.18	nsd
relative (r) CPS (150 min)	1.75	0.22	1.70	0.19	1.72	0.17	1.71	0.12	nsd
relative (r) CPS (180 min)	1.51	0.20	1.45	0.17	1.48	0.15	1.57	0.09	p=0.012
relative (r) CPS (210 min)	1.27	0.16	1.23	0.14	1.25	0.13	1.40	0.09	p<0.001

rCPS values for the influenza A are in general little higher than rest of the groups as well as the total viral populations' rCPS values across the board. Influenza B and RSV on the other hand sit little closer to the total population, but little lower on most occasions. Covid-19 rCPS values are across the board between the values of total bacterial and viral population values.

Lastly for the RNA-subgroup comparison we calculated the CI-values using the formula discussed above. CI-value comparison was done on 0.50% serum samples which is presented

in Figure 17. As previously suggested by the results, there were no significant differences between the RNA-subgroups. Kruskal-Wallis was p=0.652. CI values for all groups are still very close to the total viral populations CI values as expected with influenza A and influenza B values sitting just a little higher than the RSV and Covid-19 values.

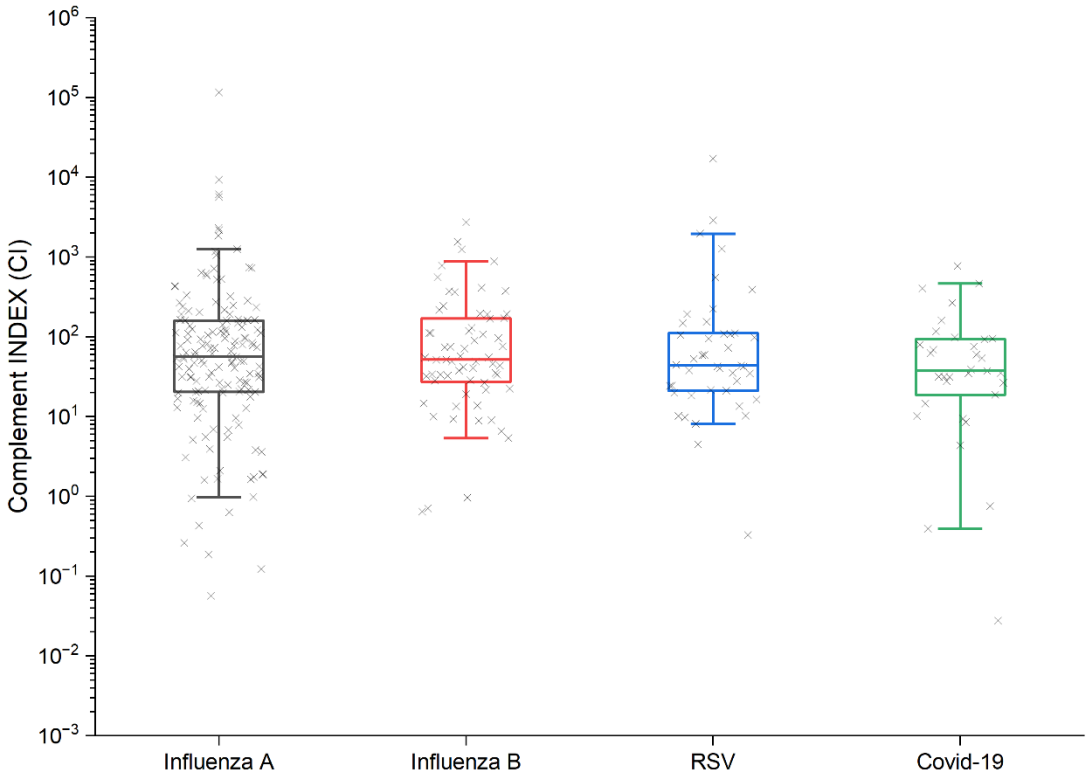

Figure 17. Comparison of complement index values (CI) between influenza A, influenza B, RSV and Covid-19 serum samples with 0.50% serum. CI value was calculated from the 0.50% samples' relative bioluminescence values (rCPS) and peak times of the 210min measurement of *E.coli*-lux exposed to serum samples. There were no significant differences between the groups. Significant differences were calculated using Kruskal-Wallis test.

5.7 Comparison between the clinically diagnosed and confirmed infections

We start the comparison as previously by comparing the peak times of clinically diagnosed bacterial serum samples (n = 81) with the peak times of confirmed bacterial infection serum samples (n = 191). We also compare together clinically diagnosed viral infection serum samples (n = 20) and confirmed viral infection serum samples (n = 288). In addition, we compare in this comparison all the above with each other with the focus on the clinical versus confirmed. Figure 18 presents 0.50% serum samples, Figure 19 the 0.25% serum samples and Figure 20 the 0.125% serum samples.

On 0.50% serum samples, peak times of clinical bacteria were significantly lower than peak times of confirmed bacteria (Figure 18). There were no differences between the confirmed and clinical viruses. Clinical bacteria peak times were also significantly lower than the peak times of both viruses and clinical viruses. P-values for the pairwise comparisons were as follows: bacteria and clinical bacteria p= 0.024, viruses and clinical viruses p=0.539, bacteria and viruses p<0.001, bacteria and clinical viruses p=0.136, clinical bacteria and viruses p<0.001 and clinical bacteria and clinical viruses p=0.009. Kruskal-Wallis was p<0.001.

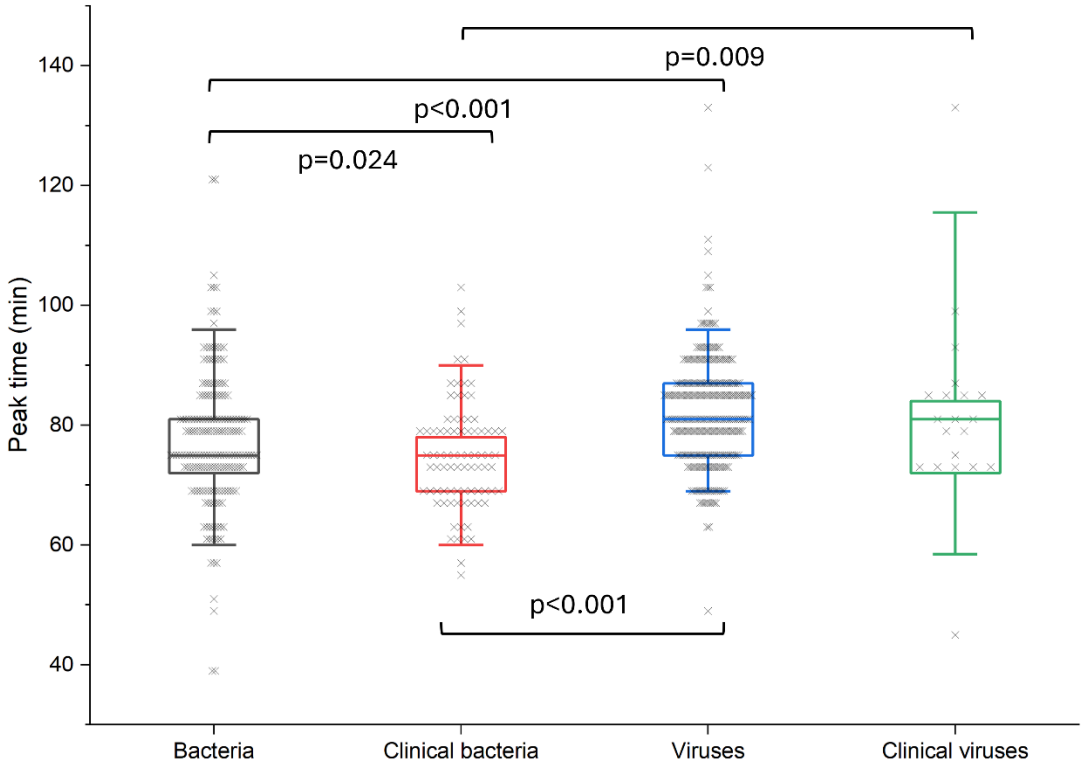

Figure 18. Peak time values of individual 0.50% serum samples rCPS values during the 210min measurement in confirmed bacterial, clinically diagnosed bacterial, confirmed viral and clinically diagnosed viral serum samples. Most importantly, there were significant differences between confirmed bacterial and clinical bacterial serum samples with clinical bacteria having lower peak times, but no significant differences between confirmed viral and clinical viral serum samples. Significant differences were calculated using Kruskal-Wallis test.

On 0.25% serum samples (Figure 19) there were no significant differences between confirmed and clinical infections. Confirmed viruses serum samples' peak times were however significantly higher than those of both clinical and confirmed bacteria's. P-values for the pairwise comparisons were as follows: bacteria and clinical bacteria p=0.063, viruses and clinical viruses p=0.107, bacteria and viruses p<0.001, bacteria and clinical viruses p=0.803,

clinical bacteria and viruses p<0.001, clinical bacteria and clinical viruses p=0.451. Kruskal-Wallis was p<0.001.

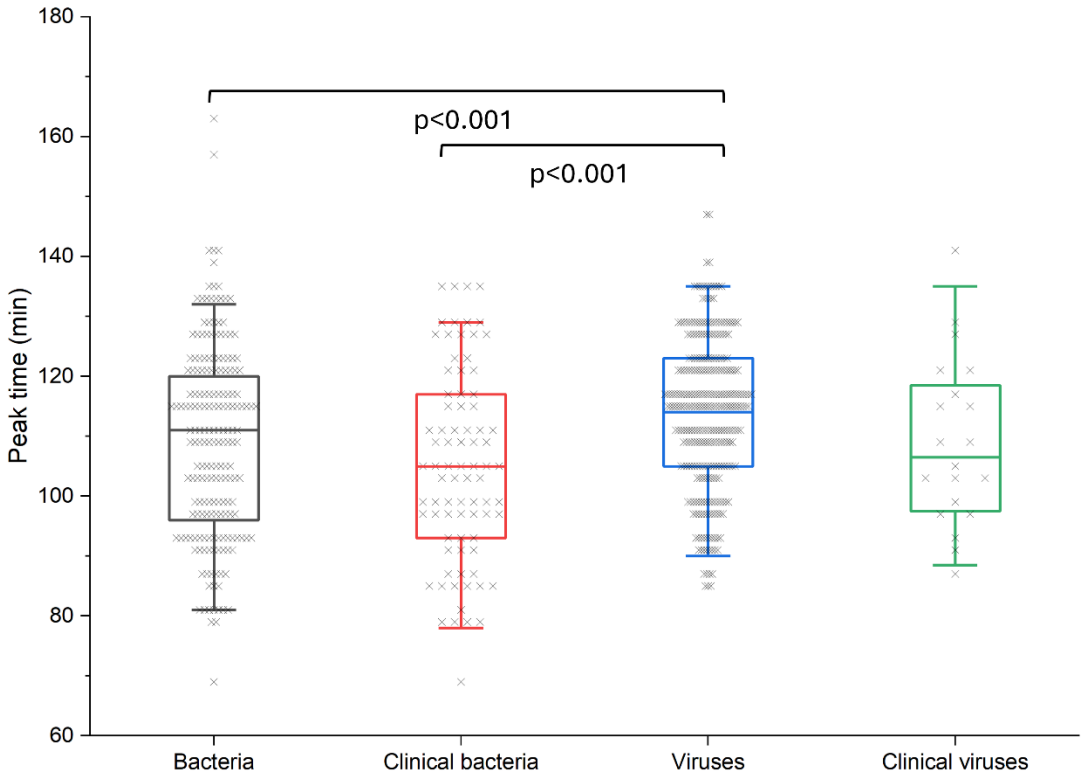

Figure 19. Peak time values of individual 0.25% serum samples rCPS values during the 210min measurement in confirmed bacterial, clinically diagnosed bacterial, confirmed viral and clinically diagnosed viral serum samples. There were no significant differences between the confirmed and clinical bacteria nor between the viruses. Confirmed viruses did have higher peak times than both confirmed and clinical bacteria. Significant differences were calculated using Kruskal-Wallis test.

On 0.125% serum samples (Figure 20) there were no significant differences between confirmed and clinical infections. On this dilution viruses also only had significantly higher peak times compared to the confirmed bacteria. P-values for the pairwise comparisons were as follows: bacteria and clinical bacteria p=0.137, viruses and clinical viruses p=0.527, bacteria and viruses p=0.002, bacteria and clinical viruses p=0.531, clinical bacteria and viruses p=0.444, clinical bacteria and clinical viruses p=0.841. Kruskal-Wallis was p=0.019.

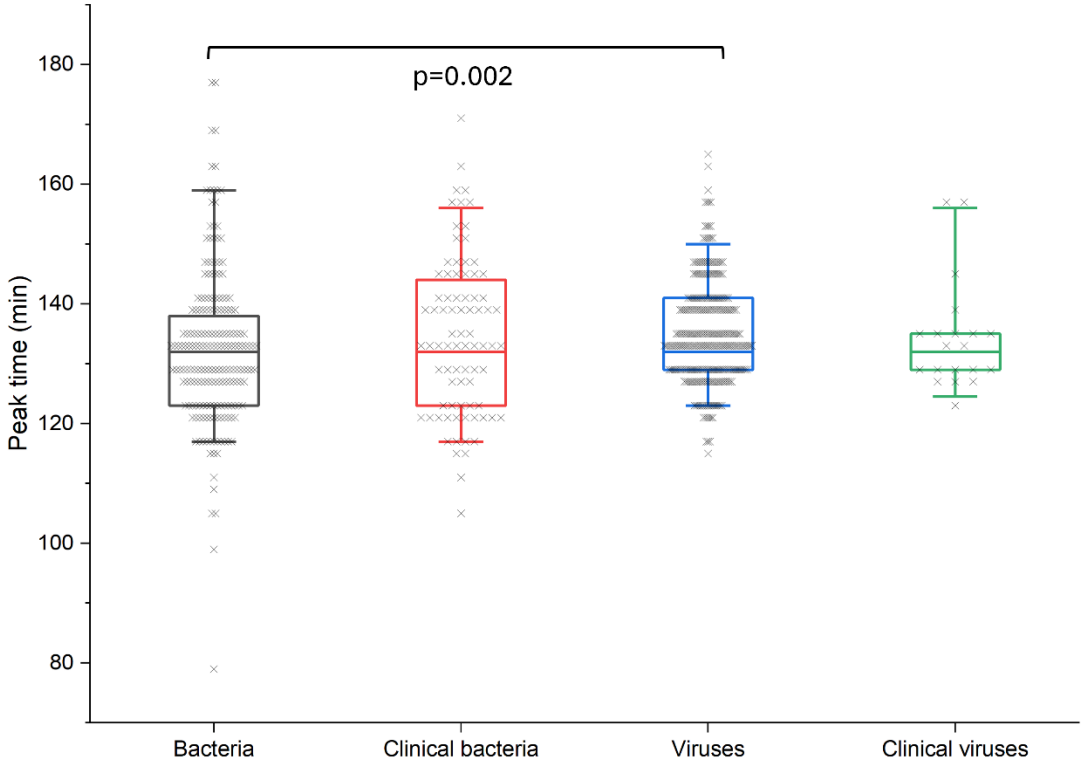

Figure 20. Peak time values of individual 0.125% serum samples rCPS values during the 210min measurement in confirmed bacterial, clinically diagnosed bacterial, confirmed viral and clinically diagnosed viral serum samples. There were no significant differences between the confirmed and clinical bacteria nor between the viruses. Significant differences were calculated using Kruskal-Wallis test.

Clinical bacterial peak times while being quite close to the total bacterial populations CI values are little lower across all dilutions. Compared to the bacterial subgroups they are little lower as well as expected. Clinical viral peak times are quite the same, sitting close to the total viral populations values but the differences are even smaller with 0.50% serum samples' peak times sitting very close but little lower, 0.25% with a bit more difference sitting still lower and 0.125% sitting little higher with little less of a difference.

Then we compared the peak time values together with the rCPS values at 60min mark and onwards every 30min during the measurement. Comparisons were presented on Table 5. Most relevantly bacteria and clinical bacteria differed significantly from each other on both peak times and from 90min mark to 150min mark rCPS values with confirmed bacterial serum samples having both higher peak times and higher rCPS values consistently. There were not

many significant differences between confirmed viruses and clinical viruses, but when they did have significant differences, confirmed viruses values were higher across the board.

Table 5. 0.50% serum, 0.25% serum and 0.125% serum samples' rCPS peak times and rCPS values, every 30min from 60min mark onwards, between clinical bacterial, bacterial, clinical viral and viral serum samples. Most notably the values were significantly higher for bacteria than clinical bacteria on peak times as well as on rCPS values between 90min and 150min mark on 0.50% serum samples. There were only few other significant differences between confirmed and clinical infections on all other dilutions for both bacteria and viruses with confirmed infections always having higher values for both peak times and rCPS values across the board. Significant differences were calculated using Mann-Whitney U test.

0.5% serum sample										
	Bacteria		**Clinical bacteria**			**Viruses**		**Clinical viruses**		
	MEAN	SD	MEAN	SD	T-TEST	MEAN	SD	MEAN	SD	T-TEST
peak time (min)	77	11	74	9	p=0.024	81	9	81	16	nsd
relative (r) CPS (60 min)	1.49	0.26	1.44	0.24	nsd	1.48	0.23	1.39	0.22	p=0.039
relative (r) CPS (90 min)	1.49	0.47	1.29	0.42	p<0.001	1.65	0.39	1.55	0.40	nsd
relative (r) CPS (120 min)	0.81	0.53	0.61	0.40	p=0.003	1.02	0.49	1.01	0.51	nsd
relative (r) CPS (150 min)	0.39	0.42	0.26	0.26	p=0.015	0.53	0.41	0.56	0.53	nsd
relative (r) CPS (180 min)	0.19	0.30	0.11	0.15	nsd	0.26	0.28	0.29	0.44	nsd
relative (r) CPS (210 min)	0.10	0.22	0.05	0.08	nsd	0.13	0.18	0.16	0.35	nsd
0.25% serum sample										
	Bacteria		**Clinical bacteria**			**Viruses**		**Clinical viruses**		
	MEAN	SD	MEAN	SD	T-TEST	MEAN	SD	MEAN	SD	T-TEST
peak time (min)	109	16	105	16	nsd	113	13	108	14	nsd
relative (r) CPS (60 min)	1.26	0.17	1.22	0.15	nsd	1.23	0.15	1.19	0.11	nsd
relative (r) CPS (90 min)	1.62	0.28	1.54	0.22	nsd	1.63	0.23	1.55	0.22	nsd
relative (r) CPS (120 min)	1.64	0.38	1.50	0.31	p=0.002	1.71	0.29	1.59	0.33	p=0.038
relative (r) CPS (150 min)	1.35	0.47	1.15	0.43	p=0.001	1.47	0.37	1.30	0.43	p=0.021
relative (r) CPS (180 min)	0.97	0.48	0.76	0.43	p<0.001	1.09	0.38	0.89	0.41	p=0.010
relative (r) CPS (210 min)	0.69	0.43	0.51	0.38	p<0.001	0.78	0.35	0.60	0.36	p=0.012
0.125% serum sample										
	Bacteria		**Clinical bacteria**			**Viruses**		**Clinical viruses**		
	MEAN	SD	MEAN	SD	T-TEST	MEAN	SD	MEAN	SD	T-TEST
peak time (min)	135	13	134	13	nsd	135	10	134	9	nsd
relative (r) CPS (60 min)	1.17	0.14	1.14	0.11	nsd	1.15	0.11	1.12	0.07	nsd
relative (r) CPS (90 min)	1.48	0.23	1.42	0.18	p=0.039	1.49	0.19	1.43	0.14	nsd
relative (r) CPS (120 min)	1.68	0.27	1.59	0.23	p=0.012	1.73	0.22	1.66	0.19	nsd
relative (r) CPS (150 min)	1.65	0.24	1.57	0.21	p=0.012	1.73	0.20	1.65	0.17	nsd
relative (r) CPS (180 min)	1.44	0.23	1.38	0.19	p=0.048	1.50	0.18	1.40	0.17	p=0.008
relative (r) CPS (210 min)	1.23	0.21	1.17	0.16	p=0.041	1.27	0.16	1.17	0.16	p=0.006

On rCPS values, clinical bacteria have little lower peak times than confirmed total bacterial as well as clinical viruses having lower rCPS values than confirmed total population of viruses. Clinical viruses still staying quite close to the rCPS values of viruses and clinical bacteria to rCPS values of bacteria.

Lastly, we calculated and compared the CI values (Figure 21). Most importantly there were no differences between the clinical bacteria and confirmed bacteria or between the clinical viruses and confirmed viruses. As partially previously confirmed on comparison between bacterial and viral infections there are significant differences between both clinical and

confirmed bacteria with both clinical and confirmed viruses with both bacterial populations being significantly higher than both viral populations. Kruskal-Wallis was $p<0.001$. CI values of clinical bacteria are pretty much identical to the total bacterial population while clinical viruses having little lower CI value mean with still staying well within the same value range of total viruses.

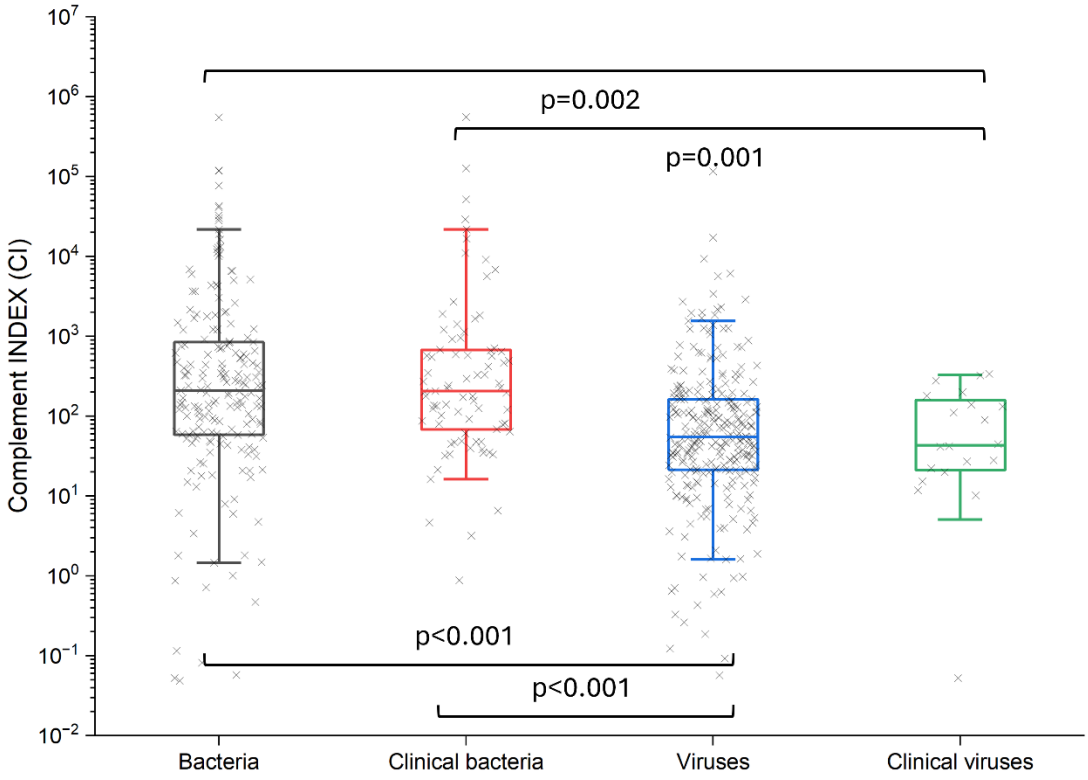

Figure 21. Comparison of complement index values (CI) between confirmed bacterial, clinically diagnosed bacterial, confirmed viral and clinically diagnosed viral serum samples. CI value was calculated from the 0.50% samples' relative bioluminescence values (rCPS) and peak times of the 210min measurement of *E.coli*-lux exposed to serum samples. Notably there were no significant differences between the confirmed and clinical serum samples on bacteria and on viruses. Significant differences were calculated using Kruskal-Wallis test.

6 Discussion and conclusions

6.1 Relevance and expectations

Importance of differentiating quickly between bacterial and viral infections grows by the day as antibiotic resistance grows. Big part of it is too hastily given prescription of antibiotics without confirmed knowledge of the effectiveness of the treatment and as such this method might prove useful in addition to pre-existing methods on confirming the nature of the infection. Method used in this study can be performed fast and without complicated setups but would benefit from further investigation to optimize it.

The hypothesis was that there are differences between bacterial and viral infections since subset of the samples were measured during a pilot study. Although the hypothesis was confirmed correct, there were no notable significant differences between the other provided sample groups based on different diagnosis. We did not include clinically diagnosed infections to the main groups of bacteria and viruses as some might have had some other pathogens contributing to the test results. However, the CI value comparison didn't show differences between the confirmed and clinically diagnosed infections therefore it would have been only singular outliers even when included.

6.2 Viability and possible error margins

The overarching method using *E.coli*-lux to measure anti-bacterial activity has been proven effective prior to this study (Atosuo et al. 2013, 2021). Error margin for human error for the measurement is quite small as it is a simple procedure however there might always be some variations based on the differences between test samples' incubation times and variations between batches of *E.coli*-lux. Incubation varied slightly (usually under 2 minutes) based on how long was needed to thaw the frozen *E.coli*-lux. Although variation was hard to completely negate, the fact that the CI values were calculated from the rCPS values meant that it should not affect the CI values significantly as the rCPS values were compared to the bacterial baseline BL signal of the same measurement which had the same incubation time between the samples.

Another possible error margin would be that the sample sizes for some of the sample groups were quite small (smallest was $n = 20$ for clinical viruses). If there would be further studies between the subpopulations of viral and bacterial infections, even larger test group would be

beneficial for that aspect. Another angle for studying the subpopulation differences would be to do measurements blocking different pathways functions during the measurement. However, the activities of complements different pathways were not measured separately at all in this study due to time constraints.

There might also be some underlying effectors on individual samples that would affect the complement activity besides the pathogen. It would probably be negligible comparing the bigger sample sizes for example for the main comparison between viral and bacterial infections, but it could affect the comparisons governing the smaller sample sizes present in this study like clinical comparisons or Covid-19 comparisons.

6.3 Future

There was too much overlap between bacterial and viral samples to use the method on its own in clinical setting, but the significant difference we can see between bacterial and viral samples in CI value is very much a possible avenue for clinical application with further research. However, with current knowledge provided by this study differentiating between other subpopulations covered here is not possible with this method.

For future studies it would be good to continue optimizing and providing good framework for automating the method as previously mentioned. The measurements of individual pathways activities that we did not have time to execute in this study would also be interesting to see. This could be achieved at least partially by blocking classical and lectin pathways with EGTA. It binds Ca^{2+} of which CP and LP are dependent. (Atosuo et al. 2021) Overall the study provides a good baseline for future research.

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