Institute of Immunology and Molecular Biology
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Freie Universität Berlin

Thermostability of selected viruses in the presence of liquid egg yolk and distribution of virus during acetone extraction and microfiltration of egg yolk phospholipids

Inaugural Dissertation
to obtain the academic degree of
Doctor of Veterinary Medicine (Dr. med. vet.)
at the
Freie Universität Berlin

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Berlin 2012

Journal Nr.: 3624

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Descriptors (according to CAB Thesaurus):
heat resistance, microfiltration, acetone, extraction, real time PCR, phospholipids, egg yolk, avian influenza viruses, swine influenza viruses, Newcastle disease virus, Feline calicivirus, Porcine parvovirus

Day of graduation: 12.04.2013

Bibliografische Information der *Deutschen Nationalbibliothek*
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <http://dnb.ddb.de> abrufbar.

ISBN: 978-3-86387-298-4
Zugl.: Berlin, Freie Univ., Diss., 2012
Dissertation, Freie Universität Berlin
D 188

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verlag@menschundbuch.de – www.menschundbuch.de

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List of Abbreviations

AA	acrylamide
AIV	avian influenza virus
APS	ammonium persulfate
ATCC	American Type Culture Collection
B19	human parvovirus B19
BAEE	benzoyl-L-arginine ethyl ester
BPV	bovine parvovirus
Ca	calcium
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
CEF	chicken embryo fibroblasts
CFSPH	Center for Food Security & Public Health
C_L	constant domain of light chain of IgY or IgG
CPE	cytopathogenic effect
CRFK	Crandell Reese feline kidney cells
ddH_2O	double distilled water
DMEM	Dulbecco's Modified Eagle Medium
dNTP	deoxyribonucleotide triphosphate
DPBS	Dulbecco's Phosphate Buffered Saline
EDTA	ethylenediaminetetraacetic acid
EID_{50}/g	egg infectious doses
ELD_{50}/ml	egg lethal doses
ELISA	Enzyme Linked Immunosorbent Assay
EMEM	Eagle's minimum essential medium
EPIA	Egg Products Inspection Act
EtOH	ethanol
EU	European Union
Fc-receptor	Fc (Fragment, crystallisable) region of immunoglobulin
FCS	foetal calf serum
FCV	feline calicivirus
h	hour
HA	haemagglutinin
HAV	hepatitis A virus
HI test	haemagglutination inhibition test
HPAIV	highly pathogenic avian influenza virus

IgG	immunoglobulin G (gamma)
IgY	immunoglobulin Y
kb	kilo base
LCPUFA	long chain polyunsaturated fatty acids
M1	matrix protein
MDBK	Madin-Darby bovine kidney cells
MDCK II	Madin-Darby canine kidney cells II
Med	medium
MEM Eagle	Minimum Essential Medium Eagle
Mg	magnesium
min	minute
MOI	multiplicity of infection
MVM	minute virus of mice (parvovirus)
µm	micro meter
NA	neuraminidase
NaCl	sodium chloride
NDV	Newcastle disease virus
NEP/NS2	nuclear export protein
NLV	Norwalk-like viruses
nm	nanometer
NP	nucleoprotein
NV	Norwalk virus
OIE	World Organisation for Animal Health (Office International des Epizooties)
ORF	open reading frames
PBS	phosphate-buffered saline
pfu	plaque-forming unit
pH	(decimal logarithm of the reciprocal of the hydrogen ion activity)
PK_{13}	porcine kidney cells
ppt	parts per trillion
PPV	porcine parvovirus
qRT-PCR	quantitative real-time polymerase chain reaction
RF	reduction factor
RNA	ribonucleic acid
RNP	ribonucleoprotein
RO	reverse osmosis

rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
SIV	swine influenza virus
SMEDI	stillbirth, mummification, embryonic death and infertility
SPF	specific pathogen free
$TCID_{50}$	50% tissue culture infective dose
TEMED	tetramethylethylenediamine
TMB substrate	3,3′,5,5′-tetramethylbenzidine substrate
TNE buffer	100 mM Tris, 2.0 M NaCl, 10 mM EDTA buffer at pH 7.4
UK	United Kingdom
USDA	United States Department of Agriculture
UV-light	ultraviolet light
V_H	variable domain of heavy chain of IgY or IgG
V_L	variable domain of light chain of IgY or IgG
VP	viral proteins
w/o	without
w/v	weight/volume

1. Introduction

The ability of viruses to remain infectious for extended periods of time in the environment influences their transmission potential (Stallknecht & Brown, 2009). The presence of viruses such as avian influenza virus in the environment depends on cumulative effects of both physical and chemical factors, for instance (1) stability in dry, humid or liquid environments, (2) influence of organic compounds, (3) the pH, (4) salt concentration and (5) temperature (Davidson et al., 2010). Therefore this study was undertaken to evaluate the stability of several viruses belonging to different virus families, namely avian influenza virus, Newcastle disease virus, feline calicivirus, porcine parvovirus and swine influenza virus in various environments.

Hen egg has an excellent nutritive value and constitutes a traditional food used in many basic and formulated preparations. Besides its important content of easily digestible proteins, lipids, vitamins and minerals, the egg contains molecules with numerous health-promoting and biological properties (Anton & Nys, 2006). With a rising income, the demand for animal protein increases and the efficient conversion of feed into eggs stimulates animal protein consumption (FAO, 2010). The term “egg products” refers to those eggs that have been removed from shells for further processing at facilities that are called breaker plants (USDA, 2011b; FAO, 2010). Basic egg products include whole eggs, whites, yolks and various blends with or without non-egg ingredients that are processed and pasteurized (USDA, 2011b). The trade in egg products, especially that in dried egg, is similar to the trade of in-shell or liquid eggs, with a rising trend in recent years. Most of the trade is conducted among EU members (FAO, 2010). With the increase in global trade, egg products could create potential biosafety problems and affect international trade in liquid and dried egg products (Chmielewski et al., 2011). Moreover, some poultry viruses such as highly pathogenic avian influenza virus (HPAIV) and Newcastle disease virus (NDV) are notifiable to national authorities as well as to OIE and are included in their List A of animal diseases (OIE, 2012b). Influenza viruses as well as NDV can be transmitted by contaminated eggs either vertically via infected eggs or horizontally by virus-contaminated egg surfaces (Swayne & Beck, 2004). Contaminated eggs could be a concern since some consumers may disregard or be unaware of the risk of infection when preparing foods such as mayonnaise with raw eggs (Spickler et al., 2008). Pasteurization of egg products has been practiced since the 1930s, primarily as a food safety measure to inactivate contaminating *Salmonella* present in the faeces on the eggshell surface or in the internal contents of the egg (Vandepopuliere, 1996). However, a side benefit of pasteurization could be inactivation of viruses as well as other bacterial species or fungi that could affect human or possibly animal health (Swayne & Beck, 2004). Therefore it is necessary to determine the stability of these viruses in eggs or egg products during pasteurization.

Additional production steps such as solvent extraction and filtration might be performed in order to obtain defined egg products. For instance, triglycerides and cholesterol are preferentially extracted by acetone, and phospholipids are subsequently purified by ethanol extraction from such intermediates (Nielsen, 2001). The filtration has two main purposes: to remove unwanted solids from suspension in a fluid (the fluid itself might be a wanted product or a waste product that needs cleaning prior to disposal) and to recover a wanted solid product from a suspension (often following a prior crystallization or precipitation step) (Sutherland, 2008). To my knowledge, there are no published data about the virus-inactivating potential of the solvent (acetone) extraction and filtration processes used by the egg-processing industry. Thus, this comparative study on viral stability also includes an assessment of selected extraction procedures with organic solvents.

The objectives of this study were as follows:

- To compare the heat stability of avian influenza virus (AIV), Newcastle disease virus (NDV), feline calicivirus (FCV) and porcine parvovirus (PPV), and to determine the influence of liquid egg yolk on the stability of AIV, NDV and FCV,

- To evaluate and compare the heat stability of swine influenza virus (SIV) and AIV,

- To determine the influence of egg yolk and water content on virus inactivation during acetone extraction,

- To evaluate the removal of virus by microfiltration and to compare the distribution of the viral genome and infectivity in different fractions after partitioning through microfiltration.

2. Literature

2.1 Egg

Eggs of a variety of avian species have been recognized as a valuable source of nutrients for humans for a very long time (Li-Chan & Kim, 2008). Today they are also understood to contain substances with biological functions more than just basic nutrition, and on-going research identified and characterized these biologically active components (Kovacs-Nolan et al., 2005). The production of hen eggs was increased with the growth of the world population. In 1970, the egg production was 19,538,000 tons and rose to 59,233,000 tons in 2005. The number was tripled within a few years and the production volume will be higher than that of beef and veal if the growth rate remains constant (Windhorst, 2007).

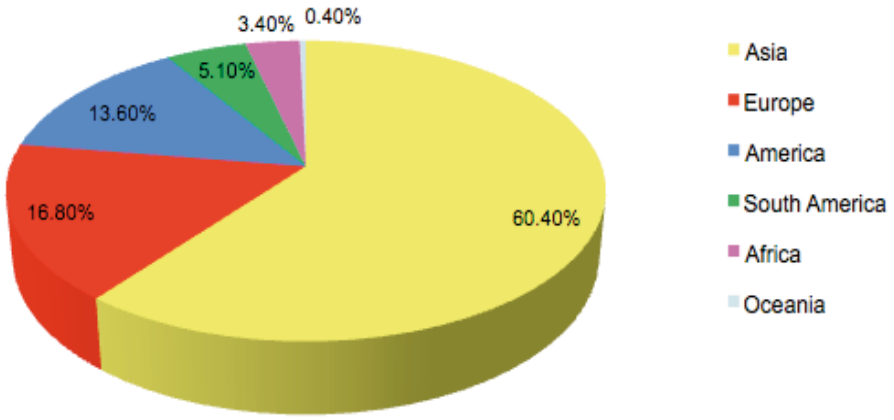
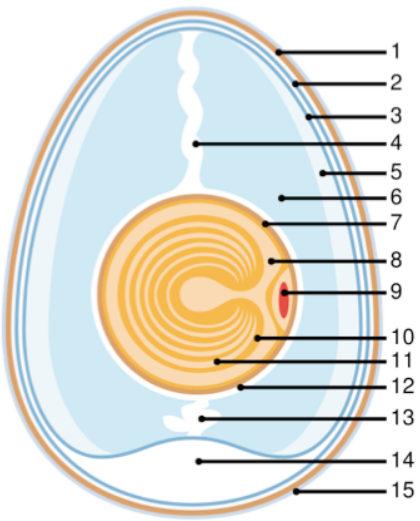


Figure 1: Worldwide distribution of hen egg production in 2005 (adapted from Windhorst, 2007)

The worldwide distribution of hen egg production in 2005 is presented in Figure 1. According to Windhorst (2007), the highest volume of egg production was contributed by Asia which produced 60.4% of the global volume. The share of Europe, North and Central America, South America, Africa and Oceania were 16.8%, 13.6%, 5.1%, 3.4% and 0.4%, respectively. When compared with 1970, the egg production volume in Asia has increased while Europe and North and Central America lost large amounts of their previous market share. South America, which until 1990 had been able to hold a higher market share, has not been able to keep their position in spite of an almost continuous growth of the production volume.

2.1.1 Structure and composition

The avian egg is a reproductive structure that has been developing through evolution to resist physical, microbial and thermal attacks from an external and possibly aggressive environment, while satisfying the needs of the developing embryo (Hincke et al., 2008).



Schematic of a chicken egg:

1. Eggshell
2. Outer membrane
3. Inner membrane
4. Chalaza
5. Exterior albumen (outer thin albumen)
6. Middle albumen (inner thick albumen)
7. Vitelline membrane
8. Nucleus of pander
9. Germinal disk (blastoderm)
10. Yellow yolk
11. White yolk
12. Internal albumen
13. Chalaza
14. Air cell
15. Cuticula

Figure 2: Structure of hen egg ("Egg (food)", 2012)

There are three main parts in an egg; the shell, the albumen and the yolk (Kovacs-Nolan & Mine, 2004). The yolk is surrounded by an albumen layer and compartmentalized by an eggshell (Kovacs-Nolan & Mine, 2004). The egg structure was well described by Li-Chan & Kim (2008) and was summarized as follow: The eggshell is composed of a foamy layer of cuticle, a calcite or calcium carbonate layer and two shell membranes. The most external layer of eggs is the water-insoluble cuticles; it is approximately 10–30 μm thick and covers the pore canals. It helps protect the egg from moisture and microbial invasion. The eggshell membrane structure is composed of inner and outer membranes that reside between the albumen and the inner surface of the shell. Eggshell membranes add to shell strength by serving as a reinforcement of the crystalline part of the shell. There are four layers in albumen or egg white: an outer thin white one next to the shell membrane, a viscous or outer thick white one, an inner thin white one and a chalaziferous or inner thick one. The chalaziferous layer is twisted, forming a thick rope-like structure, termed the chalazae cord, which suspends the yolk between the upper and lower end of the egg. The chalazae are slightly elastic and permit limited rotation of the yolk (Romanoff and Romanoff, 1949). The yolk is a complex system which contains a variety of particles (yolk spheres, free-floating granules, low-density lipoprotein globules and myelin figures) suspended in a protein solution and encircled

by a vitelline membrane. Most of the yolk consists of yellow yolk composed of layers of alternate light and deep yellow yolks. Less than 2% of the total is white yolk which originates from the white follicle maturing in the ovary (Li-Chan & Kim, 2008).

The weight and composition of a hen egg depends on heredity, age, season, diet and other factors (Ren et al., 2009). Ahn et al. (1997) found that the solid content of the whole egg increases according to the age of the hen, and the breed of hens had a significant effect on the solid content of the whole egg, both white and yolk. However, the strain effect on yolk/white ratio was not significant. The chemical composition of the edible portion of raw and dried components of whole egg, egg white and egg yolk is presented in Table 1. The egg consists of approximately 58.5% albumen, 31% yolk and 10.5% shell (Ren et al., 2009). Although the yolk represents about one-third of the egg weight, the yolk of one large egg contains as many as 55 calories while the albumen has about 17 calories (Katz, 2003). The albumen contains primarily protein (and 88% water), whereas the yolk contains both lipids and proteins (2:1 ratio) (Ren et al., 2009). Egg protein contains different amino acids, including all amino acids essential for humans, while fat (lipid) comprises about 10% of the total weight of an egg (Katz, 2003). The lipids include triglycerides, phospholipids, cholesterol, cerebroside and some minor lipids. Carbohydrates are a minor component of hen eggs. Their average content is about 0.5 g per egg, 40% of which is present in the yolk (Kovacs-Nolan et al., 2005). Furthermore, eggs contain most vitamins and minerals that are necessary for human nutrition such as vitamins A, B12, D, E and K, plus riboflavin, folic acid, iron, zinc, phosphorus, selenium and choline (Kovacs-Nolan et al., 2005).

Table 1: Chemical composition of the edible portion of raw and dried whole egg, egg white and egg yolk

Nutrient	Value per 100 g						Units
	Raw, Fresh			Dried			
	Whole egg	Egg white	Egg yolk	Whole egg	Egg white	Egg yolk	
Approximate composition							
Water	76.15	87.57	52.31	3.10	5.80	2.95	g
Protein	12.56	10.90	15.86	47.35	81.10	34.25	g
Total lipid (fat)	9.51	0.17	26.54	40.95	0.00	55.80	g
Ash	1.06	0.63	1.71	3.65	5.30	3.40	g
Carbohydrate, by difference	0.72	0.73	3.59	4.95	7.80	3.60	g
Sugar, total	0.37	0.71	0.56	3.08	5.40	3.60	g
Minerals							
Calcium, Ca	56	7	129	231	62	284	mg
Iron, Fe	1.75	0.08	2.73	6.79	0.15	5.42	mg
Magnesium, Mg	12	11	5	42	88	13	mg
Phosphorus, P	198	15	390	831	111	920	mg
Potassium, K	138	163	109	493	1125	244	mg
Sodium, Na	142	166	48	523	1280	135	mg
Zinc, Zn	1.29	0.03	2.30	5.28	0.10	4.93	mg
Copper, Cu	0.072	0.023	0.077	0.196	0.114	0.012	mg
Manganese, Mn	0.028	0.011	0.055	0.125	0.007	0.119	mg
Fluoride, F	1.1	ND*	ND	ND	ND	ND	µg
Selenium, Se	30.7	20.0	56.0	119.6	125.1	86.8	µg
Vitamins							
Thiamin	0.040	0.004	0.176	0.195	0.005	0.290	mg
Riboflavin	0.457	0.439	0.528	1.540	2.530	1.880	mg
Niacin	0.075	0.105	0.024	0.305	0.865	0.095	mg
Pantothenic acid	1.533	0.190	2.990	5.905	0.775	7.765	mg
Vitamin B6	0.170	0.005	0.350	0.388	0.036	0.660	mg
Folate, total	47	4	146	171	18	244	µg

(Table 1 cont.)

Nutrient	Value per 100 g						Units
	Raw, Fresh			Dried			
	Whole egg	Egg white	Egg yolk	Whole egg	Egg white	Egg yolk	
Choline, total	293.8	1.1	682.3	1007.2	ND	1388.3	mg
Betaine	0.3	0.3	0.9	ND	ND	ND	mg
Vitamin B12	0.89	0.09	1.95	3.95	0.18	5.33	µg
Retinol	160	0	371	270	0	779	µg
Carotene, beta	0	0	88	41	0	186	µg
Carotene, alpha	0	0	38	0	0	79	µg
Cryptoxanthin, beta	9	0	33	36	0	70	µg
Vitamin A, IU	450	0	1442	997	0	3031	IU
Lutein + zeaxanthin	503	0	1094	1329	0	2299	µg
Vitamin E (alpha-tocopherol)	1.05	0.00	2.58	3.88	0.00	5.42	mg
Tocopherol, beta	0.01	ND	0.05	ND	ND	ND	mg
Tocopherol, gamma	0.50	ND	1.33	ND	ND	ND	mg
Tocopherol, delta	0.06	ND	0.06	ND	ND	ND	mg
Vitamin D (D2+D3)	2	0	5.4	8.0	ND	11.4	µg
Vitamin D3 (cholecalciferol)	2	ND	5.4	8.0	ND	11.4	µg
Vitamin D	82	0	218	321	ND	458	IU
Vitamin K (phylloquinone)	0.3	0.0	0.7	1.2	0.0	1.5	µg
Dihydrophylloquinone	0.1	0.0	0.1	ND	ND	ND	µg
Lipids							
Fatty acids, total saturated	3.126	0.000	9.551	12.727	0.000	17.154	g
4:0	0.040	0.000	0.000	0.000	0.000	0.000	g
8:0	0.004	0.000	0.009	0.000	0.000	0.000	g
10:0	0.006	0.000	0.009	0.000	0.000	0.000	g
12:0	0.000	0.000	0.009	0.000	0.000	0.000	g
14:0	0.033	0.000	0.104	0.139	0.000	0.180	g
15:0	0.008	ND	0.013	ND	ND	ND	g
16:0	2.231	0.000	6.86	9.233	0.000	12.559	g

(Table 1 cont.)

Nutrient	Value per 100 g						Units
	Raw, Fresh			Dried			
	Whole egg	Egg white	Egg yolk	Whole egg	Egg white	Egg yolk	
17:0	0.022	ND	0.051	ND	ND	ND	g
18:0	0.811	0.000	2.417	3.291	0.000	4.326	g
20:0	0.003	ND	0.032	ND	ND	ND	g
22:0	0.004	ND	0.038	ND	ND	ND	g
24:0	0.000	ND	0.009	ND	ND	ND	g
Fatty acids, total monounsaturated	3.658	0.000	11.738	15.337	0.000	21.129	g
14:1	0.007	ND	0.024	ND	ND	ND	g
16:1 undifferentiated	0.201	0.000	0.918	1.009	0.000	1.373	g
17:1	0.012	ND	ND	ND	ND	ND	g
18:1 undifferentiated	3.411	0.000	10.701	14.162	0.000	19.536	g
20:1	0.027	0.000	0.086	0.116	0.000	0.149	g
20:2 n-6 c,c	0.018	ND	ND	ND	ND	ND	g
22:1 undifferentiated	ND	0.000	0.009	0.000	0.000	0.000	g
Fatty acids, total polyunsaturated	1.911	0.000	4.204	5.804	0.000	7.895	g
18:2 undifferentiated	1.555	0.000	3.538	4.614	0.000	6.423	g
18:3 undifferentiated	0.048	0.000	0.103	0.111	0.000	0.126	g
18:4	0.000	0.000	0.000	0.056	0.000	0.068	g
20:3 undifferentiated	0.023	ND	ND	ND	ND	ND	g
20:4 undifferentiated	0.188	0.000	0.438	0.569	0.000	0.837	g
20:5n-3 (EPA)	0.000	0.000	0.011	0.278	0.000	0.203	g
22:4	0.013	ND	ND	ND	ND	ND	g
22:5n-3 (DPA)	0.007	0.000	0.000	0.000	0.000	0.000	g
22:6n-3 (DHA)	0.058	0.000	0.114	0.176	0.000	0.239	g
Fatty acids, total trans	0.038	ND	ND	ND	ND	ND	g
Cholesterol	372	0	1085	1507	0	2052	mg

(Table 1 cont.)

Nutrient	Value per 100 g						Units
	Raw, Fresh			Dried			
	Whole egg	Egg white	Egg yolk	Whole egg	Egg white	Egg yolk	
Amino acids							
Tryptophan	0.167	0.125	0.177	0.567	0.999	0.400	g
Threonine	0.556	0.449	0.687	2.273	3.685	1.819	g
Isoleucine	0.671	0.661	0.866	2.583	4.581	1.732	g
Leucine	1.086	1.016	1.399	4.046	6.838	3.009	g
Lysine	0.912	0.806	1.217	3.402	5.515	2.718	g
Methionine	0.380	0.399	0.378	1.477	2.790	0.849	g
Cystine	0.272	0.287	0.264	1.099	2.102	0.614	g
Phenylalanine	0.680	0.686	0.681	2.515	4.736	1.463	g
Tyrosine	0.499	0.457	0.678	1.932	3.153	1.523	g
Valine	0.858	0.809	0.949	2.886	5.164	1.907	g
Arginine	0.820	0.648	1.099	2.841	4.412	2.444	g
Histidine	0.309	0.290	0.416	1.121	1.830	0.888	g
Alanine	0.735	0.704	0.836	2.636	4.684	1.759	g
Aspartic acid	1.329	1.220	1.550	4.758	8.253	3.348	g
Glutamic acid	1.673	1.550	1.970	6.190	10.770	4.340	g
Glycine	0.432	0.413	0.488	1.591	2.842	1.058	g
Proline	0.512	0.435	0.646	1.886	3.153	1.430	g
Serine	0.971	0.798	1.326	3.523	5.593	2.926	g

*ND= No data available

Adapted from USDA National Nutrient Database for Standard Reference, Release 23 (2010)

2.1.2 Chicken egg yolk antibodies (IgY)

The immune system of newly hatched chickens is not fully mature, therefore it is not capable of providing complete protection against pathogens during its first encounter with the external environment after hatching (Davison et al., 2008). Maternal antibodies transferred via the egg help protect the offspring until their own adaptive immune responses become fully effective (Davison et al., 2008). For example, maternal antibodies to NDV transferred via the egg yolk protect chicks for 3–4 weeks after hatching as the maternal antibodies have a half-life of approximately 4.5 days (MacLachlan & Dubovi, 2010).

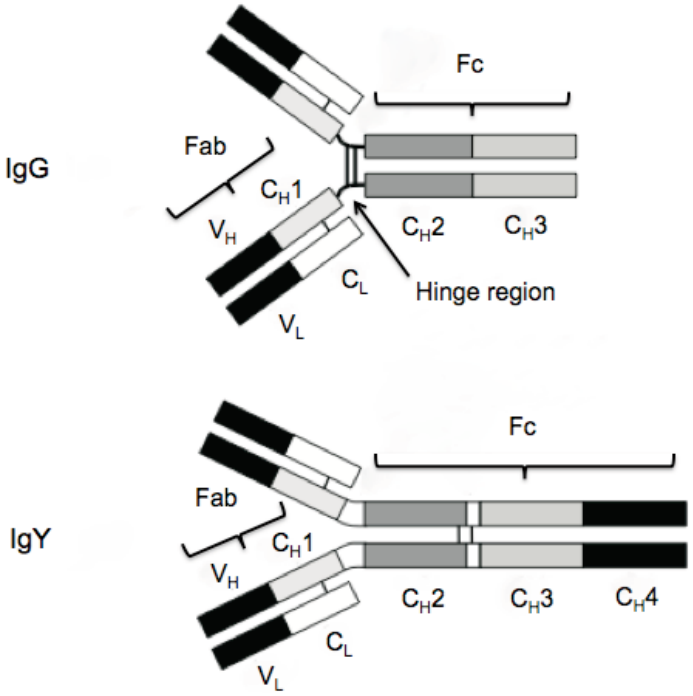


Figure 3: Structure of IgY and IgG (adapted from Kovacs-Nolan & Mine, 2004)

IgY is the major low molecular weight serum immunoglobulin in oviparous (egg-laying) animals (Michael & Meenatchisundaram, 2010). The general structure of IgY was well described in Chalghoumi et al. (2009) as follows: the IgY molecule contains two identical heavy (H) chains and two identical light (L) chains which are linked by a disulfide bridge. The light chain of IgY consists of one variable (V_L) and one constant domain (C_L) like mammalian IgG, but intra-chain disulfide linkage between the V_L region and C_L region of L-chain is absent in the IgY L-chain. The heavy chain of IgY contains one variable domain (V_H) and four constant domains (C_H1, C_H2, C_H3 and C_H4), unlike mammalian IgG which has three constant domains (C_H1, C_H2 and C_H3). In the heavy chain of IgG, the C_H1 and the C_H2 domains are separated by a hinge

region which gives considerable flexibility to the Fab fragment (the portion which contains the antigen-binding activity). In contrast, the heavy chain of IgY does not have a hinge region, but there are regions near the boundaries of the C_H1-C_H2 and C_H2-C_H3 domains that contain proline and glycine residues. These regions have the potential to confer limited flexibility on the molecule. Comparison of C-domain sequences in IgG and IgY has shown that the C_H2 and C_H3 domains of IgG are the equivalents of the C_H3 and C_H4 domains of IgY, respectively. The equivalent of the C_H2 domain of IgY is absent in the heavy chain of IgG. The content of β-sheet structure in C domains of IgY is lower than that of mammalian IgG; therefore the conformation of IgY domains is more disordered in comparison to that of mammalian IgG.

Antigen-specific chicken antibodies have been successfully raised against a variety of proteins, peptides, lipid hormones and carbohydrate components from a huge variety of species, including viruses, bacteria, fungi, plants and animals (Schade et al., 1996). Passive immunization using avian IgY can also be used in organisms that are non-responsive to antibiotic therapy (Chalghoumi et al., 2009). In this case, antibodies are usually administered in the feed in several forms: whole eggs powder, whole yolks powder, water-soluble fraction powder or purified IgY. There are advantages of using chickens for antibody production as opposed to mammals. It represents both a reduction and a refinement in animal use. It helps reduce the number of animals used because hens produce larger amounts of antibodies than laboratory rodents. It is a refinement because the collection of blood can be replaced by antibody extraction from egg yolk, thus minimizing the distress for the laboratory animals (Karlsson et al., 2004). Furthermore, IgY does not activate the human or mammalian complement system or human Fc-receptors which are all well-known cell activators and mediators (Nguyen et al., 2010). Furthermore, IgY is relatively stable and there was no change in protective activity after up to 13 months of storage at 4°C. Furthermore, lyophilization does not affect activity, making the production of IgY practical (Nguyen et al., 2010).

2.1.3 Use of egg

Eggs are important in human diets because of their excellent nutrition profile as a nutrient-rich food that contains high-quality protein and a substantial quantity of a variety of essential minerals, lipids and vitamins (Kapoor et al., 2010). In modern times, the eggs industry plays an important role in the food industry and represents a powerhouse of nutrition (Kapoor et al., 2010). Food manufacturers prefer egg products such as liquid whole egg, liquid egg white, liquid egg yolk and powdered forms over shell eggs because they are easier to handle and have a lower risk of being contaminated by microorganisms (Nemeth et al., 2011). Since egg products can be pasteurized, institutional food service operators such as fast food chains,

restaurants, hospitals and nursing homes use such egg products to ensure their high level of food safety (USDA, 2011a, 2011b).

Moreover, a number of biological activities have been related to egg components, including antibacterial and antiviral activity, immunomodulatory activity and anti-cancer activity. All this indicates the importance of eggs and egg components for human health and disease prevention and treatment (Mine & Kovacs-Nolan, 2004). The potential of some of these biologically active components has already been realized, including egg white lysozyme and avidin, and yolk IgY and lecithin, which are currently produced on an industrial scale and are being applied for the prevention and treatment of various medical conditions (Mine & Kovacs-Nolan, 2004). For instance, egg yolk phospholipids are used as a component of special dietetic products and food supplements, primarily to provide n-3 LCPUFA and n-6 LCPUFA which may be deficient under specific circumstances (Nielsen, 2007). Besides, egg yolk phospholipids are also used in the pharmaceutical and liposome industry because of their high entrapment efficiency, and they are also applied in many cosmetics products as well as in foodstuffs because of their desirable impact on final products (Nielsen, 2007). Additionally, eggs can add many positive attributes to food products such as emulsification, leavening, smoothness and flavour (Jones, 2007).

2.2 Viruses

The viruses selected for this study range from the smallest one to the middle size and from low to high sensitivity to inactivation. Two virus species were chosen because they are known to infect a huge number of species of wild birds as well as domestic poultry (Guan et al., 2009): avian influenza virus (AIV) and Newcastle disease virus (NDV). Both virus species are able to induce zoonotic diseases (Swayne & King, 2003) and are included in the OIE Listed Diseases in 2012 (OIE, 2012b). They could possibly be transmitted via egg (Swayne & Beck, 2004), which is why they are of interest for heat inactivation kinetics studies. In the experimental study three subtypes of swine influenza virus (SIV) were used in comparison to AIV to investigate the heat stability of different variants of influenza viruses classified to the same family. Another virus species chosen was feline calicivirus (FCV) because it is a non-enveloped virus while AIV and NDV are enveloped ones. In addition, FCV is smaller than the former two viruses. FCV has been used as a model for Norwalk virus (NV) and Norwalk-like viruses (NLV) which are the major cause of gastroenteritis in humans (Cannon et al., 2006; Bae and Schwab, 2008). FCV shares a variety of biological properties with NV and NLV and can be grown to high titres in cell culture, producing a clear cytopathogenic effect (Doultree et al., 1999; Slomka & Appleton, 1998; Bidawid et al., 2003). Porcine parvovirus (PPV) was selected because it has been under consideration as an indicator virus in the temperature interval 50°C–80°C (Lund et al., 1996). As PPV is considerably heat resistant, it is

suitable for comparative studies in this temperature interval, and it would therefore be possible to follow its inactivation over a longer period (Lund et al., 1996). Moreover, PPV has been used as a model (surrogate) for the human parvovirus B19 to validate different methods for inactivating viruses during the manufacture of various plasma products (Blümel et al., 2008).

2.2.1 Avian influenza virus (AIV)

A contagious disease caused by influenza A viruses termed avian influenza, or "bird flu" (WHO, 2007), affects the respiratory, digestive and nervous system (Vegad, 2008a). In addition to chickens, avian influenza virus also infects a wide variety of wild and domestic birds, especially the free-living birds that live in or near water, such as ducks, geese, swans, shorebirds, gulls, tern (sea birds), doves and others (Vegad, 2008a). According to Capua & Terregino (2009), avian influenza viruses have been grouped into two distinct pathotypes on the basis of the severity of the disease they cause in susceptible chickens. (1) Highly pathogenic avian influenza (HPAI) viruses cause severe disease in chickens and other gallinaceous birds. (2) Low pathogenic avian influenza (LPAI) viruses usually infect wild birds asymptomatically (CFSPH, 2010) and at most cause a disease with a much milder clinical course (Capua & Terregino, 2009; CFSPH, 2010). To date, only subtypes containing H5 or H7 have been classified as being highly pathogenic; subtypes that contained other haemagglutinins have been found only in the LPAIV form. H5 and H7 LPAI viruses also exist, and these strains can evolve into highly pathogenic strains (CFSPH, 2010).

2.2.1.1 Structure and composition

Avian influenza viruses belong to the family of *Orthomyxoviridae*, genus influenza A virus. They are enveloped viruses that contain eight genome segments of single-stranded negative-sense RNA (De Benedictis et al., 2007; Jordan, 1996). Virus particles are roughly round or filamentous and have a diameter of 80–120 nm (Jordan, 1996). Based on antigenic characteristics of the two types of their surface glycoproteins, influenza A viruses are differentiated into different subtypes. The major surface glycoprotein consists of rod-shaped trimers of haemagglutinin (HA), and the second glycoprotein is composed of mushroom-shaped tetramers of neuraminidase (NA). Currently 16 different HA subtypes (H1–H16) and nine NA subtypes (N1–N9) have been documented (Shaw & Palese, 2008; Swayne & Halvorson, 2008).

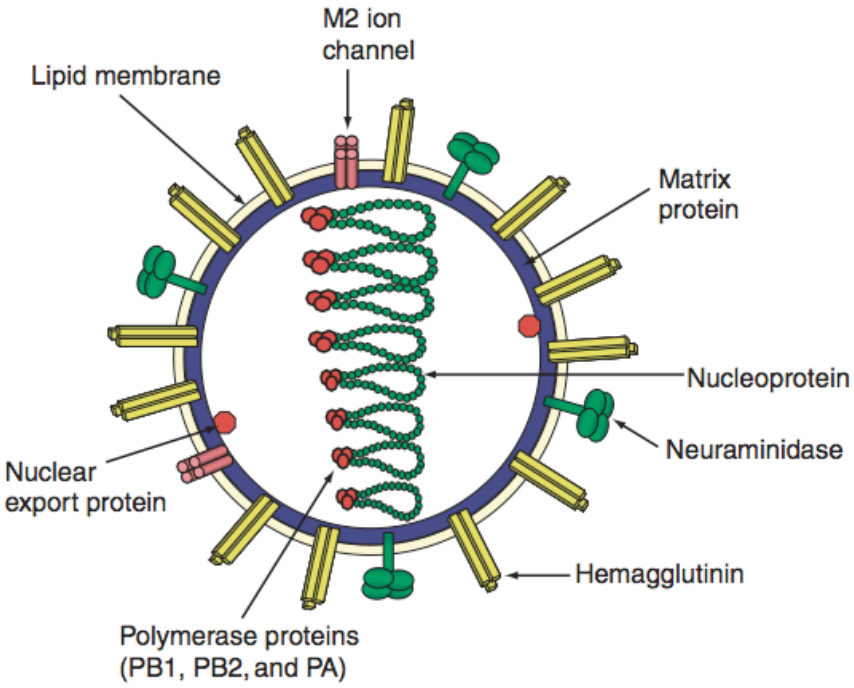


Figure 4: Structure of influenza A virus particle (Shaw & Palese, 2008)

The structural proteins of influenza A virus can be separated into the surface proteins that include HA, NA and the membrane ion channel protein M2. The internal proteins including the matrix protein (M1) lie beneath the lipid envelope and surround the ribonucleoprotein (RNP) complexes (Suarez, 2008; Shaw & Palese, 2008). RNPs consist of the viral RNAs which are coated with the nucleoprotein (NP) and associated with the polymerase complex consisting of PB1, PB2 and PA proteins (Lamb, 2008; Shaw & Palese, 2008). The nuclear export proteins (NEP/NS2) have also been found within the virus particles (Shaw & Palese, 2008).

2.2.1.2 Transmission

Virus strain, environmental factors and host species susceptibility are factors that influence transmissibility of AIV to poultry or humans (Chmielewski & Swayne, 2011). Although some HPAIV and LPAIV can be shed in the faeces and respiratory secretions of unvaccinated chickens within one to two days after infection, different viral strains appear to vary in their transmissibility as well as in the clinical symptoms (Spickler et al., 2008). In both natural and experimental infections, virulent viruses have tended to show much poorer transmission from infected to susceptible chickens and turkeys than those viruses of low pathogenicity (Alexander, 2007; Capua & Alexander, 2009).

As the natural reservoir for AIV, infected waterfowl are believed to pass AIV to domestic poultry by the faecal–oral route through contaminated water, feeding and housing facilities or shared environment. Infectious virus can be excreted by birds for up to ten days (Chmielewski & Swayne, 2011). The persistence of AIV facilitated faecal transmission in aquatic environments for prolonged periods, particularly at low temperatures (CFSPH, 2010). Faecal–cloacal transmission might also be possible (CFSPH, 2010).

Once an AIV has entered a poultry flock, it can spread on the farm by both faecal–oral route and aerosol because of the close proximity of the birds (CFSPH, 2010). Fomites can be important in transmission, and flies could act as mechanical vectors (CFSPH, 2010). Containment and disposal of infected carcasses are important in the mitigation of an infectious animal disease outbreak (Graiver et al., 2009). Moreover, lesions have been reported in the ovaries and oviducts of infected egg-laying chickens so AIV could potentially be transmitted via eggs either through virus in the internal egg contents or through virus-infected faeces on the egg surface (Swayne & Beck, 2004). This could also indicate a possible spread of virus during commercial movement of eggs (Pillai et al., 2010).

The general risk of humans to be infected by AIV is extremely low, and AIV strains vary in their ability to transmit to and infect humans (Swayne & King, 2003). AIV could be transmitted from animals to humans via two main routes: (1) directly from birds or avian virus-contaminated environments to people, and (2) through an intermediate host such as pigs (Ma et al., 2009; CDC, 2005a). H5N1 HPAIV has caused sporadic infections in humans, primarily via direct contact with infected birds. It has a high case fatality rate (morbidity) (60%) for human infections, but with limited human-to-human spread and rare transmission through raw food products (Chmielewski & Swayne, 2011).

2.2.1.3 Clinical symptoms

The clinical symptoms of avian influenza are influenced by the following factors: the strain of virus, the species and age of the host, the immune status of the host regarding the virus and regarding certain concomitant infectious agents (such as NDV, *E. coli* and mycoplasma), immunodeficiency conditions and environmental factors (such as excess ammonia and dust) (Jordan, 1996).

Mild strains (LPAIV) result in low morbidity and mortality (Shane, 2005) but may cause considerable losses, particularly in turkeys, because of anorexia, depression, decreased egg production, respiratory disease and sinusitis (MacLachlan & Dubovi, 2010). Egg production in layers can drop by as much as 45% with recovery to normal in 2–4 weeks (Animal Health Australia, 2011). When other organisms are also

present and have an exacerbative effect, or when the birds are under stress due to adverse environmental conditions, mortality may rise as high as 60–70% of the flock, and clinical symptoms show a marked increase in severity (Jordan, 1996). Ducks and geese are considered to be the natural reservoirs of LPAIV and are not known to exhibit clinical or pathological lesions following infection (Capua & Terregino, 2009).

Highly pathogenic avian influenza (HPAI) results in an acute and precipitous decline in egg production with rapidly increasing mortality and is characterized by both respiratory and central nervous symptoms (Shane, 2005). In peracute cases involving sudden death, clinical symptoms may not be perceived and death may occur as early as 24 h after the first signs of the disease, and frequently within 48 h (Animal Health Australia, 2011). Sudden death occurs in a recumbent position and is preceded by pedalling movements and gasping (Capua & Terregino, 2009). Clinical signs, which may be associated with high mortality, are cessation of egg laying, respiratory symptoms, rale, excessive lacrimation, sinusitis, oedema of the head and face, subcutaneous haemorrhage with cyanosis of the skin, particularly of the head and wattles, and diarrhoea (Jordan, 1996). Birds may show neurological signs such as tremors of the head and neck, inability to stand, torticollis (twisted neck) and other unusual postures (MacLachlan & Dubovi, 2010). In wild birds and domestic ducks, most HPAIV either do not replicate or replicate only to a limited degree and produce few clinical signs because of poor adaptation to non-gallinaceous species (Swayne & Halvorson, 2008).

Human illness due to infection with LPAIV (such as H7N7, H9N2 and H7N2) has been documented, including very mild symptoms (e.g. conjunctivitis) to influenza-like illness (CDC, 2005b). Human infections with HPAIV have ranged from mild (H7N3, H7N1) to severe and fatal disease (H7N7, H5N1) (CDC, 2005b). Factors associated with severe disease in humans following infection with H5N1 included older age, delay in hospitalization, lower respiratory tract involvement and a low total peripheral white blood cell count or lymphopenia at admission (Yuen et al., 1998). The clinical signs of human infection with AIV were well described by Shakespeare (2009) as follows: clinical onset of AIV in humans is characterized by fever with temperatures as high as 40°C that may last for up to 5 days. Associated with the fever are loss of appetite, headaches, lethargy, cough, generalized joint pain, sore throat and nasal discharge. In children, also gastrointestinal disturbance is observed. Convalescence normally does not extend beyond a period of two weeks once the major symptoms resolve; however, in elderly people and other major at-risk groups, bacterial or viral pneumonia may follow with mortality risk. Bronchitis can also arise in individuals with previous lung damage.

2.2.1.4 Tenacity of avian influenza virus

The influenza viruses are known to be relatively thermo-labile viruses which are rapidly inactivated at temperatures higher than 50°C (Shaw & Palese, 2008). The survival of AIV in the environment depends on the cumulative effects of physical and chemical factors, such as (1) stability in dry, humid or liquid environments; (2) whether the virus is mixed with organic compounds; (3) the pH; (4) salinity and (5) temperature of the environment (Davidson et al., 2010). Numerous studies have evaluated the survival of AIV in different ways as shown in Table 2 that mainly focused on heat inactivation of the virus.

Table 2: Tenacity of avian influenza virus

Samples	Viruses	Persistence of virus	Reference
Infectious allantoic fluid	Three subgroups of H9N2	At 37°C, AIV H9N2 decayed within 3–5 days, survival at 20°C was about 18 times longer and was extended at 4°C about 70 times.	(Davidson et al., 2010)
Distilled water	H3N8, H4N6, H6N2, H12N5 and H10N7	Infectious viruses were detected after 207 and 102 days at 17°C and 28°C incubation, respectively.	(Stallknecht et al., 1990b)
Egg yolk and allantoic fluid	H5N2	From an initial titre of 4.8 $\log_{10}$/ml of H5N2 in experimentally contaminated yolk, no virus was isolated after 30 min at 57°C, and in allantoic fluid an initial titre of 5 $\log_{10}$/ml of H5N2 was no longer found after 5 min at 62°C.	(King, 1991)
Distilled water	H5N1	In distilled water, the virus (10^4 and 10^6 $TCID_{50}$/ml) at three temperatures (4°C, 10°C, and 20°C) retained its infectivity beyond the 60-day study period.	(Domanska-Blicharz et al., 2010)
Infectious allantoic fluid	Three reference strains of H5N1 (2004.1, CUK-2/04 and 2004.2)	Allantoic fluid containing the three strains with virus titres of $10^{9.34}$, 10^9 and $10^{7.6}$ egg lethal doses $(ELD)_{50}$/ml, respectively totally lost their infectivity after incubation at 70°C for 60 min or 75°C for at least 45 min.	(Wanaratana et al., 2010)

Infectious allantoic fluid in leachate or reverse osmosis (RO) water	H6N2	At 21°C and 37°C, the inactivation of AIV in leachate was significantly ($p<0.01$) faster than that of AIV in RO water.	(Graiver et al., 2009)
Dried egg white with 7.5% moisture	H5N2	D-values calculated from linear regression of the survival curves at 54.4°C, 60°C, 65.5°C and 71.1°C were 475.4, 192.2, 141.0 and 50.1 min, respectively.	(Thomas & Swayne, 2009a)
Thigh and breast meat from infected chickens	H5N1 (Korea/03)	D-values for H5N1 in thigh meat were 238.8 at 57°C, 130.4 at 58°C, 80.8 at 59°C, 59.6 at 60°C and 28.6 sec at 61°C, respectively. D-values for H5N1 in breast meat were 268.7 at 57°C, 153.8 at 58°C, 76.1 at 59°C, 70.7 at 60°C and 34.1 sec at 61°C, respectively.	(Thomas & Swayne, 2007)
Suspension of infectious allantoic fluid and peptone water	H5N1	The initial virus titre of 4HA was reduced to undetectable HA activity after 30 min at 56°C and after 1 day at 28°C but the virus remained viable for more than 100 days at 4°C.	(Shahid et al., 2009)
Naturally and artificially infected chicken meat	H5N1 (Korea/03), H5N2 (PA/83) and H5N2 (TX/04)	Thermal inactivation of HPAIV (PA/83) in experimentally contaminated chicken meat was faster than that in meat from naturally infected chickens.	(Thomas et al., 2008)
Thigh and breast meat from infected chickens	H5N1 (Korea/03)	The virus titres of $10^{6.8}$ and $10^{5.6}$ egg infectious doses $(EID)_{50}$/g of infected thigh and breast meat were unchanged after samples were run through a ramp-up cycle of 25 to 30, 40, 50°C. The virus titres were reduced at 60°C and no virus was detected from infected breast and thigh meat after 70°C were reached and were held for 5 sec, respectively.	(Swayne, 2006)

Homogenized whole egg, liquid egg white. 10% salted egg yolk and dried egg white	H7N2 (LPAI/NY/94) and H5N2 (HPAI/PA/83)	HPAIV in dried egg white was the most resistant virus to inactivation at each of the time points (55, 57, 59, 61 and 63°C) while the viruses in 10% salted yolk had the most rapid and complete inactivation.	(Swayne & Beck, 2004)
Suspension of infectious allantoic fluid and phosphate buffer	Various strains of influenza A virus	There were significant differences in thermal stability between influenza A strains but no correlation between pH and thermal stability.	(Scholtissek, 1985)
Suspension of infectious allantoic fluid and distilled water	H4N6, H6N2 and H10N7	Estimated resistance of H6N2 (initial titre of 10^6 $TCID_{50}$/ml) was longest at 17°C / salinity 0 ppt / pH 8.2 (100 days) and shortest at 28°C / salinity 20 ppt / pH 8.2 (9 days).	(Stallknecht et al., 1990a)
Compost material	H7N1	The virus titre was reduced by 3.6 log_{10} after 30 min of heat treatment at 45°C.	(Elving et al., 2010)

2.2.2 Swine influenza virus (SIV)

Swine Flu (also called swine influenza, hog flu and pig flu) is an infection of a host animal by any one of several specific types of microscopic organisms called “swine influenza virus” (Prakash, 2009). Swine influenza virus infections are a common and important cause of bronchointerstitial pneumonia and respiratory disease in pigs throughout large parts of the world (Olsen et al., 2006). The primary economic impact is related to retarded weight gain resulting in an increase in the number of days to reach market weight (OIE, 2009b). Classical swine influenza is associated with influenza A virus subtypes H1N1, H1N2 and H3N2 belonging to the *Orthomyxovirus* genus of the *Orthomyxoviridae* family. Other types have been isolated from pigs but as yet have not been established as widespread endemic strains (Radostits et al., 2006).

2.2.2.1 Transmission

Pigs have been considered the “mixing vessel” for influenza viruses because of their ability to become co-infected with both avian and human strains of influenza virus (MacLachlan & Dubovi, 2010). When concurrent infection of poultry or swine with two or more strains of virus occurs, there is potential for reassortment of segments of genetic material, resulting in the development of new strains (genetic “shift”) (Neumann et al., 2009). This type of reassortment has already happened in pigs. For example, a triple reassortant containing swine, avian and human influenza genes has been circulating in the swine population of the USA since at least 1998 (WHO, 2009).

The primary route of virus transmission between pigs is through pig-to-pig contact via the nasopharyngeal route, most probably through none-to-nose contact or direct contact of mucus (OIE, 2009b). Airborne transmission through the aerosols produced by pigs coughing or sneezing is also an important means of infection (Prakash, 2009).

Human infections with swine influenza viruses (resembling seasonal influenza) are occasionally reported, usually in persons who have direct contact with infected pigs (OIE, 2009c). Swine influenza viruses subtypes H1N1, H1N2 and H3N2, which are the same subtypes used in this study, have also occasionally infected humans before. However, such zoonotic transmission events did not lead to sustained human-to-human transmission in the manner this swine-origin influenza virus has done (Jeong et al., 2010; Peiris et al., 2009). Since swine influenza is not a foodborne disease, the risk of being infected with swine influenza viruses through the consumption of pork or pork products is negligible (OIE, 2009c).

2.2.2.2 Clinical symptoms

As described in Jackson & Cockcroft (2007), the symptoms of infected pigs include fever, anorexia, jerky breathing, sneezing, painful (sometimes paroxysmal) cough and prostration. Conjunctivitis and some ocular discharge are observed. Muscular movement is painful and stiff (as in human flu). Severe weight loss occurs despite the short course of the disease. Usually, rapid recovery occurs after 4–6 days.

Decreased semen production in boars and abortions in sows may also occur due to secondary effects of fever (OIE, 2009b). Morbidity is high but mortality is low or absent in most uncomplicated outbreaks (Neumann et al., 2009). Complications may include secondary bacterial or viral infections (OIE, 2009b) such as Glasser’s disease (Jackson & Cockcroft, 2007).

The symptoms of humans infected with swine flu are similar to those of human influenza and of influenza-like illness in general, namely chills, fever, sore throat, muscle pains, severe headache, coughing, weakness and general discomfort (Prakash, 2009). Fatalities have been reported very rarely (OIE, 2009c).

2.2.2.3 Tenacity of swine influenza virus

Several studies have been conducted on the heat resistance of SIV, as shown in Table 3. However, these investigations differ in the type of sample, the viral strain and the experimental procedure.

Table 3: Tenacity of swine influenza virus

Samples	Viruses	Persistence of virus	Reference
No data	SIV	The virus can be inactivated at 56°C for a minimum of 60 min.	(OIE, 2009b)
Cell culture medium	H1N1 strain A/NWS/33 (ATCC* VR-219)	At 70°C, the initial titre of 8.08 $\log_{10}$ $TCID_{50}$ was inactivated to undetectable levels within 5 min. At 80°C, the initial titre of 8.02 $\log_{10}$ $TCID_{50}$ was inactivated to undetectable levels within 2.5 min. At 90°C, the initial titre of 8.04 $\log_{10}$ $TCID_{50}$ was inactivated to undetectable levels within 1 min.	(Jeong et al., 2010)
Virus suspension	Human influenza virus H1N1	At 60 and 65°C with relative humidity (RH) of 50 and 75%, a > 5-log reduction of influenza virus on surfaces was achieved.	(McDevitt et al., 2010)
EMEM** and slurry	SIV (Danish strain 4744)	SIV in EMEM** survives for 30 min at 55°C, but SIV in slurry survives longer (1 h). The loss of SIV infectivity occurred more rapidly than the loss of viral antigen as detected by an anti-SIV ELISA.	(Bøtner & Belsham, 2012)

*ATCC = American Type Culture Collection

**EMEM = Eagle's minimum essential medium

2.2.3 Newcastle disease virus (NDV)

Newcastle disease, a highly contagious viral disease of poultry with a wide range of clinical symptoms from mild to severe (CFSPH, 2008a), is included as a notifiable disease in the OIE listed diseases 2012 of World Organization for Animal Health (OIE, 2012a). According to Jordan (1996) and Spradbrow (1987), NDV have been divided into five large groups based on the disease caused in chickens as follow:

1. Viscerotropic velogenic strains cause a highly virulent form of disease. Characteristically, haemorrhagic lesions of the intestinal tract are observed with high mortality in chickens of all ages.
2. Neurotropic velogenic strains are highly virulent, causing respiratory and neurological symptoms with high mortality in chickens of all ages.
3. Mesogenic strains show moderate virulence, causing mortalities of up to 50% and seriously reduce egg production.
4. Lentogenic respiratory strains are strains with low virulence that cause mild or inapparent respiratory infections with low mortality except in young chicks, but which can seriously affect egg production.
5. Asymptomatic enteric strains cause inapparent enteric infection.

2.2.3.1 Structure and composition

NDV or Paramyxovirus-1 (PMV-1) is a member of the genus *Avulavirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*, order *Mononegavirales*, that consists of single-stranded, non-segmented, negative-sense RNA (Lamb et al., 2005; Alexander, 2009).

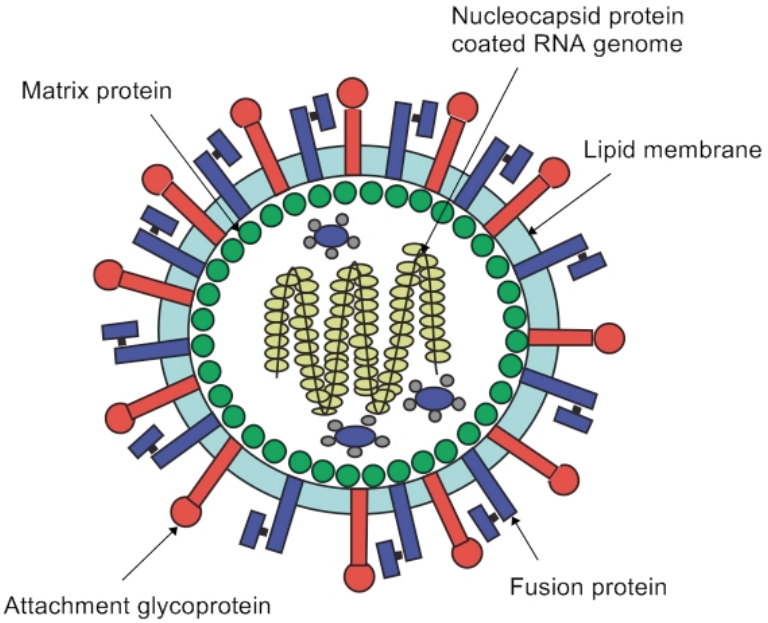


Figure 5: Structure of paramyxovirus (adapted from Dutch, 2008)

According to Dutch (2008) and Samal (2008), NDV is an enveloped virus which contains two glycosylated surface envelope proteins, the fusion (F) protein and the attachment (HN) protein that extend as spike-like projections from the virion envelope. The matrix (M) protein is the most abundant protein in the virion that lies beneath the envelope and plays a central role in virus assembly. Contained within the envelope is the viral nucleocapsid that consists of the negative-sense RNA and the viral nucleocapsid (N) protein, the large-polymerase (L) protein and the phosphoprotein (P) protein.

2.2.3.2 Transmission

Transmission between birds occurs by direct contact between birds via inhalation of aerosols and dust particles, or via ingestion of contaminated feed and water, because respiratory secretions and faeces contain high concentrations of virus (MacLachlan & Dubovi, 2010). NDV can be spread through the movement of diseased poultry, contaminated poultry products or contaminated equipment and supplies (Thomas et al., 2008). The ability of the virus to survive in the carcass or in excretions is also an important factor in the spread of the virus (Vegad, 2008b). On rare occasions, vertical transmission has been documented for lentogenic virus strains, and virus-infected chicks have hatched from virus-containing eggs (MacLachlan & Dubovi, 2010). Other routes of virus transmission to newly hatched chicks are faeces-contaminated eggshells and cracked or broken eggs (Vegad, 2008b; CFSPH, 2008a).

Human infections with NDV occur by direct contact with infected poultry or other birds (CFSPH, 2008b). Poultry crews, laboratory workers and members of vaccination teams would be at the greatest risk of potential exposure to large quantities of virus during their work (MacLachlan & Dubovi, 2010; CFSPH, 2008b). No human cases of Newcastle disease have occurred after eating poultry products (CFSPH, 2008b).

2.2.3.3 Clinical symptoms

As described above, clinical symptoms of NDV-infected chicken vary with the virulence of the strain, the avian species infected and the predilection of the infecting virus for the respiratory and gastrointestinal tracts or the CNS (Swayne & King, 2003).

According to CFSPH (2008a), velogenic strains cause severe, often fatal, disease in chickens. The clinical signs are highly variable. Most birds are lethargic and inappetent, and their feathers may be ruffled. Conjunctival reddening and oedema may be an early sign. Some birds develop watery, greenish or white diarrhoea,

respiratory signs (including cyanosis) or swelling of the tissues of the head and neck. Neurological signs may also be observed, including tremors, clonic spasms, paresis or paralysis of the wings and/or legs, torticollis (twisted neck) and circling. Neurological signs can occur concurrently with other symptoms but are generally seen later in the course of disease. Egg laying often declines dramatically, and eggs may be misshapen, abnormally coloured and rough or thin-shelled, with watery albumen. Sudden death, with few or no symptoms, is also common. Birds that survive for two weeks usually live but may have permanent neurological damage and/or a permanent decrease in egg production. The symptoms may be less severe in vaccinated birds.

NDV can produce a transitory conjunctivitis in humans within 24 h of NDV exposure to the eye (CFSPH, 2008b; MacLachlan & Dubovi, 2010). General symptoms of headache, discomfort and slight chills occur within 48 h of the initial infection, but last for only 24 h (MacLachlan & Dubovi, 2010). Human-to-human transmission of NDV has not been reported (Animal Health Australia, 2011).

2.2.3.4 Tenacity of Newcastle disease virus

Several studies have been conducted on the physical and chemical resistance of NDV as shown in Table 4. However, the composition of samples, the viral strain and the experimental procedure of these studies differ.

Table 4: Tenacity of Newcastle disease virus

Samples	Viruses	Persistence of virus	Reference
Egg yolk, albumen and infectious allantoic fluid	NDV lentogenic Ulster and Ulster-P	At 62°C, both NDV strains (Ulster and Ulster-P with initial titres of 7.0 and 7.8 $\log_{10}$/ml, respectively) in allantoic fluid were rapidly inactivated, but after 60 min at 57°C the viruses remained viable in yolk. The virus in albumen was inactivated faster than the virus in yolk. And treatment at 60°C for 30 min or less eliminated the HA and infectivity of both NDV strains.	(King, 1991)

Naturally and experimentally infected chicken meat	NDV velogenic California/02 (NDV/CA02) and lentogenic Ulster (NDV/Ulster)	Heat inactivation rates of highly pathogenic NDV (CA/02) in chicken meat infected by either route were similar. At 70°C, NDV in chicken meat was inactivated within less than 1 sec.	(Thomas et al., 2008)
Homogenized whole egg, liquid egg white. 10% salted egg yolk and dried egg white	NDV lentogenic Ulster (NDV/Ulster), lentogenic B1 vaccine (NDV/B1) and velogenic California/02 (NDV/CA02)	The viruses in 10% salt yolk had the most rapid and complete inactivation. The industry standard pasteurization protocols for egg products were effective for NDV inactivation (Froning et al., 2002).	(Swayne & Beck, 2004)
Meat homogenate	NDV strain Herts 33/56	D-values at 65°C, 70°C, 74°C and 80°C were 120, 82, 40 and 29 sec, respectively.	(Alexander & Manvell, 2004)
No data	NDV	The virus was completely inactivated at 56°C for 3 h or 60°C for 30 min.	(OIE, 2009a)

2.2.4 Feline calicivirus (FCV)

Caliciviruses are among the most common problematic infectious agents of cats, with extraordinarily high rates of infectivity, morbidity and mortality (Foley, 2009). The most commonly recognized clinical problem attributable to FCV is upper respiratory tract infection (Foley, 2009).

2.2.4.1 Structure and composition

FCV is grouped to the family *Caliciviridae*, genus *Vesivirus* (Studdert & Symes, 2008). The caliciviruses are non-enveloped, 27–40 nm in diameter, with icosahedral symmetry, and the genome is composed of a single molecule of linear positive-sense, single-stranded RNA, 7.4–8.3 kb in size (MacLachlan & Dubovi, 2010). Seal et al. (1993) described that the genome is polyadenylated at the 3' end, bound by a virus-encoded protein at the 5'-end and encodes three open reading frames (ORFs).

ORF 1 codes for the non-structural proteins including a viral protease and the RNA-dependent RNA polymerase. This polyprotein is post-translationally cleaved by the viral protease. ORF 2 codes for the major capsid protein which has been divided into six regions A–F based largely on sequence conservation (Radford et al., 2007).

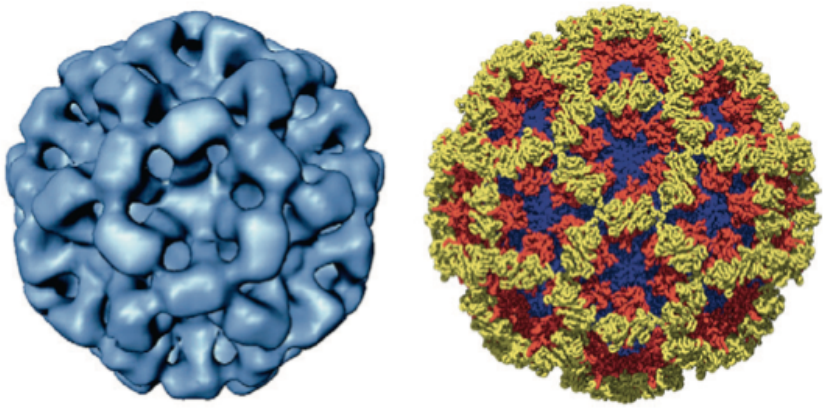


Figure 6: The structure of the calicivirus capsid exemplified by cryo-image reconstruction of recombinant Norwalk virus (NV)-like particles (rNV VLPs) (Left). X-ray structure of the NV capsid (Right) with the Shell, Protruding 1 and Protruding 2 domains coloured in blue, red and yellow, respectively (adapted from Prasad et al., 1996)

By cryoelectron microscopy, virions are approximately 40 nm in diameter with 32 cup-shaped surface structures comprising 90 arch-like capsomers arranged in *T*= 3 icosahedral symmetry (Studdert & Symes, 2008). X-ray crystallographic studies revealed that the capsid protein has the following two principal domains: a shell (S) domain, with a typical eight-stranded β-barrel fold that is commonly seen in other viral capsid proteins, and a protrusion (P) domain that can be further divided into two subdomains called P1 and P2 (Chen et al., 2004).

2.2.4.2 Transmission

FCV is usually transmitted by aerosol; the virus is introduced into cats via oral and nasal routes (Foley, 2009). Fomites also play an important role in transmission of FCV, especially of the virulent systemic FCV (Hurley et al., 2004). Moreover, Mencke et al. (2009) found that cat fleas may function as a potential vector for FCV and could spread infectious virus through their faeces or by bite.

2.2.4.3 Clinical symptoms

Due to the large number of different strains of FCV, a range of clinical signs may be seen (Radford et al., 2007). According to MacLachlan & Dubovi (2010), FCV causes respiratory disease in domestic and wild felids that is characterized by acute conjunctivitis, rhinitis, tracheitis, pneumonia and vesiculation and ulceration of the oral epithelium, including the tongue. Other common symptoms are fever, anorexia, lethargy, stiff gait and sometimes nasal and ocular discharge.

Moreover, FCV infection causes a short-lived limping syndrome in kittens infected naturally or experimentally (MacPhail, 2001). Although rarely fatal, cats may develop chronic disease and remain persistently infected (Greening & Wolf, 2010).

Cats infected with virulent FCV also exhibit subcutaneous facial and limb oedema, icterus, alopecia and striking ulceration of the nose, pinnae and feet (MacLachlan & Dubovi, 2010). Remarkably, adult cats that had been previously vaccinated showed higher mortality rates after infection with highly virulent systemic strains than did infected kittens (MacLachlan & Dubovi, 2010).

2.2.4.4 Tenacity of feline calicivirus

Generally, caliciviruses are stable in the environment and many strains show a high resistance to heat inactivation and are insensitive to certain chemicals (ether, chloroform and mild detergents) (Clarke et al., 2012). Several studies have evaluated the resistance of FCV as shown in Table 5.

Table 5: Tenacity of feline calicivirus

Samples	Viruses	Persistence of virus	Reference
Virus stocks	FCV-F9 and CaCV-48	After 1 week of incubation at 20°C, both virus titres were reduced by 3 $\log_{10}$. At 56°C and 71.3°C, a 3 $\log_{10}$ reduction was achieved after 8 min and 1 min for both viruses, resp..	(Duizer et al., 2004)
Cell culture medium and mineral water	FCV-KS20	The virus was inactivated by approximately 1 log after 15 min of incubation at 50°C. After 90 sec of incubation at 70°C, the initial virus titre of 1.5×10^7 pfu/ml was reduced by 6 log. There was no big difference between its thermostability in cell culture medium and mineral water.	(Buckow et al., 2008)

No data	FCV	The virus was inactivated in 30 min at 50°C and was fairly stable at pH 5.	(Sharma & Adlakha, 2009)
Shellfish	FCV-F9	FCV was more readily inactivated in shellfish than hepatitis A virus (HAV), which confirmed that current heating recommendations to the UK shellfish industry are adequate.	(Slomka & Appleton, 1998)
No data	FCV-Deuce, FCV-Kaos, FCV-5, FCV-127, FCV-796, FCV-F9	The individual isolates displayed minor differences in their sensitivity to heat inactivation and there was no correlation with virulence. However, the F9 vaccine strain was most sensitive to thermal inactivation, losing all infectivity after 30 min incubation at 52.2°C. The other isolates were inactivated fully at 56.9°C, except for isolate FCV-5 (inactivated fully at 62°C).	(Ossiboff et al., 2007)
No data	FCV-F9	1-propanol was more effective than ethanol and 2-propanol for the inactivation of FCV: in tests with the 50% and 70% solutions of the different alcohols, a 10^4-fold reduction was observed with 1-propanol after 30 sec, whereas the other alcohols were effective only after 3 min of contact time.	(Gehrke et al., 2004)
No data	Vesiviruses	Heat inactivation was accelerated with high concentrations of Mg^{2+} and viruses were insensitive to treatment with ether, chloroform or mild detergents, but pH 3–5 inactivated the viruses.	(Studdert & Symes, 2008)

2.2.5 Porcine parvovirus (PPV)

Porcine parvovirus has been recognized as a cause of reproductive failure of pigs (Mengeling, 2006) and is the main cause of SMEDI (Stillbirth, Mummified foetus, Embryonic Death and Infertility) (Hungerford, 1990). Parvovirus infections are endemic in most swineherds and occur worldwide in the major pork-producing countries (Neumann et al., 2009).

2.2.5.1 Structure and composition

Porcine parvovirus (PPV) is grouped to the family *Parvoviridae*, subfamily *Parvovirinae*, genus *Parvovirus*. It is a small-sized (approximately 25 nm in diameter), non-enveloped virus and has an icosahedral capsid containing 60 copies of a mixture of viral proteins (VP) as shown in Figure 7 (Parrish, 2008; MacLachlan & Dubovi, 2010; Simpson et al., 2002). The genome consists of a single molecule of negative-sense, single-stranded DNA (Sharma & Adlakha, 2009).

2.2.5.2 Transmission

The oronasal route is a common route of transmission of PPV that could happen with swine of all ages (Neumann et al., 2009). The infected pregnant gilts that already develop a viraemia can pass the virus to the embryo or foetus through the placenta (Hungerford, 1990). Boars may play a significant role in dissemination of PPV at a critical time. During acute infection the virus is shed by various routes including semen (Mengeling, 2006). Furthermore, the contaminated facility also plays an important role of transmission because PPV is quite resistant to environmental influences and many disinfectants (Neumann et al., 2009).

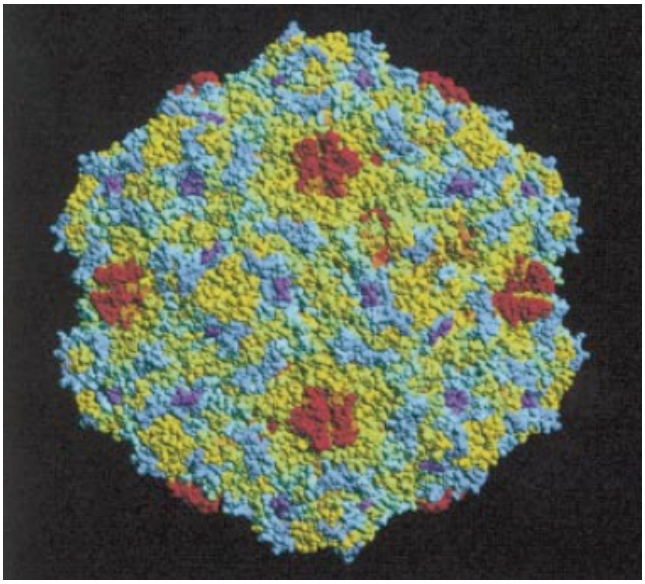


Figure 7: Conservation of surface features of PPV (Simpson et al., 2002)

2.2.5.3 Clinical symptoms

The clinical signs of infected pigs have been described by Neumann et al. (2009) and Mengeling (2006) and are summarized as follows: the infected dams may irregularly return to oestrus, fail to farrow despite being anoestrus and may have increased

numbers of mummified foetuses, reduced litter size and prolonged gestation lengths. If the infected dams develop a viraemia during the first 35 days of gestation, embryos die and are resorbed. Infection between 35–70 days of gestation causes foetal death and mummification. Foetuses that are around 70 days old or older are able to develop a protective immunity and they may survive. The increase in mummified foetuses after a normal gestation period is the hallmark of PPV.

2.2.5.4 Tenacity of porcine parvovirus

Several studies have been carried out on the resistance of PPV to physical and chemical treatment, as shown in Table 6. However, these studies differ in the type of sample, the viral strain and the experimental procedure.

Table 6: Tenacity of porcine parvovirus

Samples	Viruses	Persistence of virus	Reference
No data	PPV	The virus survives at 56°C for 30 min and was stable at pH 3–9 for 90 min.	(Sharma & Adlakha, 2009)
No data	PPV	PPV persisted for at least four months on contaminated premises.	(Neumann et al., 2009)
No data	Bovine parvovirus (BPV) strain Haden	Exposure to dry heat at 40°C for 1 h did not influence the infectivity of BPV, and the infectivity of this virus could only be influenced slightly by 0.9±0.15 log reduction after exposure to a temperature of 95°C for 2 h.	(Sauerbrei & Wutzler, 2008)
Human albumin	Human parvovirus B19 and PPV strain NADL-2	Heat resistance of human parvovirus B19 markedly differs from that of animal parvoviruses. B19 was inactivated after 10 min at 60°C by a factor of >4 $\log_{10}$ while animal parvoviruses survive the pasteurization of albumin.	(Blümel et al., 2002)

Human albumin	Human parvovirus B19 and PPV	At 100°C and low residual moisture (0.30%–0.35%), inactivation of B19 and PPV was very similar, while at higher residual moisture (0.85%–1.19%), B19 was more rapidly inactivated than PPV. Inactivation of PPV by more than 5 logs was observed after treatment at 80°C for 72 h for PPV and of human parvovirus B19 under all conditions and at 100°C for 30 min for B19 only at 1% residual moisture. Inactivation of PPV was limited to 2–3 logs at 100°C per 30 min under either high or low residual moisture, thus B19 is less resistant than PPV.	(Blümel et al., 2008)
No data	PPV: ATCC VR-742	PPV had high resistance to dry heat (80°C for 10 min and 90°C for 1 min) and 70°C moist heat for 10 min. After treatment at 80°C and 90°C moist heat for 10 min, the virus titres were reduced by a factor of 5.9±0.1 and >6.1 $\log_{10}$ $TCID_{50}$, respectively.	(Eterpi et al., 2009)
Factor VIII	Human parvovirus B19, PPV and BPV	B19 proved to be the most susceptible to inactivation at 80°C for 72 h of dry heat treatment by >4.7 $\log_{10}$ reduction, followed by BPV (2.9 $\log_{10}$ reduction) and PPV (2.0 $\log_{10}$ reduction).	(Roberts et al., 2006)
EMEM and slurry	PPV Danish strain, 893	PPV with an initial titre of 10^6 $TCID_{50}$/ 50 μl in EMEM could survive for 4 weeks, 14 days, 7 days and 5 days at 40°C, 45°C, 50°C and 55°C, respectively, and PPV could survive longer in slurry.	(Bøtner & Belsham, 2012)

2.3 Method for virus inactivation/removal

According to WHO (2004), well-recognized methods of virus inactivation are pasteurization, dry heat, vapour heat, solvent/detergent and low pH, and well-recognized methods of virus removal are precipitation, chromatography and nanofiltration. Heat inactivation, acetone (solvent) extraction and microfiltration were chosen to be the methods for inactivation or removal of viruses in this study.

2.3.1 Solvent inactivation

A number of different extraction methods for egg yolk lipids have been described, and most of them are based on the use of organic solvents (Aro, 2007). The efficient extraction of cholesterol from egg yolk by acetone has been studied especially with the aim of preparing an egg yolk product with reduced cholesterol content for nutritional and dietetic use (Nielsen, 2001). The first step of IgY separation from egg yolk usually involves the extraction of IgY from yolk, and solvents such as acetone (Sriram & Yogeeswaran, 1999) or chloroform (Ntakarutimana et al., 1992) have been used to isolate lipids and lipoproteins from egg yolk (Ko & Ahn, 2007). Moreover, most industrial methods for the production of egg phospholipids, the so-called egg lecithin, are based on schemes of more or less selective solvent extraction performed batch-wise. For this, the different solubility of neutral lipids and polar lipids in various organic solvents is utilized, such as acetone, diethylether, hexane, chloroform and ethanol (Schneider, 1989). Triglycerides and cholesterol are preferentially extracted by acetone, and phospholipids are subsequently extracted by ethanol in a state of high purity (Nielsen, 2001), because phospholipids are soluble in hydrocarbons and other organic solvents such as chloroform and diethyl ether, while they are typically acetone insoluble (Rossi, 2007). For oral administration, the product may be used simply after evaporation of ethanol. However, because of contaminating proteins further purification must be performed to allow its use as component of nutrients for parenteral administration (Nielsen, 2001). However, to my knowledge, there are no published data about the inactivating potential of the solvent (acetone) extraction processes used by the egg product industry. So this study was intended to investigate the effect of the acetone extraction processes on AIV and NDV, using different concentrations of commercial egg yolk powder, and to determine the influence of water concentration on virus inactivation through acetone extraction.

2.3.2 Heat inactivation

Heat treatment is commonly used to inactivate a variety of pathogenic and non-pathogenic viruses, bacteria, fungi and protozoa in food products derived from poultry (Thomas & Swayne, 2007). Heat treatment is usually accomplished using pasteurization or the cooking process (Thomas & Swayne, 2007). The 1970 Egg Products Inspection Act (EPIA) requires that all egg products distributed for consumption have to be pasteurized (USDA, 2011b; Froning et al., 2002). It means that they must be rapidly heated and held at a minimum required temperature for a specific length of time (USDA, 2011b). *Salmonella* is the target pathogen for which egg pasteurization treatments were designed (Buchner, 2005). The pasteurization destroys *Salmonella,* but it does not cook the eggs or affect their colour, flavour, nutritional value or use (USDA, 2011b). Research by Hank et al. (2001) illustrated that the in-shell pasteurization process by hot-air oven at 55°C for 180 min had no

effect on the protein quality of albumen. Dried egg whites are pasteurized by heating in the dried form, again at a minimum required temperature and for a specified time (USDA, 2011a). Recommended pasteurization temperatures for liquid eggs that have received no chemical additives vary from 55.6°C to 69°C, and the times of exposure vary from 10 min to 1.5 min, respectively (Buchner, 2005). Pasteurization requirements for yolks are higher than those for other egg products, as *Salmonella* and other microorganisms are more heat resistant in yolks due to the protective effects of the higher protein contents and lipid levels; a typical process would involve heating at 60°C–65C for 3.5–6.5 min (Shebuski & Freier, 2009). USDA regulates the precise minimum times and temperatures at which egg products must be held during the pasteurization process as termed a ‘‘command-and-control’’ to producers (Latimer et al., 2008). Current USDA guidelines (9 CFR 590.570) mandate that liquid egg yolk must be pasteurized at 60°C (140°F) for 6.2 min or at 61.1°C (142°F) for 3.5 min. The albumin is the most sensitive part; it is denatured within a few minutes at or above 60°C. Homogenized whole egg and yolk are reasonably stable at this temperature (Buchner, 2005). Moreover, a new technology, i.e. long-term (6–24 h) heat treatment of liquid egg products at temperatures lower than conventional pasteurization (53°C to 55°C), was applied to extend the shelf life of liquid egg products containing no preservatives (Nemeth et al., 2011).

The probability of pathogen transmission to a susceptible host is influenced by the number of infectious viruses in the environment, which is affected by the thermostability of individual virus particles (Negovetich & Webster, 2010). Therefore there are several studies on the tenacity of viruses in different environments, and an overview of AIV tenacity is given in Stallknecht & Brown (2009). Although a small number of studies have examined the tenacity of NDV, FCV, SIV and PPV, to my knowledge no investigations have been published on the tenacity of these viruses in liquid egg yolk at 60°C. Therefore the objectives of this study were to determine and compare the effect of thermal inactivation on SIV and AIV, to evaluate the resistance of AIV, NDV, FCV and PPV to heat treatment at different temperatures (40°C, 60°C and 80°C) and the influence of liquid egg yolk on AIV, NDV and FCV. One of the specific aims of the study was to provide information that may help design the pasteurization process to improve the safety of liquid egg yolk products.

2.3.3 Filtration

Filtration operates fully on the size of particles or droplets (and, to some extent, their shape). Particles smaller than a certain size pass through the pores, and larger ones are retained on or in the barriers (Sutherland, 2008). The operation of a filter usually needs a pressure differential across the filter membrane (Sutherland, 2008). This technique is used in many processes with purposes like recovery of a valuable solid component (while the liquid is discarded), recovery of the liquid (while the solids are

discarded), recovery of both the solids and liquid phases or recovery of neither phase (e.g. when a liquid is being cleaned prior to disposal, as in the prevention of water pollution) (Wakeman & Tarleton, 1999). The size of separated particles is used to define the terms used for various filtration processes. Therefore ‘macrofiltration’ is used for separating particles in the approximate range of 1 mm in size down to 5 μm (with ‘screening’ used for particles above 1 mm in size, without an upper limit), ‘microfiltration’ is used for sizes of 5 μm down to about 0.1 μm, and below this the term ‘ultrafiltration’ is applied (Sutherland, 2008).

The established industrial technology of classic extraction consists of repeated mixing of yolk with a solvent, followed by filtration or centrifugation to recover a clear extract free of denatured protein (Nielsen, 2001). In this study, microfiltration with a membrane of 0.2 μm pore size was used as filtration method. The pore size of microfiltration was not small enough to reject particles smaller than tens of nanometres. However, if the viruses are aggregated and the size of aggregated viruses exceeds the pore size of the microfiltration membrane, the aggregated viruses are retained by the microfiltration membrane (Matsushita et al., 2005). Thus the goal of this study was to investigate and compare the distribution of viral genome and infectivity in fractions after partitioning through filtration.

3. Materials and methods

3.1 Materials

3.1.1 Equipment

96-well cell culture plates	Greiner Bio-One GmbH, Frickenhausen/Germany
96-microwell plates V-bottom	Greiner Bio-One GmbH, Frickenhausen/Germany
Beaker, 10 ml	Rasotherm, Wertheim/Germany
Beaker, 25 ml	Duran Group GmbH, Wertheim/Germany
Beaker, 50 ml	Rasotherm, Wertheim/Germany
Beaker, 150 ml	Rasotherm, Wertheim/Germany
Beaker, 250 ml	Rasotherm, Wertheim/Germany
Beaker, 400 ml	Rasotherm, Wertheim/Germany
Beaker, 1000 ml	Rasotherm, Wertheim/Germany
Cell scraper, 18 cm handle/1.8 cm blade	BD Falcon™, Bedford, MA/USA
Cryo tubes, 2 ml	Kisker Biotech GmbH & Co. KG, Steinfurt/Germany
Cryo tubes, 5 ml	Kisker Biotech GmbH & Co. KG, Steinfurt/Germany
Erlenmeyer flask, 500 ml	Rasotherm, Wertheim/Germany
Filters RC 0.2 µm	Sartorius, Göttingen/Germany
Laboratory bottle, 1000 ml	Duran Group GmbH, Wertheim/Germany
Multiple PCR cap strip of 8	Biozym Scientific GmbH, Hessisch Oldendorf/Germany
Multiple PCR microtube strip of 8, 0.2 ml	Biozym Scientific GmbH, Hessisch Oldendorf/Germany
Multichannel pipette reservoir	PZ HTL S.A., Warsaw/Poland

Neubauer cell chamber	Carl Roth GmbH + Co. KG, Karlsruhe/Germany
Open-top thin-wall polyallomer tube, 38.5 ml, 25 x 89 mm	Beckman Coulter, Inc., Brea, CA/USA
Pipette tips 10 µl	Carl Roth GmbH + Co. KG, Karlsruhe/Germany
Pipette tips 20 µl	Peqlab Ltd., Erlangen/Germany
Pipette tips 100 µl	Peqlab Ltd., Erlangen/Germany
Pipette tips 200 µl	Greiner Bio-One GmbH, Frickenhausen/Germany
Pipette tips 300 µl	Kisker Biotech GmbH & Co. KG, Steinfurt/Germany
Pipette tips 1000 µl	Peqlab Ltd., Erlangen/Germany
Pipette tips 1000 µl	Greiner Bio-One GmbH, Frickenhausen/Germany
Plastic pipette, 25 ml	Greiner Bio-One GmbH, Frickenhausen/Germany
Polycarbonate syringe filter holders, 13 mm	Sartorius, Göttingen/Germany
Safeseal tips professional, 10 µl	Biozym Scientific GmbH, Hessisch Oldendorf/Germany
Sterican® Gr. 18 needle	B. Braun Melsungen AG, Melsungen/Germany
Syringe, 1 ml	B. Braun Melsungen AG, Melsungen/Germany
Syringe, 2 ml	B. Braun Melsungen AG, Melsungen/Germany
TC-tube, sterile, 12 ml, screw cap	Greiner Bio-One GmbH, Frickenhausen/Germany
Tissue culture flasks, 75 cm²	Greiner Bio-One GmbH, Frickenhausen/Germany
Tissue culture flasks, 125 cm²	Greiner Bio-One GmbH, Frickenhausen/Germany

Tubes, 15 ml, PP, graduated, conical bottom, blue screw cap, sterile	Greiner Bio-One GmbH, Frickenhausen/Germany
Tubes, 50 ml, PP, graduated, conical bottom, blue screw cap, sterile	Greiner Bio-One GmbH, Frickenhausen/Germany
UHU glue	UHU GmbH & Co. KG, Bühl/Germany

3.1.2 Organic material

Chicken red blood cells	Institute of Poultry Diseases, Free University (FU) Berlin, Berlin/Germany
Dried egg yolk	Commercial market, Berlin/Germany
Lecithin	Commercial market, Berlin/Germany
Liquid egg yolk	Commercial market, Berlin/Germany

3.1.3 Viruses

Avian influenza virus (AIV: H7N1) (A/chicken/FPV/Rostock/1934)	Institute of Immunology and Molecular Biology, FU Berlin/Germany
Feline calicivirus (FCV)	Institute of Immunology and Molecular Biology, FU Berlin/Germany
Newcastle disease virus (NDV)	Institute of Immunology and Molecular Biology, FU Berlin/Germany
Porcine parvovirus (PPV)	Institute of Immunology and Molecular Biology, FU Berlin/Germany
Swine influenza virus (SIV: H1N1, H1N2, H3N2)	Institute of Immunology and Molecular Biology, FU Berlin/Germany

3.1.4 Cell lines

Chicken Embryo Fibroblasts (CEF)	Institute of Poultry Diseases, FU Berlin/Germany
Crandell Reese feline kidney cells (CRFK)	American Type Culture Collection (ATCC): CCL-94™
Madin-Darby bovine kidney (MDBK) cells	Institute of Immunology and Molecular Biology, FU Berlin/Germany

Madin-Darby canine kidney cells II (MDCK II)	University of Marburg, Marburg/Germany
Porcine kidney cells (PK_{13})	ATCC: CRL-6489™

3.1.5 Media

Dulbecco's Modified Eagle Medium (DMEM)	PAN-Biotech GmbH, Aidenbach/Germany
Minimum Essential Medium Eagle (MEM Eagle)	PAN-Biotech GmbH, Aidenbach/Germany

3.1.6 Reagents and Markers

Acetone	Carl Roth GmbH + Co. KG, Karlsruhe/Germany
Acrylamide	Carl Roth GmbH + Co. KG, Karlsruhe/Germany
APS	National Diagnostics, Munich/Germany
Bisacrylamide	Carl Roth GmbH + Co. KG, Karlsruhe/Germany
Coomassie Brilliant Blue	Bio-Rad Laboratories, Munich/Germany
Formaldehyde solutions	Carl Roth GmbH + Co. KG, Karlsruhe/Germany
Giemsa stain	Carl Roth GmbH + Co. KG, Karlsruhe/Germany
DPBS (10X) without Magnesium, without calcium	PAN-Biotech GmbH, Aidenbach/Germany
DPBS (10X) with Magnesium, with calcium	PAN-Biotech GmbH, Aidenbach/Germany
ProSieve® Protein Marker	Lonza Group Ltd., Basel/Switzerland
Research Grade Fetal Bovine Serum	Thermo Fisher Scientific Inc., Bonn/Germany
SDS	AppliChem GmbH, Darmstadt/Germany
Sodium Chloride	AppliChem GmbH, Darmstadt/Germany
TEMED	National Diagnostics, Munich/Germany

Tinctura Jodi (Ethanolic iodine solution)	Caesar & Loretz GmbH, Hilden/Germany
Trypsin/EDTA (10X)	PAN-Biotech GmbH, Aidenbach/Germany

3.1.7 Kits

RTP® DNA/RNA Virus Mini Kit	Invitek GmbH, Berlin/Germany
DyNAmo™ cDNA Synthesis Kit	Thermo Fisher Scientific Inc., Schwerte/Germany
IDEXX ELISA kit	IDEXX Laboratories Inc. Ludwigsburg/Germany

3.1.8 Instruments

Avanti™ J-25 high performance centrifuge	Beckman Instruments Inc., Fullerton, CA/USA
Beckman L7-65 ultracentrifuge	Beckman Coulter Inc., Fullerton, CA/USA
BSS 420 incubator	Grumbach Brutgeräte GmbH, Asslar/Germany
Centrifuge labofuge 400R	Heraeus, Hanau/Germany
CO_2 incubator	Heraeus, Hanau/Germany
Diagonal condenser R-8	Büchi Labortechnik AG, Essen/Germany
Diaphragm vacuum pump MZ 2C	Vacuubrand, Wertheim/Germany
Digital Dry Bath	Süd-Laborbedarf GmBH, Gauting/Germany
Discovery Comfort Variable Volume Multichannel Pipette, 20–200 µl	PZ HTL S.A., Warsaw/Poland
Freezer (-20°C)	Liebherr, Biberach an der Riss/Germany
Freezer (-80°C)	Heraeus, Hanau/Germany
Fusion-SL	Vilber Lourmat GmbH, Eberhardzell/Germany
Fume hood Variolab Mobilien W90	Waldner Holding GmbH & Co. KG, Wangen/Germany
iQ5 real-time PCR Detection System	Bio-Rad Laboratories, Munich/Germany
Laboratory balance MJ-500	YMC Co. Ltd., Kyoto/Japan
Microbiological safety cabinet LB 72 C	Heraeus, Hanau/Germany

Microcentrifuge	Bachofer-Laboratoriumsgeräte, Reutlingen, Germany
Microscope	Helmut Hund GmbH, Wetzlar/Germany
Minigel-Twin Electrophoresis	Biometra GmbH, Göttingen/Germany
Multiple Magnetic Stirrer Poly 15	Variomag, Daytona Beach, FL/USA
Pipette Controller Accu jet pro	Brand, Wertheim/Germany
Pipette Research, 0.1–2 µl	PEQLAB Ltd., Erlangen/Germany
Pipette Research, 0.5–10 µl	Eppendorf, Hamburg/Germany
Pipette Research, 0.5–10 µl	PEQLAB Ltd., Erlangen/Germany
Pipette Research, 2–20 µl	PEQLAB Ltd., Erlangen/Germany
Pipette Research, 10–100 µl	Eppendorf, Hamburg/Germany
Pipette Research, 10–100 µl	PEQLAB Ltd., Erlangen/Germany
Pipette Research, 100–1000 µl	Eppendorf, Hamburg/Germany
Pipette Research, 100–1000 µl	PEQLAB Ltd., Erlangen/Germany
PCR-Mastercycler Gradient	Eppendorf, Hamburg/Germany
Qualitron® DW-41 microcentrifuge	Qualitron Inc., Lahore/Pakistan
Refrigerator	Siemens, Munich/Germany
Rotary evaporator Rotavapor R	Büchi Labortechnik AG, Essen/Germany
Rotary shaker CMV-1	Fröbel, Lindau/Germany
Sigma 3k12 centrifuge	Sigma Laborzentrifugen GmbH, Osterode am Harz/Germany
Thermomixer 5436	Eppendorf, Hamburg/Germany
Thermostatic cabinet	Liebherr, Biberach an der Riss/Germany
Universal oven	Memmert, Schwabach/Germany
Vortex Genie 2	Scientific Industries Inc., Bohemia, NY/USA
Water bath E100	Lauda Dr. R. Wobser GmbH & Co. KG, Lauda-Königshofen/Germany
Water bath W-240	Büchi Labortechnik AG, Essen/Germany
Water bath WB14	Memmert, Schwabach/Germany
ZBE70-35 laboratory ice machine	Ziegra Eismaschinen GmbH, Isernhagen/Germany

3.1.9 Disinfectants

Aerodesin®	Lysoform Dr. Hans Rosemann GmbH, Berlin/Germany
Aldasan® 2000	Lysoform Dr. Hans Rosemann GmbH, Berlin/Germany
BARRYCIDAL® 36 Disinfectant	Interchem Hygiene GmbH, Zürich/Switzerland
Clorina®	Lysoform Dr. Hans Rosemann GmbH, Berlin/Germany
Incuwater-Clean™	AppliChem GmbH, Darmstadt/Germany

3.2 Methods

3.2.1 Preparation of virus stocks

3.2.1.1 Virus propagation in embryonated chicken eggs

The 37°C incubator was prepared by placing a plate of water into the bottom to provide moisture to prevent the eggs from drying out. Embryonated eggs were placed into the incubator trays at room temperature for 4 h for slow warming. Afterwards, the eggs were transferred into the 37°C incubator and candled daily, starting on the fifth day of age of the embryo by candling lamp to check the viability of the embryo. Viable embryos responded to the light by moving and had blood vessels within the allantoic membrane. Dead embryos and unfertilized eggs were discarded.

On the tenth day of age, the eggs were candled, a line was drawn on the shell to locate the air sac area and the inoculation site was marked by pencil. The eggs were placed with blunt ends up into the egg trays and incubated in the 37°C incubator in preparation for inoculation. The virus stock was removed from the -80°C freezer and thawed, then diluted with 0.9% NaCl solution to the required concentration. For example, if there were 100 eggs for inoculation and one egg was injected with 0.1 ml of inoculum and the required concentration of virus was 1:10,000, 1 µl of virus was used for dilution to 10 ml of inoculum. If the volume of virus was too low, dilution of virus was required before the virus was inoculated.

The egg trays were transferred to the biological safety cabinet. Cotton wool and Povidone-iodine were used to swab the inoculation site and the air sac. The eggs were allowed to dry, and two small holes (one on the defined air sac area and one hole at an inoculation site on the shell) were punched by sterilized hole puncher for all eggs except for the negative control one. 1 ml of inoculum (for 10 eggs) was aspirated into the tuberculin syringe and a needle of the size 18 was attached. The needle was inserted horizontally through the inoculation hole, then moved 45° vertically, and 0.1 ml of inoculum per egg was injected into the allantoic cavity. All eggs were inoculated using the same procedure, except for the ones that were used for negative control. The holes in the shell were sealed with glue. Both inoculated eggs and the negative control egg were placed into the 37°C incubator for 1 h to allow the glue to dry before the inoculated eggs were transferred into the incubator tray. All eggs were incubated in the 37°C incubator for one day and candled after 24 h post inoculation to check the viability of the embryo. Eggs were incubated for a further 24 h at 37°C.

Afterwards, the eggs were transferred to a 4°C refrigerator overnight to kill the embryo and reduce blood contamination of the allantoic fluid throughout harvesting. After cooling, the eggs were placed into a biological safety cabinet. Sterile scissors were used to break and remove the eggshell above the air sac area. The allantoic membrane was pulled back, the embryo was moved to one side by using a sterile forceps and a sterile pipette was used to harvest the allantoic fluid from the eggs (about 10 ml per egg). The allantoic fluid of all eggs, except for five inoculated eggs and the negative control egg, was pooled in an Erlenmeyer flask. Approximately 8 ml of pooled allantoic fluid were collected to perform an HA test. The allantoic fluid of the five inoculated eggs mentioned above was harvested into five separate sterile tubes.

The allantoic fluid was transferred to centrifuge tubes and centrifuged for 30 min at 3,500 rpm to sediment particles including the red blood cells. Next, a small amount of allantoic fluid (50 µl) was collected for analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The rest of the clear allantoic fluid was transferred to new sterile centrifuge tubes and ultra-centrifuged for 2 h at 15,000 rpm. The supernatant was removed and discarded. The pellet was dissolved in a small volume of TNE buffer (the amount of TNE buffer depended on the size of the pellet). The concentrated virus was aliquoted into cryogenic vials, and 4 µl and 5 µl of the concentrated virus were collected for performing SDS-PAGE and the HA test, respectively. The vials were stored at -80°C in a freezer until required for experiments.

Method to determine the concentration for the propagation of virus in eggs

Each egg was inoculated with 0.1 ml of a serial dilution of the virus (undiluted, diluted 1:10, 1:100, 1:1,000 and 1:10,000). After the appropriate incubation time to allow the virus to propagate in the eggs (see above), the allantoic fluid from each egg was

harvested into separate tubes and the HA test was performed. The concentration of virus with the highest HA titre was used to produce a high-titre virus stock.

3.2.1.1.1 Quality control for NDV and AIV

Virus quality was controlled by SDS-PAGE using allantoic fluid and concentrated virus.

Procedure
50 µl of allantoic fluid and 4 µl of concentrated virus were added to 25 µl and 15 µl of reducing agent, respectively. These two samples were heated at 100°C for 3 min and held at room temperature until application to the PAGE. The glass plates were cleaned with ethanol and set up with spacer by clips. The 5% acrylamide resolving gel was prepared by mixing the ingredients as shown in Table 7 except for APS and TEMED because the gel would be polymerized quickly after adding, therefore the latter ingredients were added last. The gel was poured into prepared glass plates and ethanol was added to cover the gel to avoid an oxidation. The gel was allowed to stand for 20 min. While waiting, a 12% stacking gel was prepared by mixing the ingredients as shown in Table 8, again except for APS or TEMED. After 20 min, ethanol was removed and distilled water was used for rinsing off the ethanol. The interplate surface was dried using Kleenex. For polymerization of the stacking gel, APS and TEMED were then added to the mixture. The stacking gel was filled on top of the polymerized resolving gel and the comb was inserted straight into the gel.

After allowing the plate to stand for 30 min, the spacer was removed and the plates were assembled to the electrophoresis apparatus using clips. The Tris-glycine electrophoresis buffer was filled into the upper and lower chambers of the electrophoresis apparatus. The bubbles under the gel were removed with a syringe. The comb was removed and the wells were flushed with the syringe to remove un-polymerized polyacrylamide and bubbles. The samples were centrifuged for 3–5 min at 14,000 rpm. 10 µl of each sample and 20 µl of marker (ProSieve®) were loaded into the wells. The gel was run at 80 mV for 20 min, and then the voltage was increased to 180 mV. The gel was monitored and the electrophoresis was stopped before the band ran out of gel.

Afterwards the gel was stained with Coomassie brilliant blue overnight. The dye was removed and a solution consisting of 10% acetic acid, 10% ethanol and water (v/v) were used to destain the gel. The destaining solution was discarded and the process was repeated until the gel was clear and the bands were observed as blue bands. The results were recorded using Fusion-SL.

Table 7: Composition of 5% acrylamide resolving gel

Reagent	10 ml gel
30% AA/0.8% Bis	1.3 ml
4x Tris-HCl/SDS pH 6.8	2.5 ml
Bidest	6.1 ml
TEMED	0.015 ml
APS (10%)	0.08 ml

Table 8: Composition of 12% stacking gel

Reagent	15 ml gel
30% AA/0.8% Bis	6.0 ml
4x Tris-HCl/SDS pH 6.8	3.75 ml
Bidest	5.25 ml
TEMED	0.015 ml
APS (10%)	0.08 ml

3.2.2.1 Virus propagation in cell culture

In this part of the study, two viruses (feline calicivirus and porcine parvovirus) were propagated in cell culture using different methods and cell lines. The Crandell Reese feline kidney (CRFK) cell line was used for propagation of FCV, and the pig kidney (PK_{13}) cells were used for propagation of PPV.

Reviving CRFK cells from the -80°C freezer

A vial (1 ml) of CRFK cells was removed from the -80°C freezer and thawed in a beaker that contained water at room temperature. The growth medium (EMEM with 5% foetal calf serum [FCS]) was prepared. 11 ml of growth medium and all of the content of the vial were filled into a T-75 tissue culture flask and mixed by tilting the flask back and forth. The flask was incubated in the 37°C incubator with humidity and 5% CO_2 for one day. The next day, the medium was replaced by 12 ml of fresh growth medium. The flask was incubated again in the 37°C incubator with humidity and 5% CO_2 until cells formed a confluent monolayer. The cells were observed daily under the microscope, and the growth medium was changed every two days.

Splitting the CRFK cells for propagation

The T-75 flask with the cell monolayer was transferred to the safety cabinet. Medium was removed and cells were washed twice with phosphate-buffered saline (PBS). 3 ml of trypsin/EDTA were added to the flask, and the flask was tilted to distribute trypsin/EDTA over the entire cell surface. The flask was incubated for 30 sec at room

temperature and transferred to 37°C with humidity and 5% CO_2 incubator for 3–5 min to increase the trypsin/EDTA activity or until all cells were detached from the surface. The flask was removed from the incubator and patted on the side to dislodge the cells. 5 ml of growth medium was added to the flask (the proteolysis reaction of trypsin can be terminated by FCS), and the medium was pipetted up and down onto the cell surface to reach all of the cells and to break up cell clumps. 20 ml of growth medium were added to a new T-125 tissue culture flask, then all of the cell suspension was transferred to it, and the new flask was tilted back and forth. Afterwards, it was incubated in the 37°C incubator with humidity and 5% CO_2 for 2–3 days or until cells formed a confluent monolayer.

FCV propagation in CRFK cells and determination of infectious FCV
The amount of virus for propagation was calculated and multiplicity of infection (MOI) of 0.001 was used to propagate FCV in cell culture.

- One T-125 monolayer flask had approximately $2x10^7$ cells. The titre of the virus stock was $6x10^7$/ml, and the cells in the T-125 flask formed a confluent monolayer. Then one flask was infected with $2x10^5$ virus particles, so 3.3 µl of virus were used. The viruses were diluted with medium because the volume of virus was too low for infection.

The T-125 flask was transferred to a safety cabinet and the medium was removed. The cells were washed twice with maintenance medium (EMEM without FCS). Later, 10 ml of maintenance medium and the required viruses were added to the flask, and the flask was tilted back and forth to spread the virus over the entire cell monolayer. Next, it was incubated in the 37°C incubator with humidity and 5% CO_2 for 2 h and tilted every 15 min.

After 2 h, the medium was removed and discarded. The cells were washed once with EMEM w/o FCS, and 25 ml of growth medium were added to the flask. The flask was incubated again in the 37°C incubator with humidity and 5% CO_2 for 2–3 days or until nearly 80% of the cells showed a cytopathogenic effect (CPE). The flask was checked daily for CPE and the condition of cells. After incubation, the medium containing the cells in the flask was pipetted up and down onto the cell surface to get all of the content, and this content was transferred to a sterile 50 ml centrifuge tube. Then the collected fluid was centrifuged at 5,000 rpm at 4°C for 20 min. Then the supernatant was transferred to the ultracentrifuge tube and the cell pellet was discarded. Ultracentrifugation was performed at 28,000 rpm at 4°C for 2 h 30 min, and then the supernatant was removed and discarded. The pellet was dissolved in a small volume of PBS w/o Ca & Mg (100 µl of PBS per ultracentrifuge tube). The concentrated viruses were aliquoted into cryogenic vials and were frozen at -80°C until required for experiments.

Reviving PK_{13} cells from the -80°C freezer

A vial (1 ml) of PK_{13} cells was removed from the -80°C freezer and thawed in a beaker containing water at room temperature. The growth medium (DMEM with 10% FCS) was prepared. 11 ml of growth medium and all of the content in the vial were added into a T-75 tissue culture flask and mixed by tilting the flask back and forth. The flask was incubated in the 37°C incubator with humidity and 5% CO_2 for one day. The next day, the medium was replaced by 12 ml of fresh growth medium. The flask was incubated again in the 37°C incubator with humidity and 5% CO_2 until cells formed a confluent monolayer which usually will take approximately 5–7 days. After that, the cells were observed daily under the microscope, and growth medium was changed every two days.

Splitting the PK_{13} cells for propagation

The monolayer T-75 flask was transferred to the safety cabinet. Medium was removed and cells were washed twice with PBS w/o Ca & Mg. Next, 3 ml of trypsin/EDTA were added to the flask, and the flask was tilted to distribute trypsin/EDTA over the entire cell surface. The flask was incubated for 30 sec at room temperature and transferred to the 37°C incubator with humidity and 5% CO_2 for 3–5 min to increase trypsin/EDTA activity or until all cells were detached from the surface. Later, the flask was removed from the incubator and patted on its side to dislodge the cells. 10 ml of growth medium were added (to terminate the proteolysis reaction of trypsin), and the medium was then pipetted up and down onto the cell surface to get all cells and to break up cell clumps. After that, a small amount of the cell suspension was transferred by pipette into a chamber on the haemocytometer and was counted under the microscope with a 4X objective. The volumes of the cell suspension and of the growth medium were calculated in order to obtain $2*10^6$ cells per T-125 tissue culture flask. Next, the cell suspension and the growth medium were transferred into a new T-125 tissue culture flask. Then it was mixed by tilting back and forth and incubated in the 37°C incubator with humidity and 5% CO_2 for one day.

PPV propagation in PK_{13} cells and concentration of PPV

The amount of the viruses for propagation was calculated, and 0.1 MOI were used to propagate PPV in cell culture.

- One T-125 monolayer flask had approximately $2*10^7$ cells. The titre of the virus stock was $2*10^7$/ml, and the cells in the T-125 flask formed a confluent monolayer. Then one flask was infected with $2*10^6$ virus particles, therefore 100 µl of virus were used.

The T-125 flask was transferred to the safety cabinet and the medium was removed. The cells were washed twice with maintenance medium (DMEM w/o FCS). Then 10 ml of maintenance medium and the viruses as required were added to the flask. The flask was tilted back and forth to spread the virus over the entire cell layer. The

flask was incubated in the 37°C incubator with humidity and 5% CO_2 for 2 h and tilted every 15 min. After 2 h, the medium was removed and discarded. The cells were washed once with the maintenance medium, and 25 ml of growth medium were added to the flask. The flask was incubated again in the 37°C incubator with humidity and 5% CO_2 for 5–7 days or until the cells showed nearly 80% CPE. The flask was checked daily for CPE and the condition of cells. The growth medium with 5% FCS was changed every two days.

Afterwards, all cells were scraped off using a cell scraper, and the medium containing the cells was pipetted up and down in the flask onto the cell surface in order to get all of the content and then the content was transferred to a sterile 50 ml centrifuge tube. The cell suspension was frozen and thawed 3 times. Next, the cell suspension was centrifuged at 5,000 rpm at 4°C for 20 min, and then the supernatant was transferred to the ultracentrifuge tube and the cell pellet was discarded. Ultracentrifugation was performed at 28,000 rpm at 4°C for 2.5 h. Then the supernatant was removed and discarded. The pellet was dissolved in a small volume of PBS w/o Ca & Mg (100 µl of PBS per ultracentrifuge tube). The concentrated viruses were aliquoted into cryogenic vials, and the vials were stored at -80°C freezer until required for experiments.

3.2.2.1.1 Quality control for FCV and PPV

After the virus was propagated in the specific cell line, the concentrated FCV and PPV were analysed by SDS-PAGE as described above (see 3.2.1.1.1).

3.2.2 Quantitative assessment of virus

3.2.2.1 Determination of the 50% Tissue Culture Infective Dose ($TCID_{50}$)

This endpoint titration assay was used to determine the titre of infectious virus required to produce 50% cytopathogenic effect of infected tissue culture cells. CPE means the morphological changes of the cells such as cell rounding, cell swelling or shrinking, detachment from the surface, inclusion bodies and apoptosis etc. resulting from the viral infection. Specific cell lines were required for the different viruses in order to see the CPE. The $TCID_{50}$ per millilitre was calculated according to the formula by Spearman and Kärber (Kärber, 1931; Spearman et al., 1974).

Table 9: Cells used for propagation and quantification of viruses

Virus	Cell line
Newcastle disease virus (NDV)	Chicken Embryo Fibroblasts (CEF)
Avian influenza virus (AIV: H7N1)	Chicken Embryo Fibroblasts (CEF)
Feline calicivirus (FCV)	Crandell-Reese feline kidney cells (CRFK)
Porcine parvovirus (PPV)	Porcine kidney cells (PK_{13})
Swine influenza virus (SIV: H1N1, H1N2)	Madin-Darby bovine kidney cells (MDBK)
Swine influenza virus (SIV: H3N2)	Madin-Darby canine kidney cells II (MDCK II)

Table 10: Growth medium, cell density/well and incubation time performing $TCID_{50}$ for different cell lines and viruses

Cell line	Growth medium	Cell density/well	Virus	Incubation time
CEF	EMEM[a] + 5% FCS[b]	5.0×10^4	AIV	2 days
CRFK	EMEM + 10% FCS	2.5×10^4	FCV	2 days
PK_{13}	DMEM[c] + 10% FCS	6.0×10^3	PPV	3 days
MDBK	DMEM + 5% FCS	3.0×10^4	SIV H1N1, H1N2	3 days
MDCK II	DMEM + 5% FCS	2.5×10^4	SIV H3N2	5 days

[a] EMEM, Eagle's minimum essential medium
[b] FCS, Foetal calf serum
[c] DMEM, Dulbecco's Modified Eagle Medium

$TCID_{50}$ procedure for NDV, AIV, FCV and PPV

The 96-well microtitre plates were prepared by seeding the required number of cells (cell density for a specific virus and the cell culture medium are shown in Table 10) into each well. The plates were incubated in an incubator at 37°C, 5% CO_2 for 2 h. The virus stocks or the samples from the experiment were thawed on ice and the 12-well dilution racks were treated under UV-light for 20 min. 900 µl of serum-free medium were filled into each well of the 12-well dilution rack except for the first well.

1 ml of sample from an experiment or 1 ml of 1:100 diluted virus stock with medium was added to the first well of the 12-well dilution rack. The ten-fold serial dilutions were performed using a micropipette. 100 µl from the first well were transferred to the second well. A separate tip was used to mix the second well by pipetting the solution 10 times up and down, and 100 µl of the second well were transferred to the third well. The ten-fold serial dilutions were continued across the dilution rack until the second to last well (the two last wells were the negative control). The multi-channel micropipette was used to fill 100 µl of the diluted sample in the dilution rack into the 96-well microtitre plate. Therefore each dilution was used to infect 8 wells in the same row. The plates were then incubated in an incubator at 37°C, 5% CO_2 for the desired time.

Afterwards, each well of the plates was fixed with 150 µl of a 10% formalin solution for 20 min. After removal of the fixative, cells were stained with 75 µl of Giemsa solution for 20 min. Then the staining solution was removed, and the plates were rinsed with tap water until the water was clear and colourless. The plates were tapped on Kleenex to remove excess water and dried in the 40–45°C incubator overnight. The results were read by examining the CPE under the light microscope and calculated using the Spearman and Kärber method (Spearman et al., 1974).

Determination of $TCID_{50}$ for influenza viruses see above (Swine influenza viruses H3N2, H1N1 and H1N2) using modified medium

The medium (DMEM) containing 4 benzoyl-L-arginine ethyl ester (BAEE) units of trypsin per millilitre was prepared. Every well of the plates was filled with 100 µl of DMEM containing 4 BAEE units of trypsin per millilitre. After the plates had been incubated in the 37°C incubator with humidity, 5% CO_2 for 3 days, 100 µl from each well of the plates were removed and replaced by 100 µl of DMEM containing 4 BAEE units of trypsin per millilitre and incubated in the 37°C incubator with humidity, 5% CO_2 for another day. Cells were fixed and stained as described above.

Cytopathogenic effect (CPE) of different cell lines

The micrographs (light microscope) show the uninfected cell lines and infected cells after infection with the specific viruses for the required periods of incubation.

CEF (control)

CPE on the 2nd day after infection

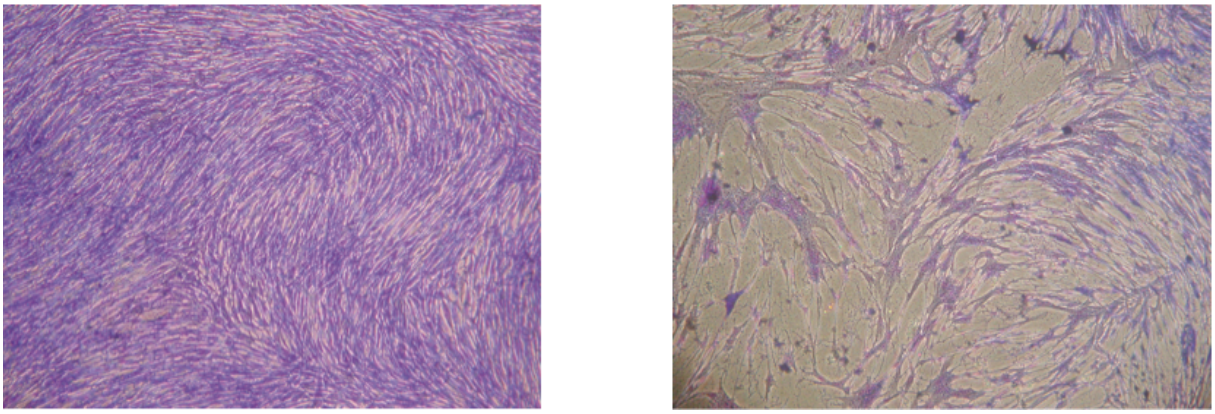


Figure 8: A comparison of confluent monolayer of chicken embryo fibroblasts (CEF) and CPE of cells infected with AIV

CRFK

CPE on the 2nd day after infection

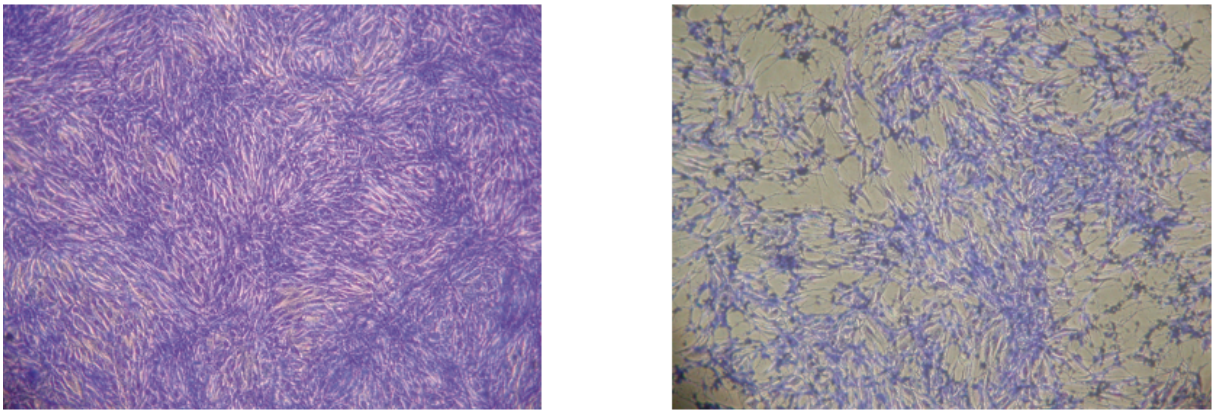


Figure 9: A comparison of confluent monolayer of Crandell Reese feline kidney cells (CRFK) and CPE of cells infected with FCV

PK_{13} CPE on the 3rd day after infection

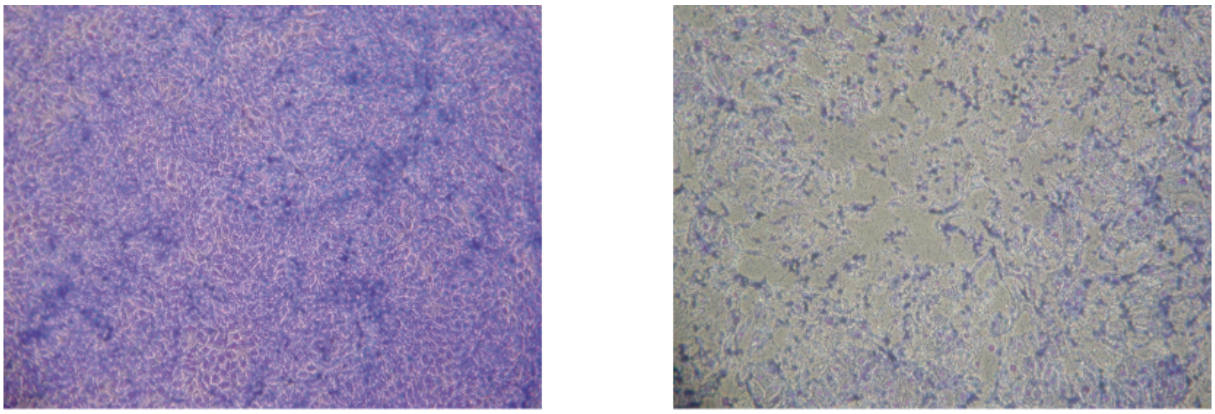


Figure 10: A comparison of confluent monolayer of porcine kidney cells (PK_{13}) and CPE of cells infected with PPV

MDBK CPE on the 3rd day after infection

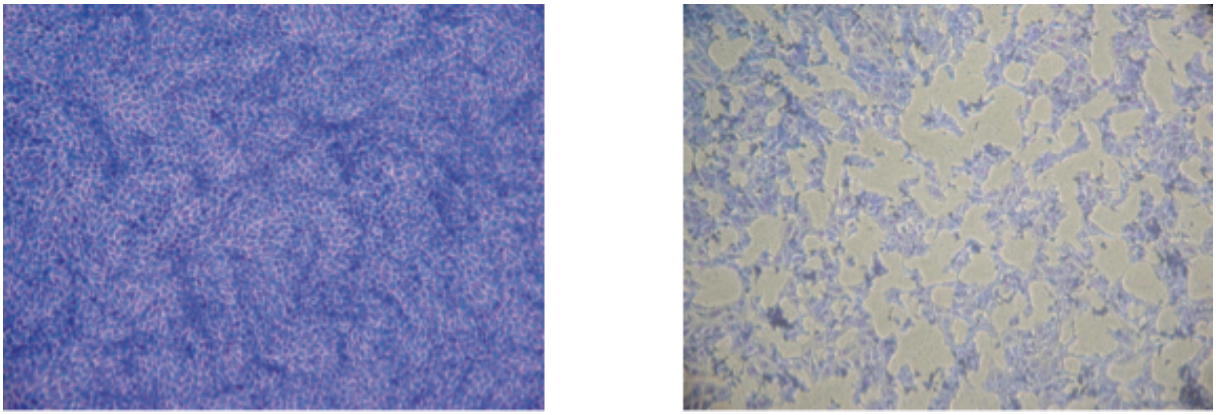


Figure 11: A comparison of confluent monolayer of Madin-Darby bovine kidney (MDBK) cells and CPE of cells infected with SIV (H1N1)

MDCK II CPE on the 5th day after infection

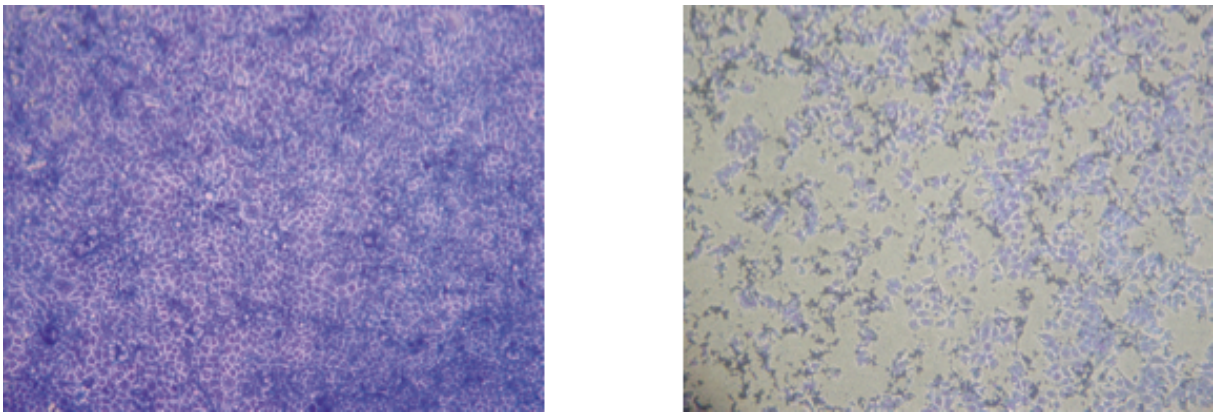
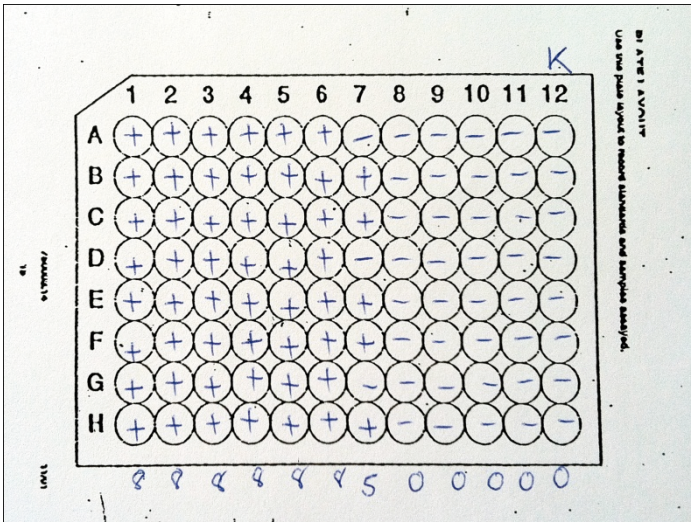


Figure 12: A comparison of confluent monolayer of Madin-Darby canine kidney cells II (MDCK II) and CPE of cells infected with SIV (H3N2)

Calculation of $TCID_{50}$ using the Spearman and Kärber method

The plates were read and the number of positive wells showing CPE and the negative wells was recorded as shown in Figure 13.



+ : positive well showing CPE

- : negative well

Figure 13: The 96-well microtitre plate data sheet for recording wells that show CPE and negative wells

After scoring the plates, the $TCID_{50}$/ml and log_{10} $TCID_{50}$ were calculated using the Microsoft Office Excel software.

*initial dilution:	1											
*dilution factor:	10											
*ml per well:	0.1											
Dilution factor:	**1.00E+00**	**1.00E-01**	**1.00E-02**	**1.00E-03**	**1.00E-04**	**1.00E-05**	**1.00E-06**	**1.00E-07**	**1.00E-08**	**1.00E-09**	**1.00E-10**	**Control**
*total wells:	8	8	8	8	8	8	8	8	8	8	8	8
*positive wells:	8	8	8	8	8	8	5	0	0	0	0	0
negative wells:	0	0	0	0	0	0	3	8	8	8	8	8
cum. positive:	53	45	37	29	21	13	5	0	0	0	0	0
cum. negative:	0	0	0	0	0	0	3	11	19	27	35	43
% infected:	100.00	100.00	100.00	100.00	100.00	100.00	62.50	0.00	0.00	0.00	0.00	0.00
prop dist (PD):	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0
$TCID_{50}$/dilution:							6.31E-07					
$TCID_{50}$/ml:							1.58E+07					
10^x $TCID_{50}$/ml							7.2					
$TCID_{50}$/ml (Spearman & Kärber)			1.33E+07									
Log_{10} $TCID_{50}$/ml			7.1									

Figure 14: Microsoft Office Excel software used to calculate $TCID_{50}$/ml and log_{10} $TCID_{50}$

3.2.2.2 Haemagglutination test (HA test)

After the viruses were propagated in specific pathogen-free (SPF) embryonated eggs the haemagglutination (HA) test was used for the rapid quantification of NDV and AIV.

Preparation of washed chicken red blood cells

The chicken blood was provided by the Institute of Poultry, Freie Universität Berlin. 6–7 ml of chicken blood (citrate blood) was transferred to a centrifuge tube and centrifuged for 5 min at 2,500 rpm at 20°C (Sigma 3k12 centrifuge). The supernatant was removed carefully in order not to disturb the pellet by using pipette and micropipette. 10 ml of a 0.9% NaCl solution were added carefully to the blood in the centrifuge tube. The centrifuge tube was mixed gently by tilting up and down and centrifuged again with the same procedure. The supernatant was removed and discarded. The red blood cells were washed 2–3 times until the supernatant was clear.

Preparation of 1% solution of chicken red blood cells

After the red blood cells had been washed with 0.9% NaCl solution and the clear supernatant was discarded, 1 ml of packed red blood cells was added to a glass container containing 99 ml of 0.9% NaCl solution. The glass container was tilted gently to suspend the packed red blood cells. This adjusted the concentration of red blood cell suspension to 1%. The 1% solution of chicken red blood cells in 0.9% NaCl solution can be kept for one week at 4°C. A new suspension was prepared in case there was a haemolysis of cells.

Method of HA test
A multi-channel micropipette was used to add 50 µl of 0.9% NaCl solution to all wells of the 96-microwell plate. A micropipette was used to fill 50 µl of the samples (allantoic fluid from inoculated eggs, allantoic fluid from the negative control egg and the concentrated virus) into the wells of the first column. A separate tip was used for each sample and for each dilution step. Two-fold serial dilutions were performed by using the multi-channel micropipette to mix all wells in the first column by pipetting the solution 10 times up and down. 50 µl of all the wells of the first column were transferred to the second column with the same tip. The two-fold serial dilution was continued across the plate using new tips for each dilution until the second to last well of the column (column 11). The 50 µl of the suspension of column 11 were discarded. The last wells of the column (column 12) were used as the red blood cells controls for auto-agglutination. The 50 µl of 1% red blood cells suspension were added to each well and mixed by patting the sides of the plate. The plate was covered with a lid and placed for 45 min at room temperature. The plate was read and the results were recorded. All the allantoic fluid from the negative control egg and red blood control wells should be HA negative. The titration end point was considered the highest dilution exhibiting a definite pattern of haemagglutination.

3.2.3 Determination of viral genome copy numbers (qRT-PCR)

The quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was applied to quantify AIV (see 3.8).

RNA extraction
RNA was extracted using the RTP® DNA/RNA Virus Mini Kit (Invitek GmbH, Berlin) following the protocol from the manufacturer. First, the Elution Buffer R was pre-warmed to 80°C for an elution of RNA.

200 µl of each sample were prepared in the extraction tube. 200 µl of ddH_2O were added to the prepared samples and then mixed by vortexing. The samples were incubated at 65°C for 15 min and at 95°C for 10 min in the shaking thermomixer. Then 400 µl of the binding solution were added to the samples and mixed with vortex. The samples were transferred to the Spin Filter Set (Invitek) and centrifuged at 10,000 rpm for 1 min. Afterwards the RTA Receiver Tubes (Invitek) with filtrates were discarded and replaced with new ones.

For the first washing step, 500 µl of Wash Buffer R1 were filled into the RTA Spin Filters and centrifuged at 10,000 rpm for 30 sec. The RTA Receiver Tubes with filtrates were discarded and replaced with new ones. For the second washing step, 700 µl of Wash Buffer R2 were filled into the RTA Spin Filter and centrifuged at 10,000 rpm for 30 sec. The RTA Receiver Tubes with filtrates were discarded and

were again replaced by new RTA Receiver Tubes, and then the samples were centrifuged at 10,000 rpm for 4 min. The RTA Receiver Tubes with filtrates were discarded. The RTA Spin Filters were assembled into the elution tubes and 60 µl of Elution Buffer R were filled on top of the RTA Spin Filters, and then incubated for 3 min at room temperature. Subsequently, the samples were centrifuged at 10,000 rpm for 1 min. The RNA was stored at -20°C until required for cDNA synthesis.

cDNA Synthesis

The RNA was reverse transcribed into cDNA before qRT-PCR was performed. The DyNAmo™ cDNA Synthesis Kit was used for cDNA synthesis. In the first step, all reagents were added as shown in Table 11 except for the RNA template to create the master mix. The amounts of reagents were calculated up to the number of the samples (RNA templates). Then the cDNA was synthesized by using 13 µl of master mix and 7 µl of RNA template of each sample. The incubation procedure was following the protocol from the manufacturer shown in Table 12. The cDNA was stored at -20°C until required for qRT-PCR.

Table 11: Reaction setup for cDNA synthesis (DyNAmo™)

Components	20 µl reaction
2x RT buffer	10 µl
Random hexamer primer set	1 µl
Template RNA	7 µl
M-MuLV RNase H^+ reverse transcriptase	7 µl
Total volume	20 µl

Table 12: Incubation protocol for reverse transcription (DyNAmo™)

Step	Purpose	Temperature	Time
1	cDNA synthesis	37°C	30 min
2	Reaction termination	85°C	5 min
3	Cooling of the sample	4°C	Hold

qRT-PCR

The influenza A virus matrix gene-specific PCR primer set and the hydrolysis probe according to Spackman et al. (2002) were used as shown in Table 13. The master mix was prepared by adding H_2O, 5x reaction buffer, 50 mM of $MgCl_2$, dNTPs, forward primer, reverse primer, hydrolysis probe and *Taq* polymerase. For a 25-µl qRT-PCR reaction, the volume of each reagent is listed in Table 14. The volumes of

reagents needed were calculated from the number of reactions. 20 µl of master mix were added to each PCR tube, followed by 5 µl of cDNA template of each sample. The mixtures were mixed by vortex and bubbles were removed by short spin. Afterwards all PCR tubes were placed into the real-time PCR machine (Bio-Rad cycler). The qRT-PCR conditions as shown in Table 15 were followed. The viral genome copy numbers were quantified by the results obtained by qRT-PCR and the standard curve method.

Table 13: PCR primer and hydrolysis probe used for AIV quantification (qRT-PCR) (Spackman et al., 2002)

Specificity	Primer/ probe	Sequence[a] (5'–3')
Influenza A virus	M + 25	AGA TGA GTC TTC TAA CCG AGG TCG
	M – 124	TGC AAA AAC ATC TTC AAG TCT CTG
	M + 64	FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA

[a] FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine

Table 14: Reaction setup for qRT-PCR

Components	25 µl reaction
H_2O	11.25 µl
5x reaction buffer	5 µl
50 mM $MgCl_2$	1.5 µl
dNTPs	0.5 µl
M + 25	0.5 µl
M – 124	0.5 µl
M + 64	0.5 µl
Taq polymerase	0.25 µl
Template cDNA	5 µl
Total	25 µl

Table 15: qRT-PCR conditions

Protocol	Temperature	Time	Remarks
Cycle 1 (1x)			
Step 1	95°C	10 min	
Cycle 2 (45x)			
Step 1	95°C	15 sec	Data collection enabled
Step 2	60°C	30 sec	Data collection and real-time analysis enabled
Cycle 3 (37x)			
Step 1	59–95°C	10 sec	Increase set point temperature after cycle 2 by 1°C

3.2.3 Heat inactivation kinetics of viruses in the presence of or without liquid egg yolk

3.2.3.1 Heat inactivation kinetics of four different viruses

Virus

The four viruses used in this part of the study were Newcastle disease virus (NDV), avian influenza virus (AIV), feline calicivirus (FCV) and porcine parvovirus (PPV). All virus strains were obtained from the Institute of Immunology and Molecular Biology, Freie Universität Berlin. Working stocks of NDV and AIV were propagated by allantoic sac inoculation of 10-day-old SPF embryonated chicken eggs and stored at -80°C. Working stocks of FCV and PPV were propagated in cell culture and stored at -80°C.

Experimental procedure

Temperatures were selected to represent the natural temperature of chickens (40°C), the temperature for pasteurization (60°C) and the temperature for processing the egg products (80°C). For NDV, AIV and FCV, at 40°C five time points: 20 min, 1 h, 6 h, 24 h and 48 h were evaluated (Table 16). At 60°C and 80°C four time points: 1 min, 5 min, 20 min and 60 min were evaluated (Table 17). For PPV, at 40°C, 60°C and 80°C three time points: 6 h, 24 h and 72 h were evaluated (Tables 16 and 17). Duplicate runs were performed for each of the time points.

Table 16: Time points at 40°C of four different viruses (NDV, AIV, FCV and PPV)

Viruses	Heat time points at 40°C					
	20 min	60 min	6 h	24 h	48 h	72 h
NDV	X	X	X	X	X	
AIV	X	X	X	X	X	
FCV	X	X	X	X	X	
PPV			X	X		X

X = sample collected at the respective time point

Table 17: Time points at 60°C and 80°C of four different viruses (NDV, AIV, FCV and PPV)

Viruses	Heat time points at 60°C and 80°C						
	1 min	5 min	20 min	60 min	6 h	24 h	72 h
NDV	X	X	X	X			
AIV	X	X	X	X			
FCV	X	X	X	X			
PPV					X	X	X

X = sample collected at the respective time point

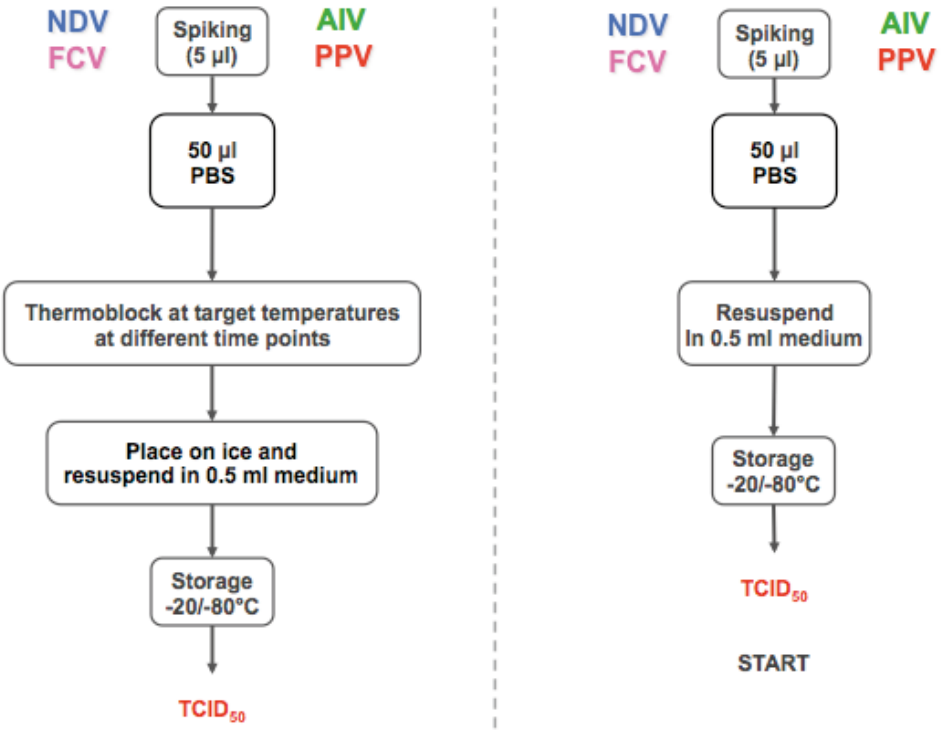


Figure 15: Flow chart of heat inactivation procedure without liquid egg yolk (the right part of the diagram shows the chart for the control)

Assessment of each of the four viruses was done on a different day. Sufficient aliquots of each virus were prepared, kept at -80°C and thawed on ice prior to use. The start samples (0 min) were prepared by spiking 5 µl of virus into 50 µl of PBS and both were mixed by vortexing. The samples were then re-suspended in 0.5 ml of medium (EMEM for NDV, AIV and FCV and DMEM for PPV) and stored at -20°C until the heat treatment of each virus at each temperature was finished. The heating block of the thermocycler was set to the target temperatures (40°C, 60°C and 80°C). During the experiment, each temperature did not vary by more than 1°C. The samples were prepared by spiking 5 µl of virus into 50 µl of PBS and both were mixed by vortexing. The samples were incubated in the thermocycler heating block for a specified length of time. After each time point the samples were removed and immediately cooled on ice. Then the samples were re-suspended in 0.5 ml of specified medium (EMEM for NDV, AIV and FCV and DMEM for PPV) and frozen at -20°C. After the heat treatment of each virus at each temperature was finished, all samples were transferred and stored at -80°C until required for end-point titration ($TCID_{50}$ assay).

Virus titration

The determination of $TCID_{50}$ assays was performed using 96-well microtitre plates containing the respective cells for the specified viruses as described in section 3.2.2.1. Virus titres were calculated according to Spearman and Kärber (Kärber, 1931; Spearman et al., 1974).

Detection limit

Depending on the amount of material investigated and the dilution of the sample, the detection limit was estimated by the calculation according to Spearman and Kärber.

This calculation method is statistically only valid if ≥ 90% of the cultures with the highest virus concentration investigated are positive and if at least one higher dilution shows ≤10% positive cultures. Due to these restrictions, a statistically valid $TCID_{50}$ virus titre can only be calculated if at least ≥ 8 wells per plate are positive, corresponding to ≥ 3.16 IU/0.1 ml ≥ 31.6 IU/ml (1/10 dilution step).

3.2.3.2 Heat inactivation kinetics of different virus species in the presence of liquid egg yolk

Egg yolk preparation

Large eggs were purchased from a local market in Berlin. Three eggs were broken and egg yolks were separated from egg white. All egg yolks were pooled and then homogenized by vortex. The homogenized egg yolks were stored at 4°C prior to use.

Experimental procedure

The temperature of interest was the one used in pasteurization (60°C). For NDV, AIV and FCV, four time points: 1, 5, 20 and 60 min of exposure to heat were evaluated (Table 18). Duplicate runs were performed at each of the time points.

Table 18: Harvesting of samples after incubation at 60°C in the presence of liquid egg yolk

Virus	Incubation time at 60°C			
	1 min	5 min	20 min	60 min
NDV	X	X	X	X
AIV	X	X	X	X
FCV	X	X	X	X

X = sample collected at the respective time point

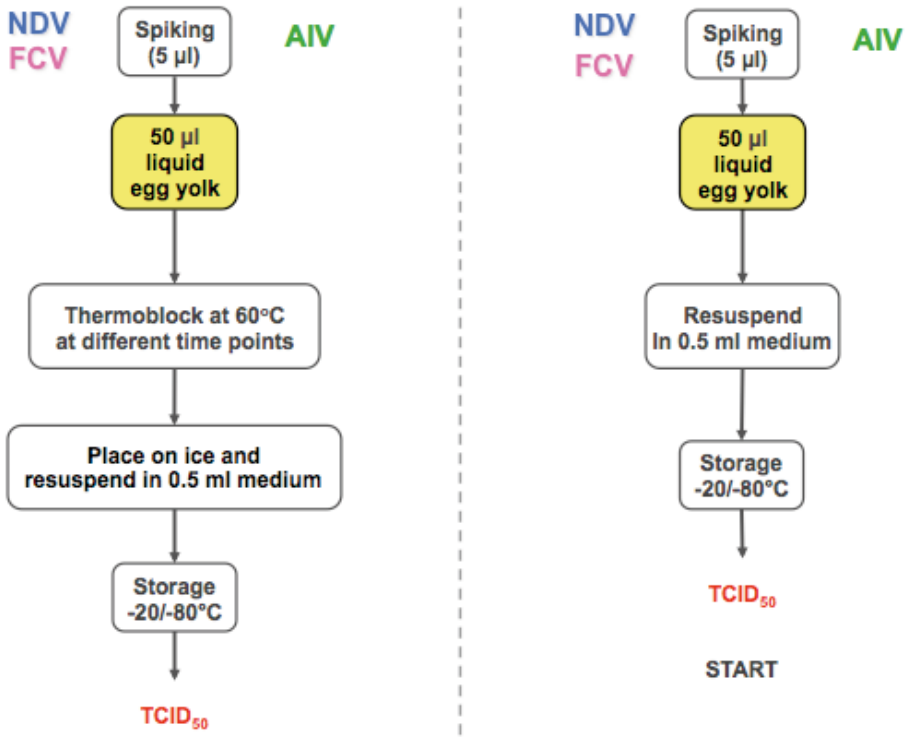


Figure 16: Flow chart of the heat inactivation procedure in the presence of liquid egg yolk (the right part of the diagram shows the chart for the control)

Sufficient aliquots of each virus were prepared and kept at -80°C and thawed on ice prior to use. The homogenized egg yolks were removed from 4°C and placed at room temperature for 2 h before using. The start samples (0 min) and the control samples were prepared by spiking 5 µl of virus into 50 µl of liquid egg yolk and both were mixed by vortexing (Fig. 16). The control samples were re-suspended in 0.5 ml

of EMEM and stored at -20°C until the heat treatment of each virus was performed (Fig. 16 left chart). The heating block of the thermocycler was set to the target temperature (60°C). During the experiment, the temperature did not vary by more than 1°C. The samples were prepared by spiking 5 µl of virus into 50 µl of liquid egg yolk, and both were mixed by vortexing. The samples were incubated in the thermocycler heating block for the specified length of time. After each time point the samples were removed and immediately cooled in ice. Then the samples were re-suspended in 0.5 ml of EMEM and frozen at -20°C. After all the heat treatments of each virus were finished, all the samples were transferred and stored at -80°C until required for an end-point titration ($TCID_{50}$ assay).

3.2.4 Heat inactivation kinetics of AIV and different subtypes of SIV

Virus

The four viruses used in this part of the study were AIV (H7N1) and three subtypes of SIV (H1N1, H1N2 and H3N2). All virus strains were obtained from the Institute of Immunology and Molecular Biology, Freie Universität Berlin. Working stocks of AIV were propagated by allantoic sac inoculation of 10-day-old SPF embryonated chicken eggs and stored at -80°C.

Experimental procedure

The temperature selected was 40°C. For all of the viruses five time points: 3 h, 6 h, 12 h, 24 h and 48 h were evaluated (Table 19). Triplicate runs were performed at each of the time points.

Table 19: Incubation of AIV and different subtypes of SIV at 40°C

Viruses	Incubation time at 40°C				
	3 h	6 h	12 h	24 h	48 h
AIV (H7N1)	X	X	X	X	X
SIV (H1N1)	X	X	X	X	X
SIV (H1N2)	X	X	X	X	X
SIV (H3N2)	X	X	X	X	X

X = sample collected at the respective time point

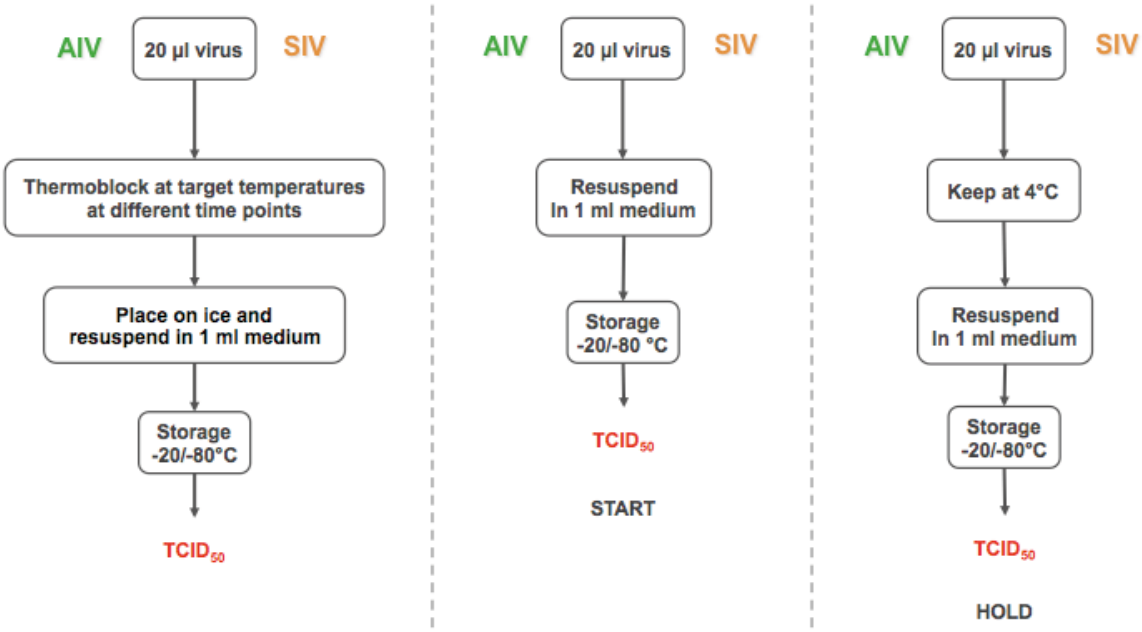


Figure 17: Flow chart of the heat inactivation procedure of AIV and different subtypes of SIV (left panel). As controls served the virus suspended in medium (middle panel, frozen immediately after mixing) and the virus suspension incubated in parallel to the heat treated sample incubated at 4°C (right panel of the diagram)

Assessment of each of the four viruses was done on a different day. Sufficient aliquots of each virus were prepared and kept at -80°C and thawed on ice prior to use. The control samples (0 min) were prepared by filling 20 µl of virus into tubes, virus was re-suspended in 1 ml of medium (EMEM for AIV and DMEM for SIV) and stored at -20°C until the heat treatment of each virus was finished (Fig. 17 rmiddle chart). The hold samples were prepared by transferring 20 µl of virus into tubes which were kept at 4°C until the heat treatment of each virus was finished (Fig. 17 right chart). The hold samples were then re-suspended in 1 ml of specified medium. The heating block of the thermocycler was set to the target temperature (40°C). During the experiment, the temperature did not vary by more than 1°C. The samples were prepared by transferring 20 µl of virus into tubes, and then these tubes were placed in the thermocycler heating block for the specified length of time (Fig. 17 left chart). After each time point the samples were removed and immediately cooled on ice. Then the samples were re-suspended in 1 ml of specified medium and frozen at -20°C. After all heat treatments of each virus were finished, all the samples including the start and hold samples were transferred and stored at -80°C until required for titration.

3.2.5 Assessment of antibodies present in egg yolk starting material

Haemagglutination Inhibition Test (HI test)
The presence of antibodies to NDV in egg yolk power was determined by the haemagglutination inhibition test (HI test).

Method of HI test
All three batches of egg yolk powder were diluted 1:4, 1:10 and 1:100 in PBS. 25 µl of PBS were filled into each well of the V-bottom 96-microwell plates, and 25 µl of each sample including the positive control serum were added. The multi-channel micropipette was used to perform two-fold serial dilutions along the row until the third to last well of the column (column 10). 25 µl of Newcastle disease virus antigen (4 HA units, see determination of the HA units) were added to each well except the antigen and red blood cells control wells (columns 11 and 12) and were mixed by patting the plates' sides. Then the plates were covered with the lids and placed for 30 min at room temperature. After that, 50 µl of 1% red blood cell suspension were added into each well and mixed as mentioned before. The plates were again covered with the lids and placed for 45 min at room temperature. The 96-microwell plates were then read, and the settling patterns in each well were recorded.

Enzyme Linked Immunosorbent Assay (ELISA)
To detect avian influenza virus antibodies, the ELISA serological test was performed with the commercial IDEXX ELISA kit, following the protocol and interpretation of the results recommended by the manufacturer.

Three different batches of egg yolk powder were resuspended in PBS (w/v 1:4). The samples were further diluted 1:500 with the PBS. As negative control two wells of a 96-well polystyrene plate were each filled with 100 µl of diluted chicken serum non-reactive for anti-AIV, and as positive control 100 µl of serially diluted chicken serum positive for anti-AIV antibodies were added to 2 new wells. 100 µl of each diluted sample were added to the respective wells, and after 30 min of incubation, the plate was washed 3–4 times with distilled water. Then 100 µl of conjugate were filled into every well, and after 30 min the plates were washed. 100 µl of TMB substrate were added to all wells and the plate was incubated for 15 min. Afterwards, 100 µl of stop solution were added to each well. The absorbance values were measured at 650 nm using an ELISA plate reader (spectrophotometer).

3.2.5.1 Virus spiking and sample handling

Since the spiked material has to correspond as closely as possible to the original composition (EMA, 2009), the spiking virus in this study was added in a small volume (usually 1/10 of the incubation mixture).

Influence of egg yolk concentration on the virus titre

Different volumes of egg yolk power (Figure 18) were spiked to the same volume of virus to observe the effect of the egg yolk concentration on the virus titre. NDV and AIV were used in the experiment.

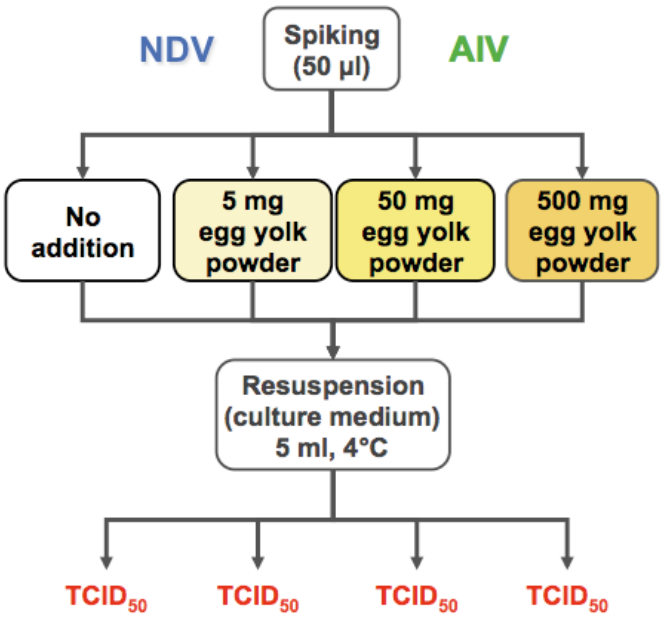


Figure 18: Flow chart of viruses spiked to different egg yolk powder quantities

Procedure

Duplicate runs were performed for each volume of egg yolk powder. The required amount (0 mg, 5 mg, 50 mg and 500 mg) was filled into the centrifuge tube, and 50 µl of virus were spiked to the prepared egg yolk powder. The sample was stirred using sterile silicone bars for 3 min to completely dissolve the virus droplet in the egg yolk powder. Afterwards the sample was incubated at room temperature for 12 min, and resuspended in 5 ml of cell culture medium. Then it was stirred by the rotary shaker for 2 h at 4°C. Afterwards, all of the samples were stored at -80°C for end-point titration ($TCID_{50}$ assay).

3.2.6 Influence of egg yolk concentration on virus inactivation through acetone extraction

Four different amounts of egg yolk power (0, 8, 80 and 800 mg) were extracted with 100% acetone. Both relevant viruses were used in this experiment (NDV and AIV). After acetone extraction of the egg yolk powder, two fractions were obtained after centrifugation, namely the residue (de-oiled egg yolk powder) and the extract (egg yolk oil). The de-oiled egg yolk powder contained the phospholipid while the egg yolk

oil contained triacylglycerol and cholesterol. The phospholipid can be extracted by further ethanol extraction.

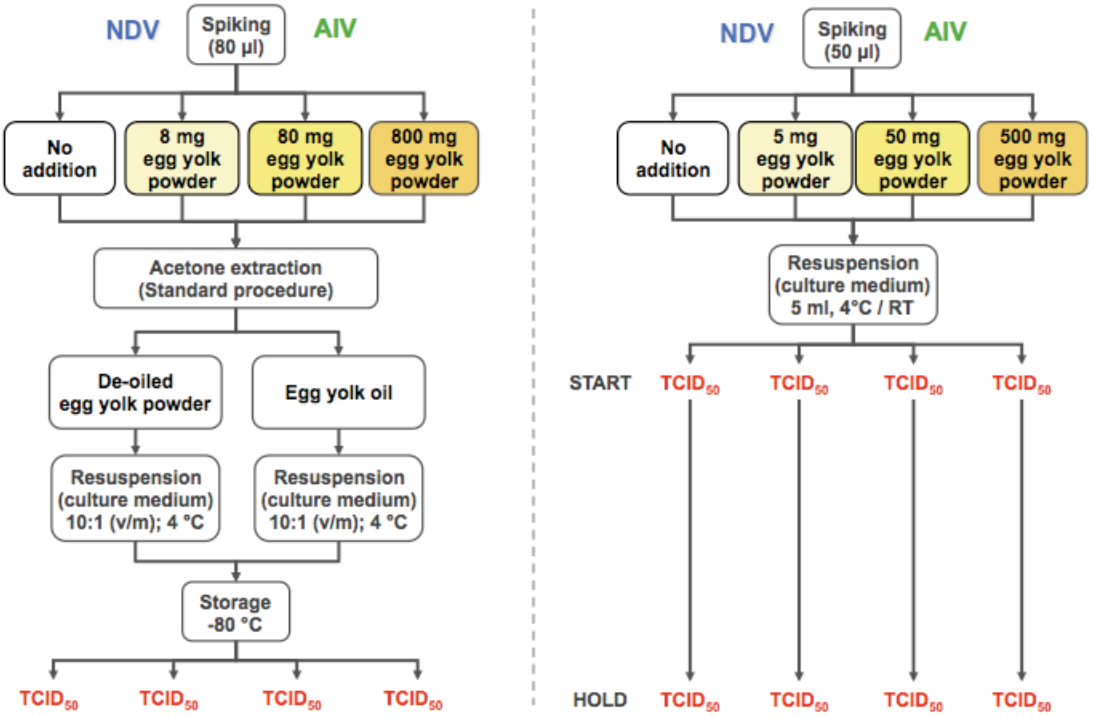


Figure 19: Flow chart of acetone extraction with different volumes of egg yolk powder (the right part of the diagram shows the chart for the control)

Procedure

According to the flow chart (Figure 19), two viruses and four different quantities of egg yolk powder were investigated. Duplicate runs were performed. The start sample, the hold sample and the test sample for acetone extraction of each of the different quantities of egg yolk powder were started at the same time. The acetone extraction was performed in centrifuge glass tubes containing the required amount of egg yolk powder (0 mg, 8 mg, 80 mg and 800 mg). Then 80 µl of virus were spiked to the prepared egg yolk powder. The test sample was stirred for 3 min using sterile silicone bars to entirely dissolve the virus droplet in egg yolk powder. After the test sample was incubated at room temperature for 12 min, four acetone extraction cycles were performed. The test sample was extracted with 8 ml of acetone, and the sterile magnetic stir bars were added to the tubes. The test sample was stirred for 8 min by magnetic mixer. Next, it was centrifuged for 5 min at 4000 U/min. Afterwards the acetone solution was removed and kept in a glass bottle with cover, 8 ml of fresh acetone were added to the tube for the second extraction and the test sample underwent the same procedure again. All acetone solutions derived from all of the acetone extractions were kept together in the covered glass bottle. The precipitate was vacuum-dried in the rotary evaporator at 40°C for 1 h. Afterwards the residue

was weighed and suspended in the ten-fold amount (weight/volume, w/v) of cell culture medium (EMEM). This mix was stirred using the rotary shaker for 2 h at 4°C. Then it was aliquoted and stored at -20°C. The combined acetone solution was placed into a rotary evaporator and the acetone evaporated. Later, the precipitate from the distillation was weighed and suspended in the ten-fold amount (w/v) of EMEM and stirred by using the rotary shaker for 2 h at 4°C. Then the suspension was aliquoted and stored at -20°C.

For the start and the hold samples the same amount of egg yolk powder was investigated. The same amount of virus as that in the acetone extraction experiment (50 µl) of test sample was used for spiking of egg yolk powder. The start and the hold samples were performed at the same time with the test sample. Duplicate runs of the start and the hold sample were performed at each quantity of egg yolk powder.

After these two samples were incubated at room temperature for 12 min, they were suspended in 5 ml of EMEM. The start sample was stirred by using the rotary shaker for 2 h at 4°C and was aliquoted and stored at -20°C. The hold sample was stirred by the shaker at room temperature until the end of the extraction procedure. Afterwards, it was aliquoted and all of the samples were stored at -80°C for end-point titration ($TCID_{50}$ assay).

3.2.7 Influence of water concentration on virus inactivation through acetone extraction

The two relevant viruses used in this experiment were NDV and AIV. During the acetone extraction with egg yolk powder, the two fractions derived from centrifugation were the residue (de-oiled egg yolk powder) and the extract (egg yolk oil). The de-oiled egg yolk powder contained the phospholipid, and the egg yolk oil contained triacylglycerol and cholesterol (Schneider, 1989). The phospholipid can also be extracted by a further ethanol extraction. The influence of water concentration on virus inactivation through acetone extraction was investigated using 800 mg of egg yolk powder.

Procedure

As can be seen in the flow chart below (Figure 20), the assessment of each of the two viruses was done at different times. Duplicate runs were performed, and the start sample, hold sample and test sample for acetone extraction were started at the same time. Centrifuge glass tubes containing 800 mg of egg yolk powder. The viruses were prepared by adding 720 µl of PBS to 80 µl of virus (10-fold dilution), and the 800-µl virus suspension was spiked to the prepared egg yolk powder. Afterwards the test sample was stirred for 3 min using sterile silicone bars to completely dissolve the virus droplet in egg yolk powder. Then it was incubated at room temperature for

12 min and when the incubation was done, four cycles of acetone extractions started as described above (section 3.2.6). The sample was extracted with 16 ml acetone and the sterile magnetic stir bar was added to the tubes. Later the test sample was stirred for 8 min by magnetic mixer and then was centrifuged for 5 min at 4000 U/min. The acetone solution was removed and kept in a covered glass bottle, then 8 ml of acetone were added again to the tubes for the second extraction, and the same procedure was repeated on the sample. All acetone solutions of acetone extraction from each cycle were kept together in the covered glass bottle. The residue was vacuum-dried in the rotary evaporator at 40°C for 1 h. Afterwards the residues were weighed and suspended in the ten-fold amount (w/v) of EMEM. The suspension was stirred for 2 h at 4°C using the rotary shaker. Then it was aliquoted and stored at -20°C. The combined acetone solution was distilled using a rotary evaporator, and the remainder from distillation was weighed and suspended in the ten-fold amount (w/v) of EMEM and stirred for 2 h at 4°C using the rotary shaker. After that, the suspension was aliquoted and stored at -20°C.

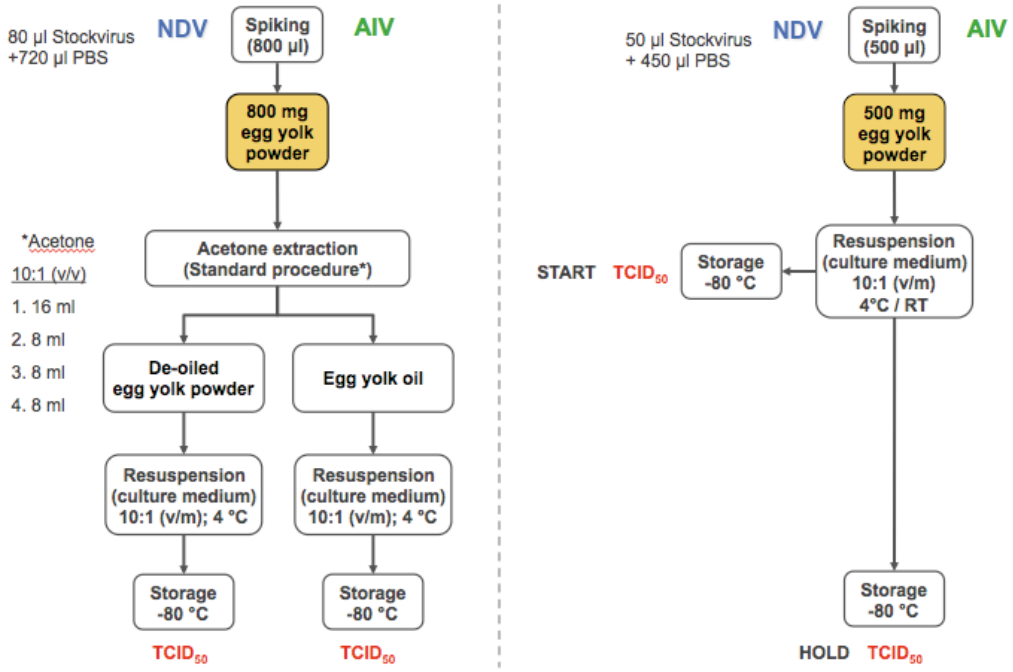


Figure 20: Flow charts of acetone extraction (the right part of the diagram shows the chart for the control)

For the start and hold samples, the concentration of egg yolk powder was downscaled to 500 mg. The same amount of virus as that in the test sample (500 µl) was used for spiking. Thus the spiked virus was prepared by adding 450 µl of PBS to 50 µl of virus. Duplicate runs were performed for the start and hold samples.

500 mg of egg yolk powder were added to the centrifuged tubes, and 500 µl of each virus were spiked to the prepared egg yolk powder. Afterwards the start and hold samples were stirred for 3 min using sterile silicone bars to completely dissolve the virus droplet in egg yolk powder. Then all samples were incubated at room temperature for 12 min. After incubation the samples were suspended with 5 ml of EMEM. The start sample was stirred by the rotary shaker for 2 h at 4°C. Then the sample was aliquoted and stored at -20°C. The hold sample was stirred by the shaker at room temperature until the end of the extraction procedure. Afterwards, they were aliquoted, and all the samples were stored at -80°C for end-point titration ($TCID_{50}$ assay).

3.2.8 Distribution of viral genome and infectivity in fractions after partitioning through filtration

In the manufacturing process of phospholipids, the filtration step is usually performed as one of the final steps during purification of the substances. Two fractions are obtained from the filtration step (filtrate and the residue on the filter). In this study, the viral infectivity and the viral genomes in the start samples and in the samples of two fractions obtained from filtration were tested and compared. AIV (H7N1) was used for spiking the samples. The titre of infectious AIV in the samples was determined by $TCID_{50}$ assay, and the viral genomes were quantified by qRT-PCR.

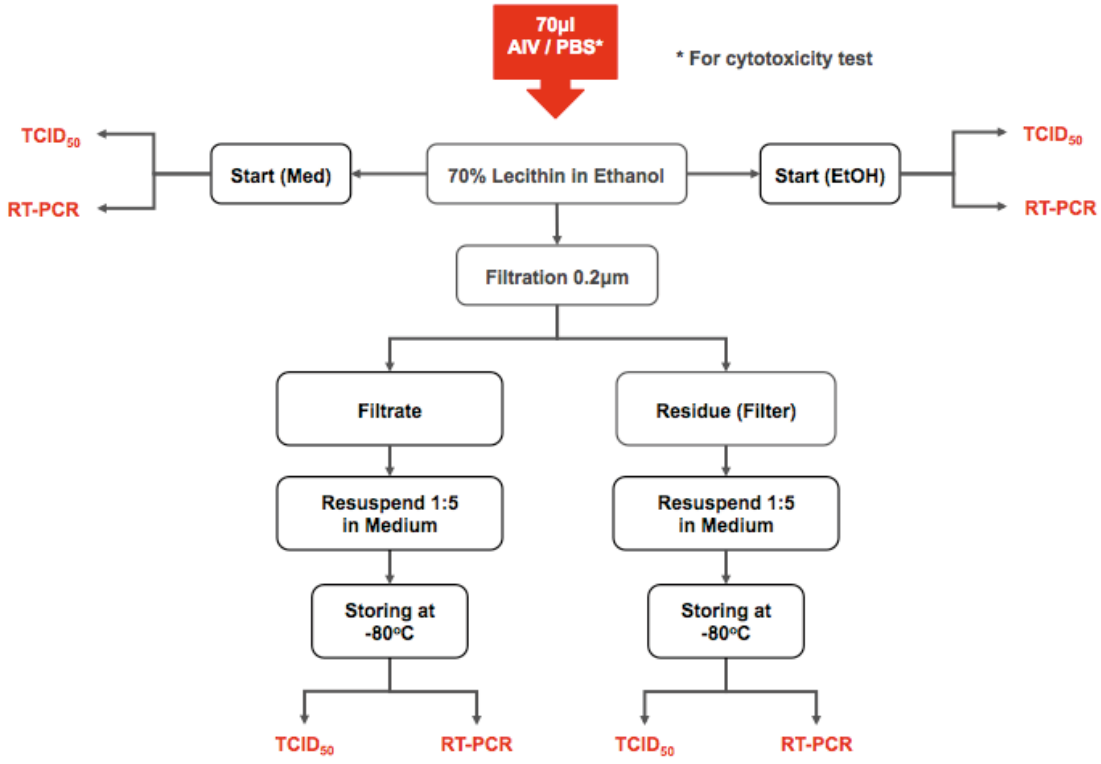


Figure 21: Flow chart of filtration experiment

Procedure

Three vials with a 70% ethanolic lecithin solution were prepared (Figure 21). AIV was thawed on ice, and the syringe inline filter holders containing a membrane with a pore size of 0.2 µm were prepared. Then the wet filters were weighed and the mean of the weight was calculated for further calculation of the residue after the filtration. After this, two types of the start samples (Medium [Med] and EtOH) were prepared. The start sample (Med) was prepared by spiking 70 µl of virus into 5 ml of EMEM. Then the virus suspension was mixed by vortexing, and the first one of the 70% ethanolic lecithin solutions (1 ml) was added and mixed again. After this, the start sample (Med) was aliquoted and stored at -20°C. As control 70 µl of virus were spiked into the second one of the 70% ethanolic lecithin solutions samples (1 ml) and mixed by vortexing. 5 ml of EMEM were added to the sample and mixed again. Afterwards samples were stored in aliquots at -20°C.

In the filtration experiment, the last one of the 70% ethanolic lecithin solutions (1 ml) was spiked with 70 µl of virus and then mixed by vortexing. Then the sample was filtrated using a syringe with an inline filter holder containing a membrane with a pore size of 0.2 µm. Then the filtrate was weighed and the weight of the precipitate on the filter calculated. The filters were then resuspended in the five-fold amount (w/v) of EMEM and aliquoted. The weight of residue (on the filter) was calculated from the weight of the filter after filtration, corrected by the weight of the wet filter prior to filtration. Afterwards all samples were stored at -80°C until required for end-point titration ($TCID_{50}$ assay) or qRT-PCR. Triplicate runs were performed in this experiment.

The cytotoxicity test was used to determine the cytotoxic effect of the samples on the cell culture. The $TCID_{50}$ assay was applied to observe the possibility of a cytotoxic effect (CPE) toward the specific cell line which could interfere with the determination of the virus titre. The same procedure as mentioned before was performed on all of the samples, but instead of virus PBS was spiked.

4. Results

4.1 Assessment of antibodies present in egg yolk starting material

The starting material (egg yolk powder) used in the viral inactivation experiments was investigated for the presence of AIV and NDV antibodies by haemagglutination inhibition (HI test) and Enzyme Linked Immunosorbent Assay (ELISA), respectively. The influence of egg yolk powder on the virus (AIV and NDV) titres was also determined by spiking the same volume of virus to the different concentrations of egg yolk powder.

4.1.1 Haemagglutination inhibition test (HI test)

The presence of antibodies to NDV in egg yolk powder was determined by HI tests. The results indicated that NDV antibodies were present in all three batches of egg yolk powder and that the HI titres were comparable in all batches. The HI titres of egg yolk powder diluted 1:4, 1:10 and 1:100 in PBS were 2^8, 2^6 and 2^3, respectively, as shown in Table 20. These results were not unexpected as egg-laying hens are usually vaccinated against NDV in production facilities.

Table 20: HI titre of three batches of egg yolk powder

Sample	Dilution	HI titre
Egg yolk powder batch 1	1:4	2^8
	1:10	2^6
	1:100	2^3
Egg yolk powder batch 2	1:4	2^8
	1:10	2^6
	1:100	2^3
Egg yolk powder batch 3	1:4	2^8
	1:10	2^6
	1:100	2^3

4.1.2 Enzyme linked immunosorbent assay (ELISA)

To determine whether antibodies against AIV were present in egg yolk used in the experiments, antibodies against influenza virus antigens were determined using the commercial IDEXX AI Ab Test kit. None of the egg yolk preparations were positive for AIV antibodies.

4.1.3 Influence of egg yolk concentration on the virus titre

Different concentrations of egg yolk powder were spiked with the same volume of virus to determine the effect of the egg yolk concentration on the virus titre. In Figure 22 the results of the influence of the egg yolk concentration on the virus titre of NDV and AIV are summarized.

The determination of antibodies against the relevant viruses NDV and AIV was important because it had to be excluded that antibodies against these two viruses interfere with the inactivation procedures investigated in this study.

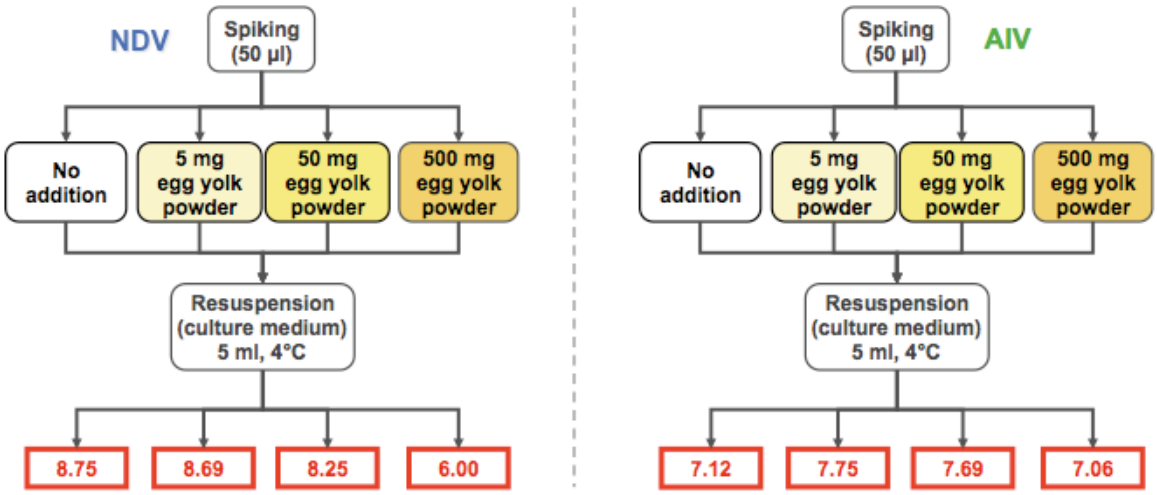


Figure 22: Influence of egg yolk concentration on NDV and AIV

The titres (log_{10} $TCID_{50}$) of the samples with 0 mg, 5 mg, 50 mg and 500 mg of egg yolk spiked with NDV were 8.8 (no egg yolk), 8.7 (5 mg), 8.3 (50 mg) and 6.00 (500 mg) log_{10} $TCID_{50}$/ml, respectively. No effect on the NDV titre was observed at lower egg yolk powder concentrations (5 mg and 50 mg), but a slight reduction of the virus titre was determined at the highest concentration of 500 mg. The reduction of the virus titre is most probably due to the presence of antibodies against NDV

surface antigens, as it has been shown that HI antibodies were detectable in egg yolk.

For AIV, the virus titres at different concentrations of egg yolk powder were nearly the same and did not decrease even in presence of the highest amount of egg yolk powder (500 mg). This confirmed the results of the antibody screening test which showed that the egg yolk powder contained no measurable amounts of AIV antibodies.

4.2 Heat inactivation kinetics of viruses in the presence of or without addition of liquid egg yolk

This experiment was divided into two parts. First, the heat inactivation experiment was done in PBS without adding egg yolk proteins with four virus species: NDV, AIV, FCV and PPV. The infectious virus titres were determined at different time points during the incubation at 40°C, 60°C and 80°C, respectively. In a second set of experiments heat inactivation was performed with three virus species, namely NDV, AIV and FCV, by spiking these viruses into liquid egg yolk instead of PBS. The heat inactivation of each of these viruses in the presence of liquid egg yolk was evaluated only at 60°C which represents the temperature used for pasteurization during the process of egg yolk production for consumption or as intermediate for the preparation of pharmaceuticals.

4.2.1 Inactivation of viruses at different temperatures

4.2.1.1 Heat inactivation of viruses at 40°C

Table 21 summarises the results of the inactivation kinetics performed at 40°C. For NDV, the mean starting virus titre of 7.50 $\log_{10}$ $TCID_{50}$/ml was reduced to 7.12, 6.87, 6.87 and 3.25 $\log_{10}$ $TCID_{50}$/ml after 0.33, 1, 6 and 24 h of heat treatment, respectively. After 48 h the titre was below the level of detection. Nonetheless, the virus titre calculated for the worst case (based on the calculation of the lower level of virus detection) was ≤0.5 $\log_{10}$ $TCID_{50}$/ml.

Table 21: Virus titres of four different viruses after heat treatment at 40°C

Treatment (h)	Virus titres ($log_{10}TCID_{50}$/ml)							
at 40°C	NDV	(RF)	AIV	(RF)	FCV	(RF)	PPV	(RF)
0	7.50		6.62		5.75		7.07	
0.33	7.12	0.38	6.38	0.24	3.87	1.88	NT*	
1	6.87	0.63	6.13	0.49	3.62	2.13	NT	
6	6.87	0.63	4.62	2.00	≤0.50	≥5.25	6.64	0.43
24	3.25	4.25	≤0.50	≥6.12	≤0.50	≥5.25	5.50	1.57
48	≤0.50	≥7.00	≤0.50	≥6.12	≤0.50	≥5.25	NT	
72	NT		NT		NT		2.00	5.07

NT = not tested; (RF) = reduction factor log_{10}

The mean starting virus titre of AIV was 6.62 log_{10} $TCID_{50}$/ml. After 0.33, 1 and 6 h of heat treatment the titre was reduced to 6.38, 6.13 and 4.62 log_{10} $TCID_{50}$/ml, respectively. It was below the level of detection (≤0.5 log_{10} $TCID_{50}$/ml) after 24 h of heat treatment.

The heat inactivation of FCV with the mean starting virus titre of 5.75 log_{10} $TCID_{50}$/ml was reduced to 3.87 and 3.62 log_{10} $TCID_{50}$/ml after 0.33 and 1 h of heat treatment, respectively. The virus titre was below the level of detection (≤0.5 log_{10} $TCID_{50}$/ml) after 6 h of heat treatment.

For PPV, the mean starting virus titre of 7.07 log_{10} $TCID_{50}$/ml was reduced to 6.64, 5.50 and 2.00 log_{10} $TCID_{50}$/ml after 6, 24 and 72 h of heat treatment, respectively.

In many publications in which the inactivation kinetics of viruses in chicken products like meat or egg white were determined the authors calculated the D-values to estimate the survival time of the agents and to validate the efficiency of the inactivation procedure. In Figure 23 the linear regressions of the inactivation kinetics for all of the viruses used in this experiment were calculated. The slope of the linear regression was used to compare the heat sensitivity between the virus species. A higher slope value indicated a more rapid inactivation of virus. At 40°C, linear regression of FCV has the highest slope value, following by AIV, NDV and PPV. These findings thus illustrate that FCV was the virus most sensitive to heat inactivation, followed by AIV and NDV, and PPV was the virus most resistant to heat inactivation.

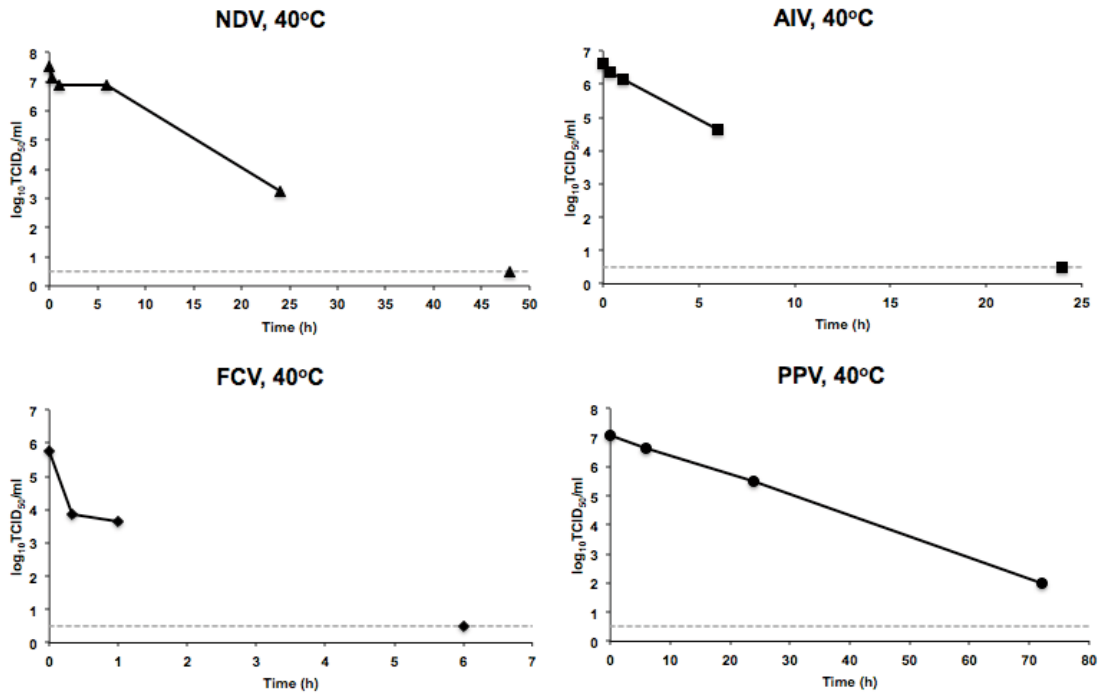


Figure 23: Kinetics of inactivation of NDV, AIV, FCV and PPV during incubation at 40°C. The dotted line shows the detection limit of the assay

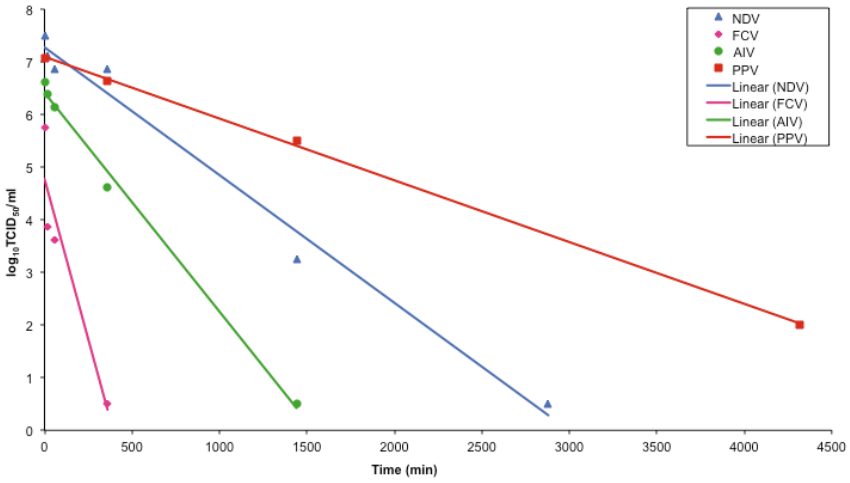


Figure 24: Comparison of linear regressions between four different viruses at 40°C

4.2.1.2 Heat inactivation of viruses at 60°C

Investigation of the inactivation of viruses at 60°C was of special interest, as in many processes pasteurization of food products or pharmaceuticals is performed at this temperature or at slightly higher ones. The results are summarized in Table 22.

Table 22: Virus titres of three different viruses after heat treatment at 60°C

Time points	Virus titres (mean log_{10} $TCID_{50}$/ml) and reduction factor (RF) in log_{10}					
at 60°C in min	NDV	(RF)	AIV	(RF)	FCV	(RF)
Control (0)	7.50		6.62		5.75	
1	2.25	5.25	≤0.50	≥6.12	3.50	2.25
5	0.75	6.75	≤0.50	≥6.12	≤0.50	≥5.25
20	≤0.50	≥7.00	≤0.50	≥6.12	≤0.50	≥5.25
60	≤0.50	≥7.00	≤0.50	≥6.12	≤0.50	≥5.25

Table 23: Virus titres of PPV after heat treatment at 60°C

Time points at 60°C in h	Virus titres (mean log_{10} $TCID_{50}$/ml)	Reduction factor (RF) in log_{10}
Control (0)	7.07	
6	≤0.50	≥6.57
24	≤0.50	≥6.57
72	≤0.50	≥6.57

For NDV, the mean starting virus titre of 7.50 log_{10} $TCID_{50}$/ml was reduced to 2.25 and 0.75 log_{10} $TCID_{50}$/ml after 1 and 5 min of heat treatment at 60°C, respectively. At 20 min, no infectious virus could be detected; nevertheless, the virus titre calculated for the worst case was ≤0.50 log_{10} $TCID_{50}$/ml.

AIV was rapidly inactivated from the mean starting virus titre of 6.62 log_{10} $TCID_{50}$/ml to ≤0.5 log_{10} $TCID_{50}$/ml (infectious virus below the level of detection) within 1 min.

FCV was inactivated from the mean starting virus titre of 5.75 log_{10} $TCID_{50}$/ml to 3.5 and ≤0.5 log_{10} $TCID_{50}$/ml (infectious virus below the level of detection) after 1 and 5 min of heat treatment, respectively.

The mean starting virus titre of PPV was 7.07 log_{10} $TCID_{50}$/ml, and after 6 h of heat treatment at 60°C no virus was detected any longer (≤0.5 log_{10} $TCID_{50}$/ml).

In two cases (AIV and NDV) the virus titres were reduced within 1 min of heat treatment by a factor of ≥5 log_{10}, nevertheless FCV was only reduced by a factor of 2.25 log_{10}.

4.2.1.3 Heat inactivation of viruses at 80°C

The results of heat inactivation of four different viruses at 80°C are shown in Tables 24 and 25. The mean starting virus titres of NDV, AIV and FCV were 7.50, 6.62 and 5.75 $\log_{10}$ $TCID_{50}$/ml, respectively. The mean starting virus titres of all viruses were rapidly reduced to ≤0.5 $\log_{10}$ $TCID_{50}$/ml (infectious virus below the level of detection) within 1 min of heat treatment.

For PPV, the mean starting virus titre was 7.07 $\log_{10}$ $TCID_{50}$/ml and after 6 h of heat treatment, no infectious virus was detectable any more (≤0.5 $\log_{10}$ $TCID_{50}$/ml). These experiments showed that the virus titres were reduced by heat treatment at 80°C by a factor of ≥5 $\log_{10}$.

Table 24: Virus titres of three different viruses after heat treatment at 80°C

Heat time points (min)	Virus titres (mean $\log_{10}$ $TCID_{50}$/ml)		
at 80°C	NDV	AIV	FCV
Control (0)	7.50	6.62	5.75
1	≤0.50	≤0.50	≤0.50
5	≤0.50	≤0.50	≤0.50
20	≤0.50	≤0.50	≤0.50
60	≤0.50	≤0.50	≤0.50

Table 25: Virus titres of PPV after heat treatment at 80°C

Time points at 80°C in h	Virus titres (mean $\log_{10}$ $TCID_{50}$/ml)
Control (0)	7.07
6	≤0.50
24	≤0.50
72	≤0.50

4.2.2 Heat inactivation kinetics of different virus species in the presence of liquid egg yolk

Heat inactivation kinetics in the presence of liquid egg yolk was performed with NDV, AIV and FCV at 60°C. Four time points at 1, 5, 20 and 60 min were evaluated (Table 26).

Table 26: Virus titres of three different viruses in the presence of liquid egg yolk after heat treatment at 60°C

Heat time points (min) at 60°C	Virus titres (mean log_{10} $TCID_{50}$/ml) and reduction factor (RF) in log_{10}					
	NDV	(RF)	AIV	(RF)	FCV	(RF)
Control (0)	7.62		6.87		5.87	
1	4.75	2.87	≤0.50	≥6.37	3.25	2.62
5	4.38	3.24	≤0.50	≥6.37	1.50	4.37
20	≤0.50	≥7.12	≤0.50	≥6.37	≤0.50	≥5.37
60	≤0.50	≥7.12	≤0.50	≥6.37	≤0.50	≥5.37

Heat inactivation in the presence of liquid egg yolk spiked with NDV showed that the virus titres were reduced by a factor of approximately 2.9 log_{10} after 1 min of treatment, and by a factor of approximately 3.2 log_{10} after 5 min. No infectious titres could be detected after a treatment of 20 min. This finding indicated that in the presence of liquid egg yolk NDV showed a higher heat resistance at 60°C than NDV without liquid egg yolk since higher reduction factors of more than 5 log_{10} were determined without the addition of egg yolk (compare Table 22).

The mean starting titres of AIV in the presence of liquid egg yolk and of AIV without liquid egg yolk were 6.9 and 6.6 log_{10} $TCID_{50}$/ml, respectively. Within 1 min of heat treatment at 60°C, the AIV titres in the presence of liquid egg yolk and without liquid egg yolk were rapidly reduced below the level of detection.

The mean starting titre of FCV in the presence of liquid egg yolk of 5.90 log_{10} $TCID_{50}$/ml was reduced to 3.25 and 1.50 log_{10} $TCID_{50}$/ml after 1 and 5 min of heat treatment, respectively. This finding showed that FCV in the presence of liquid egg yolk showed a higher heat resistance than FCV without liquid egg yolk and that the liquid egg yolk had a protective effect on FCV (compare Table 22).

4.3 Heat inactivation kinetics of AIV and different strains of SIV

The selection of virus strains used in inactivation studies was of special interest since it was shown in some investigations that differences could be observed in the susceptibility of viruses belonging to the same virus species (Pütz, 1998). In the context of utilizing egg yolk for the production of lipids for human and animal use, studies on the stability of influenza viruses are of particular interest. Accordingly, we utilized four different influenza virus strains in a comparative heat inactivation experiment: one avian strain (H7N1) and three porcine influenza virus strains (SIV-H1N1, -H1N2 and -H3N2). For all of the viruses, five time points were investigated at an incubation temperature of 40°C (3, 6, 12, 24 and 48 h) (Table 27).

Table 27: Virus titres of AIV and different strains of SIV after heat treatment at 40°C

Heat time points (h) at 40°C	Virus titres (mean log_{10} $TCID_{50}$/ml) and reduction factor (RF) in log_{10}							
	H7N1		H3N2		H1N1		H1N2	
	titre	RF	titre	RF	titre	RF	titre	RF
Start	6.46		5.71		5.33		4.87	
3 h	5.04	1.42	3.63	2.08	3.33	2.00	4.63	0.24
6 h	2.38	4.08	1.92	3.79	1.92	3.41	2.58	2.29
12 h	≤0.50	≥5.96	≤0.50	≥5.21	≤0.50	≥4.83	≤0.50	≥4.37
24 h	≤0.50		≤0.50		≤0.50		≤0.50	
48 h	≤0.50		≤0.50		≤0.50		≤0.50	
Hold	6.17		5.08		4.92		4.58	

For AIV (H7N1), the mean starting virus titre of 6.46 log_{10} $TCID_{50}$/ml was reduced to 5.04, 2.38 and ≤0.5 log_{10} $TCID_{50}$/ml after 1, 3, 6 and 12 h of heat treatment, respectively. At 12 h, no infectious virus was detectable. SIV (H3N2) was inactivated from the mean starting virus titre of 5.71 log_{10} $TCID_{50}$/ml to 3.63, 1.92 and ≤0.5 log_{10} $TCID_{50}$/ml (undetectable infectious virus) at 1, 3, 6 and 12 h of heat treatment, respectively. SIV (H1N1) was inactivated from the mean starting virus titre of 5.33 log_{10} $TCID_{50}$/ml to 3.33, 1.92 and ≤0.5 log_{10} $TCID_{50}$/ml (undetectable infectious virus) at 1, 3, 6 and 12 h of heat treatment, respectively. For SIV (H1N2), the mean starting virus titre of 4.87 log_{10} $TCID_{50}$/ml was reduced to 4.63, 2.58 and ≤0.5 (undetectable infectious virus) log_{10} $TCID_{50}$/ml after 1, 3, 6 and 12 h of heat treatment, respectively.

As shown in Table 27, the calculation of the reduction factors implies that the swine influenza strain H1N2 is more heat resistant at 40°C than the other strains investigated. Additional experiments at different temperatures will be necessary to verify this finding.

4.4 Inactivation of viruses through acetone extraction

Lecithin is used as an emulsifier in food and some pharmaceuticals. It is produced commercially from a variety of sources like plant seeds or from egg yolk. Using various fractionation methods, the different components can be selectively separated using acetone and ethanol. One of the first steps in the purification process is the de-oiling of the source material by extraction with concentrated acetone, which results in a crude lecithin preparation with a low content of oil and fatty acids (Szuhaj, 2005). To evaluate the effect of acetone on the infectivity of viruses, egg yolk powder spiked with viruses was extracted with acetone, and the precipitate as well as the

supernatant were investigated for infectious virus.

The two relevant viruses NDV and AIV were investigated. After acetone extraction of the egg yolk powder, two fractions were obtained after centrifugation. The precipitate contained the de-oiled egg yolk powder and the supernatant contained the egg yolk oil. All of the samples from the acetone extraction as well as the start and hold samples of each virus were investigated by $TCID_{50}$ assay. The detection limit of the $TCID_{50}$ assay applying the Spearman and Kärber method was ≤0.5 $\log_{10}$ $TCID_{50}$/ml.

4.4.1 Influence of egg yolk/virus ratio on virus stability during acetone extraction

The experiments investigated the influence of egg yolk quantity in relation to the volume of the virus inoculum on the stability of the viruses during acetone extraction. Samples were extracted with acetone as described in Materials and methods. The two fractions containing the de-oiled yolk powder and the acetone-soluble fraction were investigated for infectious virus. Results are shown in Figures 25 and 26.

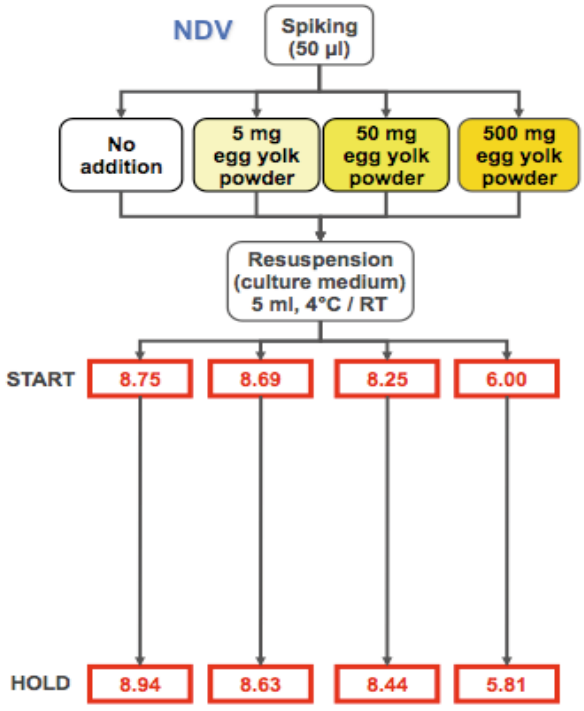


Figure 25: NDV titres of start and hold samples presented in $\log_{10}$ $TCID_{50}$/ml

In Figure 25 the results of the control experiments with NDV are summarized. The mean starting virus titres of 0, 5, 50 and 500 mg of egg yolk powder spiked with NDV were 8.75, 8.69, 8.25 and 6.00 $\log_{10}$ $TCID_{50}$/ml, respectively, and the mean holding

virus titres of 0, 5, 50 and 500 mg egg yolk powder spiked with NDV showed comparable titres of 8.94, 8.63, 8.44 and 5.81 $\log_{10}$ $TCID_{50}$/ml, respectively. Thus, the mean starting virus titres and the mean holding virus titres at same concentration of egg yolk powder were comparable which indicated that the time spent in the acetone extraction process has no or little effect on the infectivity of NDV. However, the highest quantity of egg yolk powder investigated showed a reduction of the input virus titre compared to the lower quantities of egg yolk powder. This was expected because of the presence of HI antibodies in these preparations.

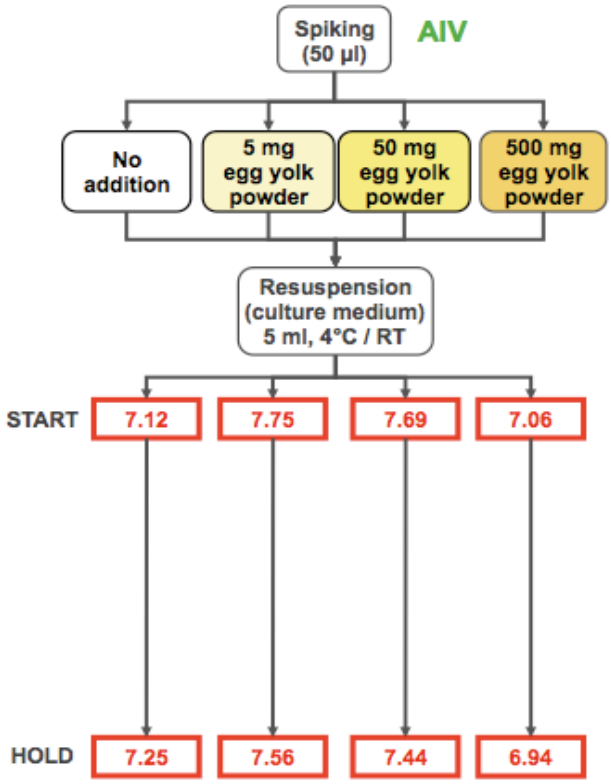


Figure 26: AIV titres of start and hold samples presented in $\log_{10}$ $TCID_{50}$/ml

Comparable results were obtained when AIV was spiked to different quantities of egg yolk powder. The mean starting virus titres of 0, 5, 50 and 500 mg of egg yolk powder spiked with AIV were 7.12, 7.75, 7.69 and 7.06 $\log_{10}$ $TCID_{50}$/ml, respectively, and the mean holding virus titres of 0, 5, 50 and 500 mg of egg yolk powder spiked with AIV were 7.25, 7.56, 7.44 and 6.94 $\log_{10}$ $TCID_{50}$/ml, respectively, as shown in Figure 26. Thus, the mean starting virus titres and the mean holding virus titres using the same concentration of egg yolk powder were nearly the same. These findings indicated that the time used in acetone extraction process did not affect the AIV titre. Furthermore, no significant reduction of virus titres was observed in any of the virus/egg yolk mixtures. This was expected since no antibodies against AIV were detectable in egg yolk powder.

In a further set of experiments the influence of the virus/egg yolk ratio on the viruses during the acetone extraction was evaluated. Figures 27 and 28 summarize the results.

The virus titres at different concentrations (0, 8, 80 and 800 mg) of egg yolk powder spiked with NDV during acetone extraction are shown in Figure 27. The mean virus titres of de-oiled egg yolk powder from 0, 8, 80 and 800 mg of the starting material spiked with NDV were ≤0.50 (infectious virus below the level of detection), 1.00, 1.50 and 2.00 $\log_{10}$ $TCID_{50}$/ml, respectively, and the mean virus titres of egg yolk oil from 0, 8, 80 and 800 mg of the starting material spiked with NDV were ≤0.50 (infectious virus below the level of detection), 1.50, 1.00 and 2.00 $\log_{10}$ $TCID_{50}$/ml, respectively. These results indicated that the concentration of the starting material was related to the virus titre of the residue. The higher virus titre in the sample with the lowest virus/egg yolk concentration (80 µl/800 mg) demonstrated that the water content in the preparation influenced the inactivation of the virus. Moreover, using acetone extraction, the mean starting virus titres of all concentrations of egg yolk powder were reduced by ≥ 4 $\log_{10}$ for the residue and the extract.

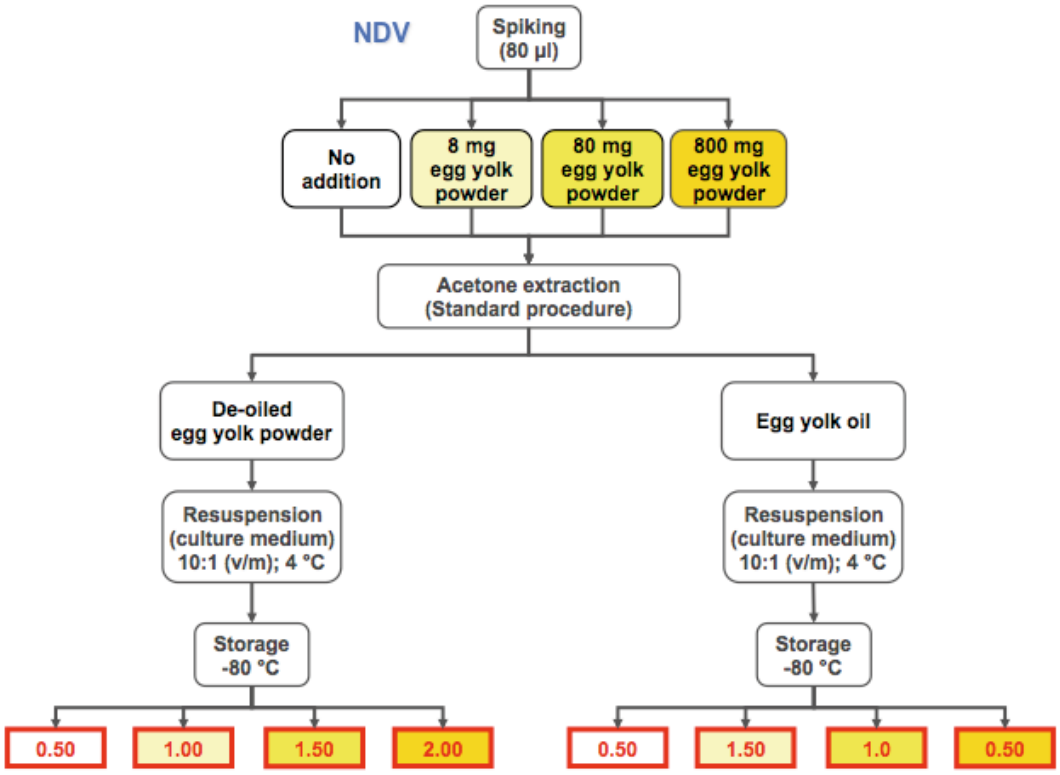


Figure 27: The virus titres at different concentrations (0, 8, 80 and 800 mg) of egg yolk power spiked with NDV during acetone extraction presented in $\log_{10}$ $TCID_{50}$/ml

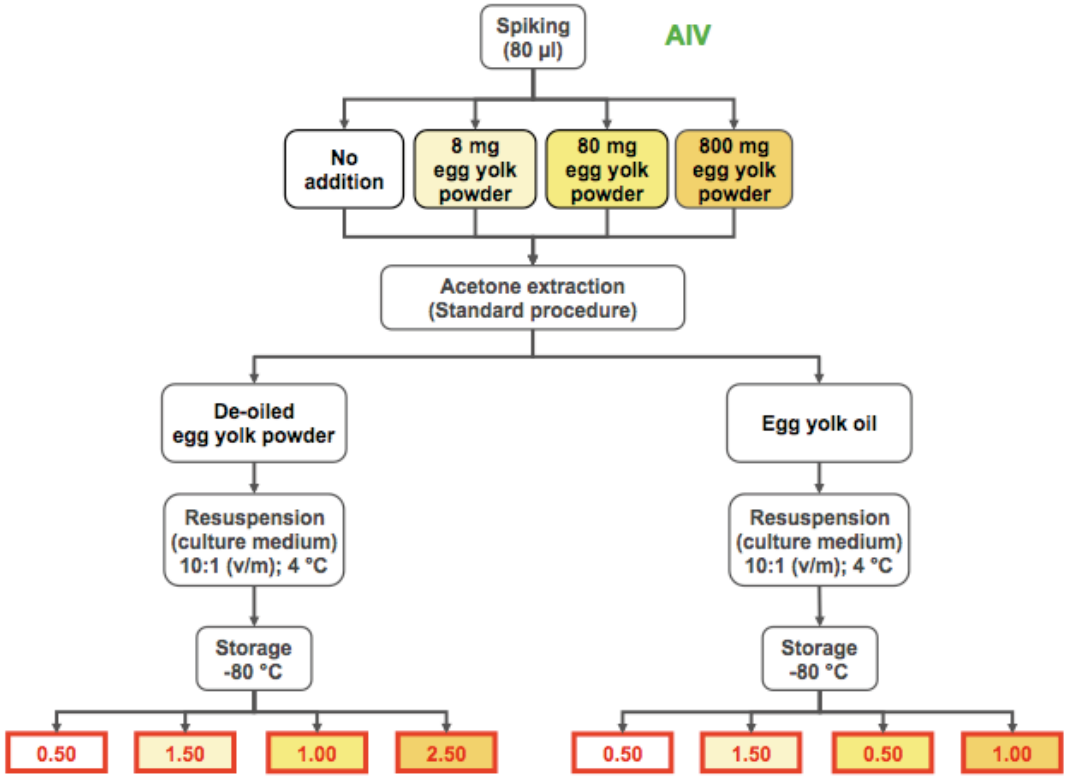


Figure 28: The virus titres at different concentrations (0, 8, 80 and 800 mg) of egg yolk powder spiked with AIV during acetone extraction presented in $\log_{10}$ $TCID_{50}$/ml

The virus titres at different concentrations (0, 8, 80 and 800 mg) of egg yolk powder spiked with AIV during acetone extraction are shown in Figure 28. The mean virus titres of de-oiled egg yolk powder from 0, 8, 80 and 800 mg of the starting material spiked with AIV were ≤0.50 (infectious virus below the level of detection), 1.50, 1.00 and 2.50 $\log_{10}$ $TCID_{50}$, respectively, and the mean virus titres of egg yolk oil from 0, 8, 80 and 800 mg of the starting material spiked with AIV were ≤0.50 (infectious virus below the level of detection), 1.50, ≤0.50 and 1.00 $\log_{10}$ $TCID_{50}$, respectively. These results indicated that the concentration of the starting material was related to the virus titre of the residue. These results are comparable to the infectious titres determined for NDV, showing that the ratio of the volume of the virus inoculum in comparison to the quantity of egg yolk powder has an influence on the acetone sensitivity of the virus.

4.4.2 Influence of water concentration on virus stability during acetone extraction

The egg yolk powder was spiked with the virus at a ratio of 1:1; afterwards the acetone extraction was performed. The results from this experiment were compared

with those from the investigation into the influence of egg yolk concentration (800 mg of egg yolk powder) on virus stability during acetone extraction (see 4.4.1). The results for NDV and AIV are summarized in Figures 29 and 30.

For NDV, the mean starting and holding virus titres were 6.75 and 6.13 $\log_{10}$ $TCID_{50}$/ml, respectively. After acetone extraction, the mean starting titre of 6.75 $\log_{10}$ $TCID_{50}$/ml was reduced to 1.0 $\log_{10}$ $TCID_{50}$/ml for de-oiled egg yolk powder and to ≤0.5 $\log_{10}$ $TCID_{50}$/ml (infectious virus below the level of detection) for egg yolk oil. Compared with the previous experiment (2.0 $\log_{10}$ $TCID_{50}$/ml), the virus titre of de-oiled egg yolk powder was lower in this experiment (1.0 $\log_{10}$ $TCID_{50}$/ml). This result demonstrated that the water concentration influenced NDV stability during acetone extraction, namely the higher the water concentration, the stronger the virus inactivation.

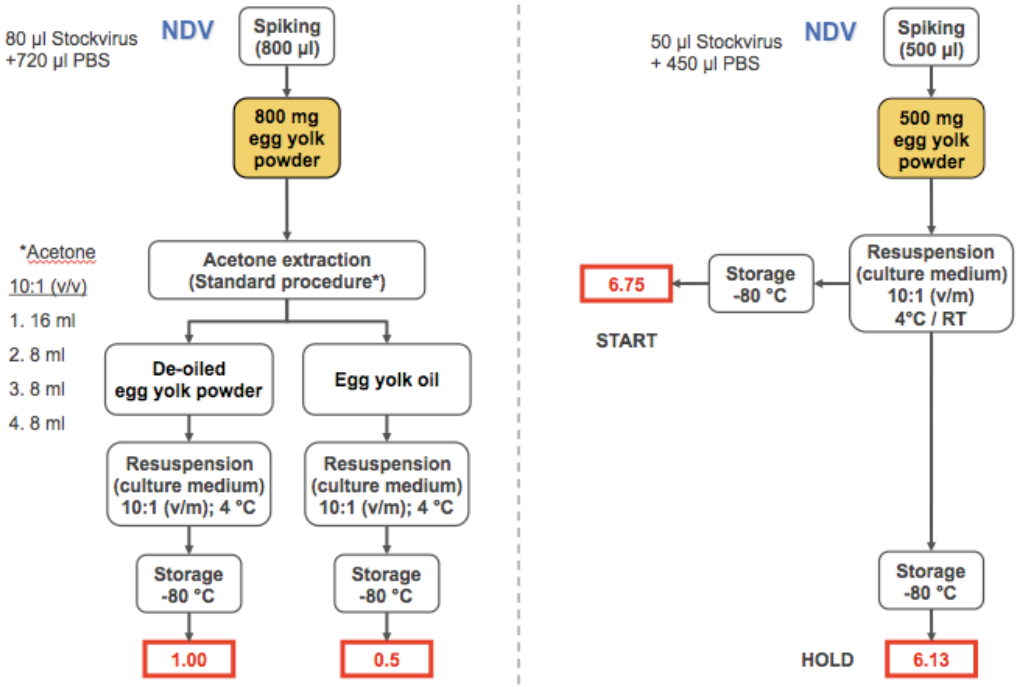


Figure 29: Results of NDV titres during acetone extraction presented in $\log_{10}$ $TCID_{50}$/ml (the right part of the diagram shows the chart for the control)

For AIV, the mean starting and holding virus titres were 7.44 and 7.69 $\log_{10}$ $TCID_{50}$/ml, respectively. After acetone extraction, the mean starting titre of 7.44 $\log_{10}$ $TCID_{50}$/ml was reduced to 1.0 $\log_{10}$ $TCID_{50}$/ml for de-oiled egg yolk powder and to ≤0.5 $\log_{10}$ $TCID_{50}$/ml (infectious virus below the level of detection) for egg yolk oil. Compared with the previous experiment (2.5 $\log_{10}$ $TCID_{50}$/ml), the virus titre of de-oiled egg yolk powder was lower in this experiment (1.5 $\log_{10}$ $TCID_{50}$/ml). The results depicted in Figure 30 demonstrate that the water concentration influenced AIV stability during acetone extraction, namely the higher the water content, the stronger the virus inactivation.

The experiments described above were performed in small volumes. To obtain further information about the role of the water content on the sensitivity of enveloped viruses against acetone, the experiments were repeated with higher quantities of egg yolk powder, keeping an equivalent ratio of 1/10 (v/w). Three samples of 3 g of egg yolk powder were spiked with 300 µl of the stock virus preparations (NDV, AIV). Each sample was stirred for 2 min with a sterile silicone spatula to disperse the virus solution completely in the powder. After incubation for 15 min one sample was used as control to determine the start titre. 30 ml of cell culture medium were added to the egg yolk powder/virus mixture. The suspension was stirred for 2 h at 4°C on a rotary shaker and aliquots were stored at -80°C.

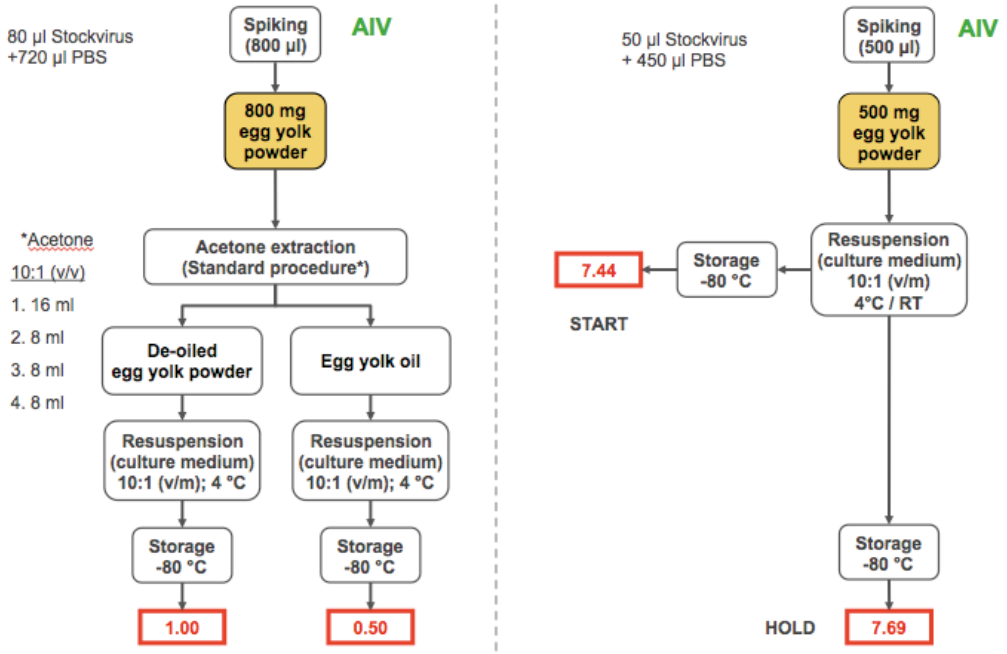


Figure 30: Results of AIV titres during acetone extraction presented in log_{10} $TCID_{50}$/ml (the right part of the diagram shows the chart for the control)

The second sample was extracted using acetone. The resulting intermediates were dried, and the de-oiled yolk powder as well as the dry residue of the acetone extract were weighted, dissolved in the tenfold amount of cell culture medium (w/v) and stirred for 2 h at 4°C on a rotary shaker. Aliquots were stored at -80°C until use.

A second control comparable to the start sample was incubated in parallel to the acetone extraction. Two independent experiments were performed, the titres determined and the reduction factors calculated (Table 28).

Table 28: Extraction of egg yolk powder with acetone

Virus	Sample	$TCID_{50}$ (log_{10})	Reduction factor (log_{10})
Newcastle Disease Virus (NDV)	start sample	7.8	
	hold sample	7.9	
	de-oiled yolk powder	4.6	3.3
	acetone extract	3.1	4.8
Avian Influenza Virus (AIV)	start sample	8.2	
	hold sample	8.1	
	de-oiled yolk powder	6.5	1.7
	acetone extract	2.4	5.8

It is noteworthy that acetone treatment of virus/egg yolk powder mixtures with a low water content resulted in a low reduction factor in the protein fraction of de-oiled egg powder as well as in the acetone extract.

4.5 Distribution of viral genome and infectivity in fractions after partitioning through filtration

The virus used in this experiment was AIV (H7N1). After filtration, the two fractions obtained were the filtrate and the filter (residue). The infectious AIV titres in the samples were determined by $TCID_{50}$ assay and the viral genome was quantified by RT-PCR. The detection limit of $TCID_{50}$ assay applying the Spearman and Kärber method was ≥0.5 log_{10} $TCID_{50}$/ml.

The standard curve was generated by plotting the threshold cycle values (Ct value) against the log concentration of copy numbers as shown in Figure 31. The regression coefficient (R^2) was 0.99. The copy numbers in the samples were calculated by the standard curve method.

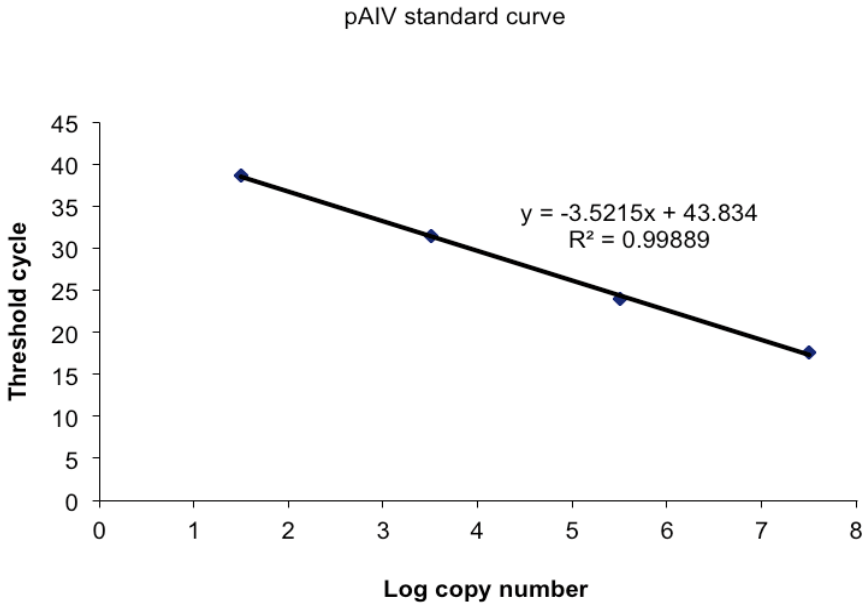


Figure 31: Standard curve of AIV

Table 29: Comparison of the results from the end-point titration and qRT-PCR

Samples	End-point titration		qRT-PCR	
	$TCID_{50}$/ml (mean)	Log_{10} $TCID_{50}$/ml (mean)	Copy number	Log copy number
AIV stock	2.31E+07	7.36	1.20E+07	7.08
Start (Med)	1.00E+06	6.00	5.61E+05	5.75
Start (EtOH)	3.16E+01	1.50	6.69E+05	5.83
Filtrate	3.16E+01	1.50	6.44E+05	5.81
Filter (residue)	3.16E+01	1.50	2.64E+05	5.42
Cytotoxicity test				
Start (Med)	3.16E+01	1.50	NT*	NT
Start (EtOH)	3.16E+01	1.50	NT	NT
Filtrate	3.16E+01	1.50	NT	NT
Filter	3.16E+01	1.50	NT	NT

*NT = not tested

The results from the $TCID_{50}$ assay indicated that the filtrate and the filter (residue) from the cytotoxicity test could affect the virus titre by 3.16E+01 $TCID_{50}$/ml or 1.5 log_{10} $TCID_{50}$/ml.

The mean virus titres of the filtrate and the filter (residue) from the samples tested were 3.16E+01 $TCID_{50}$/ml or 1.5 log_{10} $TCID_{50}$/ml, therefore these findings could be due to the cytotoxic effect on the cell culture. The titre of the AIV stock and mean starting (Med) virus titre were 2.31E+07 and 1.00E+06 $TCID_{50}$/ml, respectively, and these corresponded to 1.20E+07 and 5.61E+05 viral particles, respectively. The start sample (EtOH/egg yolk powder solution) contained 6.69E+05 viral particles while the mean virus titre determined using the $TCID_{50}$ assay was only 3.16E+01 $TCID_{50}$/ml. Inoculation of a virus suspension into a 70% ethanolic lecithin solution led to an immediate loss of infectivity while the number of genome equivalents was only partially affected. It can be assumed that the envelope of the virus particles is solubilized by the 70% ethanolic solution, releasing the nucleoprotein/RNA complex into the solution. The filtrate and the filter (residue) had 6.44E+05 and 2.64E+05 viral particles, respectively. These results showed that during filtration the viral particles or most probably the nucleoprotein/RNA complex passed through the filter and only part of the complex was trapped on the filter.

5. Discussion

Transmission of infectious diseases by food and pharmaceuticals has initiated investigations to evaluate production procedures for their capacity to inactivate the agents or to include procedures into the manufacturing process which inactivate pathogens without influencing the nutritive value or the potency of the pharmaceutical. National and international authorities as well as national and international societies have developed standards and guidelines which are thought to guarantee the quality and safety of the products. The transmission of *Salmonella* by egg products initiated the development of safety measures which on the one hand deal with the production and handling of the egg as a source of egg products and on the other hand introduce inactivation procedures. Examples are the Codes of Practice published by the Australian Egg Corporation (http://www.aecl.org/system/attachments/210/original/Egg_Products_Code_of_Practice_2008.pdf) or the European Guide to good manufacturing practice for "liquid, concentrated, frozen and dried egg products" used as food ingredients (http://ec.europa.eu/food/food/biosafety/hygienelegislation/docs/guide_good_manufacturing_practice_en.pdf). Furthermore, a manual for the pasteurization of egg products was published (Froning et al., 2002). However, this manual deals only with the inactivation of *Salmonella* in egg products and not with the inactivation of viruses. An update of the recommendations considering the contamination of eggs with highly pathogenic influenza virus was published in 2009 (Thomas & Swayne, 2009b).

Furthermore, in their publications concerning avian influenza virus in egg products, the OIE recommended incubation temperatures and incubation times for the inactivation of HPAIV (OIE, 2011) which are suitable for the inactivation of influenza viruses in egg products (Table 30).

Table 30: Incubation temperatures and incubation times recommended for egg products regarding inactivation of HPAIV

	Core temperature (°C)	Time
Whole egg	60	188 sec
Whole egg blends	60	188 sec
Whole egg blends	61.1	94 sec
Liquid egg white	55.6	870 sec
Liquid egg white	56.7	232 sec
10% salted yolk	62.2	138 sec
Dried egg white	67	20 h
Dried egg white	54.4	513 h

From: http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_1.10.4.htm; Article 10.4.25

In recent years many new and different types of egg products have been developed which encouraged governmental authorities and the industry to evaluate the effectiveness of the pasteurization processes as well as purification procedures for the isolation of specific components. Two major steps in the production processes of egg yolk components were experimentally evaluated in this investigation: heat inactivation and the de-oiling process of egg yolk with acetone.

For these investigations the two relevant viruses avian influenza virus and Newcastle disease virus were chosen, because it is known that these viruses are able to infect humans and that both can be vertically transmitted. As both viruses are enveloped, two additional non-enveloped viruses were investigated which are often used as models for highly inactivation-resistant viruses.

5.1 Heat inactivation kinetics

The biological safety of food and pharmaceutical products is of major public interest for consumers. There are several studies concerning the heat stability of AIV and NDV in egg products (Swayne & Beck, 2004; Thomas & Swayne, 2009a; Chmielewski et al., 2011).

Many investigations revealed that heat treatment generally appears to follow biphasic inactivation kinetics, with a more rapid decline at the beginning of the heat treatment, followed by a slower decline in the second phase of the treatment (Thomas and Swayne, 2009a; Burge et al., 1983).

To calculate the time needed to inactivate pathogens by heat treatment below a recommended level, authors sometimes prefer to use D_{10}-values. In principle, the estimation of D_{10} values is used for the calculation of the dose (radiation) required to inactivate 90% of a population (bacteria, virus). This calculation requires a linear regression curve. As discussed above, heat inactivation of viruses follows a biphasic inactivation curve. As targets for the inactivation of viruses by heat treatment (pasteurization), functional proteins serve as receptors on the surface of virus particles or viral enzymes like polymerases (Nuanualsuwan & Cliver, 2003; Mitra & Ng, 1986), whereas the target for radiation is the nucleic acid. Jeong et al. (2010) described that high temperatures (≥ 70°C) can initially cause the denaturation of structural and functional proteins, unwinding of nucleic acids and destabilization of the surface structure of viral envelopes. These effects cause loss of viral infectivity. As the temperature increases further, proteins and other biomolecules will precipitate, leading to further loss of structure and/or function. Furthermore, one report in the literature demonstrated that inactivation of human parvovirus B19 by heat or low pH is not mediated by capsid disintegration but by the conversion of the infectious virions into DNA-depleted capsids (Mani et al., 2007). Eterpi et al. (2009)

suggested that the intrinsic resistance differences between the parvoviruses (PPV and minute virus of mice [MVM]) might be due to variations in capsid structure rather than differences in DNA stability within the capsid. The high instability of the viral DNA in its encapsidated state is an exclusive feature of human parvovirus B19, which explains its lower resistance to inactivation treatments (Mani et al., 2007).

The inactivation kinetics of viruses may be influenced by many factors, like salt concentration, pH or stabilisers (proteins, sugar or amino acids) (Nissen et al., 1996).

In some publications investigating the heat sensitivity of viruses in egg products, the authors calculate the D_{10} value on the basis of the inactivation kinetics. However, this allows only an estimation of the time needed to inactivate viruses. Furthermore, it has to be considered that the concentration of proteins, sugar, salt, amino acids and other components as well as the pH of the target solution play a significant role concerning the inactivation kinetics. In many investigations it was shown that the water content of the target influences the inactivation kinetics, and that optimal residual moisture has to be determined to efficiently inactivate viruses without influencing the components which should be protected against denaturation.

In this investigation the influence of the egg yolk concentration on the inactivation kinetics was evaluated. It could be shown that viruses in the presence of egg yolk showed a higher stability than viruses without protective proteins and other components present in egg yolk. However, it has to be stressed that with increasing incubation temperatures a more rapid inactivation of viruses was seen, which is in agreement with many evaluations of the pasteurization process.

It was of interest that enveloped NDV showed a higher stability at elevated temperatures than the non-enveloped feline calicivirus (FCV). This finding underlines the necessity to investigate the relevant viruses in validation studies if possible or to use validated models when assumptions about the virus safety of a product are declared (EMA, 1996; 2009).

Few studies have been published for SIV inactivation by heat treatment. Scholtissek (1985) concluded that there were significant differences in thermal stability between influenza A virus strains, but there was no correlation between pH and thermal stability. Studies on the inactivation of a variety of influenza viruses in this investigation have shown obvious differences in the inactivation kinetics of AIV and SIV. This underlines again the necessity to choose the optimal, most resistant virus isolate or strain for inactivation studies and not a virus with a high sensitivity against the inactivation procedure (Pütz, 1998).

It is of interest that there are differences between samples derived from naturally infected chickens and chicken samples artificially contaminated with viruses. One

report in the literature demonstrated that the thermal inactivation rate of HPAIV (PA/83) in artificially contaminated chicken meat was faster than that in naturally infected chicken meat. However, the thermal inactivation rates of highly pathogenic NDV (CA/02) in chicken meat samples infected via both routes were similar, and the NDV-infected chicken meat was inactivated within less than 1 sec at 70°C (Thomas et al., 2008). Thomas & Swayne (2007) studied the thermal inactivation of H5N1 in naturally infected chicken meat and found that the D-values for H5N1 in thigh meat at 57–61°C were 238.8, 130.4, 80.8, 59.6 and 28.6 sec, respectively, and the D-values of H5N1 in breast meat at 57–61°C were 268.7, 153.8, 76.1, 70.7 and 34.1 sec, respectively. Moreover, the virus titres of $10^{6.8}$ and $10^{5.6}$ egg infectious doses (EID_{50})/g of H5N1-infected thigh and breast meat were unchanged after the samples ran through a ramp-up cycle from 25 to 30, 40, 50°C. The virus titres were reduced at 60°C and no virus was detected in infected breast or thigh meat after incubation at 70°C and held at 70°C for 5 sec (Swayne, 2006). Another previous study reported that D-values of NDV strain Herts 33/56 in artificially contaminated meat homogenate at 65°C, 70°C, 74°C and 80°C were 120, 82, 40 and 29 sec, respectively.

5.2 Inactivation of viruses through acetone extraction

Several extraction methods for egg yolk lipids have been developed, and most of them are based on the use of organic solvents (Aro, 2007). Acetone causes an almost complete elution of triglycerides and cholesterol together with a small amount of phospholipids from egg yolk powder (about 15% of total phospholipids). This conforms to the well-known facts that neutral lipids are easily soluble in acetone and bind only weakly to proteins, whereas phospholipids exhibit the opposite characteristics (Nielsen, 2001). In this study, both fractions were evaluated for the virus inactivation capacity using acetone extraction of various concentrations of egg yolk powder. The influence of the egg yolk/virus spike ratio on the infectious virus titres was evaluated.

To my knowledge, this study represents the first information available evaluating the virus infectivity during acetone extraction of egg yolk powder and the influence of the water content of egg yolk powder on the stability of NDV and AIV.

It could be shown that NDV and AIV were inactivated during the extraction of egg yolk powder with acetone when the water content due to the spiking volume was relatively high. When the spiking volume did not exceed the recommended spiking volume of 10% (EMA, 1996) infectious virus could be recovered. These experiments showed that investigations regarding the evaluation of inactivation procedures have to be designed carefully and carried out by standardized and well-controlled procedures. Furthermore, partial inactivation of viruses by acetone was also observed when studying the safety of laboratory methods regarding the inactivation

of viruses by fixatives like acetone. Residual HIV infectivity was found in samples prepared for serological studies when infected cells were treated with acetone (Fauvel & Ozanne, 1989; Grund, 1991). Fixation of samples containing infectious agents has to be carefully controlled, as it was also shown that the length of fixation time and the conditions of storage are important factors when fixing infected cell monolayers with acetone before staining with fluorescent conjugated antibody in the immunofluorescent cell-counting assay of viral and rickettsial agents (Hahon & Zimmermann, 1969; Bardell, 1975). The effect of organic solvents like alcohol or acetone on the infectivity of viruses was studied during the evaluation of disinfectants. It was shown that the alcohol concentration is a critical factor. Optimal inactivation of feline calicivirus was observed at a concentration of approximately 70% alcohol, whereas higher concentrations were less effective (Gehrke et al., 2004). Alcohols and other organic solvents might lead to a rapid dehydration of some viral proteins, resulting in a fixation of the particle structure and stabilization of the infectivity.

The experiments presented in this thesis show that experimental evaluation of virus inactivation studies have to be carefully planned, keeping in mind that variations in the composition of the test substances might have a major impact on the inactivation behaviour of viruses. Therefore such evaluations have to use conditions as close as possible to those used in the actual production process.

5.3 Distribution of viral genome and infectivity in fractions after partitioning through filtration

Microfiltration is generally used to separate particles suspended in liquid media (Mukhopadhyay et al., 2009; Lewis & Grandison, 2012), and filtration is therefore considered as one of the methods suitable for virus removal (WHO, 2004). Avian influenza A virus particles are roughly round or filamentous with an average size of 80–120 nm (Jordan, 1996). However, it should be considered that spiking of the ethanolic lecithin solution leads to a disintegration of the lipid-containing virus particles. The 70% ethanolic lecithin solution spiked with 7.4 $\log_{10}$ $TCID_{50}$/ml of AIV was microfiltered using a membrane with a nominal pore size of 0.2 µm. Using a 0.2 µm pore size membrane led to a passage of the particles that are less than 200 nm in size, and larger particles were retained by the microfiltration membrane. As the pore size of the microfiltration membrane was larger than the AIV, it could be predicted that AIV particles pass through the membranes and would be contained in the filtrate.

In the 70% ethanolic lecithin solution spiked with AIV, no infectious virus could be determined either in the filtrate or on the filters. The observed cytopathogenic effect with a titre of 3.16E+01 $TCID_{50}$/ml was most probably due to the residual alcohol in

the test samples. However, the results from qRT-PCR in this study showed that after microfiltration AIV genomes or genome equivalents could be determined in both fractions. The presence of viral genomes might be explained by the attachment of nucleoprotein/RNA complexes to the filter membrane. Matsushita et al. (2005) described that the aggregated virus is retained by the microfiltration membrane if the size of the aggregates exceeded the pore size of the microfiltration membrane. Furthermore, one previous investigation suggested that membranes with pore sizes larger than the virus particles could be used to remove host cell fragments and attached viruses, enabling a further purification of compounds (Wickramasinghe et al., 2005).

6. Summary

The present study was designed to
(1) compare the stability of avian influenza virus (AIV), Newcastle disease virus (NDV), feline calicivirus (FCV) and porcine parvovirus (PPV) at 40°C, 60°C and 80°C and to assess the influence of egg yolk ingredients on the thermal inactivation of AIV, NDV and FCV;
(2) examine and compare the thermal inactivation between three subtypes of swine influenza virus (SIV; H1N1, H1N2 and H3N2) and one avian influenza virus (AIV; H7N1);
(3) determine the influence of egg yolk powder and water concentration on virus inactivation during acetone extraction; and
(4) evaluate the removal of virus from ethanolic extracts by microfiltration and compare the distribution of viral genome with virus infectivity in fractions after partitioning.

The results revealed that
(1) FCV was the virus most sensitive to heat inactivation at 40°C, followed by AIV and NDV. PPV was found to be the virus most resistant to heat inactivation. In two cases (AIV and NDV) the virus titres were reduced within 1 min of heat treatment at 60°C by a factor of ≥5 $\log_{10}$, nevertheless FCV was only reduced by a factor of 2.25 $\log_{10}$. After 6 h of heat treatment of PPV at 60°C no virus was detectable (≤0.5 $\log_{10}$ $TCID_{50}$/ml). The mean starting virus titres of all viruses species (AIV, NDV, FCV) were rapidly reduced to ≤0.5 $\log_{10}$ $TCID_{50}$/ml within 1 min and PPV after 6 h of heat treatment at 80°C, respectively. These experiments showed that the virus titres were reduced by heat treatment at 80°C by a factor of ≥5 $\log_{10}$.

2) Comparison of the viral persistence between different strains of influenza viruses (SIV and AIV) showed that the swine influenza strain H1N2 was the most heat resistant one at 40°C, followed by SIV strain H1N1, SIV strain H3N2 and AIV strain H7N1. These experiments indicate that the selection of virus strains used for inactivation experiments can be of importance for the validation of production processes with regard to the inactivation capacity of individual production steps.

3) During treatment at 60°C viruses in the presence of egg yolk showed a higher stability than the control virus without egg yolk. It is suggested that proteins and other components present in egg yolk provide partial protection of virus particles during heat exposure. During acetone extraction the egg yolk quantity in relation to the volume of the virus inoculum influenced the stability of the viruses (AIV and NDV). The mean starting virus titres of all concentrations of egg yolk powder were reduced by ≥ 4 $\log_{10}$ for the residue and the extract. Inactivation of NDV and AIV was stronger when the water concentration was high.

4) No infectious influenza virus could be detected after partitioning of virus-spiked ethanolic extracts in the filtrate or on the filter parts. However, after microfiltration AIV genomes or genome equivalents were detected in both fractions.

Zusammenfassung

Hitzestabilität von ausgewählten Viren in flüssigem Eigelb und Verteilung der Viren in den verschiedenen Fraktionen bei der Acetonextraktion und Mikrofiltration von Eigelb-Phospholipiden

In dieser Arbeit sollten verschiedene Fragen zur Virussicherheit von Eiprodukten, die für pharmakologische Präparate eingesetzt werden können, beantwortet werden. Dazu wurden einzelne Reinigungsschritte für die Herstellung von Ei-Lecithin untersucht:

1) Geklärt werden sollte die Hitzestabilität von aviären Influenza-Viren (AIV), Newcastle Disease Virus (NDV), Calizivirus der Katze (feline calicivirus [FCV]) und das Schweineparvovirus (porcine parvovirus [PPV]) bei verschiedenen Inkubationstemperaturen: 40°C, 60°C und 80°C. Zudem sollte der Einfluss von Eidotterproteinen auf die Inaktivierungskinetik von AIV, NDV und FCV während der Hitzebehandlung untersucht werden.

2) In vergleichenden Untersuchungen sollte der Frage nachgegangen werden, inwieweit Unterschiede in der Inaktivierungskinetik verschiedener Influenzavirus-Stämme bestehen. Hierfür wurden drei Subtypen von Schweineinfluenzaviren (SIV-Subtypen H1N1, H1N2 und H3N2) sowie ein Geflügelinfluenzavirus (AIV Subtyp H7N1) verglichen.

3) Ein wichtiger Schritt bei der Reinigung von Lecithin ist die Extraktion des Pulvers aus Eidotter. Untersucht werden sollte der Einfluss der Eidotterproteine sowie der Wasserkonzentration auf das Inaktivierungsverhalten der verschiedenen Viren.

4) Bei der Herstellung von pharmazeutischen Präparaten werden in der Regel Filtrationsschritte eingesetzt. Von Interesse war daher, inwieweit Viren sich durch die eingesetzten Filter nach Ethanolextraktion entfernen lassen und wie sich Infektiosität bzw. Viruspartikel auf die verschiedenen Fraktionen verteilen.

Folgende Ergebnisse wurden in diesen Untersuchungen erzielt:

1) FCV wurde durch Hitzebehandlung bei 40°C schneller inaktiviert als AIV und NDV. PPV war recht stabil.

Wurden die Viren bei einer Temperatur von 60°C behandelt, sank der Titer von AIV und NDV innerhalb von 1 min um einen Faktor von ≥5 $\log_{10}$, während FCV unter diesen Bedingungen nur um etwa einen Faktor von 2,25 $\log_{10}$ inaktiviert wurde. PPV erwies sich wieder als stabilstes Virus, dessen Titer erst nach einer sechsstündigen Inkubation unter die Nachweisgrenze gesunken war (≤0,5 $\log_{10}$ $TCID_{50}$/ml).

Inkubation der Virussuspensionen bei 80°C führte bei AIV, NDV und FCV innerhalb einer Minute zum vollständigen Verlust der Infektiosität (Nachweisgrenze ≤0,5 $\log_{10}$ $TCID_{50}$/ml). Auch hier erwies sich PPV als ausgesprochen stabil, und der Titer war erst nach sechsstündiger Inkubation unter die Nachweisgrenze gefallen. Es konnte somit gezeigt werden, dass die Hitzebehandlung bei 80°C zu einem Titerabfall um einen Faktor von ≥5 $\log_{10}$ führt.

2) Die vergleichenden Untersuchungen zur Hitzestabilität verschiedener Influenzavirus-Stämme (SIV, AIV) zeigten, dass SIV Subtyp H1N2 während der Inkubation bei 40°C den geringsten Titerverlust aufwies, gefolgt von den Influenzaviren SIV H1N1, SIV H3N2 und AIV H7N1.

Diese Ergebnisse belegen, dass die Auswahl von Virusisolaten zur Beurteilung von Inaktivierungsverfahren der Herstellung von pharmazeutischen Produkten von wesentlicher Bedeutung sein kann.

3) Bei der Herstellung pharmazeutischer Produkte wird häufig eine Hitzebehandlung bei 60°C durchgeführt. Die vorliegenden Untersuchungsergebnisse zeigen, dass Eidotterpulver die Stabilität der Viren bei dieser für die Pasteurisierung typischen Temperatur erhöht. Es wird diskutiert, dass Proteine oder andere Komponenten, die im Eidotter vorhanden sind, die Inaktivierung der Viren verzögern.

4) Der Versuchsaufbau ist für die Bewertung der Virussicherheit pharmazeutischer Produkte von großer Bedeutung. So hatte das Verhältnis von Eidotterpulver und Volumen an Virussuspension einen erheblichen Einfluss auf die Stabilität der Infektiosität von AIV und NDV. Obwohl unter allen Versuchsbedingungen eine Reduktion des Titers um einen Faktor von ≥ 4 $\log_{10}$ erreicht wurde, konnte nachgewiesen werden, dass bei höheren Wasseranteilen im Behandlungsansatz die Inaktivierung der Viren ausgeprägter war.

5) Ethanol inaktivierte Influenza bis unter die Nachweisgrenze, und infektiöses Virus konnte weder im Filtrat noch auf dem Filter nachgewiesen werden. Jedoch konnten mit der quantitativen PCR sowohl im Filtrat als auch auf dem Filter Virusgenomfragmente nachgewiesen werden.

Die Validierung von drei typischen Schritten zur Reinigung von Lecithin aus Hühnereigelb zeigte, dass das Parvovirus (als Modell für nicht umhüllte Viren) unter den Versuchsbedingungen relativ stabil ist. Hingegen lassen sich die relevanten Viren AIV und NDV sowohl durch Hitzebehandlung, wie sie bei der Herstellung von Eigelbpulver angewendet wird (Pasteurisierung), als auch durch Reinigung von Lecithin aus Eigelb mit organischen Extraktionsmitteln effektiv inaktivieren.

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Acknowledgments

First of all, I would like to thank my family who are always there to support me in all aspects. I am loved and encouraged. I would not have come this far without them.

My sincere gratitude goes to Univ.-Prof. Dr. Michael F. G. Schmidt who has been helping me with everything. I feel thankful for his supportive encouragement and kind supervision. He played such an important role in getting this project successfully done.

Also, I would like to thank Univ.-Prof. Dr. Georg Pauli sincerely for co-supervising my thesis. His advice and guidance have further helped complete this thesis.

Another great support came from Univ.-Prof. Dr. Hafez Mohamed Hafez. He has been giving me a lot of terrific advice. He helped me broaden my abilities to cope with problems that arose during the working period.

Thanks to Dr. Michael Burwinkel who gave me great guidance so I could head in the right direction. I appreciate his good lead from the early stages to the completion of this project.

I would like to thank to Dr. Dörte Lüschow who willingly provided me with chicken red blood cells, one of the most important materials of this project, and also other materials and necessary equipment. Not only that, she also helped me regarding many laboratory techniques.

I am also thankful to Christiane Palissa, Bettina Esch and Ingrid Poese for their help with laboratory techniques. They not only taught me the techniques but also kindly gave me useful advice and helped me prepare laboratory materials and equipment. My work went smoothly with the help of these persons.

My heart goes out sincerely to all of my colleagues who have created a friendly work environment. I appreciate their friendship and support. The experiences with them are memorable.

Special thanks to my special friend, Juthamas Chaiwan, who is always there to support me in every rhythm of life.

Lastly, I would like to thank again all of the above-mentioned persons. My project could not have been completed without them.

I hereby confirm that the work presented here is original and the result of my own investigations and has not been submitted before, either in part or whole, for a degree at this or any other university.

Berlin, 12.04.2013

Ratchaya Vachirachewin