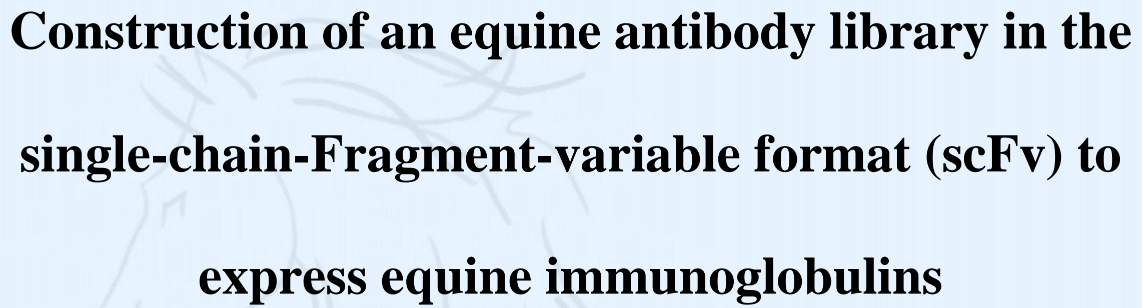

Construction of an equine antibody library in the single-chain-Fragment-variable format (scFv) to express equine immunoglobulins

DISSERTATION

to attain the doctoral degree (Dr. rer. agr.)

of the Faculty of Agricultural Science Georg-August-University Göttingen

submitted by

Franziska Isabella Pilger

born in Frankfurt am Main, Germany

Göttingen, December, 2020

D7

1st Referee: Prof. Dr. Jens Tetens

2nd Referee: Prof. Dr. Uta König von Borstel

3rd Referee: Sascha Knauf, PhD

Date of submission: 18.12.2020

Date of Thesis defence: 19.02.2021

List of content

LIST OF CONTENT I

LIST OF TABLES IV

LIST OF FIGURES V

LIST OF ABBREVIATIONS VII

1 INTRODUCTION 1

2.1 References (Vancouver) 4

3 LITERATURE REVIEW 8

3.1 Equine Herpesvirus 8

3.1.1 Classification and Structure 8

3.1.2 Prevalence, Transmission & Epidemiology 10

3.1.3 Pathogenicity and clinical signs 15

3.1.4 Latency & Immune evasion strategies 18

3.1.5 Diagnosis 22

3.1.6 Prevention & Treatment 24

3.2 Recombinant Antibodies 29

3.2.1 Antibody structure and function 29

3.2.2 Antibody development and diversification 32

3.2.3 Recombinant antibodies and antibody libraries 34

3.2.4 Phage Display 40

3.2.5 Clinical Relevance 45

3.3 Aim of the thesis 52

3.4 References (Vancouver) 53

4 STUDIES PERFORMED 65

4.1 Manuscript I: PCR primer for the construction of an equine immunoglobulin library in the single-chain fragment variable (scFv) format 65

4.1.1 Abstract 66

4.1.2 Introduction 67

4.1.3 Material and Methods 70

4.1.4 Results 80

4.1.5 Discussion 96

4.1.6 Conclusion 102

4.1.7 Acknowledgements 102

4.1.8 References (Vancouver) 103

4.2 Manuscript II: Isolation and characterization of an equine anti-EHV-1 single chain antibody fragment (scFv) from an equine phage display library 109

4.2.1 Abstract 110

4.2.2 Introduction 111

4.2.3 Material and Methods 113

4.2.4 Results 125

4.2.5 Discussion 135

4.2.6 Conclusion 141

4.2.7 References (Vancouver) 143

5 GENERAL DISCUSSION 147

5.1 Conclusion & Outlook 154

5.2 References (Vancouver) 155

6 ZUSAMMENFASSUNG & SUMMARY 158

7 APPENDIX 164

7.1 Authors contributions 164

7.1.1 Manuscript I: PCR primer for the construction of an equine immunoglobulin library in the single-chain fragment variable (scFv) format 164

7.1.2 Manuscript II: Isolating a high-affinity, equine, single-chain antibody fragment (scFv) from an equine phage display library neutralizing Equine Herpesvirus-1 164

7.2 Supplementary files: 165

7.2.1 Manuscript I: PCR primer for the construction of an equine immunoglobulin library in the single-chain fragment variable (scFv) format 165

7.2.2 Manuscript II: Isolating a high-affinity, equine, single-chain antibody fragment (scFv) from an equine phage display library neutralizing Equine Herpesvirus-1 186

7.3 Curriculum vitae 195

7.4 Acknowledgement 196

8 DECLARATION 197

LIST OF TABLES

Table 1: Equine herpesvirus classification and resulting disorder in host species upon infection. Adapted from Paillot et al. (2).

Table 2: Vaccines licensed for use in Germany by Paul-Ehrlich-Institute (accessed 30.08.2020).

Table 3: Overview of all five equine immunoglobulin isotypes, including their structure and function.

Table 4: Overview of all scFv generated by phage display approved in the EU and or USA and their applications in human medicine according to www.antibodysociety.org (accessed 23.10.2020). INN - International non- proprietary name.

Table 5: Summary of recombinant antibodies under imvestigation for potential use in veterinary medicine. Adapted from Bustamante-Córdova et al. (84).

LIST OF FIGURES

Figure 1: EHV-1 virion shown schematically. Modified according to Paillot et al. (2). dsDNA – double-stranded DNA. Created with biorender.com.

Figure 2: Modes of transmission of EHV-1. Adapted from Kapoor et al. (1). Created with biorender.com.

Figure 3: Transmission cycle of EHV-1 infection among horses. Adapted from Kapoor et al. (1). Created with biorender.com.

Figure 4: Distribution of EHV-1 outbreaks. EHV-1 outbreaks have been reported in countries marked in red according to scientific literature, the OIE WAHIS interface and the FEI from 2000 to 2020 (8, 10, 16-18, 20-33). Created with biorender.com.

Figure 5: Schematic overview of SNP in EHV-1 Pol gene within ORF30 and resulting viral strains. Created with biorender.com.

Figure 6: Schematic overview of all five equine immunoglobulin isotypes. Created with biorender.com.

Figure 7: Map of the equine IGHC region depicting all immunoglobulin isotypes; in particular seven subclasses of IgG. Boxes indicate IGHC genes. Map is adapted from Wagner et al. (75) and Sun et al. (76). Created with biorender.com.

Figure 8: Schematic antibody structure based on IgG. Created with biorender.com

Figure 9: Germline configuration of equine immunoglobulin heavy and light chain loci, including number of gene segments available to horses. IG – immunoglobulin; H - heavy chain, K – κ light chain, L – λ light chain. V – Variable, D – diversity J – joining, C - constant. Created with biorender.com.

Figure 10: On the top, Emil von Behring. On the bottom, Pierre Emile Roux producing antitoxic sera from horses to treat diphtheria.

Figure 11: Illustration of recombinant antibody formats compared to a full-size IgG antibody. Adapted from Bustamante-Córdova et al. (84). Created with biorender.com.

Figure 12: Infection of *E. coli* cell by M13 bacteriophage. Phage surface protein G3P binds to F pilus on *E.coli* cell. F pilus disassembles and transports phage closer to bacterial surface. Phage interacts with TolA receptor and induces uptake of phage genome (106). Created with biorender.com.

Figure 13: Scheme of scFv phage display selection and screening adapted from Russo et al. (113). Created with biorender.com.

LIST OF ABBREVIATIONS

ADCC	Antibody dependent cell cytotoxicity
bp	Base pairs
C	Constant
CDR	Complementarity determining regions
CG	Chorionic gonadotropin
CNS	Central nervous system
CTL	Cytotoxic T lymphocytes
D	Diversity
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic nucleoside triphosphate
dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid
EHM	Equine herpesvirus myeloencephalopathy
EHV	Equine herpesvirus
Fab	Fragment antibody
FACS	Fluorescence-activated cell sorting
Fc	Fragment constant
FDA	Food and Drug Administration
FEI	Fédération Equestre Internationale
FR	Frame
Fv	Fragment variable
HAMA	human anti-mouse antibody
IE	Immediate early
IFN	Interferon
IG	Immunoglobulin
J	Joining
K	Kappa
L	Lambda
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
NGF	Nerve growth factor
NK	Natural killer cells

OD	Optical density
OIE/WAHIS	World Organisation for Animal Health
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PhaNGS	Phage Next Generation Sequencing
Pol	Polymerase
REA	Restriction enzyme analysis
RSS	Recombination signal sequences
scFv	Single-chain-Fragment-variable antibody fragment
sdAb	Single domain antibody
SHM	Somatic hypermutations
SNP	Single nucleotide polymorphism
SOE	Splicing by overlap extension
ssDNA	Single-stranded DNA
TGF	Transforming growth factor
TRS	Terminal regulatory sequence
U_L	Unique long
URT	Upper respiratory tract
U_S	Unique short
USA	United States of America
V	Variable
VEEV	Venezuelan equine encephalitis virus
VN	Virus neutralizing
VZV	Varicello Zoster virus
WEEV	Western equine encephalitis virus

1 INTRODUCTION

Equine populations worldwide are exposed to a large variety of infectious diseases. While these are detrimental to horse health and welfare, and potentially cause significant economic shortfalls, one must bear in mind that each infectious agent also carries a risk of zoonotic potential (1). Several outbreaks of highly pathogenic zoonotic diseases affecting humans in recent years underlines the interconnectedness of humans, animal and environmental species (1). Hence, equine health concerns a broad audience beyond equine professionals and enthusiasts. The most prevalent infectious equine diseases and infections, as listed by OIE, include infection with equid herpesvirus-1 (EHV-1), equine influenza, equine encephalomyelitis (Western) or Venezuelan equine encephalomyelitis among many others (2). EHV-1 is of particular interest as it is does not only cause rhinopneumonitis, but also abortions and neonatal foal death as well as the severe neurological condition known as Equine Herpesvirus myeloencephalopathy (EHM). It is easily transmissible via nasal secretions and highly contagious (3-6). On top of that, EHV-1, like most Herpesviruses, establishes a life-long latency within the equine host by evading immune responses, rendering the host a silent shedder of the virus (7). In that manner, it has been greatly successful pursuing its epidemiological strategy to ensure its spread and survival within the host population (3, 6, 8, 9). At this point in time, at least 60 % of the worldwide horse population are considered latently infected with EHV-1 (10). Current therapeutic options are non-existent and the main focus is on costly preventative measures including biosecurity or vaccination regimens (10). However, vaccinations are limited in their application spectrum as they fail to alter viremia or prevent latent infections (10, 11). Anti-viral therapeutics proven successful in human medicine are currently under investigation for use in horses (4, 10, 12, 13). This lack of anti-viral therapeutics, along with the existence of hardy pathogens with pandemic potential and the ever increasing antibiotic resistance have (re-)-ignited the necessity for antibody treatments,

including monoclonal antibodies as well as recombinant antibodies (14). Moreover, antibody reagents would greatly add to the armoury of biological reagents elucidating antigenic properties (15). The general consensus among EHV-1 experts considers equine-specific, recombinant antibodies to be a viable option to initially provide reagents and consequently, protection against EHV-1 (4, 10).

In human medicine, recombinant antibodies have been developed over the past 30 years, resulting in numerous antibody preparations approved for treatments including infectious diseases, cancer, and auto immune diseases (16). Veterinary medicine has caught up and recently, two veterinary recombinant antibody preparations have received approval to be utilized in the treatment of pain and autoimmune diseases in cats and dogs (17). A major advantage of recombinant antibody formulation is the species-specificity of the formulation since immunogenicity to foreign proteins is thereby avoided and supports the healing process (16-19). Since the beginning of recombinant antibody generation a clear trend towards highly species-specific antibodies is observable (18): in the 1990ies merely 11,5 % of antibody candidates were fully human; ten years later a markedly higher percentage of 45 % are fully human (18). The two veterinary preparations have also been adjusted to be better tolerated in the target species and formulation have therefore been caninized and felinized (20, 21).

State-of-the-art of recombinant antibody generation is the *in vitro* construction of species-specific antibody libraries containing the immunoglobulin repertoire of all available immunoglobulin data of the target species mimicking the naturally occurring antibody repertoire (19, 22-24). Libraries are usually generated within certain antibody formats whereby the naturally occurring antibody is fragmented into smaller, still functioning, antibody molecules that have proven to be advantageous in the diagnosis and treatment of certain conditions (16). The single-chain-Fragment-variable (scFv) format has achieved popularity due to a large range of advantages including outstanding tissue penetration characteristics, higher

sensitivities and a high customizability (15, 19, 23, 25, 26). Most of these aspects are ascribable to a scFv fragments smaller size since it only contains the antigen binding domains of an antibody linked to each other via a glycine-serine linker (15, 19, 23, 25, 26).

Recombinant antibodies raised in antibody libraries, are selected on a particular antigen through a microbial display system, whereby microbes display the antibody sequence on their surface (27). Exposure to the antigen of interest during so-called biopanning rounds determines well-binding antibody sequences (23). The most robust and widely used display method is phage display technology which has been involved in drug discovery since the beginning of recombinant antibody generation in both, human and veterinary medicine and research (16). Many human and non-human primate phage display libraries as well as veterinary scFv phage display libraries (e.g. chicken, camel, cattle, and rabbit) exist (28-33), but no equine scFv phage display library to isolate scFv antibodies exists to date.

The present dissertation aims to meet this lack by constructing an equine scFv phage display library to isolate scFv antibodies to provide prognostic, diagnostic and therapeutic alternatives to investigate and treat equine conditions. The first study establishes and validates a primer set designed to capture the vast repertoire of equine immunoglobulin genes based on published data and genomic organisation (34-45) for the construction of an equine recombinant antibody library in the recombinant scFv format. The second study, is a proof-of-principle study, in which the suitability of phage display technology is assessed as a method to screen the library for an equine scFv antibody against the highly infectious EHV-1 virus and will include characterisation of isolated scFv in terms of production and binding capacities (18). Results of this dissertation will contribute to discovery and development of novel, alternative equine and veterinary prognostic and therapeutic agents against a vast variety of equine pathogens. The newly established library represents a starting point for future research and development in equine and veterinary drug development improving animal health and welfare.

2.1 References (Vancouver)

1. Middleton D, Pallister J, Klein R, Feng Y-R, Haining J, Arkinstall R, et al. Hendra virus vaccine, a one health approach to protecting horse, human, and environmental health. Emerg Infect Dis. 2014;20(3):372-9.

2. (OIE) WOfAH. Animal Health Information. Accessed on 15.09.2020.

3. Loving NS. All Horse Systems Go: The Horse Owner's Full-color Veterinary Care and Conditioning Resource for Modern Performance, Sport, and Pleasure Horses: Trafalgar Square Books; 2014.

4. Stokol T, Soboll Hussey G. Editorial: Current Research in Equid Herpesvirus Type-1 (EHV-1). Frontiers in Veterinary Science. 2020;6(492).

5. Pusterla N, Hatch K, Crossley B, Wademan C, Barnum S, Flynn K. Equine herpesvirus-1 genotype did not significantly affect clinical signs and disease outcome in 65 horses diagnosed with equine herpesvirus-1 myeloencephalopathy. The Veterinary Journal. 2020;255:105407.

6. Oladunni FS, Horohov DW, Chambers TM. EHV-1: A Constant Threat to the Horse Industry. Front Microbiol. 2019;10(2668).

7. Wilcox GE. Latent equine herpesviruses. 2004.

8. Patel J, Heldens J. Equine herpesviruses 1 (EHV-1) and 4 (EHV-4)–epidemiology, disease and immunoprophylaxis: a brief review. The Veterinary Journal. 2005;170(1):14-23.

9. Ma G, Azab W, Osterrieder N. Equine herpesviruses type 1 (EHV-1) and 4 (EHV-4)--masters of co-evolution and a constant threat to equids and beyond. Vet Microbiol. 2013;167(1-2):123-34.

10. Lunn D, Davis-Poynter N, Flaminio M, Horohov D, Osterrieder K, Pusterla N, et al. Equine herpesvirus-1 consensus statement. Journal of Veterinary Internal Medicine. 2009;23(3):450-61.

11. (OIE) WOfAH. Equine Rhinopneumonitis (Infection with Equid Herpesvirus-1 and -4). 2017.

12. Maxwell LK, Bentz BG, Gilliam LL, Ritchey JW, Pusterla N, Eberle R, et al. Efficacy of the early administration of valacyclovir hydrochloride for the treatment of neuropathogenic equine herpesvirus type-1 infection in horses. American journal of veterinary research. 2017;78(10):1126-39.

13. Tallmadge RL, Žygelytė E, Van de Walle GR, Kristie TM, Felippe MJB. effect of a histone Demethylase inhibitor on equine herpesvirus-1 activity In Vitro. Frontiers in veterinary science. 2018;5:34.

14. Kaufmann SH. Emil von Behring: translational medicine at the dawn of immunology. Nature Reviews Immunology. 2017;17(6):341.

15. Ahmad ZA, Yeap SK, Ali AM, Ho WY, Alitheen NBM, Hamid M. scFv Antibody: Principles and Clinical Application. Clinical & Developmental Immunology. 2012.

16. Frenzel A, Kügler J, Helmsing S, Meier D, Schirrmann T, Hust M, et al. Designing Human Antibodies by Phage Display. Transfusion Medicine and Hemotherapy. 2017;44(5):312-8.

17. Berger D. The Therapeutic Power of Monoclonal Antibody Therapy https://todaysveterinarypractice.com/; 2020 [Available from: https://todaysveterinarypractice.com/the-therapeutic-power-of-monoclonal-antibody-therapy/.

18. Ponsel D, Neugebauer J, Ladetzki-Baehs K, Tissot K. High affinity, developability and functional size: the holy grail of combinatorial antibody library generation. Molecules. 2011;16(5):3675-700.

19. Hoogenboom HR. Selecting and screening recombinant antibody libraries. Nat Biotechnol. 2005;23(9):1105-16.

20. Michels GM, Walsh KF, Kryda KA, Mahabir SP, Walters RR, Hoevers JD, et al. A blinded, randomized, placebo-controlled trial of the safety of lokivetmab (ZTS-00103289), a caninized anti-canine IL-31 monoclonal antibody in client-owned dogs with atopic dermatitis. Veterinary Dermatology. 2016;27(6):505-e136.

21. Bustamante J, Jankauski M, Daniel TL. Closed loop Monte Carlo models of abdominal contribution to insect flight control. Integr Comp Biol. 2018;58:E26-E.

22. Solemani Zadeh A, Grässer A, Dinter H, Hermes M, Schindowski K. Efficient Construction and Effective Screening of Synthetic Domain Antibody Libraries. Methods and protocols. 2019;2(1):17.

23. Crivianu-Gaita V, Thompson M. Aptamers, antibody scFv, and antibody Fab' fragments: An overview and comparison of three of the most versatile biosensor biorecognition elements. Biosensors and Bioelectronics. 2016;85:32-45.

24. Dias-Neto E, Nunes DN, Giordano RJ, Sun J, Botz GH, Yang K, et al. Next-Generation Phage Display: Integrating and Comparing Available Molecular Tools to Enable Cost-Effective High-Throughput Analysis. Plos One. 2009;4(12):e8338.

25. Wang XZ, Coljee VW, Maynard JA. Back to the future: recombinant polyclonal antibody therapeutics. Curr Opin Chem Eng. 2013;2(4):405-15.

26. Sandhu JS. Protein Engineering of Antibodies. Critical Reviews in Biotechnology. 1992;12(5-6):437-62.

27. Lou J, Marzari R, Verzillo V, Ferrero F, Pak D, Sheng M, et al. Antibodies in haystacks: how selection strategy influences the outcome of selection from molecular diversity libraries. Journal of immunological methods. 2001;253(1-2):233-42.

28. Kügler J. TF, Frenzel A., Hust M. Construction of Human Immune and Naive scFv Libraries. . 2018. In: Phage Display Methods in Molecular Biology [Internet]. New York, NY: Humana Press.

29. Avril A, Miethe S, Hust M, Pelat T. Construction of macaque immune-libraries. Phage Display: Springer; 2018. p. 83-112.

30. Diesterbeck US. Construction of Bovine Immunoglobulin Libraries in the Single-Chain Fragment Variable (scFv) Format. Methods in molecular biology (Clifton, NJ). 2018;1701:113-31.

31. Nguyen TTH, Lee JS, Shim H. Construction of rabbit immune antibody libraries. Phage Display: Springer; 2018. p. 133-46.

32. Romão E, Poignavent V, Vincke C, Ritzenthaler C, Muyldermans S, Monsion B. Construction of High-Quality Camel Immune Antibody Libraries. In: Hust M, Lim TS, editors. Phage Display: Methods and Protocols. New York, NY: Springer New York; 2018. p. 169-87.

33. Fehrsen J, Wemmer S, van Wyngaardt W. Construction of chicken antibody libraries. Phage Display: Springer; 2018. p. 189-203.

34. Helms CM, Allen PZ. Studies on equine immunoglobulins—I the antigenic structure of horse IgG, its fragments and subunits. Immunochemistry. 1970;7(5):401-12.

35. Pahud JJ, Mach JP. Equine Secretory IgA and Secretory Component. International Archives of Allergy and Immunology. 1972;42(2):175-86.

36. Rockey JH, Klinman NR, Karush F. Equine antihapten antibody.I. 7S beta-2A- and 1OS gamma-1- components of purified anti-beta-lactoside antibody. J Exp Med. 1964;120:589-609.

37. Sandor G, Korach S, Mattern P. 7S globulin, immunologically identical to 19S gamma-1 (beta-2)-M-globulin, a new protein or horse serum. Nature. 1964;204:795-6.

38. Vaerman JP, Querinjean P, Heremans JF. Studies on the IgA system of the horse. Immunology. 1971;21(3):443-54.

39. Wagner B, Miller WH, Morgan EE, Hillegas JM, Erb HN, Leibold W, et al. IgE and IgG antibodies in skin allergy of the horse. Vet Res. 2006;37(6):813-25.

40. Walther S, Rusitzka TV, Diesterbeck US, Czerny C-P. Equine immunoglobulins and organization of immunoglobulin genes. Developmental & Comparative Immunology. 2015;53(2):303-19.

41. Weir RC, Porter RR, Givol D. Comparison of the C-terminal amino-acid sequence of two horse immunoglobulins IgG and IgG(T). Nature. 1966;212(5058):205-6.

42. Zolla S, Goodman JW. An aggregating immunoglobulin in hyperimmune equine anti-pneumococcal sera. J Immunol. 1968;100(4):880-97.

43. Sun J, Han Y, Wang Y. [Serum antibody response to different doses of hepatitis B vaccine made by recombinant DNA techniques in yeast and Hansenula polymorpha yeast]. Zhongguo Yi Miao He Mian Yi. 2010;16(2):140-2.

44. Wade CM, Giulotto E, Sigurdsson S, Zoli M, Gnerre S, Imsland F, et al. Genome sequence, comparative analysis, and population genetics of the domestic horse. Science. 2009;326(5954):865-7.

45. Ford JE, Home WA, Gibson DM. Light chain isotype regulation in the horse. Characterization of Ig kappa genes. J Immunol. 1994;153(3):1099-111.

3 LITERATURE REVIEW

3.1 Equine Herpesvirus

3.1.1 Classification and Structure

Classification

Equine herpesviruses (EHVs) have been classified under the order *Herpesvirales* and the family of *Herpesviridae* (1, 2). The family of *Herpesviridae* is further subdivided into three subfamilies: α-, β- and γ- subfamilies (2). Division occurred according to tissue tropism, pathogenicity and behaviour in cell culture (1-4). EHVs however only belong to two of three subfamilies - α and γ (1, 2). In total, nine EHVs exist, of which EHV-1, -3, -4, -8 and -9 belong to the α-subfamily and are allocated to the genus *Varicelloviruses* (1). EHV-2, -5 and -7 belong to the γ-subfamily and are assigned to the genus *Percaviruses* (1, 2). Out of nine EHVs only five (EHV-1, -2, -3, -4 and -5) can cause diseases in horses (1, 2). While EHV-3 causes coital exanthema and EHV-2 and -5 are not associated with specific diseases but with respiratory problems and general malaise (5), EHV-1 and -4 cause the most detrimental health issues resulting in substantial economic deficits (1, 2). Both viruses affect the respiratory tract, yet only EHV-1 can causes further disorders including abortion, neonatal foal death and neurological disorders (1, 2). Table 1 shows an overview of EHV classification and disorders caused by EHVs.

Table 1: Equine herpesvirus classification and resulting disorder in host species upon infection. Adapted from Paillot et al. (2).

Host species	Name	Subfamily	Disorder
Equus caballus	EHV-1	α	Respiratory, abortion, neurological
	EHV-2	γ	Associated with: respiratory, general malaise, poor performance
	EHV-3	α	Coital exanthema
	EHV-4	α	Respiratory
	EHV-5	γ	Associated with: respiratory, general malaise, poor performance
Equus asinus	EHV-6	α	Coital exanthema
	EHV-7	γ	Associated with: respiratory, general malaise, poor performance
	EHV-8	α	Rhinitis
Gazella thomsoni	EHV-9	α	Neurological

Structure of EHV-1

The EHV-1 virion has an icosahedral nucleocapsid, containing the viral DNA, embedded into globular tegument and is encased by a shapeless envelope, which is surrounded by 12 glycoproteins (Figure 1) (1, 2, 5). The diameter of the virion is reported to be approximately 150 nm (1, 5). Similar to most herpesviruses, the EHV-1 nucleocapsid is composed of six proteins (2). Its structure is made up of 162 capsomers and twelve portal proteins, which form a ring-like structure within the capsid; these portal proteins are utilized by viral genomic information (DNA) to penetrate the capsid (2). Viral DNA is made up of a single, linear, double-stranded DNA molecule with a size of 150,223 bp and a GC content of 57% (1, 2, 5, 6). The genome incorporates a unique long (U_L) region adhering to a unique short (U_S) region, bordered by internal (IRS) and terminal (TRS) sequences (1, 5). Whole genome sequencing of a purified EHV-1 strain Ab4 clone (7) revealed the genome to consist of 80 open reading frames (ORFs), which includes 76 unique genes (5). The nucleocapsid containing the EHV-1 genome, is embedded into the tegument, which contains 12 viral proteins and enzymes responsible for replication mechanisms (5). The shapeless envelope, surrounding the nucleocapsid and the tegument, presents 11 glycoproteins on its surface – gB, gC, gD, gE, gG,

gH, gI, gK, gL, gM, gN, gp2 (2). Glycoproteins play a vital role during the adsorption and penetration process during virus pathogenicity including cell-to-cell spread, cell penetration, virus attachment and egression (5).

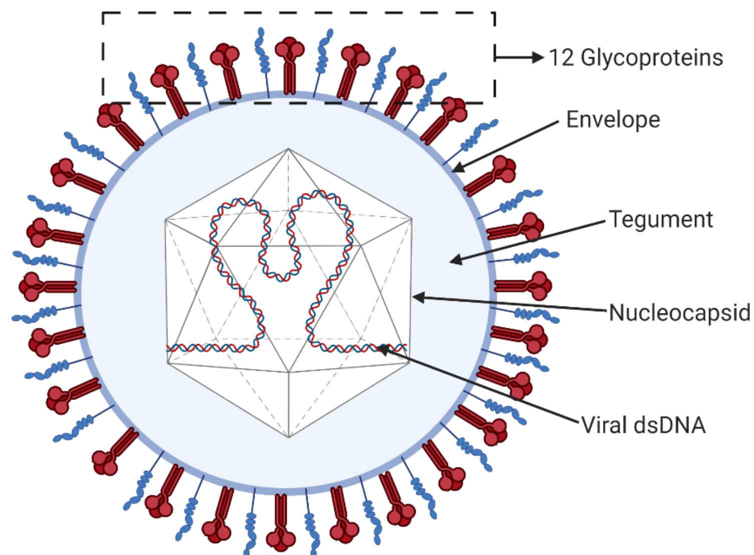

Figure 1: EHV-1 virion shown schematically. Modified according to Paillot et al. (2). dsDNA – double-stranded DNA. Created with biorender.com.

3.1.2 Prevalence, Transmission & Epidemiology

Prevalence and Transmission

EHV-1 affects the family *Equidae* including horses, mules, zebras and donkeys (8). Horses of all ages and sexes can be affected, however, young foals, older horses and immune-compromised horses are at a higher risk of infection (9). The prevalence is influenced by geographic factors, management practices, testing technology as well as sampled tissue (9). Reports claim outbreaks mostly occur at equine enterprises such as race tracks, riding facilities and veterinary clinics where animals from different locations and management styles congregate and a high stocking density is inevitably created (9).

EHV-1 is extraordinarily contagious and transmitted by a variety of routes (5): the direct and most critical transmission route is horse-to-horse contact (Figure 2; (1, 5)). Indirect transmission occurs through inanimate objects by coming in contact with virus-contaminated nasal secretions and aerosols, aborted foetuses or foetal fluids as well as latently infected equines (Figure 2; (1, 5)). The survivability of EHV-1 in the environment is reported to range from less than one week to up to one month (1). A recent study has found it to be durable and remain transmittable in water for weeks (Figure 2; (8, 10)).

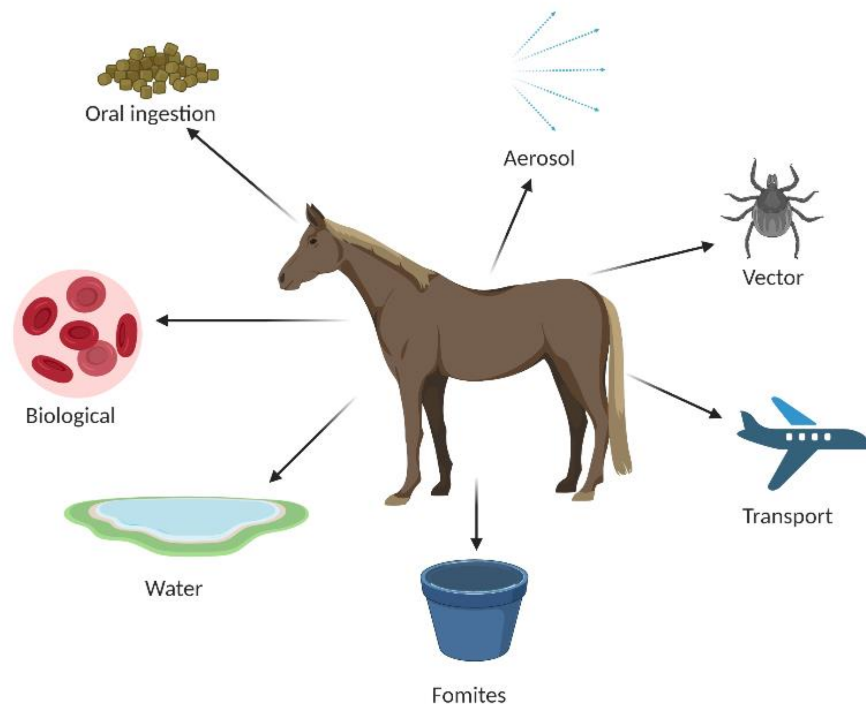

Figure 2: Modes of transmission of EHV-1. Adapted from Kapoor et al. (1). Created with biorender.com.

Reproductive transmission plays a crucial role as well: while trans-placental transmission of EHV-1 is well documented according to Hebia et al. (11), the venereal transmission route was proposed possible by one study as well since they detected EHV-1 in

semen of naturally infected stallions for up to 20 days post infection (12). Additionally, equine embryo transfer poses a risk for EHV-1 transmission to recipient mares, since recommended washing steps prior to inoculation have not proven successful in removing virus from embryos (11).

Latently infected horses have been identified as one of the principal reservoirs of EHV-1 transmission (5). They harbour the virus in trigeminal ganglia or lymphoid tissue presenting as source of viral particles which could be reactivated under stress (13). Stressors include strenuous physical exercise, transportation, pre-existing disease and a suppressed immune system (5). Once the virus has been reactivated and extended into the respiratory epithelium, it can be shed once more through nasal secretions thereby creating a source of EHV-1 infection to susceptible horses (refer to Figure 3) (13).

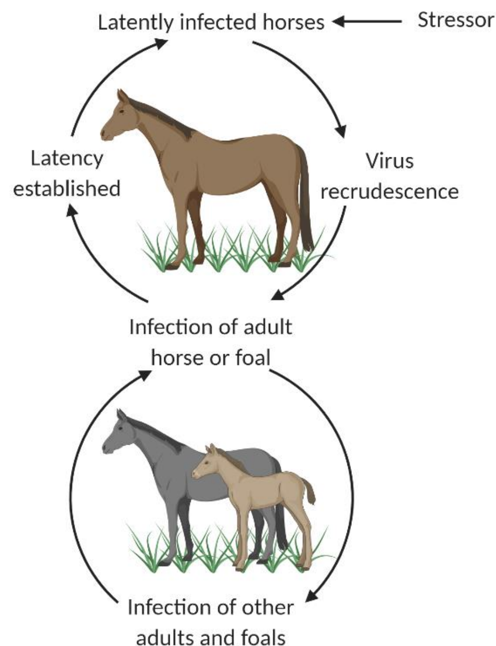

Figure 3: Transmission cycle of EHV-1 infection among horses. Adapted from Kapoor et al. (1). Created with biorender.com.

While Herpesviruses are usually species-specific and equids still remain the natural and definite host of EHV viruses, there is a small number of reports and occurrences on inter-species

transmission (14): EHV-1 has been detected in other equids including Persian onagers, various zebra species and Thomson's gazelles (15). Donkeys are a potential host for EHV-1 and serve as silent reservoirs as well (16). Highly unexpected transmission of EHV-1 has occurred to two polar bears in a German zoo with one case resulting in fatality (14). Another study found EHV-1 to have jumped to a female pregnant Indian rhinoceros, which aborted mid-pregnancy and suffered from severe neurological symptoms; this course of infection also resulted in fatality (14). Viral DNA isolated from this rhinoceros demonstrated 99% similarity with zebra-borne EHV-1 (14). Transmission in this case is proposed to have occurred through contaminated fomites, clothing of staff, aerosol transmission, or rodent vectors as the rhinoceros and zebra enclosures where not located close to each other and contact among animals can be excluded (14). Other species reported to have been infected with EHV-1 include deer, alpaca, gazelle and antelope, lamas as well as cattle according to Lecollinet et al. (8) and are considered as potential reservoir of the virus.

Epidemiology

EHV-1 infection occurs world-wide and can be considered to be one of the most successful pathogens of horse populations (8). While the majority of scientific examination of EHV-1 is focussed on the characterisation of the virus, epidemiological studies have been neglected (17). This is most likely due to regulations concerning reporting EHV-1 outbreaks. In Germany, EHV-1 infection is not mandatory to be reported to the authorities (18). In the USA however, outbreaks of the neurological disease are mandatory to report (19). Independently of these obligations, a number of studies are available that detected EHV-1 in individual countries or continents; a visual summary is given in the map below (Figure 4). The map indicates the distribution of EHV-1 outbreaks reported in the scientific literature, by the OIE WAHIS (World Animal Health Information System) and the Fédération Equestrian international (FEI) (18) since 2000 until 2020.

Figure 4: Distribution of EHV-1 outbreaks. EHV-1 outbreaks have been reported in countries marked in red according to scientific literature, the OIE WAHIS interface and the FEI from 2000 to 2020 (8, 10, 16-18, 20-33). Created with biorender.com.

Recently, in early 2019, the FEI (34) reported 70 new outbreaks in several European countries as well as Canada and the USA, which led them to adjust their veterinary regulations. Horses showing clinical signs or are known to have been in contact with diseased horses, are excluded from FEI events and may not be allowed to compete until FEI health requirements are fulfilled (18).

Due to a high contagiousness, it is estimated that at least 60 % of the world-wide horse population is latently infected with EHV-1, leaving the equine industry confronted with large economic impacts (8, 9, 19, 35). Biosecurity measures and quarantine interrupt the efficient workflow on breeding enterprises, training and competition schedules of sport horses as well as sales and auction events (9). While the occurrence of rhinopneumonitis and abortion due to EHV-1 infection are decreasing, the incidence of EHM outbreaks has increased over the past 20 years in most parts of the world (8, 21). EHM is now classified as an evolving disease of

horses (35). Once the disease has progressed to EHM or it being the sole symptom, the mortality rate is up to 50% (20, 21).

But not only well-developed countries suffer losses from this infection, in developing countries EHV-1 in equids such as donkeys and mules are detrimental to human survival as these equids present means of transport for humans and agricultural goods (16). They are at a greater risk of contracting and succumbing to the infection as they are subjected to heavy workloads, long distance travelling, poor condition, heavy parasite burdens and seasonally dependent availability of pastures (16).

3.1.3 Pathogenicity and clinical signs

EHV-1 infection is spread throughout the host's body by intercellular routes and involves many cell types (2). Cell infection and replication begins with EHV-1 utilizing glycoproteins to bind to glycosaminoglycans on the surface of permissive cells (2). Viral glycoprotein gD and gM are recruited for the entry into cells (2). Once the virus is attached, it penetrates the cell either by fusion of the virus envelope with cell membranes or by non-classical endocytosis (2). This process causes a discharge of viral nucleopcapsid and tegument proteins into the cell (2). The viral nucleocapsid binds to the nucleopore-complex of the cell thereby translocating viral DNA into the cell's nucleus, while the nucleocapsid remains in the cytoplasm (2). Immediate early (IE) genes are stimulated by tegument proteins and in turn activate transcription of early and late genes (2). Early genes stimulate virus replication, late genes translate viral structural proteins (2). Within the cell nucleus, the new EHV-1 nucleocapsid is assembled around scaffolding proteins and new viral DNA becomes encapsidated (2). The new nucleocapsid, surrounded by tegument proteins, leaves the nucleus and becomes enveloped in the inner nuclear membrane, including glycoproteins (2). This envelop dissolves when the new viral nucleocapsid buds through the outer nuclear membrane

(2). It now becomes enveloped again in cytoplasmic membranes which contain all viral surface glycoproteins (2). The new mature viral particle migrates through the secretory pathway (Golgi apparatus) and is released either into extracellular space or infects other cells by virus induced cell fusion (2).

First site of infection are nasal and nasopharyngeal epithelial cells in absence of mucosal antibodies (2). Epithelial cell necrosis and acute inflammatory responses lead to erosion within the first week after infection (2). The virus can now spread quickly and be shed to other horses (2). The next type of cells to be infected are leucocytes and endothelial cells of blood vessels and lymphatic vessels due to cell-to-cell spread from the respiratory epithelium (2). The endothelium of blood vessels becomes infected within two to four days post infection (2). Respiratory symptoms in young horses include fever, serous to mucopurulent nasal discharge, coughing and lymphadenopathy (2). In older horses respiratory symptoms are expressed mildly or subclinically (2). EHV-1 infection mostly affects the upper respiratory tract (URT) but disorders of the lower respiratory tract are possible e.g. bronchopneumonia (2). If submandibular, retropharyngeal and bronchial lymph node cells become infected (2, 5, 19), during the second stage of illness, cell-associated viremia is established and the virus can spread rapidly (2, 5). The infection is amplified by lymph node cells including different types of leucocytes (e.g. macrophages, endothelial cells), which will circulate throughout the blood and the lymphatic systems, thereby reaching distant locations for instance the uterus and central nervous system (CNS) via cell-associated viremia (2, 5, 19, 36, 37).

In 1932 in Kentucky, EHV-1 was first reported to be the responsible agent for abortion as reported by Paillot et al. (2). EHV-1 is transported to endometrial endothelial cells inducing thrombosis and ischemia in microcotyledons of the placenta resulting in premature separation of the placenta from the endometrium followed by death of the foetus, late-gestation abort of a virus-negative foal, stillbirth or weak neonatal foals (36). *In vitro* studies have observed that

chorionic gonadotrophin (CG), a major hormone released during pregnancy, reactivates latent EHV-1 (2). The higher susceptibility of pregnant mares is therefore believed to be linked to CG expression by the endometrium (1, 36, 38). Interleukin-2 is also considered to have an effect on reactivation since both, interleukin-2 and CG have been found to initiate reactivation of the virus from venous lymphocytes in an indirect fashion by releasing mediators form adherent cells which reactivate EHV-1 from T-cells (38). The less severe vasculitis may allow focal transfer of virus across the utero-placental barrier, infecting the foetus and cause multi-organ failures and macroscopic and microscopic lesions leading to abortion as well (1, 2, 36). If affected foals are born, they usually succumb to respiratory failure (2).

Cell-associated viremia can also transport EHV-1 to the vasculature of the CNS (2). Endothelial cells of the CNS become infected, small blood vessels in brain and spinal cord experience vasculitis and thrombosis (2). Clinical signs of involvement of the CNS range from mild hind limb ataxia to quadriplegia and usually occur one week after infection (2).

The severity of neuropathogenicity and abortigenic potential is considered to be strain-dependent (37, 39). A single nucleotide polymorphism (SNP) in the viral polymerase (Pol) gene within (ORF30) is contemplated the decisive indicator for the neuropathogenic aptitude of the strain (Figure 5) (16, 39, 40).

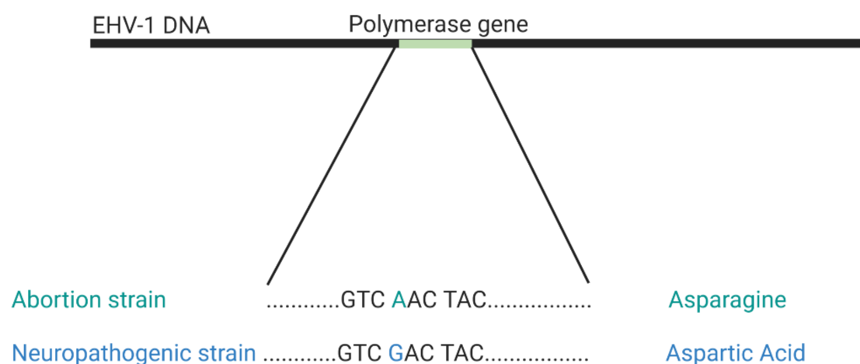

Figure 5: Schematic overview of SNP in EHV-1 Pol gene within ORF30 and resulting viral strains. Created with biorender.com.

There is a difference between A_{2254}/N_{752} and G_{2254}/D_{752} Pol variants in regards to their capability to cause EHM disease outbreak (16, 39, 40). While G_{2254}/D_{752} variant is mostly isolated from EHM cases, A_{2254}/N_{752} is mainly isolated from abortion incidences rather than EHM incidences (39). Neuropathogenic EHV-1 strains reproduce faster and achieve 10-fold elevated levels of leucocyte-associated viremia as compared to non-neuropathic strains (16). Other factors that are affecting the risk of developing EHM are breed, sex, age or vaccination status (39). Most studies and reviews report EHM outbreaks in horses older than 2 years of age (39). The neuropathogenic variant G_{2254}/D_{752} Pol has become more predominant in recent years according to Walter et al. (39). Furthermore, EHV-1 strains are classified into high virulence strain and low virulence strain depending on their effect on high levels of viremia and endotheliotropism (39). The high virulence strain Ab4 has been known to cause abortion and neurological disease on countless occasions (37). The low virulence strain V592 on the other hand, has reported to merely result in level viremia and restricted endotheliotropism (37). Nowadays genetically differing strains of EHV-1 are in circulation simultaneously (37).

3.1.4 Latency & Immune evasion strategies

Latency

The ability to establish life-long latency in their respective host is one of the trademark properties of herpesviruses (41). Alphaherpesviruses utilize long-term latency as an epidemiological strategy avoiding an active host immune system to ensure their spread and survival within the host population (35, 36, 42, 43). During latency, the virus is expressed at a low, nearly undetectable rate, yet is able to re-infect the host and in turn be spread to other horses while the host is not eliciting symptoms, obvious virus shedding or cell-associated viremia (2, 35, 42). Latently infected horses are termed silent shedders and pose a threat to susceptible horses by transmitting the virus through nasal secretions or aborted foetuses (2, 35,

36, 42). EHV-1 infection can periodically be re-activated from the latent state either spontaneously or after induction by external stimuli e.g. relocation and transport, training and competitions, illnesses, operations and treatment (2, 35, 36, 41, 44, 45). In particular, treatment with corticosteroids has shown to re-activate latent virus (2, 35, 36, 41, 44, 45).

Generally, a productive EHV-1 infection activates viral gene expression (35). During latency viral gene expression is restricted and synthesis of viral factors is not possible, hence infectious viral particles are absent (35). The primary site harbouring circulating latent EHV-1 are considered to be lymphocytes as established in numerous studies (35, 46, 47). Eighty percent of CD5+/CD8+ have been verified to enable latency; CD8-/CD4- have also been found to enable establishment of latency (35, 38). Other studies have proven latent EHV-1 to be harboured within the sensory nerve cell bodies in the trigeminal ganglia (35, 45, 48, 49). During latency, the viral genome is concealed while latency-associated transcripts, antisense to IE viral genes or regulatory early viral genes are detectable (2, 35, 38, 41, 49). The exact molecular and physiological mechanisms are still poorly understood and transformation to a latent stage is considered a deliberate biological behaviour (35). Suggestion however include, that lymphocytes advance toward active transcription resulting in DNA restoration and active virus replication (45) or IE gene promoter being trans-activated in presence of another equine Herpesvirus (38). Understanding molecular and physiological factors reactivating the virus are vital to comprehend, not only to grasp reactivation and reproduction thereafter, but also for epidemiological reasons (43).

Immune evasion strategies

The conventional immune response to epithelial cells becoming infected by a respiratory virus begins with the induction of synthesis of interferon (IFN) and interleukin-6 (2). These induce the up-regulation of major histocompatibility complex (MHC) class 1 molecules, which will bind viral peptides and present them on a virus-infected cell's surface for recognition by

lymphocytes (2). Meanwhile, antiviral resistance in uninfected cells is stimulated by IFN and interleukin-6 (2). Pro-inflammatory cytokines are released by macrophages and neutrophils, thereby restraining infection, elevating body temperature and recruiting phagocytic and natural killer (NK) cells (2). Macrophages will also synthesise IFNα and interleukin-12, which recruit further NK cells, which will exert their cytotoxic activity on infected cells (12). NK cells synthesise IFNγ, thereby urging the development of the adaptive immune system elicited in nasal associate lymphoid tissue and mucosal associated lymphoid tissue (2). The synthesis of serum or mucosal antibodies, is induced by viral antigen in lymphoid tissue and are exerting their neutralising activity and enhance humoral antibody dependent cell cytotoxicity (ADCC) (2). Virus-specific cytotoxic T- Lymphocytes (CTL) are stimulated as well and lyse virus-infected cells (2). All of these immune effector mechanisms are orchestrated by chemical messengers termed cytokines (50, 51).

Throughout evolution, herpesviruses, including EHV-1, co-evolved with their host and developed immune evasion strategies to circumvent each of the effector mechanisms as well as the cytokine orchestration (2, 50): during the early phase of infection, customarily, immediate protection is provided by the innate immune response. EHV-1 developed strategies to avoid this innate or non-specific immune response by tempering with its recognition mechanisms: glycoprotein C of EHV-1 binds to a protein within the immune system which usually activates the complement pathway and would, under normal circumstances, generate a membrane-attack-complex. By binding to this protein, this mechanism is blocked.

EHV-1 circumvents the specific immune response by avoiding the virus neutralizing antibodies by becoming intracellular and undetectable rapidly within a few hours after infection (43, 50). Moreover, EHV-1 glycoproteins form a complex mimicking an antibody and thereby blocking antibody–mediated functions such as complement activation or ADCC (43, 50). EHV-1 infected peripheral blood mononuclear cells (PBMCs) cannot express the virus on their

surface, also leaving these cells undetectable to the immune system and insensitive to ADCC and CTL (50). A further evasion strategy of EHV-1 is the interference with NK cell-mediated lysis (50): if cells do not present viral proteins or MHC-1 post infection and are rendered insensitive to ADCC and CTL recognition and destruction, they may remain vulnerable to lysis by NK cells (50). Yet, herpesviruses have developed evasion strategies to avoid detection and activation by NK cells as well (50): EHV-1 is able to lower the concentration of MHC-1 presented on the cell surface, thereby avoiding recognition by NK cells (43, 50, 51). Downregulation of MHC-1 has been found to be strain dependent (43): while EHV-1 viral Ab4 induced significant downregulation, viral strain RacL11 only led to mild downregulation (43). Additionally, it is not only virus strain dependent but also locus or allele dependent (43).

EHV-1 also evades or interferes with the cytokine network by either mimicking cytokines or blocking their activity: usually, cytokines act as chemical messengers between cells during an innate or adaptive immune answer (43, 50). Upon secretion, they bind to cytokine receptors immune cells and induce their functions (43, 50). Viruses might mimic these cytokines' activities by emitting cytokine binding proteins themselves or introducing cytokines with inhibitory action on immune cells (50). EHV-1 employed an immune suppressive activity in ponies which was believed to be connected with a circulating cytokine, transforming growth factor (TGF), exerting multiple inhibitory effects on immune cells (50). Once again, it is the viral surface proteins interfering with cytokine mediated immune response (50). Viral surface protein gB has the ability to bind to certain chemokines and thereby obstructs interaction with receptors, proper signal transduction and migration (43, 50). Ultimately, it will inhibit chemokine-mediated inflammatory reactions (50).

3.1.5 Diagnosis

The most widely used and preferred method to confirm EHV-1 infection is polymerase chain reaction (PCR) as it has proven to be a reliable, rapid and sensitive method, which does not depend on the infectivity of the virus (8, 52, 53). Templates are easily obtained by aseptical collection of nasal or nasopharyngeal swabs during viral replication in the nasal mucosa or whole blood (EDTA) samples (8, 52, 53). It is recommended to obtain swabs during the acute, febrile phase of the disease via the nares (53). Extraction of DNA can be carried out using commercially available kits (8, 52, 53).

Since the nineties researchers have developed a number of PCR protocols: Borchers and Slater (54) developed a nested PCR that enabled detection as well as differentiation between EHV-1 and EHV-4. Since nested PCR is prone to cross-contamination, the sensitive rapid one-step protocol developed by Lawrence et al (55) was preferred for differentiation (53). Varrasso et al (56) developed a semi-nested multiplex PCR for the distinction of EHV-1 and EHV-4 before Diallo et al. (57) developed a real-time PCR that could detect EHV-1 without further need for restriction enzyme analysis (REA) and was less prone to cross-contamination (57). Diallo et al. (58) also developed a multiplex PCR that would differentiate EHV-1 and -4 in a single reaction (58), which is the most widely used protocol to date and the current standard method at the World Organisation for Animal Health (OIE) reference laboratory (20, 53).

The next step in PCR diagnosis was the development of strain typing methods to differentiate between EHV-1 strains carrying the ORF30 neuropathogenic marker SNP (A_{2254} to G_{2254}) to become aware of the risk of neurological complications in outbreak situations (53). For this purpose, Allen et al (59) developed a single-step quantitative RT-PCR, that could differentiate neuropathogenic from non-neuropathogenic EHV-1 strain (59). Fritsche and Borchers (60) also developed a conventional PCR with adjacent REA enabling strain-typing

(60). Samples most useful for strain-typing are obtained from aborted foetuses, adult horses' cerebrospinal fluids, placenta, lung and liver, EDTA blood and nasal swabs (61).

PCR results should be complemented with laboratory isolation of the viral agent in cell culture, as PCR may not be sufficient and should be interpreted in correlation to virus isolation results (8, 53, 56). For virus isolation techniques, viral material is best obtained from nasal or nasopharyngeal swabs obtained during the febrile phase of URT infection, but also from placenta, liver, lungs, spleen or thymus of aborted foetuses or corpses, or from the leukocyte fraction of acutely infected horses (53). EHV-1 is easily propagated in equine as well as non-equine cells (53); this ability, viral propagation in non-equine cells, is a convenient method to distinguish EHV-1 from EHV-4, since EHV-4 can only be isolated from equine cells (53).

Other methods aiding EHV-1 detection and diagnosis include direct immunofluorescence in cryostat sections of placental and foetal tissue for rapid, yet presumptive diagnosis (53). It is not a type-specific method and must be combined with PCR or virus isolation (53). Placental and foetal tissue are also useful in post-mortem examination when histopathological lesions are observable (53). Histopathological examination can detect intra-nuclear inclusion bodies, characteristic for herpesvirus infection as well as miscroscopic lesions (53). Serology is another detection method for EHV-1 infection, however, results are to be interpreted with care, since most horses are either acutely or latently infected, most horses will demonstrate a positive antibody titre (52). Further considerations include vaccination history and possible cross-reactivity with EHV-4 (52). A type-specific ELISA has been developed but is not available for commercial use yet (62). Other laboratory methods that have been used in recent studies include ORF30 sequencing as well as Multi Locus Sequence Typing to determine and emphasize diversity of EHV-1 strains encountered (10).

Some challenges present themselves when diagnosing EHV-1 infection: latently infected animals are not shedding infectious virus during the latent stage and the titre may be

below detection limits (52). Latent infection cannot be excluded from a seronegative horse (52). Moreover, latently infected cells are difficult to obtain since they are harboured in the trigeminal ganglion (52). Neurological disease detection is challenging, since there is no one specific method to diagnose it (52). However, sampling cerebrospinal fluid of febrile horses and testing it for EHV-1 infection would meet this gap of EHM diagnosis in the alive horse, but are challenging to obtain (52). Diagnosis of EHM before death is presumptive and must take clinical, epidemiological and laboratory results into consideration including the exclusion of additional possible origins of neurological disease (52). Similarly to diagnosing respiratory disease caused by EHV-1, it must be kept in mind, that several pathogens can cause URT disease and the possibilities of detecting subclinical EHV-1 is likely, when in fact another pathogen is aetiologically involved in the development of respiratory disease (52). Diagnosis of all three main diseases (respiratory, abortion, EHM) should be a combination of clinical history, appearance and laboratory results in nasal swabs, blood, and foetal tissue by the abovementioned laboratory techniques (52).

3.1.6 Prevention & Treatment

Biosecurity

Prevention and control measures can be divided into several sections: biosecurity through managerial strategies and vaccination. The goals of managerial control measures are either prevention or reduction of the likelihood of outbreaks or limiting transmission of disease once outbreak occurs (9). Actions to attain biosecurity goals include isolation of new or returning horses arriving at the enterprise before introducing them to resident horses (9, 52). Additionally, dividing herds into smaller herds according to age and risk of disease development e.g. high shedders such as foals and weanlings, could be advantageous (9, 52). In case of an outbreak, strict biosecurity measures have to be put into place as soon as possible to

stop virus spreading (8): isolation and quarantine of horses exhibiting clinical symptoms or respiratory disease is mandatory to screen and separate potential spreaders or virus shedders from unaffected horses (8, 52); enlistment of specific care takers for affected horses and provision of staff with distinct supplies such as gloves, boots, coats and provision of a footbath at entrance and exit ways (8). If it is not possible to enlist specific care takers, healthy horses should be handled before isolated horses, ideally with separate or disinfected equipment (52). Disinfection of all tools and buildings should occur on a regular basis (52). Three weeks of quarantine is advised once the last reported case is declared free of symptoms (8). Preventing recrudesce, minimization of stressors such as transport, incompetent handling, competition for food and shelter or insufficient pasture quality must be afforded (9, 52). Additionally, development and adherence to a vaccination schedule is strongly advised (9, 52)

Vaccines

EHV-1 vaccines were first introduced in the 1960ies (1, 2). Vaccines offer a high degree of safety since they stimulate an immune responses that limit virus replication, infection and development of disease as well as cell-associated viremia and re-activation of latent virus (1, 2, 9). Currently licensed vaccines against EHV-1 (see Table 2) have proven to decrease clinical symptoms of rhinopneumonitis, viral load in nasal mucus, reduce transmission and aid in the prevention of abortions (1, 2, 8, 53). These vaccines are either inactivated vaccines or live attenuated vaccines and contain different permutations of EHV-1 targeting envelope glycoproteins of the virus (1, 2, 8, 53). Different vaccines have different aims: while one vaccine aims to prevent EHV-1 related rhinopneumonitis, another vaccine aims to prevent abortion; a few vaccines claim to aid protection against both (2, 53). All currently available vaccines do not provide protection against EHM but enable decreased viral load and transmission in a vaccinated population, when a schedule of two primers at a one month interval (3 to 4 months

concerning the live attenuated vaccines) and half-yearly to yearly booster vaccination is adhered to (8, 9, 53). Vaccination schedules can however vary with vaccine (53).

Table 2: Vaccines licensed for use in Germany by Paul-Ehrlich-Institute (accessed 30.08.2020).

Vaccine type	Vaccine	Marketing authorization holder
Inactivated	BIOEQUIN H®	BIOVETA a.s. Czech Republic
		Vetcool B.V. Netherlands
	EquiShield EHV	Dechra Regulatory B.V: Netherlands
	EQUIP EHV1,4®	Zoetis Deutschland GmbH
Live attenuated	PREVACCINOL® (only licensed in Germany)	MSD Animal Health
		Intervet Deutschland GmbH

Nowadays, inactivated vaccines are widely used and have been reported to elicit a strong humoral response by eliciting complement fixation and virus neutralising (VN) antibody production (1, 2). Additional advantages of inactivated vaccines are absence of pathogenicity, virus replication and subsequent spreading (2). Disadvantageous outcomes of vaccination with inactivated vaccines might be local intolerance, fever and the risk remaining of incomplete inactivation and subsequent possible infection (2). Despite its wide spread use, inactivated vaccines remain problematic since numerous studies have generated controversial results: while some report success in reducing clinical symptoms and transmission (63), others report no response to vaccination in vaccinated mares and vaccinated foals (64). Moreover, abortion storms still occur despite vaccination (65).

Live attenuated vaccines consist of live microorganisms or genetically modified organisms used as vector for the pathogen's antigen and elicit an immune response similar to natural infection (1). They are often administered intra-nasally, to induce a local immune response at the primary site of infection (1, 9). Live attenuated vaccines do not establish virulence or latency, yet remain immunogenicity and stability; however the risk of reversion to virulence is apparent and remains a problem (1).

Since currently available vaccines have generated controversial results, they are slowly but surely being replaced by other vaccine compositions. Complex-based subunit or split vaccines against EHV-1, containing fragments of a pathogen, have been trialled *in vitro* in rodents and produced induction of a humoral response (66). Further vaccine compositions currently under investigation include, DNA vaccines (67), non-infectious EHV L particles (68) as well as poxvirus-based vector vaccines (69); these are either being trialled or improved, as they proofed immune-stimulation.

Inducing protective immunity against EHV-1 remains a substantial challenge and a general conclusion is that protection against EHV-1 will require neutralizing antibodies as well as CTL response (9).

Treatment options

Despite vaccination schedules, outbreaks might still occur as they cannot completely eliminate viral shedding, prevent viremia or the outbreak of EHM (70). On top of that, treatment options and their efficacy are currently only of theoretic value. The only currently recommended treatment options consist of anti-inflammatory agents as well as antiviral agents. Anti-inflammatory medication includes dexamethasone and prednisolone as well as free-radical scavengers (52). Aspirin in combination with Vitamin E supplementation has also been suggested to be efficacious (52, 71). Other supportive treatments such as antimicrobials can also be implemented depending on the individual horse's condition (52). Antimicrobials have been reported to reduce viral loads after infection with Epstein Barr virus, a human *Herpesvirus* (72).

Antiviral medication in connection with EHV-1 has been proposed to reduce viral shedding and found to improve the clinical outcome of EHM (52, 70). Currently available antiviral medication for the treatment of *Alphaherpesvirinae* infection in humans and equines are nucleoside analogues (70). These have been found to reduce the convalescent period as well

as rate of transmission by decreasing the amount of infectious viral particles (70). The key feature of nucleoside analogues is the interaction with the DNA polymerase of the virus, which under normal circumstances would be heavily involved in virus replication (70). The nucleoside analogues thereby interfere with the replication process by either competing with the natural deoxyribose nucleoside triphosphate (dNTP) substrate or being incorporated as alternative substrate, both essential mechanisms for antiviral activity (70). Antiviral agents Acyclovir and Ganciclovir have been developed in the late 1970s and were highly specific towards herpes simplex virus and Varicello Zoster virus (70, 72). Since Acyclovir proved highly efficacious in humans, it has been trialled against EHV-1 infection on numerous occasions and also been found effective in *in vitro* studies and proposed to potentially decrease severity of EHM thereby increasing survival rates (52, 70, 72). Acyclovir was also successful in reducing the number of viral genome copies (72). The next generation, prodrug, of Acyclovir, Valacyclovir has been proposed as an alternative nucleoside analogue, with improved bioavailability over acyclovir, and may provide an option for treatment of EHM-diseased animals and is currently recommended for treatment in the case of an outbreak (52, 70).

Other antiviral medications have also achieved promising results in *in vitro* studies: Ganciclovir has proven to be efficacious against three differing strains of EHV-1 *in vitro* and presented more effective than Acyclovir overall (70, 72). A further antiviral agent trialled against EHV-1, Penciclovir, has limited virus replication in respiratory tissues and reduced viremia in murine models in *in vitro* studies (73). Cidofovir, has shown promising results against equid herpesviruses but only *in vitro* so far (70). Many more compounds are being trialled in the antiviral sector such as Foscarnet or Guanosine analogues A-5021, deemed to be more potent than Acyclovir (70). Other compounds targeting other replication mechanisms such as helicase primase are also under development (70). A recent study also detected Spironolactone, a mineralocorticoid and antagonist, to be a potential new inhibitor of EHV-1 (72). However, no antiviral therapeutic agent has had an effect in horses *in vivo* yet (70, 72).

Results in regards to EHV-1 remain controversial and further support that research and protection against EHV-1 will require equine-specific antibodies to elucidate viral mechanisms (9).

3.2 Recombinant Antibodies

3.2.1 Antibody structure and function

Antibodies are soluble immunoglobulins (Igs) found in blood of all vertebrates. Most vertebrates , including the horse, have five main classes of Igs: IgG, IgA, IgM, IgD and IgE (74). This classification is based upon differences in structure and function, which is depicted in Figure 5 and summarised in Table 3 (74).

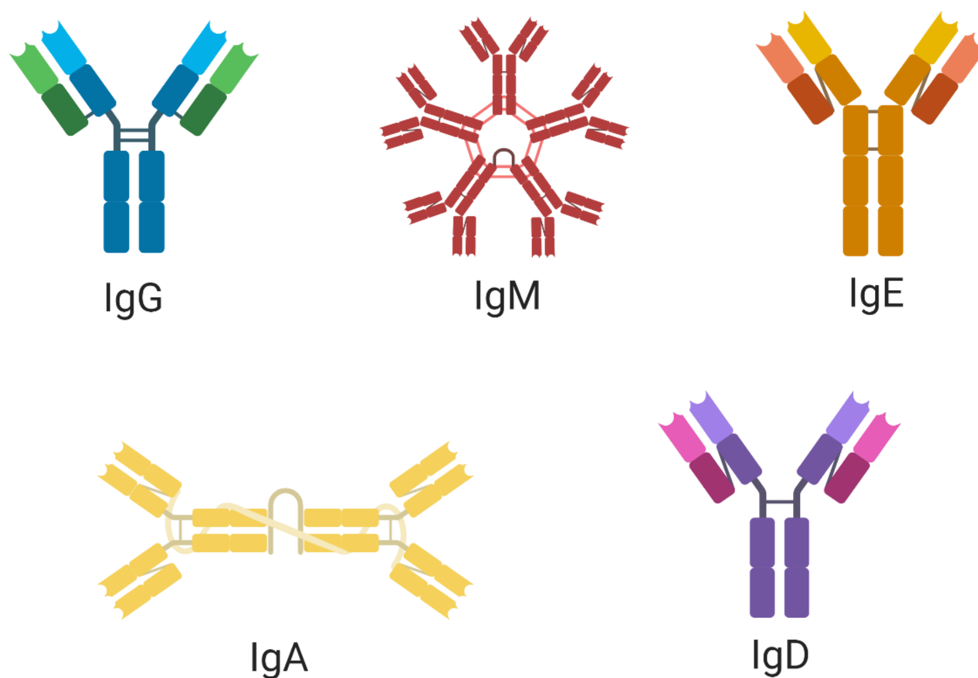

Figure 6: Schematic overview of all five equine immunoglobulin isotypes created with biorender.com.

Table 3: Overview of all five equine immunoglobulin isotypes, including their structure and function.

Immunoglobulin	Structure	Function
IgM	Monomer, Pentamer	Primary Immune response; agglutinates invading pathogens, promotes lysis and phagocytosis
IgD	Monomer	B-cell receptor and B-cell eliminator
IgG	Monomer	Enhances phagocytosis, neutralizes toxins, inactivates virus, kills bacteria, initiates ADCC
IgA	Monomer, Dimer	"Gate-keeper"; prevents further dissemination of pathogen
IgE	Monomer	Mediator of allergic reactions, lysis and degradation of protozoa, helminths and arthropods; initiates inflammation

These main classes are further divided into subclasses which are differentiated according to the number of disulphide bonds connecting antibody structures (74). Each Ig class has a certain amount of subclasses, which varies in between species. Horses are outstanding in this context since they possess seven subclasses of IgG (75-77). IgG is by far the most versatile of all isotypes and can offer all antibody functions such as antigen binding, complement fixation and binding to various cells and contributes 75% of all antibodies (77). This versatility is further enhanced by the large amount of subclasses, IgG1 to IgG7, which are classified according to differences within their genetic composition (75-77). Each subclass retains differing antigenic defence properties and thereby takes specific roles in protective immunity (77). For instance, IgG1, IgG4 and IgG7 play a key role in limiting the spread and severity of EHV-1 (77).

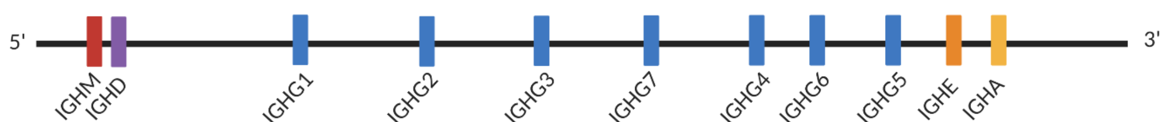

Figure 7: Map of the equine IGHC region depicting all immunoglobulin isotypes; in particular seven subclasses of IgG. Boxes indicate IGHC genes. Map is adapted from Wagner et al. (75) and Sun et al. (76). Created with biorender.com.

The antibody structure is best explained based on the most abundant IgG molecules (74): there are four subunits to its Y-shape structure composed of two identical heavy chains

and two identical light chains (refer to Figure 7) (74). Light chains either belong to the κ-or λ-type (74). Constant regions of the heavy chains belong either to α, γ, μ, δ or ε types (74). All subunits connected by covalent disulphide bridges in between cysteine remnants and participate in building the antibody molecule (74). Some domains of the subunits remain constant, while others domains differ from molecule to molecule (74). Hence one refers to the constant region and the variable regions of an antibody (refer to Figure 7) (74). The constant region is mainly responsible for effector functions such as complement activation, while the variable regions (Fv) is mainly responsible for specific antigen binding (74). Forces driving this binding procedure are either electrostatic, van-der-Walls forces or hydrogen bonds and are expressed as the affinity between antibody and antigen (74). The higher the affinity of an antibody, the less the necessary concentration to reach equilibrium, meaning saturation of all antigens by antibodies (74).

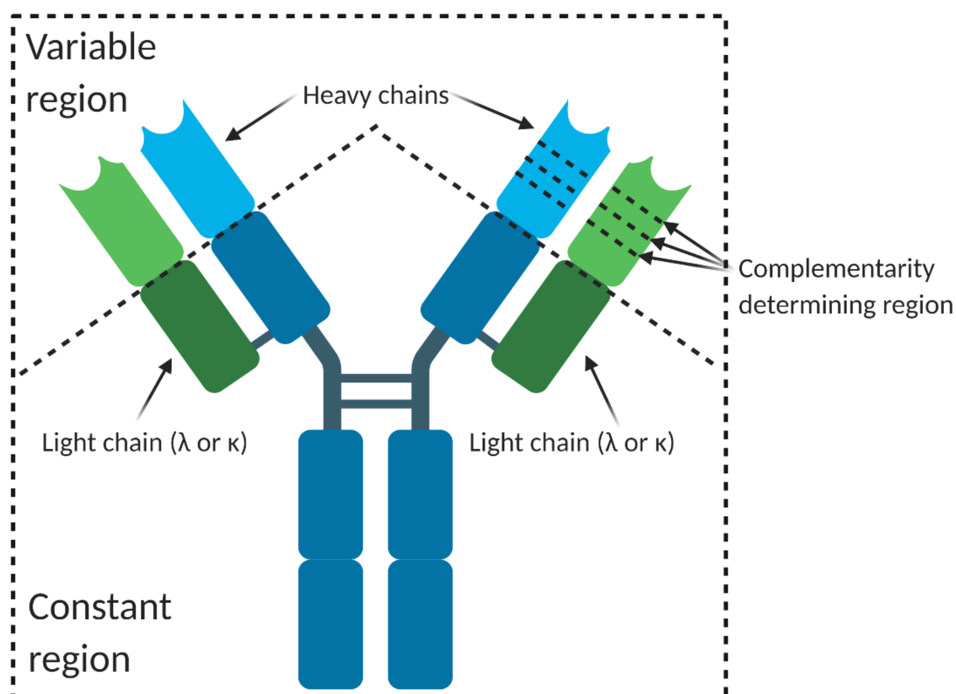

Figure 8: Schematic antibody structure based on IgG. Created with biorender.com

3.2.2 Antibody development and diversification

The constant region as well as the variable region of each chain are encrypted by individual gene segments (78): variable (V) gene segments, diversity (D) gene segments and joining (J) gene segments and constant gene segments (C) (78). The heavy chain is usually arranged into a VDJC pattern and the light chain into VJC. (76). The variable region is further divided into three hypervariable regions, the complementarity determining regions (CDRs) embedded in between four stable framework (FRs) regions (78). CDRs are epitope-specific paratopes of an antigen, which enclose the epitope of the antigen, similarly to a key-lock-principal (74).

The number of gene segments is species-specific and horses stand out among mammals since they possess 50 immunoglobulin heavy chain V genes (IGHV) of which 12 are functional genes, 33 are pseudogenes and five are ORF, 40 immunoglobulin heavy chain D genes (IGHD) genes, 11 immunoglobulin heavy chain C genes (IGHC) genes and eight immunoglobulin heavy chain J genes (IGHJ) genes on heavy chain gene loci, contig Un0011, along with two further functional IGHV genes, one ORF and one pseudogene on contig Un0038 (75-77). Further IGHC are available on chromosome 24 comprising 11 genes (75-77).

Horses express two different types of light chains, kappa (κ) and lambda (λ); their differences lie within their amino acid residue motifs, antigenic properties and chemical structure (77). Equine λ light chain genes are located on *Equus caballus* (ECA) chromosome 8 and contain 144 IGLV genes, seven IGLJ genes and seven IGLC genes (75-77). Equine IGLV genes are divided into two clusters according to their transcriptional orientation, both containing functional and pseudogenes. Presenting two clusters is an exclusive feature only occurring in equines (75-77). Equine κ light chain genes are located in one cluster on ECA15, containing 60 IGKV genes, five IGKJ genes and one IGKC gene. Ninety-five percent of equine serum antibodies combine λ light chains with heavy chains (77).

Due to these large numbers of available V gene segments, horses have enhanced combinatorial possibilities: the diversity of antibodies is usually generated by V(D)J recombination, during B cell development. Gene segments are surrounded by recombination signal sequences (RSSs), which are paired at the beginning of recombination by RAG1 and RAG2 proteins, which will induce breaks between RSSs and coding segments. The non-homologous end joining pathway initiates joining of the ends of different genes segments and the RSSs to form coding and signal joints (76). Differing combinations of V, (D) and J segments combined with junctional diversity produced during imprecise processing, result in an immense repertoire of primary B cell receptors. Additionally, binding affinity of antibodies to antigen is improved when B cells undergo somatic hyper mutations (SHM) in form of nucleotide substitutions, insertions and deletions; CDRs are considered most prone to SHM (76). Horses are hypothesized to develop the majority of antibody diversity through SHM (75).

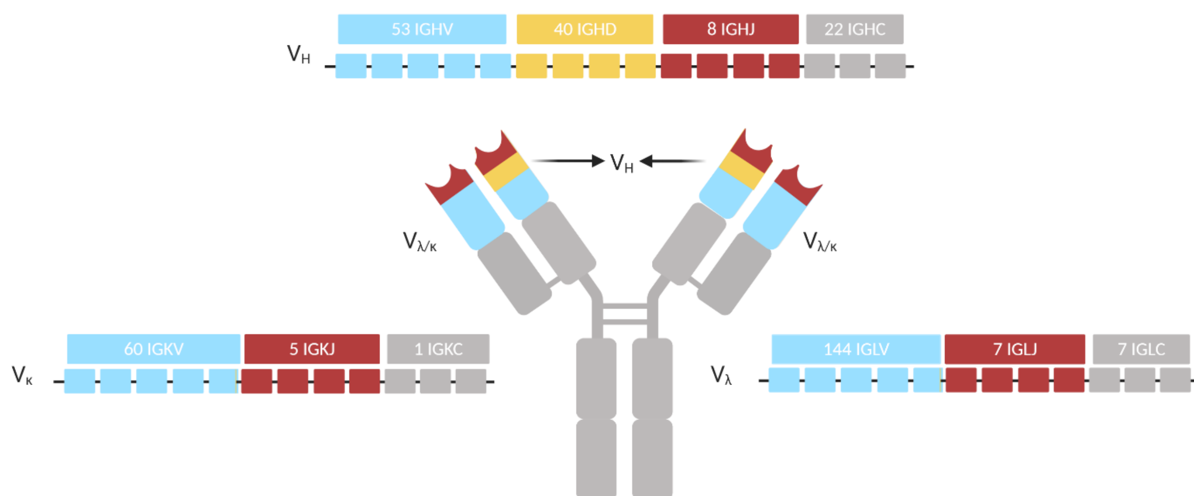

Figure 9: Germline configuration of equine immunoglobulin heavy and light chain loci, including number of gene segments available to horses. IG – immunoglobulin; H - heavy chain, K – κ light chain, L – λ light chain. V – Variable, D – diversity J – joining, C - constant. Created with biorender.com.

3.2.3 Recombinant antibodies and antibody libraries

Recombinant antibodies

Ever since Behring and Kitasato (79) discovered that specific binding molecules can be extracted from blood (80), antibodies became popular tools in research and diagnostics, due to highly specific binding affinities and great stability. Emil von Behring is since considered the founder of antibody and antitoxic serum discovery (79). Him and his colleagues immunized rats and rabbits in a laboratory setting, extracted serum and tested it in children, suffering from diphtheria with favourable results and called it antitoxic serum from thereinafter (81). Following this result, the firm Hoechst began to immunize rodents in a factory setting in 1892 and began production of the antitoxic serum for commercial use (81). Emile Roux and Louis Martin were the first to immunize horses and thus enable production of antitoxic serum on a large scale (81). In 1901 ,both Behring and Roux (Figure 10) received the Nobel Prize for their discoveries (81).

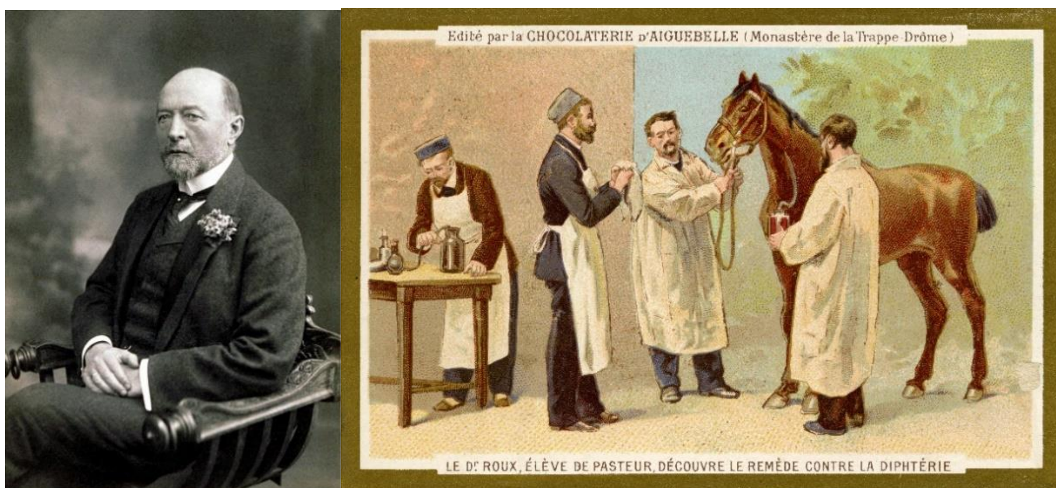

Figure 10: On the left, Emil von Behring. On the right, Pierre Emile Roux producing antitoxic sera from horses to treat diphtheria.

The classical pathway for the generation of antibodies used to begin by immunizing laboratory animals with the antigen of interest (80). Within the following weeks, desired antibodies were detected in the blood serum of these animals, so called antiserum, was extracted

(80). Antisera contain a mixture of different antibody proteins of different binding affinities including antibodies present prior to immunisation (80). The mixture consists of polyclonal antibodies, which are secreted by several B cells and react against the specific antigen, yet each identifying a different epitope of the antibody.

In the 1970ies Köhler und Milstein (82) developed a new technology to produce antibodies: hybridoma technology (80). This technology also relies on immunisation, but antibodies are not extracted from blood sera (80). Instead, B lymphocytes are isolated from spleens (80). Each B lymphocyte produces only one unique antibody, due to its unique genetic arrangement with one single binding affinity (80). Clones of this B lymphocyte will produce specific antibodies of a single specificity (80). To initiate this process, B lymphocytes are propagated in cell culture in *in vitro* production, fused with plasma cells and thereby generating hybridoma cells (80). Hybridoma cells contain properties of both, the B lymphocyte as well the plasma cell (80). They possess the immortality of the plasma cell, and produce specific antibodies similar to the B cell (80). Since these antibodies are only specific to one epitope, they are called monoclonal antibodies (mAbs) (80). MAbs have been favoured from this point on as they can be produced in unlimited amounts, easily standardized and bind to any antigen (83). Moreover, hybridoma technology results in immortal cells that can be produced and harvested repeatedly (80). However, the fusion of cell types also risks contamination with B lymphocytes producing irrelevant antibodies (80). Hence thousands of screening and selection procedures are required to test B cell clones for their specificity (80, 83). Additional difficulties arise when these mAbs are administered to treat human conditions, as they are exclusively murine in origin and could therefore induce a human anti-mouse antibody, also known as HAMA reaction, which limits the clinical application, if the antibody therapeutic is not species-specific (83). Moreover it does not produce high-affinity binders (83).

The most recent development of antibody production relies on genetic engineering by producing recombinant antibodies (80). Immunisation of laboratory animals is no longer necessary, instead antibody fragments are produced *in vitro* in bacteria or cell culture (80). Once antibody fragments are cloned into bacterial systems it is possible to select high-affinity and highly specific binders by simulating SHM during an immune response (83). This technology is advancing to replace animal immunization or hybridism technology (83). The main focus when generating recombinant antibodies is on the antigen binding regions of an antibody (80). For higher yield of antibodies, it is preferred to emit the remaining parts of the antibody and to retain the intact antigen binding sites while reducing the size of the antibody (83). Newly created recombinant antibodies no longer have the natural abilities of an antibody, yet are easily fused to other antibodies or enzymes and thereby gain new properties (80).

Popular recombinant antibody formats include single domain antibodies (sdAb), fragment variable (Fv) antibodies, fragment antibodies (Fab), and single-chain-Fragment-variable (scFv) antibodies. The smallest possible molecule are Fv antibodies. sdAb and Fvs consist solely of one variable region, either light or heavy chain (see Figure 10) yet retain antigen binding properties (80). Fvs lack covalent binding of both chains, therefore require stabilisation (80). Fab antibodies are larger molecules than Fv since they consist of variable regions of both chains, connected by respective constant regions (Figure 10) (80). scFv are generated when connecting variable regions of both chains by a peptide linker to a single protein strand, which retains antigen binding properties of both chains, yet is smaller in size than a Fab (Figure 10) (80). ScFv can be expressed in functional form in *E. coli* cells and be manipulated within this system to select high-affinity and highly specific binders (80, 83).

Smaller molecules such as the scFv antibody fragment, penetrate tumours more easily and spend less retention time in kidneys (80). Both properties are desirable when diagnosing tumours through immune scintigraphy (80). More advantages of smaller antibody molecules

include more rapid blood clearance, lower retention times in non-target tissue and lessened immunogenicity (83). The fusion of heavy and light chain genes into one gene, enables a straightforward transfection of a functioning antibody fragment into cells and the risk of integrating heavy and light chain at separate location within the genome is deleted (83). The additional risk of producing differing amounts of protein is thereby eliminated and expression of equal amounts in *E. coli* cells of both chains guaranteed (80). The winning combination of small antibody molecules produced in microbial systems enables the production of homogenous protein in amounts sufficient for diagnostic and therapeutic purposes (83).

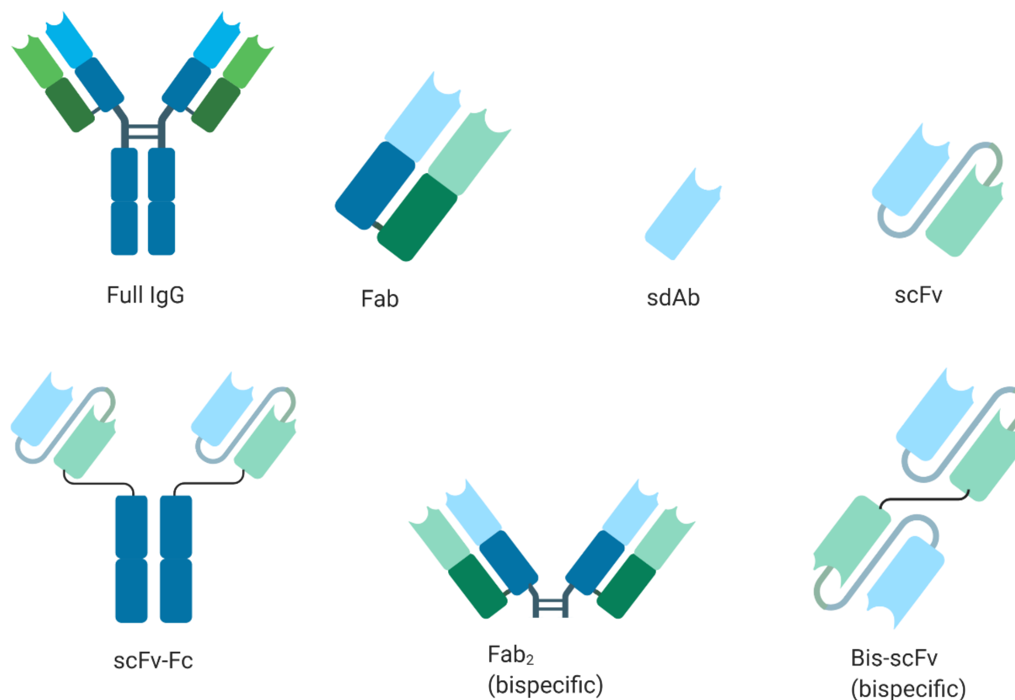

Figure 11: Illustration of recombinant antibody formats compared to a full-size IgG antibody. Adapted from Bustamante-Córdova et al. (84). Created with biorender.com.

Antibody libraries

Since the early Nineties, hundreds of antibody libraries have been constructed. This was enabled by the advances of sequence data of immunoglobulin genes and the onset of PCR technology enabling amplification of antibody repertoires (85). Four different types of libraries can be generated: immune, naïve, semi-synthetic and synthetic libraries (86). Each type of library is meant to present a vast variety of antibodies against any antigen resembling a natural immunoglobulin reservoir (86). Library quality can be assessed by estimating the size, referring to the number of individual clones, the diversity among clones, biophysical properties and production characteristic (87).

Immune libraries are usually generated by extracting immunoglobulin genes from lymphocytes of already either synthetically or naturally immunized donors (86). The likelihood to extract antigen-specific antibody genes and affinity matured clones from immune libraries is more likely due to previous exposure to the antigen of interest (86). The first immunised library consisted of the immunoglobulin repertoire of mice and was generated by PCR from immunised animals (88). Other examples of immunized libraries include a human scFv library (89), a non-human library derived from macaques, both in the scFv format (90), bovine scFv library (91), a rabbit immune library (92), a camel immune library (93) or chicken immune library in the scFv format (94). The immunization procedure prior to library generation conveys the disadvantages of being time consuming, and bears the risk of lack of efficacy of the vaccine or immune response and its unpredictability (86).

The construction of an immunized library begins with the generation of scFv fragments: this entails blood and lymphocyte collection after completed immunization procedures. Immunoglobulin genes are extracted from lymphocytes and variable regions are amplified using species-specific PCR primers (83). The variable regions of both chains are connected into scFv fragments which from thereon contain a linker in between chains (83). Newly generated

scFv fragments are ligated into plasmid vectors (80). This step should create a sheer variety of differing antibody genes, similar to random combinatorial processes within the natural host (80). Plasmid-DNA, now including the scFv fragment, is transformed into a microbial host thereby producing antibody libraries of more than 10^7 individual clones (80).

Naïve, synthetic and semi-synthetic libraries can be generated independently of exposure to antigen (86). Naïve libraries are derived by amplifying naturally occurring immunoglobulin genes (86). The amplified heavy and light chain genes are randomly recombined to create a recombinatorial scFv library (86). Contrary to scFv of an immune library, these scFv have not been exposed to antigen therefore may show lower affinities to the antigen (86). Naïve human libraries exist as well in the scFv (89) and the Fab format (95). scFv have also been selected from murine naïve libraries (96), camelids such as llama (97) and alpaca libraries (98) and production animals such as naïve chicken libraries (99).

Synthetic and semi-synthetic libraries generate immunoglobulin diversity differently to the recombinatorial immune and naïve libraries (85): semi-synthetic libraries contain scFv fragments which are created from naturally occurring diversity and artificially inserted diversity by inserting oligonucleotides (86). Since the source of antibody genes has not been exposed to antigen and parts of the antibody domain are assembled from synthetic oligonucleotides, complete or partial sequence degeneracy are introduced into the CDR loops (85). This allows a synthetic diversity that is independent of natural biases and redundancies and thereby creating a larger amount of antibodies against any desired antigen (85, 86). A well-known example of a human synthetic library is Tomlinson I (100). The first human semi-synthetic library was constructed in 1992 by Hoogenboom et al (101) and contains a scFv repertoire constructed from germline immunoglobulin gene segments rearranged *in vitro* with synthetic CDR residues (85). Since then, efforts have been made to further enhance its diversity by insertion of synthetic

oligonucleotides into CDR regions (85). Before diversity was reduced to make up for a lack of robustness of antibody fragments (85).

3.2.4 Phage Display

The isolation of antibody fragments from abovementioned antibody libraries requires i*n vitro* antibody selection methods (87). These selection methods have been developed over the past decades and include phage display technology, ribosomal display and bacterial, yeast or mammalian cell surface display serving as selection methods for antibody-antigen interaction (87). Most of these selection methods can be applied to a vast array of antigens and the selection conditions can be tailored to the requirements (87). For instance, ribosome display, developed by Hanes and Plueckthun (102) serves as a selection method for scFv fragments, but presents to be technically challenging and is limited to the scFv format only (87). Yeast display of scFv antibodies was developed by Boder and Wittrup (103) in the late Nineties and was proven effective by Feldhaus et al. (104), who isolated human scFv from a naïve library. Yeast display allows selection of therapeutic format full-size IgG molecules rather than recombinant fragments (103, 104). Moreover, it prevents impaired affinities due to antibody format changes and is compatible with fluorescence-activated cell sorting (FACS) sorting techniques, which allows further selection and parallel assessment of antibody characteristics (87, 103, 104). However, a key disadvantage of yeast display are a limited transformation efficiency, thereby significantly reducing library size (87).

Phage display technology promised to overcome these obstacles and has since become a commonly used technology to screen antibody libraries (105). The crucial element of phage display technology is a linkage between the genotype and the phenotype of a phage, where filamentous phages express a foreign protein in their genotype and their phenotype by displaying the foreign protein as a phage surface protein (105).

Crucial to phage display technology are the characteristics of the bacteriophage itself (106). Several bacteriophages are available for phage display technology including the T4 phage, lambda phage or the filamentous M13 bacteriophage of which the latter has been utilized the most (106). M13 phages belong to the group of Ff phages, the only phages that infect *E. coli* cell strains that express an F pilus on which absorption of the phage to the bacterium depends on (106). M13 phages are neither temperate nor lytic, but establish a chronic infection and repeatedly release new phages (106). The phage itself contains a genome of single-stranded DNA consisting of nine genes encoding 11 proteins (106). Five of these proteins are surface proteins, six are involved in replication and assembly of the phage (106). Of these five surface proteins, it is G3P that is mainly responsible for adsorption purposes during infection (106). Infection continues by the N2 domain of the G3P protein of the phage binding to the tip of the *E.coli* F pilus (see Figure 11) (106). Upon binding, the phage is brought closer to the surface of the bacterium (see Figure 11) and initiates the G3P-N1 domain to bind to TolA (see Figure 11), which functions as an additional receptor on the bacterium's surface (see Figure 11) (106). Three Tol proteins are present within an *E.coli* cell and all facilitate infection by depolarization of the phage coat and translocation of the phage's ssDNA into the bacterium (106). The phage's ssDNA is then synthesised into a supercoiled dsDNA phage chromosome within the *E.coli* cell, which is termed the replicative form of the phage (106).

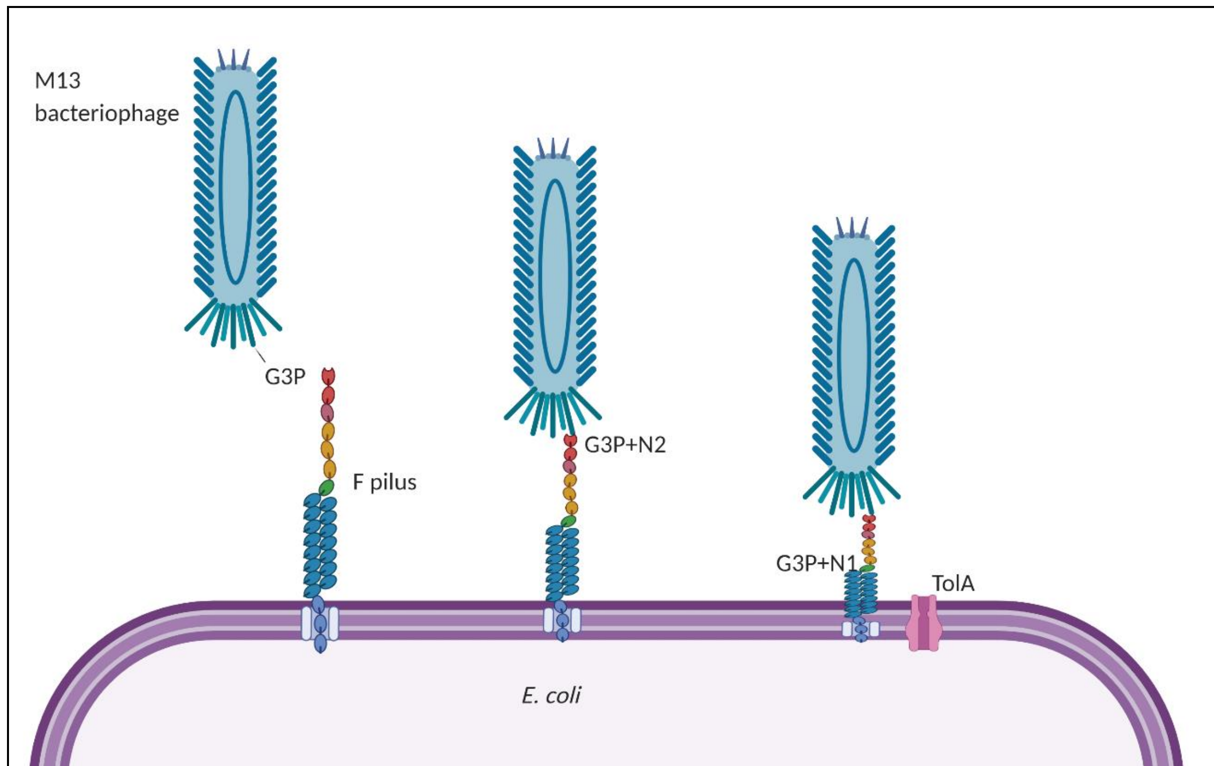

Figure 12: Infection of *E. coli* cell by M13 bacteriophage. Phage surface protein G3P binds to F pilus on *E.coli* cell. F pilus disassembles and transports phage closer to bacterial surface. Phage interacts with TolA receptor and induces uptake of phage genome (106). Created with biorender.com.

Smith was the first to successfully fuse foreign DNA into the M13 bacteriophage (106, 107). McCafferty et al were the first to fuse scFv fragments to G3P (106, 108). Since then, a large variety of differing antibody formats have been employed in the construction of antibody displaying phage libraries: human (109), camelid (93) or shark variable regions (110), bovine scFv (91), diabodies (111), Fab (95, 111, 112) just to name a few. All were fused to the G3P surface protein of M13 (106).

The actual selection of antibody fragments through phage display technology occurs by binding of phages to the desired antigen in a process called biopanning (105, 106). Biopanning rounds consist of five main events (105, 106): incubating the phage library on the immobilized antigen on a surface (refer to Figure 12) (105, 106). Immobilization of the antigen can be achieved by direct coating thus absorption or a capture system e.g. streptavidin-biotin (105,

106). The second step consist of phages displaying scFv fragments with affinities binding to the antigen, followed by several washing steps to remove low-affinity binders (refer to Figure 12) (105, 106). Following washing procedures, high-affinity binders are eluted by either pH shift or enzymatic treatment (refer to Figure 12) (105, 106). Following elution, helper phages are added to aid amplification of eluted infective phages in *E. coli* cells for a consecutive round of biopanning (105, 106). This procedure is usually repeated one to five times to enrich specifically binding phage particles and accumulate phages displaying high-affinity binders (105, 106). Each round, selection conditions become more stringent by e.g. increasing the number of washing steps, decreasing antigen coating concentrations and thereby selecting for high-affinity binders (105). Titration of phage output numbers should be monitored to observe enchrichment progress and phage ELISA to confirm affinity is increasing (106).

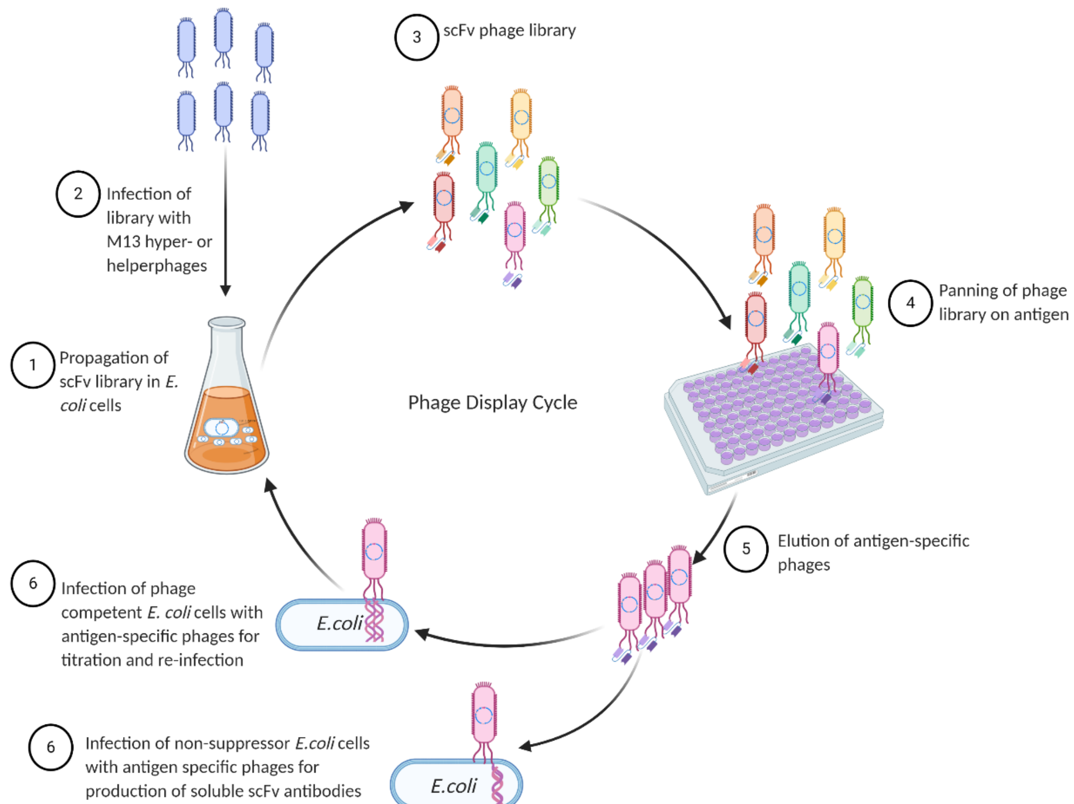

Figure 13: Scheme of scFv phage display selection and screening adapted from Russo et al (113). Created with biorender.com.

Prior to utilizing phage display technology, antibody or scFv antibody fragments have to be ligated into phage compatible phage or phagemid vectors. Since antibody fragments displayed on phage vectors are limited by their capacity to be expressed efficiently by *E.coli* cells, a second type of phage vector systems have been developed, the phagemid vector system (87). The main difference between phage and phagemid vectors lies in the display of antibodies on phage surface: in the phage vector system, each phage carries three to five copies on its surface (multivalent display) (87). In the phagemid vector system, each phage carries solely one copy on its surface (monovalent) and only less than ten percent of all phages carry an antibody fragment on their surface (87). Phagemid vectors are smaller in size and easier to clone, resulting in larger library sizes (87). Commonly both systems are used subsequently, using the avidity effect on a multivalent display to their advantage in the first biopanning round, and increasing the stringency by using a monovalent display in following rounds (87).

If the antibody library contains fragments within a phagemid system, antibody display levels can be increased by applying hyper phages instead of helper phages (87). A helper phage carries all genetic information, required for infection, including the surface protein G3P (87). A phagemid vector also contains G3P (87). In case of infecting a phagemid library with a helper phage, the majority of resulting phages will carry phagemid DNA and incorporate G3P from both the helper phage as well as the phagemid (106). This would result in a competition, and the wild-type G3P from the helper phage is preferred, yielding bald phages not displaying antibody fragments (106). The hyper phage carries a G3P deletion thus preventing a competition and opening the path for multivalent display (87).

3.2.5 Clinical Relevance

Human medicine

Recent antibody engineering combined with microbial expression systems provide a powerful source of tailor-made antibody therapeutics (83, 114). An important advantage of recombinant antibody therapeutics over conventional medication is the agent's specificity, which facilitates precise action, as well as long half-lives, which allow less frequent dosing (114). Their species-specificity prevents any adverse reactions to animal constituents within the therapeutic agent (115). More than 500 recombinant antibodies are currently being under development for several arms of the industry such as biotechnology, immunology, and human medicine, for a number of purposes such as immunotoxins, therapeutic gene delivery or anticancer intrabodies (see Table 4) (83, 114, 116). The first monoclonal antibody therapeutic was Orthoclone OKT3, approved by the Food and Drug Administration (FDA) in 1986 and used to treat the rejection of acute transplant rejection (115). Since then many more antibody therapeutics have been developed for treatment of human conditions such as cancer, autoimmune and inflammatory diseases (115). In the industry of human antibody therapeutics, one speaks of the big five, that have had a major influence (115): infliximab (Remicade; Centocor, Merck), a tumour necrosis factor specific antibody, adalimumab (Humira, Trudexa, Abbott), to treat rheumatoid arthritis, Crohn's disease and psoriasis, trastuzumab (Herceptin; Genentech, Roche), a human epidermal growth factor receptor specific antibody, and bevacizumab (Avastin, Genentech) a vascular endothelial cell specific antibody (115). Trastuzumab and Bevacizumab are used for the treatment of numerous types of cancer (115). The fifth, rituximab (Rituxan/Mabhera; Genentech, Roche, Bioen Idec) is used to treat rheumatoid arthritis as well and non-Hodgkin's lymphoma (83, 115).

Therapeutic recombinant antibodies exert their effects in either one of three mechanisms: the first mechanisms consists of binding or incorporating soluble target particles

(e.g. cytokines) before these can reach their target tissue (117). The second mechanism is an antagonistic one where the therapeutic agent binds to target receptor cells thereby blocking antigen reception and signal transduction (117). Most of human recombinant antibodies therapeutics act through this mechanisms (117). The third mechanism consists of binding of the therapeutic agent to the infectious agent or cancerous cell and induce cell lysis or clearance by ADCC (117). Many recombinant antibody therapeutics are administered by injection to prevent enzymatic breakdown and denaturation by stomach environment when administered orally (117). Once injected, their half-lives are approximately 20 days, similar to naturally occurring antibodies (117).

The market for monoclonal antibodies has grown exponentially over the past 20 years (115) and especially the scFv fragment has been of particular interest, since it retains the complete antigen-binding capacity of an antibody and provides a unique molecule particularly interesting for cancer treatment due to its reduced size (83). The small molecule, can penetrate tumours more easily and more rapidly than full Ab (83). Alternatively, scFv are considered to be able to play a role in gene therapy of cancerous patients since they can be delivered to target tissue and release their anti-cancerous genes into the affected cell, by infecting it and consequently eliminating it (83). Currently approved scFv therapeutics are summarised in Table 4.

Table 4: Overview of all scFv generated by phage display approved in the EU and USA and their applications in human medicine according to www.antibodysociety.org (accessed 23.10.2020). INN - International non- proprietary name.

INN	Brand name	Ab -format	Indication	First EU approval year	First US approval year
Adalimumab	Humira	Humanized scFv	Rheumatoid arthritis	2003	2002
Belimumab	Benlysta	scFv	Systemic lupus erythematosus	2011	2011
Blinatumomab	Blincyto	Murine bispecific tandem scFv	Acute lymphoblastic leukemia	2015	2014
Brolucizumab, brolucizumab-dbll	Beovu	Humanized scFv	Neovascular age-related macular degeneration	2020	2019
Oportuzumab monatox	(Pending)	Humanized scFv immunotoxin	Bladder cancer	NA	In review
Raxibacumab	(Pending)	scFv	Anthrax infection	NA	2012

Another promising use of recombinant antibodies produced through phage display technique is cell classification and characterisation using a technique referred to as phage-antibody next generation sequencing (PhaNGS) (116). It will allow assessment of surface protein changes in diseased cells and identification of new biomarkers and drug targets (116). Moreover, structural studies like to employ antibodies to aid in imaging procedures such as electron microscopy, cryo-electron microscopy and X-ray crystallography (116).

Veterinary medicine

The majority of recombinant antibodies have been designed for human use, yet advances in recombinant antibody design have also been made in veterinary medicine as diagnostic, prophylactic or therapeutic drugs to treat or prevent autoimmune diseases or cancerous diseases (84). The trend goes towards production animals such as pigs, cows and poultry, but also towards companion animals such as dogs and cats.

Porcine recombinant antibodies are under development for diagnostic and therapeutic purposes. For instance, recombinant antibodies are designed to diagnose porcine circovirus type II (PCV II) infection, classic swine fever virus (CSF) infection and bacterial infections (84, 118). A sdAb was developed to detect PCV 1 and yielded promising results, as no cross-reactivity was noticed (119). Further porcine recombinant antibodies have been developed for the diagnosis of classical swine fever virus and showed promising results *in vivo* in regards to neutralization of the antigen (119). Several porcine recombinant antibodies are being investigated for treatment purposes of numerous porcine infections with the majority of efforts directed toward porcine reproductive and respiratory syndrome (PRRSV), nowadays known as Betaarterivirus 1 (120-122). One particular scFv-Fc fragment was designed to fuse with PRRSV structural proteins and improved humoral and cellular response, but not fully induce protective immunity (122). Additional porcine recombinant antibodies are researched for their effect on infections with foot and mouth disease virus (FMDV), African swine fever virus (ASFV) and bacterial infections of which one sdAb proved successful in introducing a reduction of clinical symptoms and transmission (123).

Bovine recombinant antibodies have been developed for diagnostic purposes of FMDV, *Mycobacterium bovis* (*M. bovis*), bovine spongiform encephalopathy (BSE) and bovine immunodeficiency virus (BIV) (84, 124). A scFv fragment designed for detection of FMDV has proven successful in the differentiation of vaccinated and infected animals with FMDV (125). However, this particular scFv was not derived from bovine immunoglobulin genes, rather chicken and Tobacco plants genes serving as detectors of the 3ABC gene of FMDV (125). A further scFv developed meant to improve diagnosis of *M. bovis* was also not entirely of bovine origin, since it had been fused with a heavy chain derived from chickens to improve its stability while retaining functionality (84). A further chicken scFv was developed to diagnose BSE and other prion infections (126). However, a scFv produced to diagnose BIV was generated entirely from bovine Ig genes and provided more sensitive results than the gold standard mAb (127).

Bovine recombinant antibodies for treatment purposes were designed for bacterial infections, enterotoxigenic *E. coli* (ETEC), bovine mastitis and *S. aureus* infections. One of these bovine recombinant antibodies, a scFv, was evaluated *in vivo* and resulted in reduced fluid accumulation, which would otherwise be indicative of an ETEC infection (84).

scFv fragments have been developed for poultry species to diagnose and treat diseases like avian influenza virus (AIV) (128), Newcastle disease virus (NDV), infectious bursal disease virus (IBDV) and avian coccidiosis (129). Throughout, scFv antibodies showed higher sensitivities and potential as detection tool in ELISA and WB (84). Further avian recombinant antibodies for immune-prophylaxis showed reduced viral titres not only *in vivo* but also *in ovo* (130). Another avian recombinant antibodies showed neutralizing effects against NDV (131).

Concerning companion animals, a number of canine recombinant antibodies are currently under investigation to treat tumour patients, inflammatory diseases or dermatitis (84). In terms of tumour treatment, advantage was taken of high similarity between human epidermal growth factor receptor and its canine homologue and two recombinant antibodies with cross-reactivity generated; both seemed suitable for detection of malignant cells when tested to detect breast cancer cells (132).

There is one fully licensed and commercially available canine recombinant antibody product, Lokivetmab (Cytopoint™) produced by Zoetis for the treatment of canine allergic dermatitis (117). Lokivetmab exerts its action by binding to soluble antigenic targets thereby preventing it from reaching their target cell receptors and tissues (117). It is a caninized anti-interleukin-31 (IL-31) mAb which neutralizes soluble IL-31 which has shown to be involved in the development of atopic dermatitis (117). Its efficacy and safety was demonstrated in clinical studies, who reported no hypersensitivities and only mild discomfort upon the application of Lokivetmab (133). Next to this, a further therapeutic recombinant antibody for dogs has been developed to alleviate osteoarthirits-related pain, the mAb Renevetmab targeting

nerve growth factor (NGF). Its efficacy and safety have been assessed and deemed sufficient (134). A feline counterpart also relieving the animal of osteoarthirits-related pain has been developed and assessed, Frunevetmab (135). Both recombinant antibody therapeutics promise a great advance in pain-relieve therapy for dogs and cats but are not available for use yet.

In the equine sector only limited efforts have been made to identify equine recombinant antibodies as diagnostic or therapeutic agents. 20 years ago one study utilized phage display technology to identify an equine full-size IgG molecule against EHV-1 for diagnostic purposes (136). Other than that merely human-like or for human purposes designed recombinant antibodies have been investigated against equine viruses that also affects humans such as Western Equine Encephalitis Virus (137) or for non-human primates suffering from Venezuelan Equine Encephalitis Virus infection (138). In future, the use of recombinant antibodies to control or treat human as well as animal diseases will be a reality and become a part of the pharmaceutical economy. Currently investigated recombinant antibodies for veterinary purposes are summarised in Table 5.

Table 5: Summary of recombinant antibodies under imvestigation for potetntial use in veterinary medicine. Adapted from Bustamante-Córdova et al. (84).

Application	Ab format	Target Species	Indication
Diagnosis	scFv	Pig	*B .hyodysenteriae*
	scFv	Cow	FMDV
	scFv	Cow	FMDV
	scFv	Cow	BIV
	scFv	Cow	*M. tuberculosis*
	scFv	Chicken	AIV
	scFv	Birds	NDV
	scFv	Chicken	IBDV
	scFv	Chicken	*E. tenella*, avian coccidiosis
Prophylaxis	scFv-Fc	Pig	PEDV, PRRSV
	scFv-Fc	Pig	PEDV
	scFv-Fc	Pig	PEDV
	scFv	Pig	PEDV
	scFv	Pig	pAPN
	scFv	Pig	ASFV
	scFv	Cow	ETEC
	scFv	Cow	ETEC
	scFv	Cow	ETEC
	scFv	Chicken	IBV
	scFv	Chicken	IBDV
	scFv	Chicken	IBDV
	scFv	Chicken	NDV
	scFv	Chicken	*E. tenella*
	scFv	Chicken	*E. tenella*
	scFv	Sheep	Rift Vally Fever virus
Therapeutics	scFv	Cow	*S. aureus*, Bovine mastitis
	scFv	Chicken	Avian Influenza virus
	scFv	Dog	B cell malignancies

3.3 Aim of the thesis

The aim of the thesis is to generate an equine-specific antibody library to meet the lack thereof within veterinary medicine and research. The first aim was to construct scFv fragments based on Ig genes of an immunized donor and utilizing a species-specific PCR primer set. Equine scFv fragments were then ligated into a phagemid vector and the library assembled using phage-competent *E.coli* cells. This would lay the groundworks for phage display screening procedures against any desired equine pathogen. We chose to screen against EHV-1 due to its high infectivity, ubiquitous occurrence and detrimental effects on equine health and equine-related economies. The isolated anti-EHV-1 scFv fragment was propagated and its properties further characterised in terms of affinity to EHV-1.

The thesis will contribute to the development of novel, therapeutic recombinant antibody solutions for horses and presents a starting point for further developments in the search for diagnostic and therapeutic agents improving veterinary as well as human health and welfare.

3.4 References (Vancouver)

1. Kapoor S, Sharma H, Singh M, Kumar P, Ranjan K, Kumari A, et al. Equine herpesviruses: a brief review. Nine. 2014;2014:06-25.

2. Paillot R, Case R, Ross J, Newton R, Nugent J. Equine herpes virus-1: virus, immunity and vaccines. The Open Veterinary Science Journal. 2008;2(1).

3. McGeoch DJ, Dolan A, Ralph AC. Toward a comprehensive phylogeny for mammalian and avian herpesviruses. J Virol. 2000;74(22):10401-6.

4. Davison AJ. Evolution of the herpesviruses. Vet Microbiol. 2002;86(1):69-88.

5. Khusro A, Aarti C, Rivas-Caceres RR, Barbabosa -Pliego A. Equine Herpesvirus-I Infection in Horses: Recent Updates on its Pathogenicity, Vaccination, and Preventive Management Strategies. Journal of Equine Veterinary Science. 2020;87:102923.

6. Darlington RW, Randall CC. The nucleic acid content of equine abortion virus. Virology. 1963;19(3):322-7.

7. Telford E, Watson MS, Perry J, Cullinane AA, Davison AJ. The DNA sequence of equine herpesvirus-4. J Gen Virol. 1998;79(5):1197-203.

8. Lecollinet S, Pronost S, Coulpier M, Beck C, Gonzalez G, Leblond A, et al. Viral Equine Encephalitis, a Growing Threat to the Horse Population in Europe? Viruses. 2020;12(1):23.

9. Lunn D, Davis-Poynter N, Flaminio M, Horohov D, Osterrieder K, Pusterla N, et al. Equine herpesvirus-1 consensus statement. Journal of Veterinary Internal Medicine. 2009;23(3):450-61.

10. Sutton G, Garvey M, Cullinane A, Jourdan M, Fortier C, Moreau P, et al. Molecular surveillance of EHV-1 strains circulating in France during and after the major 2009 outbreak in Normandy involving respiratory infection, neurological disorder, and abortion. Viruses. 2019;11(10):916.

11. Hebia I, Fiéni F, Duchamp G, Destrumelle S, Pellerin J-L, Zientara S, et al. Potential risk of equine herpes virus 1 (EHV-1) transmission by equine embryo transfer. Theriogenology. 2007;67(9):1485-91.

12. Walter J, Balzer HJ, Seeh C, Fey K, Bleul U, Osterrieder N. Venereal Shedding of Equid Herpesvirus-1 (EHV-1) in Naturally Infected Stallions. Journal of veterinary internal medicine. 2012;26(6):1500-4.

13. Dunowska M. How common is equine herpesvirus type 1 infection? Veterinary Record. 2016;178(3):67-9.

14. Abdelgawad A, Azab W, Damiani AM, Baumgartner K, Will H, Osterrieder N, et al. Zebra-borne equine herpesvirus type 1 (EHV-1) infection in non-African captive mammals. Vet Microbiol. 2014;169(1-2):102-6.

15. Ghanem Y, Fukushi H, Ibrahim E, Ohya K, Yamaguchi T, Kennedy M. Molecular phylogeny of equine herpesvirus 1 isolates from onager, zebra and Thomson's gazelle. Archives of virology. 2008;153(12):2297-302.

16. Negussie H, Gizaw D, Tessema TS, Nauwynck HJ. Equine herpesvirus-1 myeloencephalopathy, an emerging threat of working equids in ethiopia. Transboundary and emerging diseases. 2017;64(2):389-97.

17. ATA EB, Salama A, Zaghawa A, Ghazy A, Elsify A, Nayel M, et al. Seroprevalence of equine herpes virus-1 in endemic area of Egypt with risk factors assessment. Bulgarian Journal of Veterinary Medicine. 2020;23(1).

18. Interantional FE. 2020 [

19. Stokol T, Soboll Hussey G. Editorial: Current Research in Equid Herpesvirus Type-1 (EHV-1). Frontiers in Veterinary Science. 2020;6(492).

20. McFadden A, Hanlon D, McKenzie R, Gibson I, Bueno I, Pulford D, et al. The first reported outbreak of equine herpesvirus myeloencephalopathy in New Zealand. New Zealand veterinary journal. 2016;64(2):125-34.

21. Anagha G, Gulati BR, Riyesh T, Virmani N. Genetic characterization of equine herpesvirus 1 isolates from abortion outbreaks in India. Archives of virology. 2017;162(1):157-63.

22. Stasiak K, Rola J, Ploszay G, Socha W, Zmudzinski JF. Detection of the neuropathogenic variant of equine herpesvirus 1 associated with abortions in mares in Poland. Bmc Vet Res. 2015;11(1):102.

23. Tsujimura K, Oyama T, Katayama Y, Muranaka M, Bannai H, Nemoto M, et al. Prevalence of equine herpesvirus type 1 strains of neuropathogenic genotype in a major breeding area of Japan. Journal of Veterinary Medical Science. 2011:1108020591-.

24. Silva AAd, Cunha EMS, Lara MdCCdSH, Villalobos EMC, Nassar AFdC, Mori E, et al. Low occurrence of equine herpesvirus 1 (EHV-1) as cause of abortion and perinatal mortality in Brazil. Arquivos do Instituto Biológico. 2018;85.

25. Burgess B, Tokateloff N, Manning S, Lohmann K, Lunn D, Hussey S, et al. Nasal Shedding of Equine Herpesvirus-1 from Horses in an Outbreak of Equine Herpes Myeloencephalopathy in W estern C anada. Journal of veterinary internal medicine. 2012;26(2):384-92.

26. Goehring LS, van Winden SC, Van Maanen C, van Oldruitenborgh-Oosterbaan MMS. Equine herpesvirus type 1-associated myeloencephalopathy in The Netherlands: a four-year retrospective study (1999–2003). Journal of veterinary internal medicine. 2006;20(3):601-7.

27. Garvey M, Lyons R, Hector RD, Walsh C, Arkins S, Cullinane A. Molecular characterisation of equine herpesvirus 1 isolates from cases of abortion, respiratory and neurological disease in Ireland between 1990 and 2017. Pathogens. 2019;8(1):7.

28. Barbić L, Lojkić I, Stevanović V, Bedeković T, Starešina V, Lemo N, et al. Two outbreaks of neuropathogenic equine herpesvirus type 1 with breed-dependent clinical signs. Veterinary Record. 2012.

29. Slater J, Lunn D, Horohov D, Antczak D, Babiuk L, Breathnach C, et al. Report of the equine herpesvirus-1 Havermeyer Workshop, San Gimignano, Tuscany, June 2004. Vet Immunol Immunop. 2006;111(1-2):3-13.

30. Ataseven VS, Dağalp SB, Başaran Z, Keskin S. Seroepidemiological studies of equine herpesviruses 1 (EHV-1) and 4 (EHV-4) infections in working horses from the eastern Turkey. 2010.

31. Aharonson-Raz K, Davidson I, Porat Y, Altory A, Klement E, Steinman A. Seroprevalence and rate of infection of equine influenza virus (H3N8 and H7N7) and equine herpesvirus (1 and 4) in the horse population in Israel. Journal of Equine Veterinary Science. 2014;34(6):828-32.

32. Pagamjav O, Kobayashi K, Murakami H, Tabata Y, Miura Y, Boldbaatar B, et al. Serological survey of equine viral diseases in Mongolia. Microbiology and immunology. 2011;55(4):289-92.

33. (OIE) WOfAH. Animal Health Information. Accessed on 15.09.2020.

34. <1990Horton_COE-PCR.pdf>.

35. Oladunni FS, Horohov DW, Chambers TM. EHV-1: A Constant Threat to the Horse Industry. Front Microbiol. 2019;10(2668).

36. Patel J, Heldens J. Equine herpesviruses 1 (EHV-1) and 4 (EHV-4)–epidemiology, disease and immunoprophylaxis: a brief review. The Veterinary Journal. 2005;170(1):14-23.

37. Manning F. Equine herpesvirus -1 Part 1: An update. UK Vet Companion Animal. 2006;11(6):12-5.

38. Smith DJ, Iqbal J, Purewal A, Hamblin AS, Edington N. In vitro reactivation of latent equid herpesvirus-1 from CD5+/CD8+ leukocytes indirectly by IL-2 or chorionic gonadotrophin. J Gen Virol. 1998;79(12):2997-3004.

39. Walter J, Seeh C, Fey K, Bleul U, Osterrieder N. Clinical observations and management of a severe equine herpesvirus type 1 outbreak with abortion and encephalomyelitis. Acta Vet Scand. 2013;55(1):19.

40. Pusterla N, Hatch K, Crossley B, Wademan C, Barnum S, Flynn K. Equine herpesvirus-1 genotype did not significantly affect clinical signs and disease outcome in 65 horses diagnosed with equine herpesvirus-1 myeloencephalopathy. The Veterinary Journal. 2020;255:105407.

41. Wilcox GE. Latent equine herpesviruses. 2004.

42. Loving NS. All Horse Systems Go: The Horse Owner's Full-color Veterinary Care and Conditioning Resource for Modern Performance, Sport, and Pleasure Horses: Trafalgar Square Books; 2014.

43. Ma G, Azab W, Osterrieder N. Equine herpesviruses type 1 (EHV-1) and 4 (EHV-4)--masters of co-evolution and a constant threat to equids and beyond. Vet Microbiol. 2013;167(1-2):123-34.

44. Edington N, Bridges C, Huckle A. Experimental reactivation of equid herpesvirus 1 (EHV 1) following the administration of corticosteroids. Equine veterinary journal. 1985;17(5):369-72.

45. Slater J, Borchers K, Thackray A, Field H. The trigeminal ganglion is a location for equine herpesvirus 1 latency and reactivation in the horse. J Gen Virol. 1994;75(8):2007-16.

46. Welch HM, Bridges CG, Lyon AM, Griffiths L, Edington N. Latent equid herpesviruses 1 and 4: detection and distinction using the polymerase chain reaction and co-cultivation from lymphoid tissues. J Gen Virol. 1992;73(2):261-8.

47. Welch L, Dong X, Hewitt D, Irwin M, McCarty L, Tsai C, et al. Facile quantitation of free thiols in a recombinant monoclonal antibody by reversed-phase high performance liquid chromatography with hydrophobicity-tailored thiol derivatization. J Chromatogr B Analyt Technol Biomed Life Sci. 2018;1092:158-67.

48. Borchers K, Wolfinger U, Ludwig H. Latency-associated transcripts of equine herpesvirus type 4 in trigeminal ganglia of naturally infected horses. J Gen Virol. 1999;80(8):2165-71.

49. Baxi M, Efstathiou S, Lawrence G, Whalley J, Slater J, Field H. The detection of latency-associated transcripts of equine herpesvirus 1 in ganglionic neurons. J Gen Virol. 1995;76(12):3113-8.

50. van der Meulen KM, Favoreel HW, Pensaert MB, Nauwynck HJ. Immune escape of equine herpesvirus 1 and other herpesviruses of veterinary importance. Vet Immunol Immunop. 2006;111(1-2):31-40.

51. Kydd JH, Townsend HG, Hannant D. The equine immune response to equine herpesvirus-1: the virus and its vaccines. Vet Immunol Immunop. 2006;111(1-2):15-30.

52. Dunowska M. A review of equid herpesvirus 1 for the veterinary practitioner. Part A: clinical presentation, diagnosis and treatment. New Zealand veterinary journal. 2014;62(4):171-8.

53. (OIE) WOfAH. Equine Rhinopneumonitis (Infection with Equid Herpesvirus-1 and -4). 2017.

54. Borchers K, Slater J. A nested PCR for the detection and differentiation of EHV-1 and EHV-4. J Virol Methods. 1993;45(3):331-6.

55. Lawrence G, Gilkerson J, Love D, Sabine M, Whalley J. Rapid, single-step differentiation of equid herpesviruses 1 and 4 from clinical material using the polymerase chain reaction and virus-specific primers. J Virol Methods. 1994;47(1-2):59-72.

56. Varrasso A, Dynon K, Ficorilli N, Hartley C, Studdert M, Drummer H. Identification of equine herpesviruses 1 and 4 by polymerase chain reaction. Australian Veterinary Journal. 2001;79(8):563-9.

57. Diallo IS, Hewitson G, Wright L, Rodwell BJ, Corney BG. Detection of equine herpesvirus type 1 using a real-time polymerase chain reaction. J Virol Methods. 2006;131(1):92-8.

58. Diallo IS, Hewitson G, Wright LL, Kelly MA, Rodwell BJ, Corney BG. Multiplex real-time PCR for the detection and differentiation of equid herpesvirus 1 (EHV-1) and equid herpesvirus 4 (EHV-4). Vet Microbiol. 2007;123(1-3):93-103.

59. Allen GP. Development of a real-time polymerase chain reaction assay for rapid diagnosis of neuropathogenic strains of equine herpesvirus-1. Journal of Veterinary Diagnostic Investigation. 2007;19(1):69-72.

60. Fritsche A-K, Borchers K. Detection of neuropathogenic strains of Equid Herpesvirus 1 (EHV-1) associated with abortions in Germany. Vet Microbiol. 2011;147(1-2):176-80.

61. Lechmann J, Schoster A, Ernstberger M, Fouché N, Fraefel C, Bachofen C. A novel PCR protocol for detection and differentiation of neuropathogenic and non-neuropathogenic equid alphaherpesvirus 1. Journal of Veterinary Diagnostic Investigation. 2019;31(5):696-703.

62. Hartley CA, Wilks CR, Studdert MJ, Gilkerson JR. Comparison of antibody detection assays for the diagnosis of equine herpesvirus 1 and 4 infections in horses. American journal of veterinary research. 2005;66(5):921-8.

63. Paillot R, Ellis SA, Daly JM, Audonnet JC, Minke JM, Davis-Poynter N, et al. Characterisation of CTL and IFN-γ synthesis in ponies following vaccination with a NYVAC-

based construct coding for EHV-1 immediate early gene, followed by challenge infection. Vaccine. 2006;24(10):1490-500.

64. Foote C, Love D, Gilkerson J, Whalley J. Serological responses of mares and weanlings following vaccination with an inactivated whole virus equine herpesvirus 1 and equine herpesvirus 4 vaccine. Vet Microbiol. 2002;88(1):13-25.

65. Van Maanen C, Willink D, Smeenk L, Brinkhof J, Terpstra C. An equine herpesvirus 1 (EHV1) abortion storm at a riding school. Veterinary Quarterly. 2000;22(2):83-7.

66. Tewari D, Whalley J, Love D, Field H. Characterization of immune responses to baculovirus-expressed equine herpesvirus type 1 glycoproteins D and H in a murine model. J Gen Virol. 1994;75(7):1735-41.

67. Ruitenberg K, Walker C, Wellington J, Love D, Whalley J. DNA-mediated immunization with glycoprotein D of equine herpesvirus 1 (EHV-1) in a murine model of EHV-1 respiratory infection. Vaccine. 1999;17(3):237-44.

68. Mohd LMA, Field H-J, Frazer R, Lauchlan J-M. Protective Immune Reponses Induced by Non-infectious L-particles of Equine Herpesvirus Type-1: Implication of Cellular Immunity. Journal of Microbiology. 2002;40(1):11-9.

69. Huemer HP, Strobl B, Nowotny N. Use of apathogenic vaccinia virus MVA expressing EHV-1 gC as basis of a combined recombinant MVA/DNA vaccination scheme. Vaccine. 2000;18(14):1320-6.

70. Vissani MA, Thiry E, Dal Pozzo F, Barrandeguy M. Antiviral agents against equid alphaherpesviruses: Current status and perspectives. The Veterinary Journal. 2016;207:38-44.

71. Goehring L, Van Maanen C, van Oldruitenborgh-Oosterbaan MS. Neurological syndromes among horses in The Netherlands a 5 year retrospective survey (1999–2004). Veterinary quarterly. 2005;27(1):11-20.

72. Thieulent CJ, Hue ES, Fortier CI, Dallemagne P, Zientara S, Munier-Lehmann H, et al. Screening and evaluation of antiviral compounds against Equid alpha-herpesviruses using an impedance-based cellular assay. Virology. 2019;526:105-16.

73. de la Fuente R, Awan AR, Field HJ. The acyclic nucleoside analogue penciclovir is a potent inhibitor of equine herpesvirus type 1 (EHV-1) in tissue culture and in a murine model. Antiviral research. 1992;18(1):77-89.

74. Luttmann W, Bratke K, Küpper M, Myrtek D. Der Experimentator: Immunologie: Springer-Verlag; 2014.

75. Wagner B. Immunoglobulins and immunoglobulin genes of the horse. Developmental & Comparative Immunology. 2006;30(1-2):155-64.

76. Sun Y, Wang C, Wang Y, Zhang T, Ren L, Hu X, et al. A comprehensive analysis of germline and expressed immunoglobulin repertoire in the horse. Dev Comp Immunol. 2010;34(9):1009-20.

77. Walther S, Rusitzka TV, Diesterbeck US, Czerny C-P. Equine immunoglobulins and organization of immunoglobulin genes. Developmental & Comparative Immunology. 2015;53(2):303-19.

78. Schroeder HW, Jr., Cavacini L. Structure and function of immunoglobulins. The Journal of allergy and clinical immunology. 2010;125(2 Suppl 2):S41-S52.

79. Behring Ev. Ueber das zustandekommen der diphtherie-immunität und der tetanus-immunität bei thieren. 1890.

80. Breitling F, Dübel S. Rekombinante Antikörper: Spektrum Akademischer Verlag Heidelberg Berlin; 1997.

81. Macnalty AS. Emil von Behring, born March 15, 1854. Br Med J. 1954;1(4863):668-70.

82. Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 1975;256(5517):495-7.

83. Ahmad ZA, Yeap SK, Ali AM, Ho WY, Alitheen NBM, Hamid M. scFv Antibody: Principles and Clinical Application. Clinical & Developmental Immunology. 2012.

84. Bustamante-Córdova L, Melgoza-González EA, Hernández J. Recombinant antibodies in veterinary medicine: an update. Frontiers in Veterinary Science. 2018;5:175.

85. Benhar I. Design of synthetic antibody libraries. Expert opinion on biological therapy. 2007;7(5):763-79.

86. Weisser NE, Hall JC. Applications of single-chain variable fragment antibodies in therapeutics and diagnostics. Biotechnology Advances. 2009;27(4):502-20.

87. Ponsel D, Neugebauer J, Ladetzki-Baehs K, Tissot K. High affinity, developability and functional size: the holy grail of combinatorial antibody library generation. Molecules. 2011;16(5):3675-700.

88. Ward ES, Güssow D, Griffiths AD, Jones PT, Winter G. Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli. Nature. 1989;341(6242):544-6.

89. Kügler J. TF, Frenzel A., Hust M. Construction of Human Immune and Naive scFv Libraries. . 2018. In: Phage Display Methods in Molecular Biology [Internet]. New York, NY: Humana Press.

90. Avril A, Miethe S, Hust M, Pelat T. Construction of macaque immune-libraries. Phage Display: Springer; 2018. p. 83-112.
91. Diesterbeck US. Construction of Bovine Immunoglobulin Libraries in the Single-Chain Fragment Variable (scFv) Format. Methods in molecular biology (Clifton, NJ). 2018;1701:113-31.
92. Nguyen TTH, Lee JS, Shim H. Construction of rabbit immune antibody libraries. Phage Display: Springer; 2018. p. 133-46.
93. Romão E, Poignavent V, Vincke C, Ritzenthaler C, Muyldermans S, Monsion B. Construction of High-Quality Camel Immune Antibody Libraries. In: Hust M, Lim TS, editors. Phage Display: Methods and Protocols. New York, NY: Springer New York; 2018. p. 169-87.
94. Fehrsen J, Wemmer S, van Wyngaardt W. Construction of chicken antibody libraries. Phage Display: Springer; 2018. p. 189-203.
95. Omar N, Lim TS. Construction of naive and immune human Fab phage-display library. Phage display: Springer; 2018. p. 25-44.
96. Sommavilla R, Lovato V, Villa A, Sgier D, Neri D. Design and construction of a naïve mouse antibody phage display library. Journal of Immunological Methods. 2010;353(1):31-43.
97. Monegal A, Ami D, Martinelli C, Huang H, Aliprandi M, Capasso P, et al. Immunological applications of single-domain llama recombinant antibodies isolated from a naive library. Protein Engineering, Design & Selection. 2009;22(4):273-80.
98. Xu Y, Xiong L, Li Y, Xiong Y, Tu Z, Fu J, et al. Anti-idiotypic nanobody as citrinin mimotope from a naive alpaca heavy chain single domain antibody library. Anal Bioanal Chem. 2015;407(18):5333-41.
99. Davies EL, Smith JS, Birkett CR, Manser JM, Anderson-Dear DV, Young JR. Selection of specific phage-display antibodies using libraries derived from chicken immunoglobulin genes. Journal of Immunological Methods. 1995;186(1):125-35.
100. Tomlinson IM, Walter G, Marks JD, Llewelyn MB, Winter G. The repertoire of human germline vH sequences reveals about fifty groups of VH segments with different hypervariable loops. J Mol Biol. 1992;227(3):776-98.
101. Hoogenboom HR, Winter G. By-passing immunisation: human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. J Mol Biol. 1992;227(2):381-8.
102. WoÈrn A, PluÈckthun A. Stability engineering of antibody single-chain Fv fragments. J Mol Biol. 2001;305(5):989-1010.

103. Boder ET, Wittrup KD. Yeast surface display for screening combinatorial polypeptide libraries. Nature biotechnology. 1997;15(6):553-7.

104. Feldhaus MJ, Siegel RW, Opresko LK, Coleman JR, Feldhaus JMW, Yeung YA, et al. Flow-cytometric isolation of human antibodies from a nonimmune Saccharomyces cerevisiae surface display library. Nature biotechnology. 2003;21(2):163-70.

105. Solemani Zadeh A, Grässer A, Dinter H, Hermes M, Schindowski K. Efficient Construction and Effective Screening of Synthetic Domain Antibody Libraries. Methods and protocols. 2019;2(1):17.

106. Ledsgaard L, Kilstrup M, Karatt-Vellatt A, McCafferty J, Laustsen AH. Basics of Antibody Phage Display Technology. Toxins (Basel). 2018;10(6).

107. Smith GP. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science. 1985;228(4705):1315-7.

108. McCafferty J, Griffiths AD, Winter G, Chiswell DJ. Phage antibodies: filamentous phage displaying antibody variable domains. Nature. 1990;348(6301):552-4.

109. Kügler J, Wilke S, Meier D, Tomszak F, Frenzel A, Schirrmann T, et al. Generation and analysis of the improved human HAL9/10 antibody phage display libraries. BMC Biotechnology. 2015;15(1):10.

110. Grzeschik J, Könning D, Hinz SC, Krah S, Schröter C, Empting M, et al. Generation of semi-synthetic shark IgNAR single-domain antibody libraries. Phage Display: Springer; 2018. p. 147-67.

111. Abraham R, Buxbaum S, Link J, Smith R, Venti C, Darsley M. Screening and kinetic analysis of recombinant anti-CEA antibody fragments. J Immunol Methods. 1995;183(1):119-25.

112. Williams MN, Freshour G, Darvill AG, Albersheim P, Hahn MG. An antibody Fab selected from a recombinant phage display library detects deesterified pectic polysaccharide rhamnogalacturonan II in plant cells. Plant Cell. 1996;8(4):673-85.

113. Russo G, Meier D, Helmsing S, Wenzel E, Oberle F, Frenzel A, et al. Parallelized antibody selection in microtiter plates. Phage Display: Springer; 2018. p. 273-84.

114. Frenzel A, Kügler J, Helmsing S, Meier D, Schirrmann T, Hust M, et al. Designing Human Antibodies by Phage Display. Transfusion Medicine and Hemotherapy. 2017;44(5):312-8.

115. Leavy O. Therapeutic antibodies: past, present and future. Nature Reviews Immunology. 2010;10(5):297.

116. Basu K, Green EM, Cheng Y, Craik CS. Why recombinant antibodies—Benefits and applications. Current opinion in biotechnology. 2019;60:153-8.

117. Berger D. The Therapeutic Power of Monoclonal Antibody Therapy https://todaysveterinarypractice.com/; 2020 [Available from: https://todaysveterinarypractice.com/the-therapeutic-power-of-monoclonal-antibody-therapy/.

118. Deckers S, Braren I, Greunke K, Meyer N, Rühl D, Bredehorst R, et al. Establishment of hapten-specific monoclonal avian IgY by conversion of antibody fragments obtained from combinatorial libraries. Biotechnology and applied biochemistry. 2009;52(1):79-87.

119. Chen S, Li S, Sun H, Li Y, Ji S, Song K, et al. Expression and characterization of a recombinant porcinized antibody against the E2 protein of classical swine fever virus. Appl Microbiol Biotechnol. 2018;102(2):961-70.

120. Subramaniam S, Piñeyro P, Tian D, Overend C, Yugo DM, Matzinger SR, et al. In vivo targeting of porcine reproductive and respiratory syndrome virus antigen through porcine DC-SIGN to dendritic cells elicits antigen-specific CD4T cell immunity in pigs. Vaccine. 2014;32(50):6768-75.

121. Ooms K, Van Gorp H, Botti S, Van Gaever T, Delputte PL, Nauwynck HJ. Evaluation of viral peptide targeting to porcine sialoadhesin using a porcine reproductive and respiratory syndrome virus vaccination-challenge model. Virus research. 2013;177(2):147-55.

122. Liu H, Wang Y, Duan H, Zhang A, Liang C, Gao J, et al. An intracellularly expressed Nsp9-specific nanobody in MARC-145 cells inhibits porcine reproductive and respiratory syndrome virus replication. Vet Microbiol. 2015;181(3-4):252-60.

123. Argilaguet J, Perez-Martin E, Gallardo C, Salguero F, Borrego B, Lacasta A, et al. Enhancing DNA immunization by targeting ASFV antigens to SLA-II bearing cells. Vaccine. 2011;29(33):5379-85.

124. Bustamante J, Jankauski M, Daniel TL. Closed loop Monte Carlo models of abdominal contribution to insect flight control. Integr Comp Biol. 2018;58:E26-E.

125. Foord AJ, Muller JD, Yu M, Wang L-F, Heine HG. Production and application of recombinant antibodies to foot-and-mouth disease virus non-structural protein 3ABC. Journal of immunological methods. 2007;321(1-2):142-51.

126. Miyamoto K, Shimamoto T, Aosasa M, Kimura S, Nakamura N, Okubo Y, et al. Development of recombinant chicken IgY from single chain fragment of variable region for diagnosis of BSE. Biologicals. 2007;35(1):31-4.

127. Bhatia S, Gangil R, Gupta DS, Sood R, Pradhan H, Dubey S. Single-chain fragment variable antibody against the capsid protein of bovine immunodeficiency virus and its use in ELISA. J Virol Methods. 2010;167(1):68-73.

128. Sengupta D, Shaikh A, Bhatia S, Pateriya A, Khandia R, Sood R, et al. Development of single-chain Fv against the nucleoprotein of type A influenza virus and its use in ELISA. J Virol Methods. 2014;208:129-37.

129. Réfega S, Cluzeaud M, Péry P, Labbé M, Girard-Misguich F. Production of a functional chicken single-chain variable fragment antibody derived from caecal tonsils B lymphocytes against macrogamonts of Eimeria tenella. Vet Immunol Immunop. 2004;97(3-4):219-30.

130. Greenall SA, Tyack SG, Johnson MA, Sapats SI. Antibody fragments, expressed by a fowl adenovirus vector, are able to neutralize infectious bursal disease virus. Avian Pathology. 2010;39(5):339-48.

131. Li B, Ye J, Lin Y, Wang M, Zhu J. Preparation and identification of a single-chain variable fragment antibody against Newcastle diseases virus F48E9. Vet Immunol Immunop. 2014;161(3-4):258-64.

132. Mazzega E, De Marco A. Engineered cross-reacting nanobodies simplify comparative oncology between humans and dogs. Veterinary and comparative oncology. 2018;16(1):E202-E6.

133. Michels GM, Walsh KF, Kryda KA, Mahabir SP, Walters RR, Hoevers JD, et al. A blinded, randomized, placebo-controlled trial of the safety of lokivetmab (ZTS-00103289), a caninized anti-canine IL-31 monoclonal antibody in client-owned dogs with atopic dermatitis. Veterinary Dermatology. 2016;27(6):505-e136.

134. Webster RP, Anderson GI, Gearing DP. Canine Brief Pain Inventory scores for dogs with osteoarthritis before and after administration of a monoclonal antibody against nerve growth factor. Am J Vet Res. 2014;75(6):532-5.

135. Gearing DP, Huebner M, Virtue ER, Knight K, Hansen P, Lascelles BD, et al. In Vitro and In Vivo Characterization of a Fully Felinized Therapeutic Anti-Nerve Growth Factor Monoclonal Antibody for the Treatment of Pain in Cats. J Vet Intern Med. 2016;30(4):1129-37.

136. I. Birch Machin SR, L. Taylor, P. Iniguez, M.Marault, L. Ceglie, S. Zientara, C. Cruciere, F. Cancelotti, G. Koptopoulos, J. Mumford, M. Binns, N. Davis-Poynter, D. Hannant. Utilisation of bacteriophage display libraries to identify peptide seqeunces recognised by Equine herpesvirus type 1 specific equine sera. J Virol Methods. 2000:89-104.

137. Hulseweh B, Rulker T, Pelat T, Langermann C, Frenzel A, Schirrmann T, et al. Human-like antibodies neutralizing Western equine encephalitis virus. MAbs. 2014;6(3):718-27.

138. Burke CW, Froude JW, Rossi F, White CE, Moyer CL, Ennis J, et al. Therapeutic monoclonal antibody treatment protects nonhuman primates from severe Venezuelan equine encephalitis virus disease after aerosol exposure. PLoS pathogens. 2019;15(12):e1008157.

4 STUDIES PERFORMED

4.1 Manuscript I: PCR primer for the construction of an equine immunoglobulin library in the single-chain fragment variable (scFv) format

Franziska I. Pilger[a], Henrike P. Ahsendorf[a,b,c], Sascha Knauf[a,d], Anja Schwarz[a,] , Stefanie Walther[a] , Claus-Peter Czerny[a,†], Ulrike S. Diesterbeck[a,e], Kim Fechner[a,f] *

[a] Department of Animal Sciences, Georg-August-University, Goettingen, Burckhardtweg 2, 37077 Goettingen, Germany

[b] Present address: Institute of Virology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany

[c] Present address: Cluster of Excellence RESIST (EXC2155), Hannover Medical School, Carl-Neuberg-Straße 1, 30625 Hannover, Germany

[d] Present address: Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Südufer 10, 17493 Greifswald, Insel Riems, Germany

[e] Present address: Ceva Innovation Center GmbH, Am Pharmapark, 06861 Dessau-Rosslau, Germany

[f] Present address: SL Stanlab, Laboratory for Bacteriology and Food Hygiene, Borsigring 8, 31319 Sehnde, Germany

[†] Passed away during completion of this study.

*Corresponding author: Kim Fechner, Department of Animal Sciences, Division of Microbiology and Animal Hygiene, Faculty of Agricultural Sciences, Georg-August-University, Burckhardtweg 2, 37077 Goettingen, Germany; kfechner@gwdg.de; kfechner87@gmail.com

Declarations of interest: none.

Prepared for submission to Veterinary Immunology and Immunopathology.

4.1.1 Abstract

Species-specific recombinant antibodies including single chain fragment variable (scFv) immunoglobulins are an increasingly popular tool to prevent or treat veterinary conditions. The ability to assemble them to match any given epitope makes them particularly versatile. Moreover, recombinant antibodies can be produced fast, inexpensively and they can be stored up to several years under cooled or frozen conditions. In this study, we design recombinant equine antibodies in the scFv format and construct a scFv immunoglobulin library, generated from a donor previously immunized against EHV-1 (strain live-attenuated RAC-H and inactivated strain 438/77). We started with a detailed analysis of the equine immunoglobulin sequence data available in the NCBI GenBank using BLAST analysis. Subsequently, universal primer sets for the construction of an equine scFv were designed to amplify variable antibody domains of an immunized Hanoverian warmblood gelding. Variable domains were fused by splicing-by-overlap-extension PCR (SOE PCR) using primer sets containing a glycine-serine linker sequence as overlap sequence. Fused variable domains were ligated into a phagemid vector and transformed into phage display compatible *E. coli* cells with a transformation efficiency of 4.8×10^8 cfu/µg. Subsequent sequence analysis revealed g3 signal sequence, equine variable region of the heavy chain, the linker to the light chain variable region, and ligation into phagemid vector pCANTAB 5E in one reading frame. Presence of conserved key amino acid residues Cys, Trp and Leu was confirmed. A 3D model of an exemplary protein structures was calculated and revealed compact molecule showing crucial antigen binding structures. These products provide the basis for the isolation of high-affinity, neutralizing scFv molecules by phage display against any desired antigenic site e.g. EHV-1.

Keywords: ***Equus caballus*, recombinant library, variable, Herpesvirus, phagemid, bacterial systems**

4.1.2 Introduction

Since the 19th century, equine antibodies have played a key role in human and veterinary immunology. Equine antisera have aided the therapy of numerous human diseases such as tuberculosis and pneumonia (1-6), organ transplants, autoimmune diseases (7, 8), or poisoning (9). Moreover, hyper-immune equine sera have been used to treat and prevent equine diseases such as neonatal foal septicaemia (10). Similar to humans, horses also express all five immunoglobulin isotypes and are heterotetrameric in structure consisting of two identical heavy chains and two light chains (11). Equine immunoglobulin heavy and light chains are functionally and genetically divided into variable and constant domains; the constant domain of the heavy chain retains effector functions (11). The variable domains of either chain are created in the natural host by random fusion of variable (V), diversity (D), and joining (J) gene segments; the variable domain of the heavy chain is subsequently combined with a constant region gene (C) (11). The variable domains of heavy and light chain contain complementary determining regions (CDR) which serve as perfect counterparts to various antigen epitope regions, as these are highly diverse. Immunoglobulin diversity within a species and an individual is generated by combinatorial and junctional processes such as imprecise joining of gene segments, non-templated (N) or palindromic (P) nucleotide insertions or somatic hyper-mutations and is further enhanced by gene conversions and isotype switches (11). Horses in particular have enhanced combinatorial possibilities as they feature large numbers of available V gene segments: with 50 immunoglobulin heavy chain V genes (IGHV; 12 functional genes, 33 pseudogenes, and 5 open reading frames (ORF)), 40 IGHD genes, eight IGHJ and 11 IGHC genes on heavy chain gene loci contig Un0011 along with two additional functional IGHV genes, one ORF and one pseudogene on contig Un0038 (12). Only the African elephant featuring 41 IGHD genes and the guinea pig with 87 IGHD genes are comparable (11). Equine IGHC genes are available on chromosome 24 comprising 11 genes, which is only comparable to pigs, who are featuring the same number of IGHC genes (11). Yet another unique property

of horses are the characteristics of equine immunoglobulin IgG. IgG is by far the most versatile of all isotypes and can offer all antibody functions such as antigen binding, complement fixation, and binding to various cells and contributes 75% of all antibodies (11). Horses possess 7 subclasses, IgG1 to IgG7 which are classified according to differences within the C domain genes (11, 12). Each subgroup retains differing antigenic defence properties and thereby takes specific roles in protective immunity (11). IgG1, IgG4, and IgG7 play a key role in limiting the spread and severity of EHV-1 (11). The genetic variation in C genes are best explained by multiple duplications, gene conversions or cross overs, which is yet another exclusive phenomenon in horses (11).

Not only heavy chains contribute to immunoglobulin diversity, but also light chains increase and contribute to it (11). Equines express two different types of light chains, kappa (κ) and lambda (λ); their differences lie within their amino acid residue motifs, antigenic properties and chemical structure (11). Equine λ light chain genes are located on *Equus caballus* (ECA) chromosome 8 and contain 144 IGLV genes, seven IGLJ genes, and seven IGLC genes (11). Equine IGLV genes are divided into two clusters according to their transcriptional orientation, both containing functional and pseudogenes; presenting two clusters is yet another unique trait only occurring in horses (11). Equine κ light chain genes are located on ECA15, containing 60 IGKV genes, organized into one cluster, five IGKJ genes and one IGKC gene. Even though both types are present, 95 % of equine serum antibodies combine λ light chains with heavy chains (11).

Even though the use of equine sera to treat human conditions has been successful and would appear to offer a great variety of possibilities to treat veterinary as well as human conditions alike, a major disadvantage in species other than horses remains that foreign proteins are immunogenic and can result in the formation of anti-species antibodies. Equine sera do, therefore, not provide a feasible option where repeated immunoglobulin therapy is indicated

(13, 14). To circumvent this problem, designing species-specific recombinant antibodies has become a reliable method of generating more compatible antibody therapeutics (15). Species-specific antibody libraries contain the immunoglobulin repertoire of all available immunoglobulin data of one species. Recombinant antibody formats considered favourable for this purpose have been trialled on numerous occasions such as Fab, dAb and Diabody (16), yet, the single-chain-Fragment-variable antibody (scFv) format has proven to show outstanding tissue penetration characteristics owing to its smaller size in comparison to other antibody formats and reduced immunogenicity *in vivo* compared to non-species-specific antibodies (17-19). The scFv antibody consists of the variable domains of an antibody heavy chain (VH) and light chains (Vλ and Vκ). Both domains are generated by PCR utilizing comprehensive primer sets, designed taking all available immunoglobulin data into consideration. Once domains are amplified they are connected by a peptide linker in a splicing-by-overlap extension PCR (SOE-PCR).

In humans, recombinant scFv have already been established and used for therapeutic intervention (20). Several studies constructed recombinant antibodies from animal species such as cattle (21), chicken (22), mice (23), rabbit (24), macaques (25), sharks (26), and camels (27), partly for human medicinal use or veterinary use. Currently, two veterinary therapeutic antibody agents for the treatment of autoimmune diseases and osteoarthritis-related pain for dogs and cats are available (28). In addition, despite the vast availability of equine immunoglobulin gene data and genomic organisation, (11, 29-40), a primer set comprising the equine immunoglobulin repertoire for the construction of recombinant equine scFvs is still not available. In this study, we aimed to establish and validate a primer set for the construction of an equine immunoglobulin library in the recombinant scFv format.

4.1.3 Material and Methods

Animal immunization

An 18-year old Hanoverian gelding received three intra muscular EHV vaccinations: live-attenuated Prevaccinol® (EHV-1, Strain RAC-H; Intervet Deutschland GmbH, Unterschleißheim, Germany), and inactivated Equip $EHV_{1,4}$ (EHV-1, Strain 438/77; EHV-4 Strain 405/76; Pfizer, Berlin, Germany) prior to the study as part of a standard immunization regimen (see Figure 1).

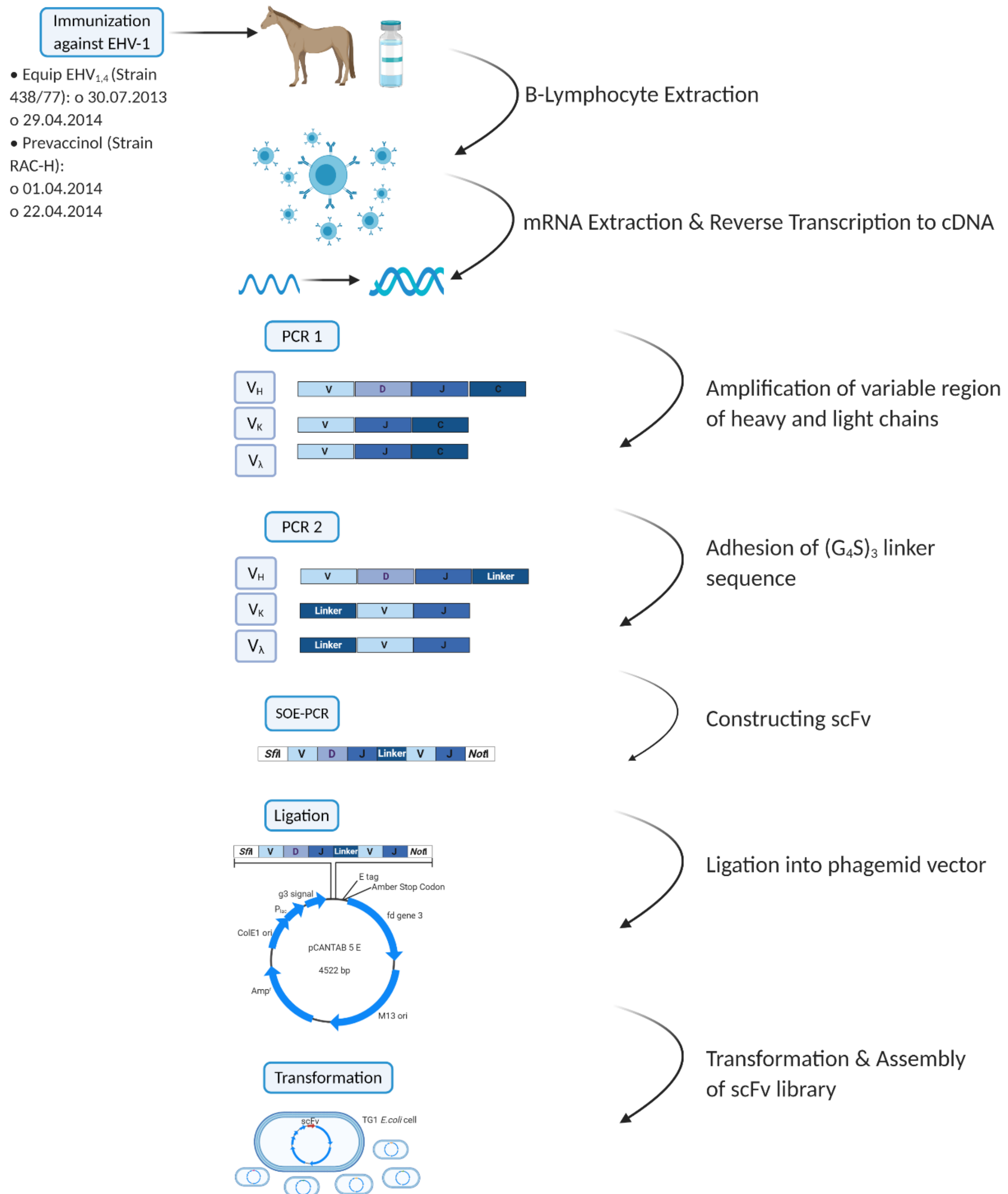

Figure 1: Schematic overview of all processes involved in constructing the equine immunoglobulin library. V – variable gene segment, D - diversity gene segment, J – joining gene segment, C – constant gene segment. VH – variable domain of heavy chain. Vλ - variable domain of lambda light chain. Vκ – variable domain of kappa light chain. Created with biorender.com

Ethics, blood collection and lymphocyte isolation

An EDTA-blood sample was collected during routine titre determination under consideration of the German codex “Good Veterinary Practice” (Bundesverband Praktizierender Tierärzte, Frankfurt am Main, Germany). The study has been approved by the Animal Welfare and Ethics Committee of the Georg-August University of Goettingen (Ref E3-20).

Density gradient centrifugation using Ficoll Paque Plus (GE Healthcare, Chalfont St. Giles, UK) was used to isolate peripheral blood mononuclear cells (PBMCs; see Figure 1) from EDTA-blood according to manufacturer’s protocol. Cells were washed with PBS (12 mM Phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4; Carl Roth GmbH&Co.KG, Karlsruhe, Germany) and erythrocytes were removed during incubation in red blood cell lysis buffer (12 mM $NaHCO_3$, 155 mM NH_4Cl, 0.1 mM EDTA; Carl Roth GmbH&Co.KG, Karlsruhe, Germany) for 5 min on ice. Isolated PBMCs were kept in liquid nitrogen until further use.

Total RNA isolation and cDNA synthesis

Total RNA was obtained from PBMCs (see Figure 1) using the RNeasy Mini Kit as described by the manufacturer (Qiagen, Hilden, Germany). Quantity and purity were verified by UV absorbance at 260 and 280 nm in a NanoDrop 1000 spectrophotometer (peQLab Biotechnology GmbH, Erlangen, Germany). Reverse transcription (see Figure 1) was achieved using the SuperScript III first-strand Synthesis Supermix (Invitrogen, Carlsbad, USA). Master mix 1 for the reverse transcription reaction contained 1 µl Annealing Buffer, 1 µl of random hexamers (50 ng/µl) and 6 µl total RNA. Master mix 1 was denatured at 65°C for 5 min followed by the addition of 10 µl of 2X First-Strand Reaction Mix and 2µl SuperScript® III/RNaseOUT™ Enzyme Mix. RNA was transcribed at 25°C for 10 min, incubated at 55°C for 50 min and finally terminated at 85°C for 5 min. cDNA was stored at -80°C until further use.

Primer Design

Primer design was established according to published equine immunoglobulin isotype IgG, subtypes IgG1-7, sequences and database entries. Using BLAST software (41), the database was screened for variable, joining, and first constant immunoglobulin gene segments of equine heavy and light chain sequences. Homologue sequences were aligned and analysed for similarity; duplicates were eliminated. Primers were designed using the resulting sequence alignment. Wobble bases were used in case of single nucleotide polymorphisms. Forward primer sets for PCR 1 were designed to bind to the 5´ end variable gene segments of the respective chains (sense). Reverse primer sets of the heavy chain binds to the 3´end of the first constant gene segments of IgG heavy chain isotypes (antisense). Reverse primers of both light chains bind to the 3´end of the respective constant regions. Generated VH fragments were expected to be 650 - 700 bp in length, while Vλ light chain fragments were expected to be approximately 600 bp in length and Vκ light chain fragments to range between 350 and 400 bp. Forward primer sets for PCR 2 of the heavy chain remained the same as for PCR 1. The reverse primer set were designed to bind to the joining region of VH and contain an overhang of 30 nucleotides coding partially for the N-terminal part of the $(Gly_4Ser)_3$ linker sequence. Forward primer sets for PCR 2 of the light chains were designed to anneal at the 5`end of variable domains and contain an overhang of 36 nucleotides coding for the C-terminal part of the $(Gly_4Ser)_3$ linker sequence. Reverse primers of the light chains were generated to bind to the 3`end of light chain joining regions. All generated fragments of all chains after PCR 2 were expected to be approximately 400 bp long. The $(Gly_4Ser)_3$ linker sequence is 45-nucleotide sequence (CAG GTG GAG GCG GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT) and connects VH with the Vλ or Vκ during SOE-PCR. Primers for the re-amplification of the SOE-PCR fusion product were designed to contain overhangs coding for restriction enzyme sites *Sfi*I (GCG GCC CAG C) and *Not*I (GCG GCC GC) for ligation purposes. Figure 1 shows a schematic overview of all gene segments amplified during all PCRs. Primers were

synthesized by Eurofins (Ebersberg, Germany). All sequences that were used for the primer design, are available in the Table S1.

Amplification of the equine heavy and light chain variable domains

Prior to this study our research group confirmed that all designed primers yield amplification products when combined individually. Out of 13 primers designed to bind to VH, only one forward primer, EqVH12_BACK, failed to amplify VH fragments when combined with reverse primer GSP2. Ten primers were designed to bind to the Vλ. EqVL9_BACK and EqVL10_BACK failed to produce amplification products when combined with IGLC1-7_FOR. EqVK6_BACK; EqVK8_BACK and EqVK12_BACK failed to amplify Vκ in combination with JK1-5_FOR. Primers that failed to amplify variable domains were excluded from this study. Primers that successfully amplified variable domains were pooled and PCR conditions optimised to determine suitable conditions for primer sets used for library construction.

Each pooled forward primer set (e.g. PCR1Hpool_FOR, PCR1Kpool_FOR) was combined with the respective reverse primer set and reactions were run in a 12-temperature-step (50°C - 70°C) gradient PCR cycler (Tprofessional, Biometra, Goettingen, Germany) to ascertain the most suitable annealing temperatures. Moreover, master mixes were prepared with differing concentrations of dimethyl sulfoxide (DMSO; Carl Roth GmbH&Co.KG, Karlsruhe, Germany), 0 %, 2.5 %, 5 %, and 7.5 % to determine optimal binding conditions for the primer pools. As a positive control for effective RNA isolation and reverse transcription into cDNA, the housekeeping gene equine glycerine-3-phosphate-dehydrogenase-housekeeping gene (eGAPDH; eGAPDH_for: 5'- TTG GCC GTA TTG GGC GCC CTG -3'; eGAPDH_rev: 3'- GGC AGG TCA GAT CCA CG -5') - was amplified and generated a fragment of 675 bp in length as well as a negative control (HPLC water; Carl Roth GmbH&Co.KG, Karlsruhe, Germany) to ascertain pureness of components. All amplifications were processed in a T3000 cycler (Biometra, Goettingen, Germany). Amplification was evaluated by agarose gel

electrophoresis on a 1% gel and DNA sequencing at a commercial sequencing facility (SeqLab, Goettingen, Germany).

cDNA encoding the V, D, J, and C gene segments of VH as well as the V, J, and C genes of Vλ and Vκ were amplified using the primers designed for PCR 1 and are summarised in Table 2. The master mixes for VH/Vλ/Vκ contained 5 µl 10x Standard Reaction Buffer with $MgCl_2$ (Biotools B&M Labs S.A., Madrid, Spain), 10 µM forward primer pool, 10 µM reverse primer pool , 2,5 % DMSO (Carl Roth GmbH&Co.KG, Karlsruhe, Germany), 10 mM dNTPs (Carl Roth GmbH&Co.KG, Karlsruhe, Germany), 1 U/µl DNA Polymerase (Biotools B&M Labs S.A., Madrid, Spain) and 2 µl cDNA. The overall reaction volume was adjusted to 50 µl with HPLC water (Carl Roth GmbH&Co.KG, Karlsruhe, Germany). The PCR was processed in a T3000 cycler (Biometra, Goettingen, Germany) and the cycling conditions for PCR 1 were as follows: 10 min at 95°C, followed by 35 cycles of 95°C for 1 min, 56°C for 1 min and 72°C for 2 min. The reaction was finalized with an elongation step at 72°C for 10 min. PCR products were purified using Min Elute Gel Extraction Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions to avoid contamination from side products during further use. The quantity and purity of the extracted DNA fragments were measured in a NanoDrop 1000 Spectrophotometer at UV absorbance of 260 and 280 nm.

Amplification of equine VH, Vλ and Vκ with linker overhang

As previously described, PCR 2 amplified the V, D, and J gene segments of VH as well as the V and J gene segments of Vλ and Vκ. Primers comprise an overlapping 45 nucleotide sequence translating a $(Gly_4Ser)_3$ linker to facilitate the joining of the variable regions in a subsequent PCR step and are summarised in Table 3. The master mix for PCR 2 is constituted of 10 µl 5x GC Reaction Buffer with $MgCl_2$ (Peqlab, Erlangen, Germany), 10 µM forward primer pool, 10 µM reverse primer pool, 10 mM dNTPs ,1 U/µl KAPA Hifi Polymerase (PeqLab, Erlangen, Germany) and 25 ng purified DNA of PCR 1. The reaction volume was

adjusted to 50 µl with HPLC water. PCR 2 reaction began with 5 min at 95°C, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 45 sec. Final elongation was appointed at 72°C for 10 min. PCR 2 was also run in a T3000 cycler (Biometra, Goettingen, Germany). PCR products of correct size were extracted and purified using the Min Elute Gel Extraction Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The quantity and purity of the extracted DNA fragments were measured at UV absorbance of 260 and 280 nm in the NanoDrop 1000 Spectrophotometer.

Generation of single chain antibody fragments VH - Vκ and VH - Vλ

The scFv fragments VH - Vκ and VH - Vλ were generated by SOE PCR. The linker overhang introduced in PCR 2 served as overlapping complementary sequence and recombines the VH with Vκ and VH with Vλ, respectively, during an initial recombination step of the SOE PCR. The fused product was re-amplified using primer sets that contained restriction enzyme sites for *Sfi*I and *Not*I (Table 4). These restriction enzyme sites are compatible with restriction enzymes sites of the phagemid vector pCANTAB 5E, which was used to construct the equine scFv immunoglobulin library.

For the first recombination step, 300 ng of gel-purified VH PCR 2 product was combined with either 300 ng of Vλ PCR 2 product for VH – Vλ recombination or 300 ng of Vκ PCR 2 product for VH – Vκ recombination. The reaction mix was constituted of 5 µl 10x Standard Reaction Buffer (Biotools B&M Labs S.A., Madrid, Spain) with $MgCl_2$, 2.5% DMSO, 10 mM dNTPs, 1 U/µl DNA polymerase (Biotools B&M Labs S.A., Madrid, Spain) and reaction volume was adjusted with HPLC water to 50 µl. The recombination was initiated at 95°C for 10 min, 15 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 2 min and a final elongation step at 72°C for 10 min. SOE-PCR was run in a T3000 cycler (Biometra, Goettingen, Germany). The recombination product was analysed by gel electrophoresis on a 1 % agarose gel and had an expected size of approximately 800 bp and was purified using QIAquick PCR

Purification Kit according to the manufacturer and re-amplified with primer pools containing the restriction cleavage sites for *Sfi*I and *Not*I (Table 4). The reaction mix contained 5 µl 10x Standard Reaction Buffer with $MgCl_2$ (Biotools B&M Labs S.A., Madrid, Spain), 10 µM forward primer pool, 10 µM reverse primer pool, 2.5 % DMSO, 10 mM dNTPs, 1 U/µl DNA polymerase and 25 ng of recombination product for VH - Vλ scFv fragments and 50 ng for VH-Vκ scFv fragments. The final reaction volume was adjusted to 50 µl with HPLC water. Re-amplification SOE PCR was initialized at 95°C for 10 min, followed by 30 cycles of 95°C for 1 min, 67°C for 1 min, 72°C for 2 min and a final elongation step at 72°C for 10 min.

Construction of scFv library in pCANTAB 5E

The phagemid vector pCANTAB 5E (GE Healthcare, Chalfont St. Giles, UK) was utilized to construct the immunized equine scFv library. This vector contains insertion sites flanked by the restriction enzyme cleavage sites for *Sfi*I and *Not*I and followed by an E-Tag sequence and an amber stop codon. The stop codon separates the E-Tag sequence from the fd-phage-gene 3, which codes for the minor coat protein III of the phage M13. The phagemid vector was extracted from dam-/dcm- *E. coli* cells (New England Biolabs GmbH, Frankfurt, Germany) using the Qiagen Spin Miniprep Kit (Qiagen, Hilden, Germany). scFv-insert DNA and vector DNA were cleaved with *Sfi*I (New England Biolabs GmbH, Frankfurt, Germany) and *Not*I (New England Biolabs GmbH, Frankfurt, Germany) prior to ligation according to the manufacturer's guidelines with some minor modification as described in Protocol S1. Digested scFv-insert DNA and dephosphorylated vector DNA were purified using the Monarch® PCR & DNA Cleanup Kit (New England Biolabs GmbH, Frankfurt, Germany) and ligated into a circular plasmid at a molar ratio of 1:5 (vector : insert). The ligation reaction mix contained 1 µl T4 Ligase (New England Biolabs GmbH, Frankfurt, Germany), 2 µl T4 Ligation Buffer (New England Biolabs GmbH, Frankfurt, Germany), 100 ng of vector DNA and the appropriate concentration of insert DNA. The reaction was incubated overnight at 16°C and heat inactivated

at 65°C for 90 Minutes. Monarch® PCR & DNA Cleanup Kit (New England Biolabs GmbH, Frankfurt, Germany) was used to purify and concentrate ligated DNA by removing salts and enzyme residues prior to electroporation. Purified, ligated DNA (100 ng) was transformed into Phage Display Electrocompetent TG1 cells (Lucigen Corporation, Middleton, USA). Electroporation was conducted using the BIO-RAD Gene Pulser® (Bio-Rad Laboratories GmbH, Munich, Germany) in a 1 mm electroporation cuvette (Bio-Rad Laboratories GmbH, Munich, Germany). Transformation was achieved using an electro pulse of 1,800 Volt, 100 Ohm, a capacitance of 50 µF and a TC of 5.0 ms. Subsequently, the transformed bacterial cells were grown in a 15 ml culture tube for 1 hour at 37°C in Recovery Medium (Lucigen Corporation, Middleton, USA). Transformation reactions were titrated in log_{10} steps until 10^{-6}, adding 100 µl into 900 µl 2x TYGA medium (16 g tryptone, 10 g yeast extract, 20 g glucose, 5 g NaCl, 100 µg/ml ampicillin, ad 1 L sterile water; all from Carl Roth GmbH & Co.KG, Karlsruhe, Germany), plated onto 2xTYGA agar plates (16 g tryptone, 10 g yeast extract, 20 g glucose, 5 g NaCl, 100 µg/ml ampicillin, 15 g agar-agar, 100 µg/ml ampicillin, ad 1 L sterile water; all from Carl Roth GmbH & Co.KG, Karlsruhe, Germany) and propagated overnight at 37°C. The dilution series of transformed bacteria on 2xTYGA agar plates was utilized to calculate the transformation efficiency (TE). Only successfully transformed bacteria propagated on these plates, since these bacteria are resistant to ampicillin. Bacteria were scraped from the plates using a Drigalski spatula with 6 ml 2xTYGA medium including 15% glycerol (Carl Roth GmbH&Co.KG, Karlsruhe, Germany). The bacterial glycerol stock was aliquoted to 1 ml and kept at -80°C. Ten clones of each sublibrary were analysed to encompass full-length inserts by colony PCR and Sanger sequencing of PCR products. Single colonies were picked for the colony PCR, and immersed into a 20 µl master mix made up of 2 µl 10x Standard Reaction Buffer with $MgCl_2$ (Biotools B&M Labs S.A., Madrid, Spain), 10 µM primer R1 (5'-CCA TGA TTA CGC CAA GCT TTG GAG CC-3') and primer 10 µM R2 (3'-CCT TTC TGC TGT TTT GAA ATC TAG C-5'; both synthesised at Eurofins Genomics Germany GmbH,

Ebersberg, Germany), 10 mM dNTPs (Carl Roth GmbH&Co.KG, Karlsruhe, Germany), 1 U/µl DNA polymerase (Biotools B&M Labs S.A., Madrid, Spain); the final volume was adjusted with HPLC water (Carl Roth GmbH&Co.KG, Karlsruhe, Germany). PCR ran in a T3000 cycler (Biometra, Goettingen, Germany) under the following settings: 94°C for 10 min, 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, followed by 72°C for 15 min. Individual clones that showed bands of expected size were proven to contain an ORF encoding full-size scFv fragments by sequencing utilizing primers R1 and R2, after plasmid DNA extraction. Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Ligations and transformations were repeated until a TE of 4.8×10^8 individual clones was achieved.

Sequence Data Analysis

Software used to import, analyse and align sequences included Lasergene EditSeq, and MegAlign (DNAStar, 12.3.1.4 Madison, USA). For primer design, homologue sequences were aligned using Lasergene MegAlign software (DNAStar, 12.3.1.4 Madison, USA) employing the ClustalW algorithm and analysed for similarity; duplicates were eliminated. Sequences from clones were also aligned using the ClustalW algorithm in MegAlign. SeqBuilder software (DNAStar, 12.3.1.4 Madison, USA) was used to translate nucleotide sequences into amino acid sequences and obtain ORF. The IMGT database (41) was used to determine CDR frameworks. A 3D model was calculated of the protein structure of a scFv antibody based on protein similarity found by the Phyre2 server (42) and annotated using Protean 3D (DNAStar, 12.3.1.4 Madison, USA). ProABC-2 was utilized to obtain probabilities of CDRs likely to interact with the antigen (43).

Fingerprint analysis

Fingerprint analysis assessed library diversity using the enzyme *Bst*NI (New England Biolabs GmbH, Frankfurt, Germany). The fingerprint analysis master mix contained 5 µl 10x NEBuffer 3.1 (New England Biolabs GmbH, Frankfurt, Germany), 0.5 µl *Bst*NI (New England Biolabs GmbH, Frankfurt, Germany), and 10 µl of colony PCR product; fifty µl HPLC water was used to adjust to the final volume. Cleaved colony PCR products were analysed by gel electrophoresis on a 2% agarose gel.

4.1.4 Results

Primer design

A detailed analysis of equine immunoglobulin genetics was conducted for the assembly of the equine scFv antibody library. Primer design was based on the up-to-date description of necessary gene loci by Sun et al (2010) and aligned with additional sequences available in the NCBI database to cover all possible genes. The analysis included database entries by Schrenzel et al. (1997), Tallmadge et al. (2003), Almagro et al. (2006), Home et al. (1992), Ford et al. (1994), and Wade et al. (2009) (12, 39, 40, 45-48). Table 1 provides an overview of the number of sequences analysed per gene segment and the number of primers designed to cover several genes per segment simultaneously. For this purpose, primers were designed to include wobble bases and primer pools were used for gene amplification, which was possible after prior confirmation of amplification success of individual primer pairings and amplification optimisation experiments for primer pools.

One IGKC gene is published and validated by our BLAST analysis, therefore only one primer was designed to cover the kappa constant gene. Furthermore, IGLC genes differed markedly, which resulted in almost an equal number of primers to the amount of genes; we designed five primers to amplify seven genes. Primers for PCR 2 were designed to contain the $(Gly_4Ser)_3$ peptide linker sequence to connect variable domains during SOE-PCR. SOE-PCR primers were

elongated with restriction enzyme cleavage sites for *Sfi*I and *Not*I. All primer pools are listed in Table 2, 3, and 4, including individual primer sequences, length, as well as accession number and binding positions of exemplary reference sequences.

Table 1: Number of equine variable gene segments per chain, including number of sequences used for analysis retrieved from NCBI data bank and the locations of these genes on the Equine Genome (EquCab 3.0) and the number of primers designed to cover all corresponding genes.

Immunoglobulin chain	Genes	Number of genes	Sequences aligned	Locations	Number of primers
Heavy	IGHV	50	73	Chromosome 24 (NC_009167.3) Contig 5705 (NW_019644314.1) Contig 80152 (NW_019645998.1) Contig 16657 (NW_019642020.1) Contig 93753 (NW_019646341.1)	13
	IGHJ	8	73		6
	IGHC	11	1	Chromosome 24 (NC_009167.3)	1
Lambda	IGLV	144	204	Chromosome 7 (NC_009150.3) Chromosome 8 (NC_009151.3)	10
	IGLJ	7	204		7
	IGLC	7	20	Chromosome 8 (NC_009151.3)	5
Kappa	IGKV	60	97	Chromosome 15 (NW_001867379.1) Contig 5625 (NW_019644222.1)	15
	IGKJ	5	97		5
	IGKC	1	100	Chromosome 15 (NW_001867379.1)	1

Table 2: Primer sets for PCR 1: primer set (forward and reverse) for amplification of the V, D, J and C gene segments of VH. Primer sets (forward and reverse) for V, J and C genes of the Vλ and Vκ. Primer names, sequence, length, accession number, and binding positions of exemplary reference sequences. Wobble bases are shown in bold and defined according to the IUPAC code.

Primer Name	Sequence (5‘- 3‘)	Length (bp)	Accession Number	Position
PCR 1 - Forward primer set for the amplification of equine VH (PCR1Hpool_FOR)				
EqVH1_BACK	CAG **R**T**R** CAA **S**TG AAG GAG TCA GG	23	HM176092	58 - 80
EqVH2_BACK	CAG GT**W** CAA **S**TG AAG GAG TC**R** GG	23	HM175938	58 - 80
EqVH3_BACK	CAG GTG CAA **S**TG **M**AG GA**S** TC**R** GG	23	HM175992	58 - 80
EqVH4_BACK	CAG GTG **S**A**R** CTG A**R**G GA**R** TC**R** GG	23	HM175979	58 - 80
EqVH5_BACK	CAG GTG CAA **M**TG AAG GAG **YY**G GG	23	HM175919	58 - 80
EqVH6_BACK	CAG GTG **MM**A CTG AAG GAG **K**C**Y** GG	23	HM176027	58 - 80
EqVH7_BACK	CAG **R**T**S** AG**S** CTG CAG GAG TC**W** G**S**	23	NW_001876796	141209 - 141509
EqVH8_BACK	CAG GTG **M**AG **Y**TG **M**AG GAG TC**R** GG	23	HM175915	58 - 80
EqVH9_BACK	CAG GT**R** CAG CTG CAG **R**AG **K**TG **R**G	23	NW_001876796	232063 - 232361
EqVH10_BACK	**S**AG GT**R** CAG CT**S** **R**TG **R**AG **Y**C**Y** GG	23	NW_001876796	283617 - 283909
EqVH11_BACK	**R**AG T**S**C A**K**C TGG TG**S** AGT CTG **R**	23	NW_001876796	211197 - 211492
EqVH13_BACK	GAG GGT CAG CTG GAA CAG TCG GG	23	NW_001876796	175891 - 176186
PCR 1 - Reverse primer set for the amplification of equine VH (PCR1Hpool_REV)				
GSP2	CTA CG**Y** TGC AGA TGT AGG TCT	21	NW_001876796	730907 - 730887
PCR 1 - Forward primer set for the amplification of equine Vκ (PCR1Kpool_FOR)				
EqVK1_BACK	G**RS** **R**TC GTG ATG A**YY** CAG **WY**T CC	23	NW_001867379.1	16739610 - 16739895
EqVK2_BACK	GAC ATC G**K**G TTG A**Y**C CAG T**Y**T CC	23	X75612.1	67 - 89
EqVK3_BACK	GAC **M**TC GTG ATG ACG CAG TCT CC	23	NW_001867379.1	17057542 - 17057828

EqVK4_BACK	GAT RTT GTG WTG ACC CAG ACT CC	23	NW_001867379.1	16599670 - 16599971
EqVK5_BACK	GAS AYT GTG HTG ACS CAG TYT CC	23	NW_001867379.1	16560248 - 16560552
EqVK6_BACK	GAA ATA ACA GTC ACA CAG TCT CC	23	NW_001867379.1	16431518 - 16431804
EqVK7_BACK	GAC GTC RTD WTG ACC CAG TCT CC	23	NW_001867379.1	16431518 - 16431804
EqVK8_BACK	GAG WYT GTG CTG ACT CAG TCT CC	23	NW_001867379.1	17071497 - 17071783
EqVK9_BACK	GAC ATC RTG WTG ACC CAG TCT CC	23	NW_001867379.1	16313053 - 16313339
EqVK10_BACK	GAC ATC GTG TTG ACC CAG TCT CC	23	NW_001867379.1	17010212 - 17010498
EqVK11_BACK	GAC ATC GTC WTG ACC CAG TCT CC	23	NW_001867379.1	16941855 - 16942141
EqVK12_BACK	GAC AKY GTA CTC ACC CAG TCT CC	23	NW_001867379.1	16855771 - 16856057
EqVK13_BACK	GAC ACA GTK TTG ACC CAG WCY CC	23	NW_001867379.1	16360142 - 16360443
EqVK14_BACK	GAC ATT ATD CTG ACC CAG TCT CC	23	NW_001867379.1	16933657 - 16933943
EqVK15_BACK	RAG ATC VTG WTG ACC CAG TCT CC	23	NW_001867379.1	16502696 - 16502982
PCR 1 - Reverse primer set for the amplification of equine Vκ (PCR1Kpool_REV)				
EqKC1_FOR	GGA AGA TGA AGG CAG ATG GCT TAG C	23	NW_001867379.1	13731499 - 13731821
PCR 1 - Forward primer set for the amplification of equine Vλ (PCR1Lpool_FOR)				
EqVL1_BACK	CAG YCT CTG ACT CAR CCC GC	20	HM176432	58 - 77
EqVL2_BACK	CAG TCT STG ACS CAG CCC GC	20	HM176296	58 - 77
EqVL3_BACK	CAG TCT GCC CTG ACT CAG CCT GC	23	NW_001867428.1	4590316 - 4590612
EqVL4_BACK	TCC TYG GAG CTG AMT CAG CCA SC	23	HM176470	58 - 80
EqVL5_BACK	TCT TCT GAG STG ACT CAG CCA MC	23	HM176469	58 - 80
EqVL6_BACK	TCT TCT AAG CTG ACT CAG CCA TC	23	HM176468	58 - 80
EqVL7_BACK	TCT TCT GCA STG ACT CAG CCR TC	23	HM176467	58 - 80
EqVL8_BACK	CAG WCT STG RCT CAG CCY GC	20	HM176394	58 - 77
EqVL9_BACK	CAG GMT GTG GTG ATY CAG GAM YC	23	NW_001867428.1	4763155 - 4763451
EqVL10_BACK	CAG TCT GTG GTG AYC CAG GAG CC	23	NW_001867428.1	4994608 - 4994903

PCR 1 - Reverse primer set for the amplification of equine Vλ (PCR1Lpool_REV)				
IGLC1_FOR	CTG CTG GAC GAT TTC CAC TG	20	JN228102	232 - 251
IGLC2_FOR	CTG CTG TAA GAT TTC CAC TG	20	NW_001867428	4139041 - 4139460
IGLC3_FOR	CTG CTG TAT GAT TTC CAC TG	20	NW_001867428	4134043 -4134062
IGLC4/6/7_FOR	CTG CTG TAC GAT TTC CAC TG	20	JN228115	232 - 251
IGLC5_FOR	CTG CTG TAC GAT TTC CAC TC	20	JN228115	232 - 251

Table 3: Primer sets for PCR 2: primer set (forward and reverse) for amplification of the V, D, and J gene segments of VH. Primer sets (forward and reverse) for V and J genes of Vλ and Vκ. Primers PCR2Hpool_REV, PCR2Kpool_FOR and PCR2Lpool_FOR include an overlapping 45-nucleotide sequence translating a $(Gly_4Ser)_3$ linker. Primer names, sequence, length, accession number and binding positions of exemplary reference sequences. Wobble bases are shown in bold and defined according to the IUPAC code. Letters in *italic* indicate the nucleotide sequence of the peptide linker.

Primer Name	Sequence (5'- 3')	Length (bp)	Accession Number	Position
PCR 2 – Reverse primer set for the amplification of equine VH and glycine-serine linker adhesion (PCR2Hpool_REV)				
EqJH1_FOR	*AGA ACC ACC TCC GCC TGA ACC GCC TCC ACC* TGA GGA GAC GGT GAC CAG G**R**T GC	23	HM175973	392 - 414
EqJH2_FOR	*AGA ACC ACC TCC GCC TGA ACC GCC TCC ACC* TGA GGA GAC GAA GAC CAG GAT GC	23	HM176051	401 - 423
EqJH3_FOR	*AGA ACC ACC TCC GCC TGA ACC GCC TCC ACC* TGA GGA **S**AC GGT GA**Y** CAG GG**Y** GC	23	HM175991	398 - 420
EqJH4_FOR	*AGA ACC ACC TCC GCC TGA ACC GCC TCC ACC* TGA GGA GAC **R**GT GAC CAG AGT GC	23	HM175957	413 - 435
EqJH5_FOR	*AGA ACC ACC TCC GCC TGA ACC GCC TCC ACC* T**R**A **S**GA GAC **R**GT GAC CAG GAT **R**C	23	HM176028	401 - 423
EqJH6_FOR	*AGA ACC ACC TCC GCC TGA ACC GCC TCC ACC* TGA GGA GAC GGT GAC CAG **W**AT GC	23	HM176011	395 - 417

PCR 2 – Forward primer set for the amplification of equine Vκ and glycine-serine linker adhesion (PCR2Kpool_FOR)				
EqVK1_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGG* **RSR** TCG TGA TGA **YY**C AG**W** **Y**TC C	58	NW_001867379.1	16739610 - 16739895
EqVK2_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGG* ACA TCG **K**GT TGA **Y**CC AGT **Y**TC C	58	X75612.1	67 - 89
EqVK3_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGG* AC**M** TCG TGA TGA CGC AGT CTC C	58	NW_001867379.1	17057542 - 17057828
EqVK4_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGG* AT**R** TTG TG**W** TGA CCC AGA CTC C	58	NW_001867379.1	16599670 - 16599971
EqVK5_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGG* ASA **Y**TG TG**H** TGA C**S**C AGT **Y**TC C	58	NW_001867379.1	16560248 - 16560552
EqVK7_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGG* ACG TC**R** T**DW** TGA CCC AGT CTC C	58	NW_001867379.1	16431518 - 16431804
EqVK9_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGG* ACA TC**R** TG**W** TGA CCC AGT CTC C	58	NW_001867379.1	16313053 - 16313339
EqVK10_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGG* ACA TCG TGT TGA CCC AGT CTC C	58	NW_001867379.1	17010212 - 17010498
EqVK11_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGG* ACA TCG TC**W** TGA CCC AGT CTC C	58	NW_001867379.1	16941855 - 16942141
EqVK13_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGG* ACA CAG T**K**T TGA CCC AG**W** C**Y**C C	58	NW_001867379.1	16360142 - 16360443
EqVK14_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGG* ACA TTA T**D**C TGA CCC AGT CTC C	58	NW_001867379.1	16933657 - 16933943
EqVK15_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CG***R** AGA TC**V** TG**W** TGA CCC AGT CTC C	58	NW_001867379.1	16502696 - 16502982
PCR 2 – Reverse primer set for the amplification of equine Vκ and glycine-serine linker adhesion (PCR2Kpool_REV)				
EqJK1_FOR	GT**B** **Y**GA **K**CT CCA GCT TGG TCC CCT	24	NW_001867379.1	13728269 - 13728232
EqJK2_FOR	**KY**T TGA T**Y**T CCA G**Y**T T**K**G TCC C**Y**T	24	NW_001867379.1	13727624 - 13727586
EqJK3_FOR	**K**TT TGA TCT CCA **MY**T TGG TCC C**Y**T	24	NW_001867379.1	13727931 - 13727894

EqJK4_FOR	G**Y**T TGA TTT CCA **R**CT TGG TCC CGG	24	NW_001867379.1	13727254 - 13727217
EqJK5_FOR	TTT TAA TGT CCA GAC GCG TCC CTT	24	NW_001867379.1	13728570 - 13728534
PCR 2 – Forward primer set for the amplification of equine Vλ and glycine-serine linker adhesion (PCR2Lpool_FOR)				
EqVL1_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGC* AG**Y** CTC TGA CTC A**R**C CCG C	55	HM176432	58 - 77
EqVL2_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGC* AGT CT**S** TGA C**S**C AGC CCG C	55	HM176296	58 - 77
EqVL3_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGC* AGT CTG CCC TGA CTC AGC CTG C	58	NW_001867428.1	4590316 - 4590612
EqVL4_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGT* CCT **Y**GG AGC TGA **M**TC AGC CA**S** C	58	HM176470	58 - 80
EqVL5_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGT* CTT CTG AG**S** TGA CTC AGC CA**M** C	58	HM176469	58 - 80
EqVL6_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGT* CTT CTA AGC TGA CTC AGC CAT C	58	HM176468	58 -80
EqVL7_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGT* CTT CTG CA**S** TGA CTC AGC C**R**T C	58	HM176467	58 - 80
EqVL8_Linker_BACK	*GTT CAG GCG GAG GTG GTT CTG GCG GTG GCG GAT CGC* AG**W** CT**S** TG**R** CTC AGC C**Y**G C	58	HM176394	58 - 77
PCR 2 – Reverse primer set for the amplification of equine Vλ and glycine-serine linker adhesion (PCR2Lpool_REV)				
EqJL1_FOR	CAG GAC G**M**T GAG GTG GGT CCC GC**Y**	24	NW_001867428.1	4146337 - 4146302
EqJL2_FOR	T**RR** GA**Y** GGT CAG **R**TG GGT **R**CC TC**Y**	24	NW_001867428.1	4141033 - 4140996
EqJL3_FOR	TAG GAC TAT CAG CTG GGT CCC TCA	24	NW_001867428.1	4135576 - 4135541
EqJL4_FOR	TG**Y** GAG GGT CAG GTG GGT GCC TCC	24	NW_001867428.1	4132448 - 4132410
EqJL5_FOR	TG**Y** GA**Y** GGT CAG GTG GGT GCC TCC	24	NW_001867428.1	4129086 - 4129050
EqJL6_FOR	T**R**C **R**AT GGT CAG GTG GGT GCC TCC	24	NW_001867428.1	4125743 - 4125707
EqJL7_FOR	TGC **R**AC GGT CAG GTG GGT GCC TCC	24	NW_001867428.1	4122272 - 4122236

Table 4: Primer sets for splicing-by-overlap-extension (SOE) PCR: primer sets (forward and reverse) for re-amplification of recombined VH - Vκ and VH – Vλ products and extension of restriction enzyme sites *Sfi*I and *Not*I for ligation. Table shows primer names, sequence, size, accession number, and binding positions. Wobble bases are shown in bold and defined according to the IUPAC code. Underlined nucleotides signify restriction enzyme recognition sites for *Sfi*I and *Not*I.

Primer Name	Sequence (5‘- 3‘)	Length (bp)
SOE – PCR Forward primer set for the amplification of the recombined product (VH +Vλ and VH + Vκ) including enzyme restriction site SfiI (SOE_H_pool)		
EqVH1BACK*Sfi*I	GTC CTC GCA ACT GCG GCC CAG CCG GCC CAG **R**T**R** CAA **S**TG AAG GAG TCA GG	50
EqVH2BACK*Sfi*I	GTC CTC GCA ACT GCG GCC CAG CCG GCC CAG GT**W** CAA **S**TG AAG GAG TC**R** GG	50
EqVH3BACK*Sfi*I	GTC CTC GCA ACT GCG GCC CAG CCG GCC CAG GTG CAA **S**TG **M**AG GA**S** TC**R** GG	50
EqVH4BACK*Sfi*I	GTC CTC GCA ACT GCG GCC CAG CCG GCC CAG GTG **S**A**R** CTG ATG GAT TC**R** GG	50
EqVH5BACK*Sfi*I	GTC CTC GCA ACT GCG GCC CAG CCG GCC CAG GTG CAA **M**TG AAG GAG **YY**G GG	50
EqVH6BACK*Sfi*I	GTC CTC GCA ACT GCG GCC CAG CCG GCC CAG GTG **MM**A CTG AAG GAG **K**C**Y** GG	50
EqVH7BACK*Sfi*I	GTC CTC GCA ACT GCG GCC CAG CCG GCC CAG **R**T**S** AG**S** CTG CAG GAG TC**W** G**S**	50
EqVH8BACK*Sfi*I	GTC CTC GCA ACT GCG GCC CAG CCG GCC CAG GTG **M**AG **Y**TG **M**AG GAG TC**R** GG	50
EqVH9BACK*Sfi*I	GTC CTC GCA ACT GCG GCC CAG CCG GCC CAG GT**R** CAG CTG CAG **R**AG KTG **R**G	50
EqVH10BACK*Sfi*I	GTC CTG CAA CTG CGG CCC AGC CGG CC**R** AGG T**S**C A**K**C TGG TG**S** AGT CTG **R**	50
EqVH11BACK*Sfi*I	GTC CTC GCA ACT GCG GCC CAG CCG GCC **R**AG GT**S** CA**K** CTG GTG **S**AG TCT G**R**	50
EqVH13BACK*Sfi*I	GTC CTC GCA ACT GCG GCC CAG CCG GCC GAG GGT CAG CTG GAA CAG TCG GG	50
SOE – PCR Reverse primer set for the amplification of the recombined product (VH + Vκ) including enzyme restriction site NotI (SOE_K_pool)		
EqJK1FOR*Not*I	GAG TCA TTC TCG ACT TGC GGC CGC GT**B** **Y**GA **K**CT CCA GCT TGG TCC CCT	48
EqJK2FOR*Not*I	GAG TCA TTC TCG ACT TGC GGC CGC **KY**T TGA T**Y**T CCA G**Y**T T**K**G TCC C**Y**T	48
EqJK3FOR*Not*I	GAG TCA TTC TCG ACT TGC GGC CGC KTT TGA TCT CCA **MY**T TGG TCC C**Y**T	48
EqJK4FOR*Not*I	GAG TCA TTC TCG ACT TGC GGC CGC G**Y**T TGC TTT CCA **R**CT TGG TCC CGG	48
EqJK5FOR*Not*I	GAG TCA TTC TCG ACT TGC GGC CGC TTT TAA TGT CCA GAC GCG TCC CTT	48

SOE – PCR Reverse primer set for the amplification of the recombined product (VH + Vλ) including enzyme restriction site *Not*I (SOE_L_pool)		
EqJL1FOR*Not*I	GAG TCA TTC TCG ACT TGC GGC CGC CAG GAC G**M**T GAG GTG GGT CCC GC**Y**	48
EqJL2FOR*Not*I	GAG TCA TTC TCG ACT TGC GGC CGC T**RR** GA**Y** GGT CAG **R**TG GGT **R**CC TC**Y**	48
EqJL3FOR*Not*I	GAG TCA TTC TCG ACT TGC GGC CGC TAG GAC TAT CAG CTG GGT CCC TCA	48
EqJL4FOR*Not*I	GAG TCA TTC TCG ACT TGC GGC CGC TG**Y** GAG GGT CAG GTG GGT GCC TCC	48
EqJL5FOR*Not*I	GAG TCA TTC TCG ACT TGC GGC CGC TG**Y** GA**Y** GGT CAG GTG GGT GCC TCC	48
EqJL6FOR*Not*I	GAG TCA TTC TCG ACT TGC GGC CGC T**R**C **R**AT GGT CAG GTG GGT GCC TCC	48
EqJL7FOR*Not*I	GAG TCA TTC TCG ACT TGC GGC CGC TGC **R**AC GGT CAG GTG GGT GCC TCC	48

Amplification of cDNA encoding equine immunoglobulin chains

The V, D, J, and C gene segments of VH as well as the V, J, and C genes of Vλ and Vκ were successfully amplified during PCR 1. Following initial confirmation that all primer combinations individually result in amplification products and excluding primer pairings that failed to amplify, primers were pooled and PCR optimisation experiments for PCR 1 primer pools have shown that distinguishable products are detectable in gel electrophoresis when 2.5 % DMSO are included in the master mix and annealing is set to 56°C. Primer pools for PCR 1 resulted in differing numbers of bands and band intensity. PCR 1 for Vκ amplified a fragment of approximately 350 - 400 bp as appeared on a 1% agarose gel. Cloning and sequencing (n=3) proved that PCR 1 Vκ products of 355 - 379 bp contained an ORF (Figure S1). PCR 1 of Vλ amplified products of approximately 600 bp. Cloning and sequencing (n=3) proved that PCR 1 Vλ fragments of 571 - 596 bp represent functional antibody sequences (Figure S1). PCR 1 of VH amplified products of approximately 650 - 700 bp. Cloning and sequencing (n=3) proved that PCR 1 VH fragments of 650 bp to contain ORFs (Figure S3). All PCR 1 products are shown in Figure 2.

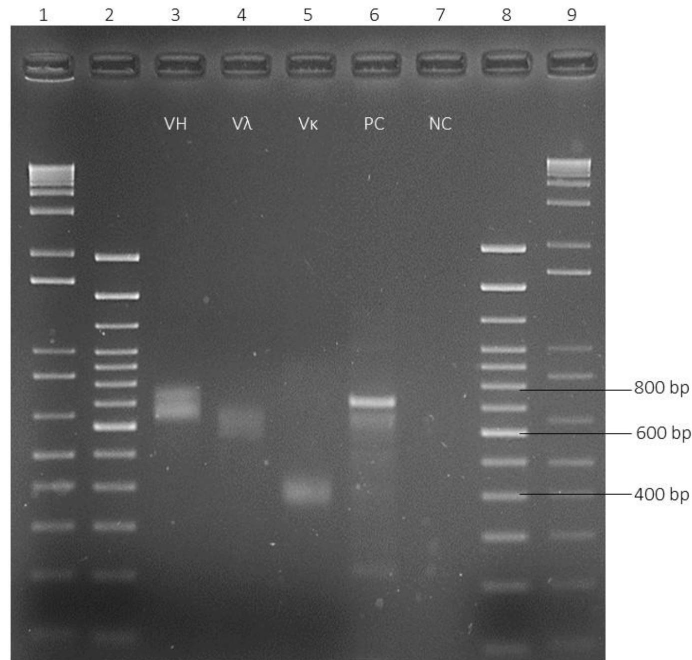

Figure 2: Amplification of equine cDNA by PCR 1 using pooled primer sets (PCR1_pool_FOR/PCR1_pool_REV of respective chain). Lane 1 and 9: 1 kb+ Ladder (Invitrogen, Carlsbad, USA). Lanes 2 and 8: 100 bp Ladder (Invitrogen, Carlsbad, USA). Lane 3: equine heavy chain (VH). Lane 4: equine lambda light chain (Vλ). Lane 5: equine kappa light chain (Vκ). Lane 6: positive control (PC; equine GAPDH). Lane 7: negative control (NC; HPCL).

Amplification of equine VH, Vλ and Vκ, linker adhesion

V, D, and J gene segments of VH as well as the V and J gene segments of Vλ or Vκ were amplified during PCR 2 and extended by $(Gly_4Ser)_3$ linker sequences on the 5' and 3' ends, respectively. PCR optimisation experiments for PCR 2 primer pools have shown that distinguishable products are detectable in gel electrophoresis when no DMSO was added to the master mix and annealing temperature was set to 60°C. PCR 2 of the VH region amplified products detectable in gel electrophoresis of approximately 400 - 500 bp as depicted in Figure 3. Cloning and sequencing (n=3) proved that PCR 2 VH fragments of 400 bp represent putative functional antibody sequences including the $(Gly_4Ser)_3$ linker sequence (Figure S1). PCR 2 for the Vλ amplified products of approximately 400 bp (Figure 3). Cloning and sequencing (n=3) proved that PCR 2 Vλ fragments of 375-395 bp represent functional antibody sequences and

include the $(Gly_4Ser)_3$ linker sequence (Figure S1). PCR 2 for the Vκ regions amplified products of approximately 400 bp (Figure 3). Cloning and sequencing (n=3) revealed that fragments of 361 - 381 bp to represent putative functional antibody sequences and to include the $(Gly_4Ser)_3$ linker sequence (Figure S1).

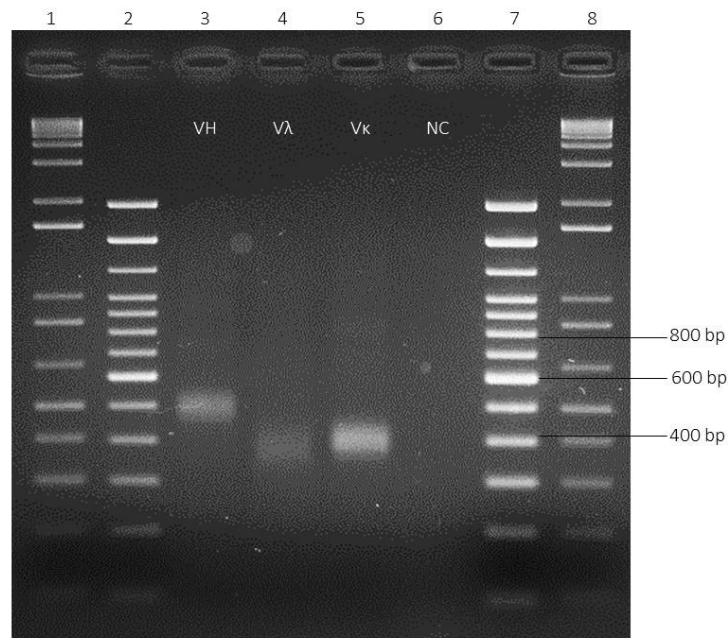

Figure 3: Amplification of equine cDNA by PCR 2 utilizing pooled primer sets (PCR2_pool_FOR/PCR2_pool_REV of respective chain). Lane 1 and 8: 1 kb+ Ladder. Lanes 2 and 7: 100 bp Ladder. Lane 3: equine heavy chain (VH). Lane 4: equine lambda light chain (Vλ). Lane 5: equine kappa light chain (Vκ). Lane 6: negative control (NC; HPLC).

Generation of scFv sequences

For the generation of scFv encoding DNA sequences, the PCR 2 products encoding VH were either recombined with Vλ or Vκ through the overlapping $(Gly_4Ser)_3$ linker sequence during SOE-PCR. Successful recombination was determined by 1 % agarose gel electrophoresis which revealed a band of 800 bp for the successfully recombined fragments (Figure 4). Sanger sequencing confirmed the products of 800 bp to represent complete scFv sequences. BLAST analysis revealed that VH and Vλ/Vκ were recombined into a single ORF,

containing the restriction enzyme cleavage sites as well as the E-tag sequence and Amber Stop codon of the phagemid vector pCANTAB 5E.

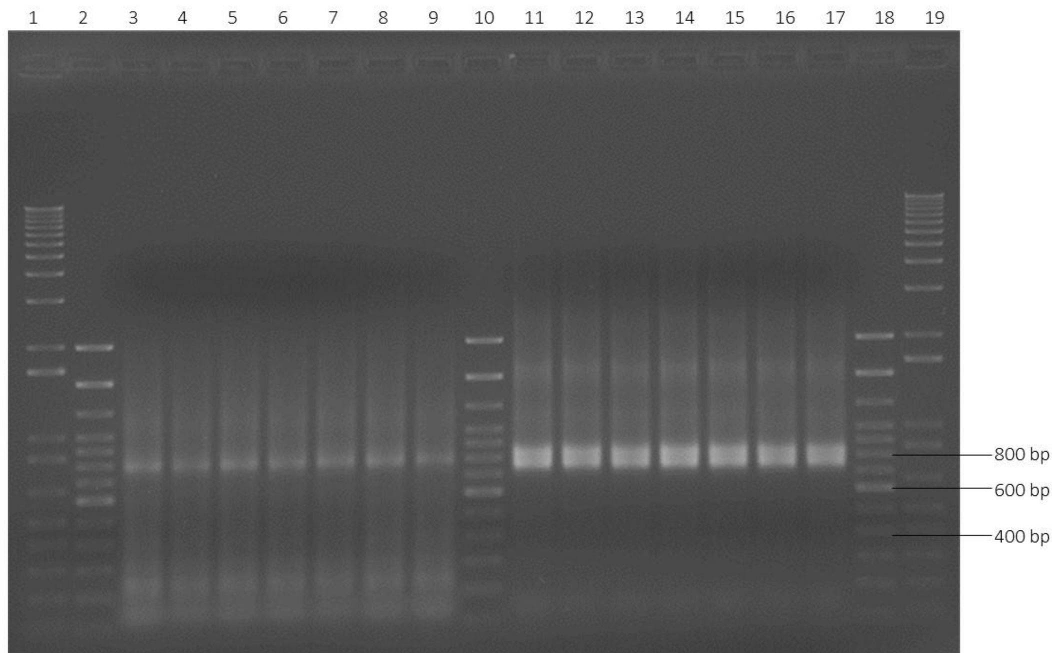

Figure 4: Recombination products of VH - Vλ and VH - Vκ. Lane 1 and 19: 1 kb+ ladder. Lanes 2, 10 and 18: 100 bp Ladder. Lane 3 to 9: VH - Vλ Splicing-by-overlap-extension (SOE) PCR recombination products. Lane 11 to 17: VH - Vκ SOE-PCR recombination products.

Construction of scFv library in pCANTAB 5E

The two sublibraries contained scFv sequences comprising recombination products of VH - Vλ and VH - Vκ, respectively. Three λ transformations with an average TE of 4.5×10^8 cfu/µg were combined with three κ transformations with an average TE of 7.8×10^8 cfu/µg. Sublibraries were considered for further use when at least 80% of selected clones revealed full-size scFv fragments as compared to empty vectors within a colony PCR. Fingerprint analysis using *BstN*I was used to evaluate clones from each sublibrary. Both sublibraries contain clones with differing restriction patterns and no duplications of one pattern (Figure 5). Therefore sublibraries can be considered to contain high sequence diversity.

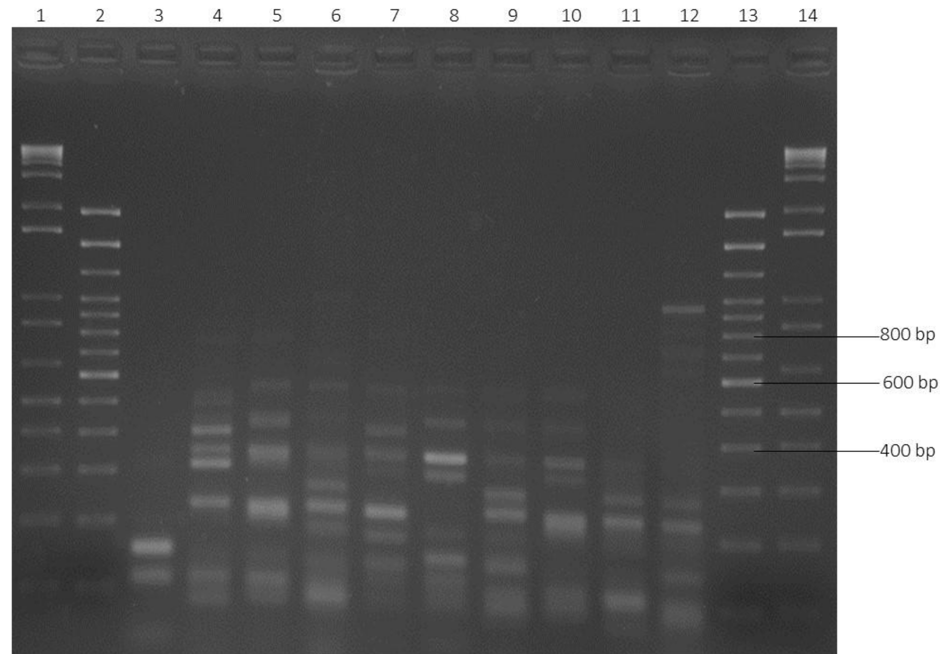

Figure 5: Colony PCR products after fingerprint analysis (*Bst*NI) of 10 bacterial clones of the λ sublibrary. Lane 1 & 14: 1kb ladder. Lane 2 & 13: 100 bp ladder. Lane 3 - 12: products of the *Bst*NI digest of 10 independent clones.

Prior to further downstream applications, vector-insert constructs were confirmed to contain an ORF, including g3 signal sequence, equine variable region of the heavy chain, the linker to the light chain variable region, and the E-Tag sequence and amber stop codon, further confirming ligation into phagemid vector pCANTAB 5E. Therefore, 3 clones per sublibrary were picked, cultivated overnight, plasmid DNA extracted and Sanger sequenced. An exemplary sequence (MW082605) is shown in Figure 6 and a 3D model is shown in Figure 7. The sequence of the exemplary scFv in Figure 6 shows the functionality of this clone as a complete scFv sequence was given since the ORF, contained all relevant sequences mentioned above. Sequence analysis of the CDRs shows both, hydrophobic as well as hydrophilic amino acid residues, yet hydrophilic residues predominate. Serine is the dominating amino acid, closely followed by an abundant amount of Glycine and Tyrosine. Analysis of amino acid residues within the ORF included Cys23 and alterations of conserved amino acid residues Trp41, Leu89, Cys104. These were substituted by Alanine, Valine, Threonine and Glycine. The

3D prediction in Figure 7 shows a molecule with the structures crucial for solvent exposed antigen binding regions (CDRs). As expected, CDR-H3 is lying at the center of the molecule and remaining CDRs are lying on the surface, close to each other, enabling antigen binding according to the key-lock model. Highest probability to interact with the antigen was obtained for CDR-H2 (Pt 0,49) and CDR-L1 (Pt 0.5) according to proABC-2 (1).

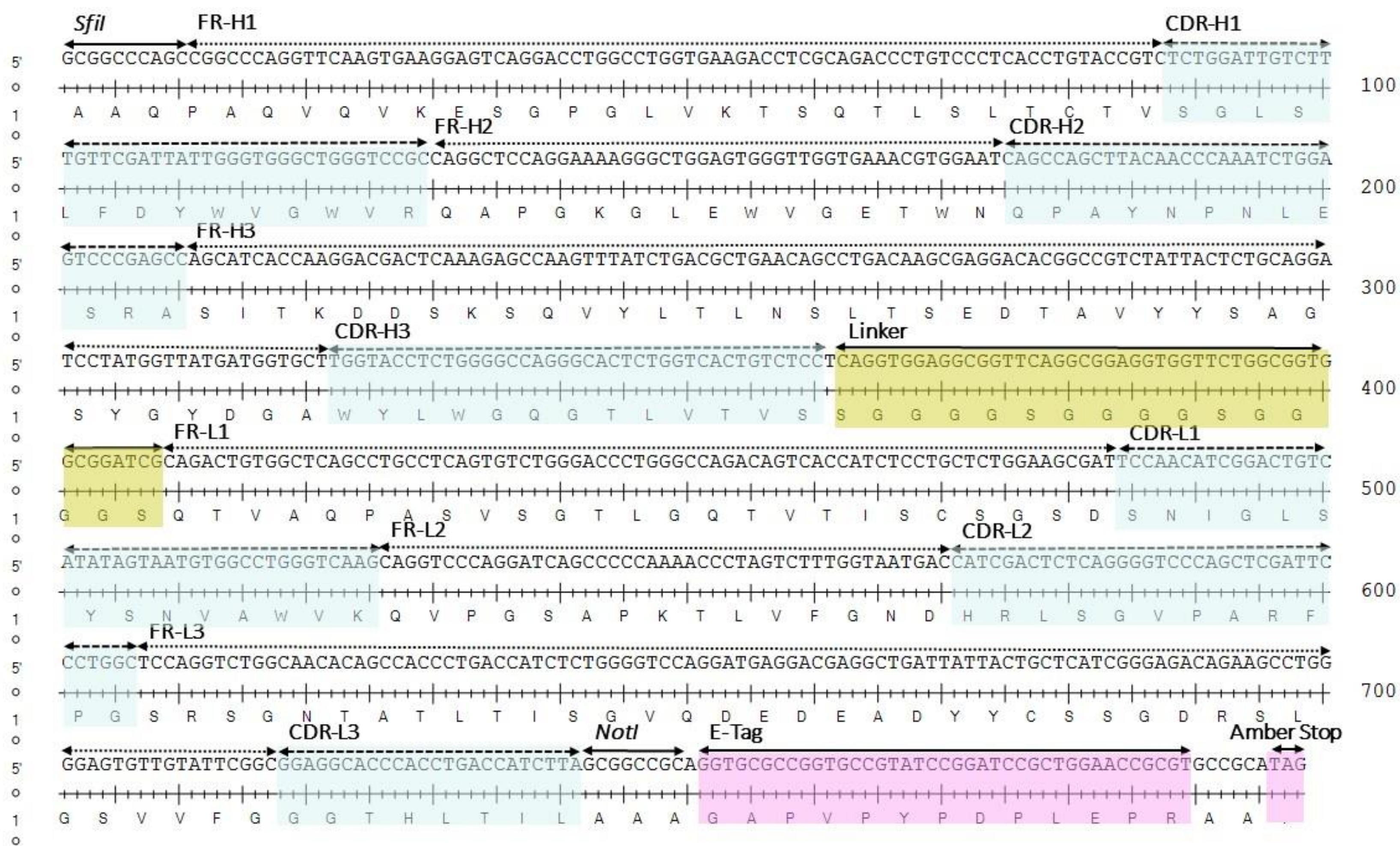

Figure 6: Nucleotide sequence of scFv clone HL1. Highlighted are CDRs (light blue) of heavy chain and λ light chain as well as the linker sequence (yellow) and E-Tag sequence and Amber Stop codon (purple).

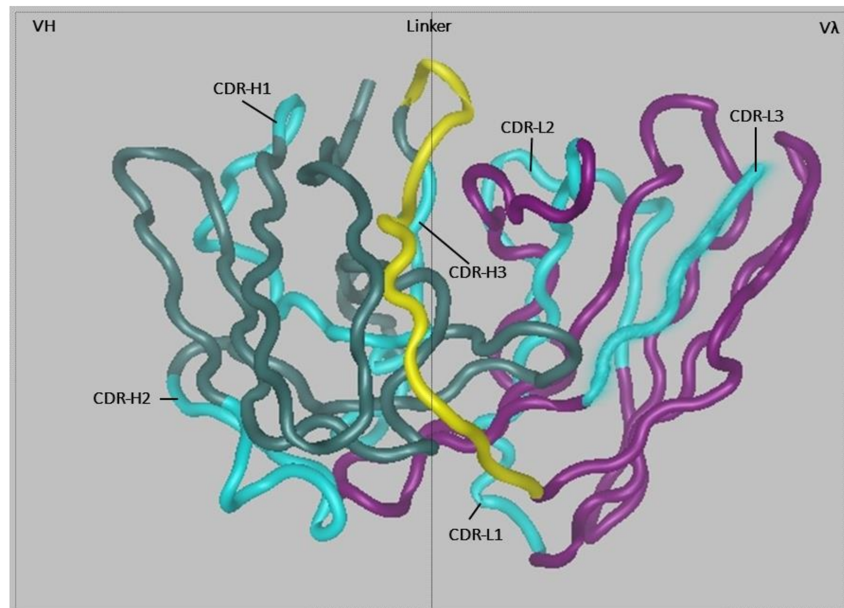

Figure 7: 3D model of exemplary scFv clone HL1. Marked are the linker and CDR regions of heavy chain and λ light chain. The heavy chain (VH) is marked in dark blue on the left side, the λ light chain (Vλ) is marked in purple on the right side. All CDRs are highlighted in light blue. The $(Gly_4Ser)_3$ linker is marked in yellow.

4.1.5 Discussion

The primer sets designed in this study to construct recombinant equine antibodies successfully amplified equine variable domains and connected them to scFv antibody fragments. Fragments were successfully cloned into phage competent *E. coli* cells and an equine scFv library was assembled providing a basis for antibody isolation by phage display technology in future studies. Phage display technology requires a high-quality antibody gene library, which is given if it contains a large gene collection, representing a vast diversity of antibodies, numerically speaking when it contains more than one million individual antibody sequences (44). While we were able to generate an equine library of that size, containing $4.8x10^8$ antibody sequences, each presumed to be unique according to fingerprint analysis results (see Figure 5), it could be argued that diversity could be increased even further. In this study, antibody encoding genes were obtained from B cells of a single warmblood gelding.

However, a large number of donors from various breeds, equine enterprises or parts of the world, would ensure a broader coverage of the species' allelic diversity (44). Human antibody library HAL4/7/8 was constructed from 44 donors, yielding five billion independent clones within their library (45). The next generation of human library of this particular group was constructed from as many as 98 donors, yielding 10 billion independent clones (46).

Individual pairings of forward and reverse primers resulted in amplification products for most combinations that were confirmed by sequencing to be equine variable segments. Unsuccessful amplification of individual primer pairings could be due to the formation of primer dimers or secondary structures consequently inhibiting amplification of target DNA sequences. Unproductive primer pairings were excluded from the primer sets for library construction.

The primer sets used in this study consist of a total of 115 primers with primer sets containing between five and 13 primers. This is comparable to the construction of human scFv antibodies to the extent that their number of primers per set also ranged between 4 and 7 (47). It is however not comparable to the total amount of primers used as it only takes a total of 33 primers to construct human scFv antibodies (47). It takes a total of 19 primers to construct rabbit scFv antibodies (48) and a total of 13 primers to construct an immunized mouse antibody library (49). A study assembling a bovine scFv library used 46 primers (50). The noticeable differences between horses and other species lies within the amount of immunoglobulin gene segments of each species. Horses have a markedly higher number of variable gene segments having 52 IGHV genes, 60 IGKV genes and 144 IGLV genes, which is contributing to the immunoglobulin diversity and the ability to respond to numerous antigens (11). In contrast, humans for example only have 38-46 IGHV genes, 34-38 IGKV genes and 29-33 IGLV genes (11). Furthermore, it is described that immunoglobulin gene usage and expression changes with age (11, 51) and among breeds as observed between Rhenish German warmblood and

Hanoverian warmblood (52). Since an intra-breed variability as well as an age variability in regards to gene expression cannot be excluded, it was important to include all equine immunoglobulin sequences that were available on the NCBI data base (53). Next to warmblood breeds, thoroughbred, Arabs, the Mongolian breed Ajinai, of different ages were available. All this information must be considered when establishing an equine IgG primer set intended for all breeds of all ages.

Primer sets generated multiple products during PCR 1 in particular during amplification of VH and Vλ. Multiple products were also observable during SOE-PCR, particularly while recombining VH with Vκ. The formation of multiple products could be due to differences in length of individual variable domain gene segments. Generally, immunoglobulin diversity of the heavy chain is characterized by the rearrangement of individual V, D, J gene segments. It has however been observed that in equines a D-D fusion recombination takes place, rendering the order to V-D-D-J, thereby elongating the fragment substantially (12). While common equine recombination products are on average 30.5 bp, D-D fusion rearrangements range from 32 to 58 bp (12). Additional findings contributing to size differences of amplification products, are N- and P- nucleotide insertions further enhancing diversity (12). On top of that, D gene segments vary in their individual sizes and can be found in three possible reading frames (RF) on each side, each RF varying in size as well; D genes of the equine heavy chain range from 18 to 48 bp (12) posing a substantial difference in size that should not to be overlooked (11).

Further consideration in regards to the formation of multiple bands should be taken in regards to wobble bases, or the GC content of a primer as they might render primers prone to the formation of secondary structures which result in either no yield at all or have adverse effects on annealing temperature and amplification. In this study, special consideration was taken to thoroughly optimising PCR conditions (54). Gel excision, cloning and sequencing of each band revealed only products of expected sizes to contain functional sequences, while other

products did not contain complete equine variable domains and were deemed unconstructive for library construction since they revealed premature stop codons within the sequence.

Successfully amplified functional variable domains were recombined to scFv antibody fragments of approximately 800 bp by SOE-PCR. This is in accordance with other studies where human and murine scFv antibody sizes are approximately 750 - 800 bp (49, 55); bovine scFv antibodies were as long as approximately 800 bp due to their extra-long CDR 3 regions (50). It is not possible to compare species-specific equine scFv antibodies constructed in this study to other studies constructing recombinant equine antibodies since to this date none have been developed. There are simply murine or human scFv that have been produced against Venezuelan Equine Encephalitis Virus (56, 57) and Western Equine Encephalitis Virus (58) however in the context of treating human conditions. In veterinary medicine, most advances in recombinant antibody production, have been made in the field of production animals such as swine, cattle, and poultry as well as companion animals (59, 60). The scFv antibody format has proven to be the most popular format (61) and has been developed to, e.g. diagnose swine dysentery or Avian Influenza; therapeutic and prophylactic agents in the scFv format have also been developed for swine, cattle, and poultry (59).

ScFv antibodies have shown to represent a suitable and commonly used recombinant format for antibody isolation by phage display along with naïve, Fab antibody fragments, and can easily be re-formatted to full sized antibodies or Fabs if necessary (44). They are producible at low cost, while exhibiting better specificity and solubility, reduced immunogenicity as well as neutralisation ability (61, 62). A scFv antibody trialled against peptides playing a role in Alzheimer's disease has proven to retain specificity and protective properties of the parental antibody, while a full-length immunoglobulin in the same trial led to adverse effects (62). ScFv antibodies are also a popular tool in the development of biorecognition elements due to their

versatility, customizability and their immobilization properties on surfaces where whole antibodies would be difficult to immobilize (63).

When constructing scFv antibodies it is important to use the peptide linker $(Gly_4Ser)_3$; Fragment variable (Fv) antibodies are unstable due to the differences of CDR 3 length (63). The glycine-serine linker provides the stability for the variable antibody fragments. Its widespread use in the construction of scFv molecules (64, 65) is due to its reliability in terms of not only stability but also flexibility. This is essential for the correct orientation of variable heavy chain and variable light chain domains in the scFv molecule (66). A study by van Rosmalen et al. (2017) has shown, that any linker longer than $(Gly_4Ser)_3$ or with a lower glycine content will result in lower efficiency and increased stiffness, preventing proper protein folding.

Another important consideration during scFv construction is the purification of amplification products. This study has shown that templates used for the recombination reaction best be gel-purified as other methods result in products that still contain amplification by-products; these could possibly inhibit proper recombination reactions.

The genetic code of a complete, recombinant, functional equine scFv fusion protein was ligated into the phagemid vector pCANTAB 5E. This phage-competent vector has been and is still used widely across human (67) and veterinary scFv production (50, 68). Moreover, phagemid vectors promise higher TE than phage vectors, resulting in larger antibody libraries exhibiting a greater diversity (69). The restriction enzyme cleavage sites in this vector ensure direct cloning of the scFv sequences. The E-Tag sequence allows for an effortless purification and detection of soluble scFv antibodies. An alternative method to ligating the full scFv construct into the vector in between the cleavage sites is using a vector that already contains the linker sequence such as pHAL14 or its modified version pHAL30 (70). Firstly, the light chain will be ligated into the vector, before the heavy chain is ligated into the construct (70).

Ligated plasmids were transformed into electro-competent TG1 *E. coli* cells through electroporation. These cells contain an F-pilus which is necessary for infection by M13 phages when constructing a phage display library later on. Multiple transformation reactions were carried out which is in accordance with recent publications and protocols that suggested performing up to 100 ligations for large libraries (70). Transformation outputs are subject to purity of DNA in relation to electro-competent transformation parameters and should be adjusted accordingly.

The overall TE of 4.8×10^8 cfu/µg was deemed satisfactory, as it is widely known that immunised antibody libraries yield a lower number of antibodies than non-immunised libraries, as the immune system of the B-lymphocyte donor has already undergone affinity maturation (71). Nian et al. (2016) constructed a human scFv library from a donor which was immunized against *Staphylococcus aureus* reaching a TE of 1.7×10^7 cfu/µg, while Li et al. (2000) reached a TE of 8.7×10^8 cfu/µg constructing an immunized scFv rabbit library.

Fingerprint analysis of the sublibraries revealed highly diverse sublibraries as clones exhibited unique restriction patterns. The sequencing result of the exemplary λ clone among others proofed that the libraries contain functional scFv clones, containing ORFs, consisting of equine variable regions and successful ligation into phagemid vector pCANTAB 5E. Sequence analysis revealed hydrophilic and hydrophobic amino acid residues, but predominantly hydrophilic residues. Hydrophilicity is advantageous as it enhances flexibility (72). Antibody sequences should contain an abundant amount of Serine, Glycine and Tyrosine and the key amino acid residues Cys23, Trp41, Leu89 and Cys104. This was the case for the sequence on hand, however Trp41, Leu89 and Cys104 were substituted by Alanine, Valine, Threonine and Glycine, which bear similar biochemical characteristics. CDR regions were confirmed to lie in their expected positions, with the CDR-H3 lying at the center of the molecule and remaining CDR-regions are lying on the surface. Highest probability to interact with the antigen was

obtained for CDR-H2 (Pt 0,49) and CDR-L1 (Pt 0.5) (43) confirming successful construction of antigen binding scFv antibodies. Yet, the exemplary scFv sequence lacks Cysteine residues within CDRs which would enhance its stability.

4.1.6 Conclusion

New PCR primers were developed to amplify variable domains of equine IgG1-7 antibody genes. The primers enable the construction of equine scFv antibodies and the construction of an immunized equine scFv antibody library in bacterial systems with a diversity of 4.8×10^8 cfu/µg. The equine antibody library provides the basis for the isolation of neutralizing scFv fragments by phage display technology. Isolates will have affinities towards EHV-1 and -4 as well as other pathogenic agents the donor had been immunized with as part of a standard immunization regimen (Influenza and Tetanus). Primers for this purpose did not exist for horses to this date and will pave the way for future recombinant equine antibody production.

4.1.7 Acknowledgements

The authors would like to thank Tamara Rusitzka for providing blood samples of her horse “William”, which had received the booster vaccinations prior to this study.

4.1.8 References (Vancouver)

1. Daniels J. Über die prophylaktische Wirkung des Diphtherieheilserums und des normalen antitoxinfreien Pferdeserums auf die Diphtherieinfektion des Meerschweinchens. Feller, Neubrandenburg. 1921.

2. Bingel A. Über Behandlung der Diphtherie mit gewöhnlichem Pferdeserum. F C W , Leipzig. 1918.

3. Behring von E. Epidemiologie, Aetiologie und Bekämpfung der Diphtherie. Beiträge zur Experimentellen Therapie. 1918.

4. Glatman-Freedman A, Casadevall A. Serum therapy for tuberculosis revisited: reappraisal of the role of antibody-mediated immunity against Mycobacterium tuberculosis. Clinical microbiology reviews. 1998;11(3):514-32.

5. Winau F, Winau R. Emil von Behring and serum therapy. Microbes and infection. 2002;4(2):185-8.

6. Cole R, Moore HF. The production of antipneumococcic serum. The Journal of experimental medicine. 1917;26(4):537-61.

7. Leleu C, Cotrel C. Body composition in young standardbreds in training: relationships to body condition score, physiological and locomotor variables during exercise. Equine Vet J Suppl. 2006(36):98-101.

8. Zand MS. B-cell activity of polyclonal antithymocyte globulins. Transplantation. 2006;82(11):1387-95.

9. Taherian AF, Mohammad; Moghadam, Ahmad Taghavi; Tebyanian, Hamid Optimization of purification procedures for horse F(ab')2 antivenom against *Androctonus crassicauda* (Scorpion) venom. Tropical Journal of Pharmaceutical research. 2018;17(3).

10. Morris DD, Whitlock RH. Therapy of Suspected Septicemia in Neonatal Foals Using Plasma-containing Antibodies to Core Lipopolysaccharide (LPS). Journal of Veterinary Internal Medicine. 1987;1(4):175-82.

11. Walther S, Rusitzka TV, Diesterbeck US, Czerny C-P. Equine immunoglobulins and organization of immunoglobulin genes. Developmental & Comparative Immunology. 2015;53(2):303-19.

12. Sun Y, Wang C, Wang Y, Zhang T, Ren L, Hu X, et al. A comprehensive analysis of germline and expressed immunoglobulin repertoire in the horse. Dev Comp Immunol. 2010;34(9):1009-20.

13. Lang J, Kamga-Fotso L, Peyrieux JC, Blondeau C, Lutsch C, Forrat R. Safety and immunogenicity of a new equine tetanus immunoglobulin associated with tetanus-diphtheria vaccine. Am J Trop Med Hyg. 2000;63(5-6):298-305.

14. Theakston RD, Warrell DA, Griffiths E. Report of a WHO workshop on the standardization and control of antivenoms. Toxicon. 2003;41(5):541-57.

15. Dias-Neto E, Nunes DN, Giordano RJ, Sun J, Botz GH, Yang K, et al. Next-Generation Phage Display: Integrating and Comparing Available Molecular Tools to Enable Cost-Effective High-Throughput Analysis. Plos One. 2009;4(12):e8338.

16. Hoogenboom HR. Selecting and screening recombinant antibody libraries. Nature Biotechnology. 2005;23:1105.

17. Ahmad ZA, Yeap SK, Ali AM, Ho WY, Alitheen NBM, Hamid M. scFv Antibody: Principles and Clinical Application. Clinical & Developmental Immunology. 2012.

18. Wang H, Yu R, Zhang X, Ren J, Xie N, Fan H, et al. [Construction and expression of recombinant prokaryotic expression vector for disulfide-stabilized anti-TeNT single-chain Fv antibody]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2013;29(1):60-4.

19. Sandhu JS. Protein Engineering of Antibodies. Critical Reviews in Biotechnology. 1992;12(5-6):437-62.

20. Bradbury ARM, Sidhu S, Dübel S, McCafferty J. Beyond natural antibodies: the power of in vitro display technologies. Nature biotechnology. 2011;29(3):245-54.

21. O’Brien P, Aitken R, O’Neil B, Campo M. Generation of native bovine mAbs by phage display. Proceedings of the National Academy of Sciences. 1999;96(2):640-5.

22. Reynaud C-A, Dahan A, Anquez V, Weill J-C. Somatic hyperconversion diversifies the single VH gene of the chicken with a high incidence in the D region. Cell. 1989;59(1):171-83.

23. Kaartinen M, Mäkelä O. Reading of D genes in variable frames as a source of antibody diversity. Immunology Today. 1985;6(11):324-7.

24. Becker RS, Knight KL. Somatic diversification of immunoglobulin heavy chain VDJ genes: Evidence for somatic gene conversion in rabbits. Cell. 1990;63(5):987-97.

25. Chassagne S, Laffly E, Drouet E, Hérodin F, Lefranc M-P, Thullier P. A high-affinity macaque antibody Fab with human-like framework regions obtained from a small phage display immune library. Molecular Immunology. 2004;41(5):539-46.

26. Liu JL, Anderson GP, Delehanty JB, Baumann R, Hayhurst A, Goldman ER. Selection of cholera toxin specific IgNAR single-domain antibodies from a naïve shark library. Molecular Immunology. 2007;44(7):1775-83.

27. Honda TI, JP), Akahori, Yasushi (Nagoya, JP), Kurosawa, Yoshikazu (Nagoya, JP), inventor; Institute For Antibodies Co., Ltd. (Nagoya-shi, JP), assignee. Methods of constructing camel antibody libraries. United States2008.

28. Berger D. The Therapeutic Power of Monoclonal Antibody Therapy https://todaysveterinarypractice.com/; 2020 [Available from: https://todaysveterinarypractice.com/the-therapeutic-power-of-monoclonal-antibody-therapy/.

29. Helms CM, Allen PZ. Studies on equine immunoglobulins—I the antigenic structure of horse IgG, its fragments and subunits. Immunochemistry. 1970;7(5):401-12.

30. Hill WC, Cebra JJ. Horse Anti-SI Immunoglobulins. I. Properties of γM-Antibody. Biochemistry. 1965;4(12):2575-84.

31. Pahud JJ, Mach JP. Equine Secretory IgA and Secretory Component. International Archives of Allergy and Immunology. 1972;42(2):175-86.

32. Rockey JH, Klinman NR, Karush F. Equine antihapten antibody.I. 7S beta-2A- and 1OS gamma-1- components of purified anti-beta-lactoside antibody. J Exp Med. 1964;120:589-609.

33. Sandor G, Korach S, Mattern P. 7S globulin, immunologically identical to 19S gamma-1 (beta-2)-M-globulin, a new protein or horse serum. Nature. 1964;204:795-6.

34. Vaerman JP, Querinjean P, Heremans JF. Studies on the IgA system of the horse. Immunology. 1971;21(3):443-54.

35. Wagner B, Miller WH, Morgan EE, Hillegas JM, Erb HN, Leibold W, et al. IgE and IgG antibodies in skin allergy of the horse. Vet Res. 2006;37(6):813-25.

36. Weir RC, Porter RR, Givol D. Comparison of the C-terminal amino-acid sequence of two horse immunoglobulins IgG and IgG(T). Nature. 1966;212(5058):205-6.

37. Zolla S, Goodman JW. An aggregating immunoglobulin in hyperimmune equine anti-pneumococcal sera. J Immunol. 1968;100(4):880-97.

38. Sun J, Han Y, Wang Y. [Serum antibody response to different doses of hepatitis B vaccine made by recombinant DNA techniques in yeast and Hansenula polymorpha yeast]. Zhongguo Yi Miao He Mian Yi. 2010;16(2):140-2.

39. Wade CM, Giulotto E, Sigurdsson S, Zoli M, Gnerre S, Imsland F, et al. Genome sequence, comparative analysis, and population genetics of the domestic horse. Science. 2009;326(5954):865-7.

40. Ford JE, Home WA, Gibson DM. Light chain isotype regulation in the horse. Characterization of Ig kappa genes. J Immunol. 1994;153(3):1099-111.

41. Lefranc M-P. IMGT, the international ImMunoGeneTics database®. Nucleic Acids Res. 2003;31(1):307-10.

42. Kelley LA, Sternberg MJ. Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc. 2009;4(3):363-71.

43. Ambrosetti F, Olsen TH, Olimpieri PP, Jiménez-García B, Milanetti E, Marcatilli P, et al. proABC-2: PRediction of AntiBody contacts v2 and its application to information-driven docking. Bioinformatics. 2020.

44. Frenzel A, Kügler J, Helmsing S, Meier D, Schirrmann T, Hust M, et al. Designing Human Antibodies by Phage Display. Transfusion Medicine and Hemotherapy. 2017;44(5):312-8.

45. Hust M, Meyer T, Voedisch B, Rülker T, Thie H, El-Ghezal A, et al. A human scFv antibody generation pipeline for proteome research. Journal of Biotechnology. 2011;152(4):159-70.

46. Kügler J, Wilke S, Meier D, Tomszak F, Frenzel A, Schirrmann T, et al. Generation and analysis of the improved human HAL9/10 antibody phage display libraries. BMC Biotechnology. 2015;15(1):10.

47. Pansri P, Jaruseranee N, Rangnoi K, Kristensen P, Yamabhai M. A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. BMC Biotechnology. 2009;9(1):6.

48. Li Y, Cockburn W, Kilpatrick JB, Whitelam GC. High affinity ScFvs from a single rabbit immunized with multiple haptens. Biochem Biophys Res Commun. 2000;268(2):398-404.

49. Dong S, Bo Z, Zhang C, Feng J, Liu X. Screening for single-chain variable fragment antibodies against multiple Cry1 toxins from an immunized mouse phage display antibody library. Appl Microbiol Biotechnol. 2018;102(7):3363-74.

50. Diesterbeck US. Construction of Bovine Immunoglobulin Libraries in the Single-Chain Fragment Variable (scFv) Format. Methods in molecular biology (Clifton, NJ). 2018;1701:113-31.

51. Tallmadge RL, Tseng CT, Felippe MJ. Diversity of immunoglobulin lambda light chain gene usage over developmental stages in the horse. Dev Comp Immunol. 2014;46(2):171-9.

52. Hara S, Diesterbeck US, Konig S, Czerny CP. Transcriptional analysis of equine lambda-light chains in the horse breeds Rhenish-German Coldblood and Hanoverian Warmblood. Vet Immunol Immunopathol. 2012;145(1-2):50-65.

53. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215(3):403-10.

54. Benita Y, Oosting RS, Lok MC, Wise MJ, Humphery-Smith I. Regionalized GC content of template DNA as a predictor of PCR success. Nucleic Acids Res. 2003;31(16):e99-e.

55. Nian S, Wu T, Ye Y, Wang X, Xu W, Yuan Q. Development and identification of fully human scFv-Fcs against Staphylococcus aureus. BMC Immunol. 2016;17(1):8-.

56. Kirsch MI, Hulseweh B, Nacke C, Rulker T, Schirrmann T, Marschall HJ, et al. Development of human antibody fragments using antibody phage display for the detection and diagnosis of Venezuelan equine encephalitis virus (VEEV). BMC Biotechnol. 2008;8:66.

57. Rülker T, Voß L, Thullier P, O' Brien LM, Pelat T, Perkins SD, et al. Isolation and Characterisation of a Human-Like Antibody Fragment (scFv) That Inactivates VEEV In Vitro and In Vivo. Plos One. 2012;7(5):e37242.

58. Hulseweh B, Rulker T, Pelat T, Langermann C, Frenzel A, Schirrmann T, et al. Human-like antibodies neutralizing Western equine encephalitis virus. MAbs. 2014;6(3):718-27.

59. Bustamante J, Jankauski M, Daniel TL. Closed loop Monte Carlo models of abdominal contribution to insect flight control. Integr Comp Biol. 2018;58:E26-E.

60. Michels GM, Walsh KF, Kryda KA, Mahabir SP, Walters RR, Hoevers JD, et al. A blinded, randomized, placebo-controlled trial of the safety of lokivetmab (ZTS-00103289), a caninized anti-canine IL-31 monoclonal antibody in client-owned dogs with atopic dermatitis. Veterinary Dermatology. 2016;27(6):505-e136.

61. Kuhn P, Fühner V, Unkauf T, Moreira GMSG, Frenzel A, Miethe S, et al. Recombinant antibodies for diagnostics and therapy against pathogens and toxins generated by phage display. PROTEOMICS – Clinical Applications. 2016;10(9-10):922-48.

62. Manoutcharian K, Perez-Garmendia R, Gevorkian G. Recombinant Antibody Fragments for Neurodegenerative Diseases. Curr Neuropharmacol. 2017;15(5):779-88.

63. Crivianu-Gaita V, Thompson M. Aptamers, antibody scFv, and antibody Fab' fragments: An overview and comparison of three of the most versatile biosensor biorecognition elements. Biosensors and Bioelectronics. 2016;85:32-45.

64. Hudson PJ, Kortt AA. High avidity scFv multimers; diabodies and triabodies. J Immunol Methods. 1999;231(1-2):177-89.

65. Chen X, Zaro JL, Shen WC. Fusion protein linkers: property, design and functionality. Advanced drug delivery reviews. 2013;65(10):1357-69.

66. van Rosmalen M, Krom M, Merkx M. Tuning the Flexibility of Glycine-Serine Linkers To Allow Rational Design of Multidomain Proteins. Biochemistry. 2017;56(50):6565-74.

67. Yang Z, Lee J, Ahn HJ, Chong CK, Dias RF, Nam HW. Western Blot Detection of Human Anti-Chikungunya Virus Antibody with Recombinant Envelope 2 Protein. Korean J Parasitol. 2016;54(2):239-41.

68. Huang J, Zhao Y, Shiraigol W, Li B, Bai D, Ye W, et al. Analysis of horse genomes provides insight into the diversification and adaptive evolution of karyotype. Sci Rep. 2014;4:4958.

69. Ledsgaard L, Kilstrup M, Karatt-Vellatt A, McCafferty J, Laustsen AH. Basics of Antibody Phage Display Technology. Toxins (Basel). 2018;10(6).

70. Kügler J. TF, Frenzel A., Hust M. Construction of Human Immune and Naive scFv Libraries. . 2018. In: Phage Display Methods in Molecular Biology [Internet]. New York, NY: Humana Press.

71. Moon SA, Ki MK, Lee S, Hong M-L, Kim M, Kim S, et al. Antibodies against non-immunizing antigens derived from a large immune scFv library. Molecules and cells. 2011;31(6):509-13.

72. Walther S, Czerny C-P, Diesterbeck US. Exceptionally long CDR3H are not isotype restricted in bovine immunoglobulins. Plos One. 2013;8(5):e64234-e.

4.2 Manuscript II: Isolation and characterization of an equine anti-EHV-1 single chain antibody fragment (scFv) from an equine phage display library

Franziska I. Pilger[a], Dorothee Arthaus[a, b], Caroline Bierschenk[a], Sebastian Schimkowiak[a]

[a] Department of Animal Sciences, Georg-August-University, Goettingen, Burckhardtweg 2, 37077 Goettingen, Germany

[b] AHRHOFF GmbH, Wissenschaftliche Abteilung, Ehmschen 17, 25462 Rellingen

Declarations of interest: none.

4.2.1 Abstract

Equine herpesvirus type 1 (EHV-1) is one of the most prevalent viral pathogens infecting horses worldwide causing respiratory diseases, abortion or severe neurological disorders. Not only is it a highly contagious virus but the most critical form of transmission are the silent shedders once the virus has undergone latency. No current prevention or treatment methods have proven ground breaking in altering duration of viremia or protecting against latent infection. Hence, it has been suggested that recombinant antibodies may contribute to protection and treatment of EHV-1. This study aimed to select a recombinant equine anti-EHV-1 antibody in the single-chain-Fragment-variable format (scFv) from a newly generated equine, immunised scFv antibody library by phage display technology and examine the scFv antibody's characteristics. The antibody library, with a diversity of 4.8 x 10^8 individual clones, was packaged into M13KO7 bacteriophages. This newly created equine phage display library was exposed to EHV-1 during three rounds of biopanning. One anti-EHV-1 scFv antibody was isolated from the library as it showed enhanced binding capacities as determined by ELISA. Its antibody capacities were confirmed by the presence of key conserved amino acid residues Cysteine, Tryptophan and Leucine. One amino acid substitution in the linker sequence was observable but the 3D-model of scFv-3H2 revealed no effects on protein folding due to the substitution. Affinity and concentration were low after purification as determined by ELISA, SDS-PAGE and western blot analysis. This study proved efficient selection of an equine scFv candidate, yet methods require optimisation. The results contribute to the broader establishment of recombinant, equine antibodies and phage display method in equine and veterinary drug development.

Keywords: recombinant, protein, horse, M13, phagemid

4.2.2 Introduction

Equine herpesvirus Type 1 (EHV-1) is one of the most prevalent viral pathogens infecting horses worldwide. It infects via the nasal passage eliciting symptoms ranging from mild signs of respiratory disease to potentially fatal neonatal pneumonia, abortion during late gestation or equine myeloencephalopathy (EHM) (1-3). EHV-1 virus is highly contagious and spreads easily among horses mainly through nasal secretions, fomites and aerosols (4). Yet the most critical form of transmission are the silent shedders once the virus has undergone latency (1). During latency, the virus is expressed at a low rate in seemingly healthy horses, yet can be re-activated during stressful periods, re-infecting the host and be shed via nasal secretions (1, 4). More than 60% of the worldwide horse population is considered to be latently infected with EHV-1 (5). Current prevention methods consist of vaccination programs that aim to reduce transmission and prevent subsequent diseases (5). Four vaccines are currently licensed by Paul-Ehrlich Institute (http:\\www.pei.de) for use in Germany. Vaccination however, does not alter duration of viremia (5) nor does it protect against latent infection (1). Therapeutic agents are being trialled against EHV-1 infection, but their efficacy remains of theoretic value at this point. Antiviral therapeutics acyclovir, valacyclovir or histones have demonstrated *in vitro* efficacy by inhibiting viral replication, however, neither drug inhibited viremia and *in vivo* studies are yet to be performed and prove effective (2, 5-7). A general consensus points toward development of equine-specific reagents e.g. monoclonal antibodies to study and subsequently prevent cellular adhesion of viral antigens, which are currently missing (2).

Species-specific, neutralizing antibodies are produced by recombining antibody fragments into new recombinant antibody formats (8). Popular recombinant formats are single-domain antibodies (sdAb), monoclonal antibodies (mAb), fragment antibodies (Fab) or single-chain-Fragment-variable (scFv) antibodies (9). The latter consist of the variable domains of the light and heavy chains, connected by a flexible glycine-serine linker (10). scFv antibodies are much smaller than mAbs or Fabs, resulting in higher sensitivities (10, 11). On top of that, they

are highly customizable and can undergo affinity maturation (10). Development of scFv antibodies begins with PCR-isolation and connection of antibody fragments and their assembly into antibody libraries containing up to 10 billion different clones (8, 10, 11). Antigen-specific scFv antibodies can be isolated from a library through selection on a particular antigen (11). This selection may occur through a number of microbial display systems e.g. ribosomal display, yeast display or phage display systems, of which the latter is the most utilized due to its robustness and versatility (10). Phage display utilizes bacteriophages for infection of a library taking advantage of phages' ability to display the gene encoding the scFv in their genome as well as on their phage coat proteins (8-10). Thereby, each phage is encoding and presenting a different antibody, thus creating an antibody phage library, allowing the simulation of an antibody repertoire similar to the natural immune system (8). This phage library is exposed to an immobilized antigen of interest whereby phages encoding high affinity scFv antibodies will bind to the antigen and weak binding phages are eliminated during a procedure termed biopanning (8, 9). High affinity scFv antibodies are either eluted for further characterisation or used for reinfection of the phage library for re-amplification and selection of binders with higher affinities under more stringent conditions (9). Typically three to five rounds of biopanning are necessary to select specifically binding phage particles (9).

Next to phage display many different selection methods have been trialled over the years: prokaryotic or mammalian cells to display antibodies, enrichment on tissue or biotinylated antigen, ribosome display, yeast display or using cell sorting procedures such as fluorescent-activated-cell-sorting (FACS) and magnetic-activated-cell-sorting (MACS) (11). Comparing these methods to phage display, phage display technique offers several advantages: it allows automation of antibody production and manipulation of biopanning conditions to enhance stringency, which can strongly influence the nature of the antibodies selected from a library (11). Furthermore, phage display technique has successfully been used for drug discovery since the nineties and by 2017, six human antibodies discovered by phage display

had been approved for use and more were in clinical trials (8). Phage display is a well-established and reliable source for the generation of therapeutic antibodies, both human and veterinary (8).

In this study, we screen an immunized, equine antibody library in the scFv format using phage display technique to isolate an anti-EHV-1 scFv antibody and compare it to an equine mAb in regards to expression and binding capacity.

4.2.3 Material and Methods

Virus production and purification

EHV–1 was obtained by infecting rabbit kidney cells (RK-13, Friedrich Loeffler Institut, Riems, Germany) with 5 ml live-attenuated Prevaccinol® (EHV-1, Strain RAC-H; Intervet Deutschland GmbH, Unterschleißheim, Germany). Infected cell culture was incubated in Minimum Essential Medium (MEM; Anprotec, Bruckberg, Germany) containing 2 % fetal calf serum (FCS; Anprotec, Bruckberg, Germany) at 37°C. After 72 hours and appearance of lytic cytopathogenic effect (CPE), virus-infected cell culture was frozen at -80°C, thawed at room temperature (RT) and centrifuged at 12500 g (Sorvall® RC-5B, Rotor GSA, Du Pont Instruments, Midland, USA) at 4°C for 30 minutes to eliminate cell debris. The supernatant was centrifuged in a SW 28-rotor at 112000 g (Combi Plus, Rotor AH-629, Du Pont Instruments, Midland, USA) for one hour. Virus pellets were re-suspended in phosphate buffered saline (PBS) buffer (8000 mg NaCl, 200 mg KCl, 120 mg KH_2PO_4, 910 mg Na_2HPO_4, ad 1 L distilled water; every constituent from Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and layered on a step-wise sucrose gradient (20%, 40% and 60%, w/w in tris(hydroxymethyl)aminomethane (TRIS) - HCl buffer pH 9.0 (1mM)). Centrifugation was carried out in a SW 40-rotor (L-60 ultracentrifuge, rotor SW 40 Ti, Beckman Coulter GmbH, Krefeld, Germany) at 4°C for 90 minutes (40000 g). Lateral puncture facilitated collection of

the virus, which was pelleted in the SW 28-rotor for one hour (112000 g) and re-suspended in TRIS buffer. Virus concentration was determined by Lowry protein assay and the optical density (OD) was measured with the Sunrise microplate reader (Tecan, Maennedorf, Switzerland) at 450 nm. The purified virus was kept at -80°C. Binding between EHV-1-specific equine monoclonal antibody mAb 6B11 (12) conjugated with α-mouse horse radish peroxidase (HRP; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was confirmed in an ELISA (Protocol S1 and results in Figure S1 and Figure S2). The virus propagation and purification were repeated for continuous supply of viral material.

Antibody selection

Library

The equine immunized scFv library contains a library diversity of 4.8×10^8 individual clones and the percentage of clones containing inserts relative to the empty phagemid vector is 80 %. Inserts are made up of equine variable gene regions of IgG heavy and light chains (λ and κ) connected to each other by a Glycine-Serine linker $(Gly_4Ser)_3$. ScFv fragments are displayed on the minor coat protein pIII in phagemid vector pCANTAB 5E (GE Healthcare, Chalfont St. Giles, UK, 25), which contains an E tag sequence and an Amber Stop Codon. The library was assembled into phage-competent TG1 *E. coli* cells (Lucigen Corporation, Middelton, USA). The library was constructed from a warmblood gelding that had been immunised with live-attenuated Prevaccinol® (EHV-1, Strain RAC-H; Intervet Deutschland GmbH, Unterschleißheim, Germany), and inactivated Equip $EHV_{1,4}$ (EHV-1, Strain 438/77; EHV-4 Strain 405/76; Pfizer, Berlin, Germany) prior to library construction. In advance of antibody selection, presence of antibodies specifically against EHV-1 was detected in an ELISA assay using EHV-1-specific equine monoclonal antibody mAb 6B11 (12); negative control (NC) consisted of a monoclonal antibody (mAb 5B4/2F2 (13)) against bovine enteric coronavirus,

conjugated with the detection antibodies α-mouse horse radish peroxidase (HRP; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) (Protocol S2, results in Figure S3).

Infection with hyper- and helperphages

A 500 µl aliquot of the library was thawed on ice and grown in 1 L 2xTY+2 % G+A medium (16 g tryptone, 10 g yeast extract, 20 g glucose, 5 g NaCl, 1 ml ampicillin (100 µg/ml), final volume was adjusted to 1 L with sterile water; all from Carl Roth GmbH & Co.KG, Karlsruhe, Germany) at 30°C with 250 revolutions per minute (rpm; Sartorius, Certomat-BS1, Göttingen, Germany) to achieve log phase (OD 0.4 – 0.6) and thereby amplify the library (as depicted in Figure 1 as Step 1). A 100 ml aliquot of the amplified library was infected with M13KO7ΔpIII hyperphages (Progen, Heidelberg, Germany) (Step 2 in Figure 1), for oligovalent display at a multiplicity of infection (MOI) of 30 and incubated stationary at 37°C for 30 minutes, followed by gentle shaking (Titramax 1000, Heidolph Instruments GmbH und Co.KG, Schwabach, Germany) at 37°C for a further 30 minutes. Throughout the following phage display rounds, libraries were re-infected with helperphages M13K07 ($1x10^{11}$ pfu/ml; New England Biolabs GmbH Frankfurt am Main, Germany) to produce a monovalent display thereby increasing selection stringency.

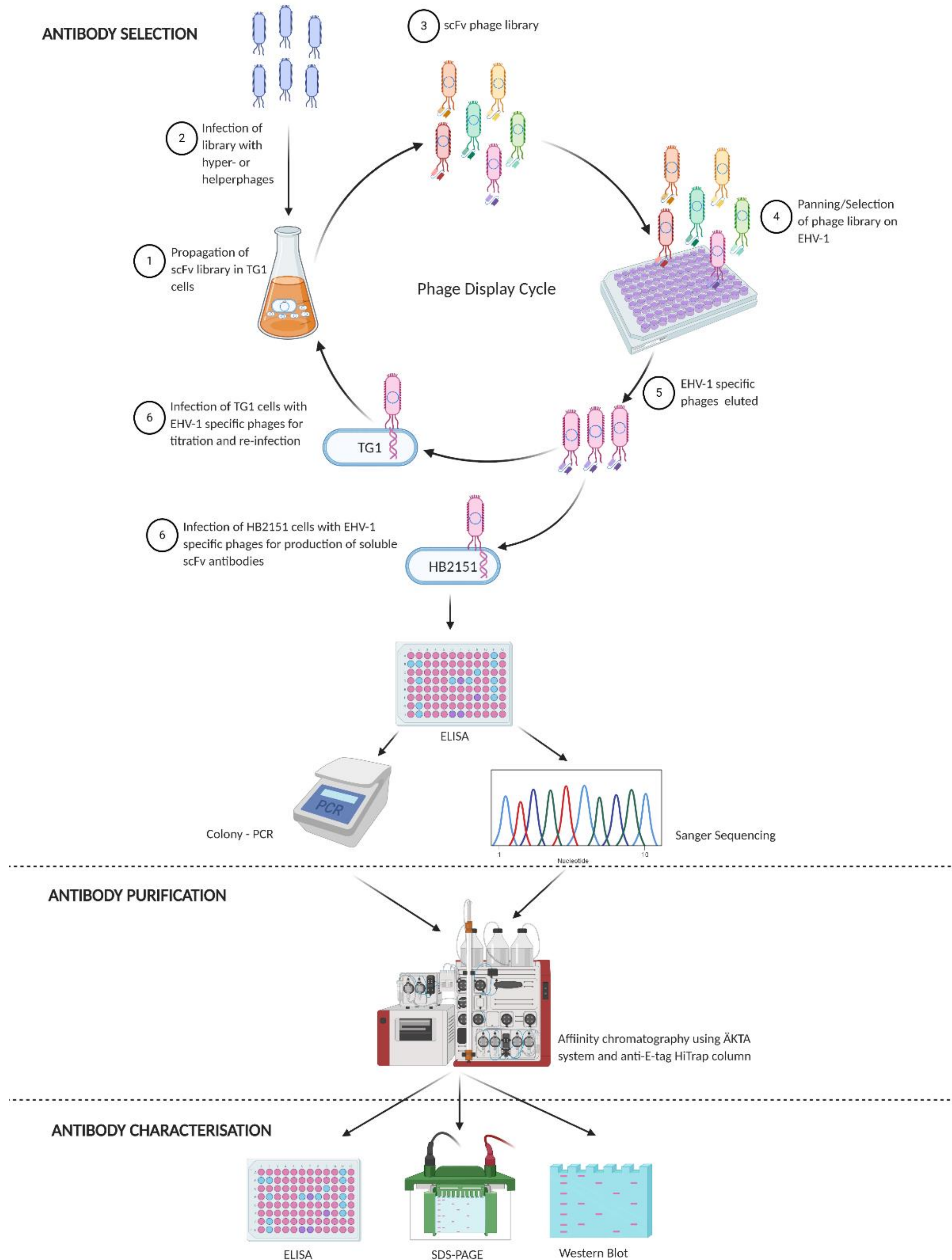

Figure 1: Schematic overview of antibody selection, purification and characterisation processes. Created with biorender.com.

The phage-infected culture was centrifuged at 4°C for 15 minutes at 3220 g (HERAEUS™ Multifuge™ 3 S-R, Rotor 7500 6445; Thermo Fisher Scientific Inc., Waltham, USA). Supernatant was discarded and the phage-infected cell pellet was re-suspended in 1 L 2xTY medium (16 g tryptone, 10 g yeast extract, 5 g NaCl, ad 1 L sterile water; every constituent from Carl Roth GmbH & Co.KG, Karlsruhe, Germany), supplemented with ampicillin and kanamycin (100 µg/ml and 50 µg/ml respectively; both from Carl Roth GmbH & Co.KG, Karlsruhe, Germany). The re-suspended pellet was aliquoted into two 1 L Erlenmeyer Flasks (Corning® Incorporated Life Science, Oneonta, USA) and propagated at 30°C and 200 rpm over night (Titramax 1000, Heidolph Instruments GmbH und Co.KG, Schwabach, Germany). Bacteria were pelleted once again at 4°C for 10 minutes at 3220 g (HERAEUS™ Multifuge™ 3 S-R, Rotor 7500 6445; Thermo Fisher Scientific Inc., Waltham, USA) to separate supernatant, now containing phages, and cell pellet, prior to phage precipitation.

Phage precipitation

The bacterial pellet was discarded and phages within the supernatant precipitated. Precipitation was initiated by addition of 8 ml of 20 % PEG (200 g of 20 % PEG8000, 146.1 g 2.5 M NaCl, ad one L distilled water; both from Carl Roth GmbH & Co.KG, Karlsruhe, Germany) at a ratio of 1:5 and gentle shaking of the solution on ice for 45 minutes, followed by static incubation on ice for 45 minutes. Precipitates were centrifuged to obtain pellets at 4°C for 1 h at 4500 g (HERAEUS™ Multifuge™ 3 S-R, Rotor 7500 6445; Thermo Fisher Scientific Inc., Waltham, USA). Subsequently, pellets were re-suspended in PBS without Ca^{2+} and Mg^{2+} (8,000 mg NaCl, 200 mg KCl, 2,890 mg Na_2HPO_4, 200 mg KH_2PO_4, 1 mg glucose, ad 1 L distilled water; all from Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and precipitation was repeated. After the second precipitation, the pellet was re-suspended in 1 ml PBS+15% glycerol and centrifuged anew at 4°C for 10 minutes at 9500 g (Biofuge Pico, Heraeus, Hanau,

Germany). The resulting supernatant, containing the phage library, was either kept short-term at 4°C or at -80°C for long-term storage.

The concentration of phages within the supernatant was determined by diluting a 10 µl aliquot in 990 µl 2xTY+2% G and preparing a dilution series (log 100). 100 µl of each dilution were added to 900 µl TG1 *E. coli* cells and incubated at 37°C for 30 minutes without shaking followed by an incubation at 37°C for 30 minutes with shaking (250 rpm; Certomat BS-1, Sartorius, Göttingen, Germany). The dilution series was plated onto 2xTY+2 % G+A agar plates and incubated at 30°C over night. A positive control was incubated alongside consisting of TG1 *E. coli* cells spread onto a 2xTY+2 % G agar plate without ampicillin. A negative control consisted of TG1 *E. coli* cells spread onto a 2xTY+2 % G agar plate including ampicillin. All plates were prepared in duplicates. The phage titre was determined the following day by counting bacterial colonies on the plates.

Biopanning

For the first panning round a 96-well NUNC MaxiSorb plate (NUNC^{TM}, Langenselbold, Germany), was coated with 5 µg/ml EHV-1 and incubated at 37°C for 4 hours (Step 4 in Figure 1). Virus coating concentration decreased during the following panning rounds (1st round: 5 µg/ml; 2nd round: 2 µg/ml; 3rd round: 1 µg/ml) to enhance selection stringency. After incubation, the plate was washed three times with PBS+0,1 % Tween (T; 8 g NaCl, 0.2 g KCl, 0.12 g KH_2PO_4, 0.91 g Na_2HPO_4, 1 ml Tween-20, ad 1 L sterile water; all constituents from Carl Roth GmbH & Co.KG, Karlsruhe, Germany) and blocked with 300 ml blocking solution (2 % skimmed milk powder (SMP; Carl Roth GmbH & Co.KG, Karlsruhe, Germany), 10 % FCS, PBS) at 37°C for 2 hours. Following incubation, washing the plate occurred three times with PBS+0,1 % T. Supernatant, containing the phage library, pre-incubated in blocking solution for 1 hour at RT, was added to the plate at an input concentration of $1x10^{11}$ cfu/ml (2nd round: $1x10^{13}$ cfu/ml; 3rd round: $1x10^{13}$ cfu/ml) and incubated at RT for 2 hours. The pre-incubation

step was omitted in all following panning rounds. After incubation, wells were washed 10 times (2nd round: 20 times; 3rd round: 30 times) with PBS+0,1 % T to remove unbound phages. Bound phages were eluted (Step 5 in Figure 1) enzymatically with 200 µl Trypsin (10 µg/ml; Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and gentle shaking at 37°C for 30 minutes.

Phage titration

Eluted phage suspension was titrated in TG1 *E. coli* cells to determine the concentration of eluted phages after each panning round and serve as selected scFv phage library for following phage display rounds (Step 6 in Figure 1). Simultaneously, scFv genes, displayed by the eluted phages, were cloned into a non-suppressor *E. coli* strain HB2151 (Amersham Biosciences Inc., Buckinghamshire, United Kingdom) to produce soluble scFv (Step 6 in Figure 1). Both strains of *E. coli* cells were cultured in 2xTY+2 % G medium. After *E. coli* cells reached log phase (OD 0.4-0.6), 10 µl of eluted phages were added to 1 ml of HB2151 *E. coli* cells and 190 µl of eluted phages were added to 10 ml of TG1 *E. coli* cells and incubated at 37°C for 30 minutes stationary, followed by incubation at 37°C for 30 minutes and 250 rpm. After incubation, 10 µl of both infected *E. coli* cell strains were diluted in 990 µl 2xTY+2 % G medium and diluted serially (log100). The dilution series were plated onto 2xTY+2 % G+A agar plates (16 g tryptone, 10 g yeast extract, 20 g glucose, 5 g NaCl, 15 g agar-agar, 100 µg/ml ampicillin, ad 1 L sterile water; all constituents from Carl Roth GmbH & Co.KG, Karlsruhe, Germany). Remaining TG1 *E. coli* cells were pelleted at 4°C for 10 minutes and 3000 g, re-suspended in 2xTY+2 % G medium and plated onto 24 cm x 24 cm 2xTY+2 % G+A agar plates (NUNC™, Langenselbold, Germany). Remaining HB2151 cells were transferred onto two 15 cm 2xTY+2 % G+A agar plates. Uninfected cells from both strains were plated onto 2xTY+2 % G+A agar plates serving as negative control and onto 2xTY+2 % G agar plates to serve as positive control. All plates were incubated at 30°C over night. Following incubation, colonies were counted and cfu/ml determined. TG1 cells were scraped from the plate with 2xTY+2 % G+A medium

containing 15% glycerol and stored at -80°C to serve as selected scFv phage library for following phage display rounds (Step 6 in Figure 1).

Production of scFv and ELISA

Infected HB2151 *E. coli* colonies were picked randomly (384 – 480 colonies per panning round) and over-night cultures were prepared: individual colonies were submerged in 2xTY+2 % G+A medium on a 96-well microtiter plate (one clone per well) and incubated at 37° over night and constant shaking at 450 rpm (Titramax 1000, Heidolph Instruments GmbH und Co.KG, Schwabach, Germany) in order to produce scFv preparations, unlinked to phages. On the following day, 0.5 µl of over-night cultures were grown in 100 µl 2xTY+0.1 % G+A medium at 30°C and 300 rpm for 4 hours (Titramax 1000, Heidolph Instruments GmbH und Co.KG, Schwabach, Germany). After 4 hours of incubation, scFv expression by the prokaryotic *lacZ* promotor in *E. coli* was induced by adding 50 µl of 2xTY+A+1M isopropyl-beta-D-thiogalacto-pyranoside (IPTG) per well (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and incubation at 30°C over night and 300 rpm (Titramax 1000, Heidolph Instruments GmbH und Co.KG, Schwabach, Germany). Induced HB2151 cells were centrifuged at 3000 g, for 10 minutes at 4°C. Supernatants were used in ELISA to assess OD of EHV-1-binding scFvs after every panning round alongside a positive control consisting of an EHV-1 specific equine monoclonal antibody (Mab 6B11; (12)), conjugated with α-mouse horse radish peroxidase (HRP 1:2000 in 2% SMP in PBS+0.1 % T; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for the positive control and α-E-Tag HRP (1:2000 in 2% SMP in PBS+ 0.1 % T, abcam, Cambridge, UK) for scFvs (detailed ELISA protocol S3 and exemplary result in Figure S4).

Colony PCR and Sequencing

Clones exhibiting OD measurements above 0.25 were analysed by colony PCR to contain full-length inserts using 10 µM of primer R1 (5´-CCA TGA TTA CGC CAA GCT TTG

GAG CC-3´), 10 µM of primer R2 (3´-CCT TTC TGC TGT TT GAA ATC TAG C-5`). For a detailed protocol and result refer to Protocol S4 and Figure S5.

The software used to import, analyse and align sequences included Lasergene EditSeq, SeqBuilder, Protean 3D and MegAlign (DNAStar, 12.3.1.4 Madison, USA). Sequences from clones as well as generated primer sets were aligned using the Clustal W algorithm. The IMGT database was used to determine CDR frameworks. A 3D model was calculated of protein structures of a scFv antibody established according to protein homology using the Phyre2 server. ProABC-2 was used to determine probabilities of CDR regions likely to interact with antigen (14). Calculations and graphs were generated using GraphPad Prism 8.0.0 (San Diego, California USA).

Antibody purification

Propagation and induction

A 10 µl aliquot of the candidate scFv supernatant was propagated in 2xTY+2 % G+A medium at 37°C and 200 rpm over night (Titramax 1000, Heidolph Instruments GmbH und Co.KG, Schwabach, Germany). The following day, the suspension was added to 750 ml 2xTY+0.1 % G+A and incubated at 37°C and 170 rpm until it reached log phase (OD 0.4 – 0.5). Once log phase was reached, a 1 ml aliquot was removed and stored to serve as control of the suspension prior to induction (from now on referred to as “pre-induction”). Once log phase was reached 250 ml of 2xTY+A+ 2mM IPTG were added and the culture was incubated at 30°C for 23 hours and 170 rpm. After 23 hours a 1 ml aliquot was removed and stored to serve as control of the suspension after induction (from now on referred to as “post-induction”). The remaining suspension was centrifuged to separate supernatant and peri-plasm at 4°C for 20

minutes and centrifuged at 12500 g (Sorvall® RC-5B, Rotor GSA, Du Pont Instruments, Midland, USA).

Precipitation of the supernatant

The supernatant was separated from the pellet and transferred to 2 L glass bottles. Saturated ammonium sulphate solution was added at a ratio of 1:1, chilled on ice and exposed to gentle rocking. After 1 hour the ammonium sulphate - supernatant solution was centrifuged at 4°C for 20 minutes and 12500 g (Sorvall® RC-5B, Rotor GSA, Du Pont Instruments, Midland, USA). Following centrifugation the supernatant was discarded and the pellet was re-suspended in 15 ml PBS. The re-suspension was centrifuged once again for 10 minutes. Supernatants were collected in a 50 ml tube (Sarsted, Nümbrecht, Germany) and stored at -80°C.

Precipitation of the peri-plasm

The peri-plasm was re-suspended in 40 ml of TES buffer (200 mM Tris-HCl, 500 mM EDTA, 500 mM Saccharose, all constituents from Carl Roth GmbH & Co.KG, Karlsruhe, Germany). Re-suspension was incubated on ice for 60 minutes and gentle shaking prior to centrifugation at 4°C for 40 minutes and 12500 g (Sorvall® RC-5B, Rotor GSA, Du Pont Instruments, Midland, USA). The supernatant was collected and the pH value adjusted to 7.5 prior to a further centrifugation step (Eppendorf, 5424, Rotor FA-45-24-11, Eppendorf, Hamburg, Germany) for 15 minutes. Supernatants were collected in 50 ml tube (Sarsted, Nümbrecht, Germany) and stored at -80°C.

Affinity chromatography

The candidate scFv molecules were purified from both, the bacterial supernatant as well as peri-plasm by affinity chromatography via the ÄKTA™ start chromatography system (GE Healthcare UK Ltd, Buckinghamshire, UK) using an anti-E-Tag HiTrap column (GE

Healthcare UK Ltd, Buckinghamshire, UK) with a flow rate of 1 ml/min. Purified scFv molecules were eluted with 0.1 M glycine (pH3; Carl Roth GmbH & Co.KG, Karlsruhe, Germany) and neutralized with 1 M TRIS and 0.13 M NaN_3 (both from Carl Roth GmbH & Co.KG, Karlsruhe, Germany). Purified scFv molecules were dialyzed against PBS and concentrated by Amicon Ultra 15 10K Centrifugal Filter Device (Merck, Darmstadt, Germany). The concentration of the scFv was determined performing a Bradford Assay in microtitre plates according to standard protocol using bovine serum albumin as a standard (Merck, Darmstadt, Germany).

Antibody characterisation

ELISA

Five columns of a 96-well ELISA plate (C96 Maxisorp NUNC-Immuno Plate, Thermo Fisher Scientific Inc., Waltham, USA) were coated with 2 µg/ml EHV-1 and incubated at 37°C for 4 hours. Remaining columns on the plate were not coated with virus. The plate was washed three times with PBS+0.1 % T and blocked with 300 µl blocking solution (2% SMP, 10% FCS in PBS+0.1 % T) at 37°C for 2 hours. Wells were emptied and 100µl of blocking solution was added to every well. The positive control consisting of an EHV-1 specific equine monoclonal antibody (Mab 6B11 (12) was added and diluted (log2) until row H. The purified candidate scFv was added and diluted as well. Columns 5, 10, 11 and 12 received blocking solution to determine the background. The plate incubated at 37°C for 1 hour before it was washed five times with PBS+0.1 % T. The detection antibodies α-mouse horse radish peroxidase (HRP; 1:2000 in 2% SMP in PBS+ 0,1 % T) for the positive control and α-E-Tag HRP (1:2000 in 2% SMP in PBS+ 0,1 % T) were added to respective wells and incubated for one hour at 37°C. Columns 5, 10, 11 and 12 only received blocking solution. Following incubation, wells were washed ten times with PBS+0.1 % T, followed by the addition of 100 µl TMB substrate (IDEXX GmbH, Ludwigsburg, Germany). After 20 minutes incubation at RT in the dark, 50 µl

HCl (IDEXX GmbH, Ludwigsburg, Germany) stop solution was added and OD was measured with the Sunrise microplate reader (Tecan, Maennedorf, Switzerland) at 450 nm.

SDS-PAGE and western blot analysis

Characterization of the purified scFv-3H2 was performed by SDS-PAGE (sodium dodecyl sulphate-polyacrylamid gel electrophoresis) and western blot analysis. The bacterial cell culture “pre-induction”, bacterial cell culture “post-induction”, supernatant, peri-plasm and purified candidate scFv were loaded onto a reducing 12% SDS-PAGE along with Precision Plus Protein Standard (Bio-Rad Laboratories GmbH, Munich, Germany). Mini Trans Blot Cell electrophoresis system (Bio-Rad Laboratories GmbH, Munich, Germany) carried out electrophoresis for 60 minutes at 150 V and 400 mA using a Power Pac Basic (Bio-Rad Laboratories GmbH, Munich, Germany). Afterwards, the gel incubated in Coomassie-Brilliant-Blue dye (Thermo Scientific™ Pierce Coomassie Brilliant Blue R-250 und G-250, Waltham, USA) for 45 minutes at RT and gentle shaking. The dye was removed and gel was washed twice with distilled water before de-colorizing solution (40 % methanol, 10 % acetic acid, 50 % H_2O; all constituents from Carl Roth GmbH&Co.KG, Karlsruhe, Germany) was added. The gel incubated in the de-colorization solution for 15 minutes, removed and the process of de-colorization was repeated until a clear contrast was observable. Once de-colorization was deemed sufficient, gels were photographed (PEQLAB Biotechnologie GmbH, Erlangen, Germany).

For western blotting, 12 % SDS-PAGE gels were prepared as above. Both gels were loaded with differing concentrations of purified EHV-1 virus in designated lanes (2µg/ml, 5µg/ml and 10 µg/ml). Segregated proteins were loaded onto a PROTRAN BA83 nitrocellulose membrane (GE Healthcare, Chalfont St. Giles, UK) and electrophoresis was performed in the electrophoresis system mentioned above for 75 minutes at 150 V and 400 mA in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol (v/v); all constituents from Carl Roth

GmbH&Co.KG, Karlsruhe, Germany). Membranes were blocked for 2 hours at RT with 5% SMP containing 10% FCS in PBS+0.1 % T, followed by washing twice with PBS+ 0.1 % T and once with PBS. The first membrane was incubated with precipitated supernatant of candidate scFv-3H2 and developed at 4°C over night. The membrane was washed three times with PBS + 0.1 % T and twice with PBS. It was incubated with polyclonal rabbit anti-E-Tag antibody (1:1000; Novus Biologicals, Littleton, USA) for two hours at RT before staining substrate was added (Bio-Rad Laboratories GmbH, Munich, Germany). The color reaction was stopped with distilled water.

A second gel was incubated with purified candidate scFv-3H2 and incubated at 4°C over night. Following washing steps on the next day, the membrane was incubated with polyclonal rabbit anti-E-Tag antibody for 2 hours at RT. Washing steps and staining steps remain unchanged of the protocol above.

4.2.4 Results

Virus production and purification

Sucrose-gradient purification of EHV-1 yielded concentrations of 1900 µg/ml after the first purification and 700 µg/ml after the second purification. Both aliquots were confirmed to bind to monoclonal antibodies in ELISA assays (Figure S1 and Figure S2).

Antibody selection

An interplay of phage input and output titre was assessed after each panning round to evaluate correct processing of phage display procedures. An increasing output tire and enriched input titre was presumed to ensure correct application of stringency strategies (Figure 2). Phage output titre was based on a TG1 dilution series. In panning round one 3,645x10^5 cfu/ml antigen-specific phages were eluted, in panning round two a decrease was observable (1,7x10^5 cfu/ml) but number of antigen-specific phages rose in panning round three (8,32x10^5 cfu/ml).

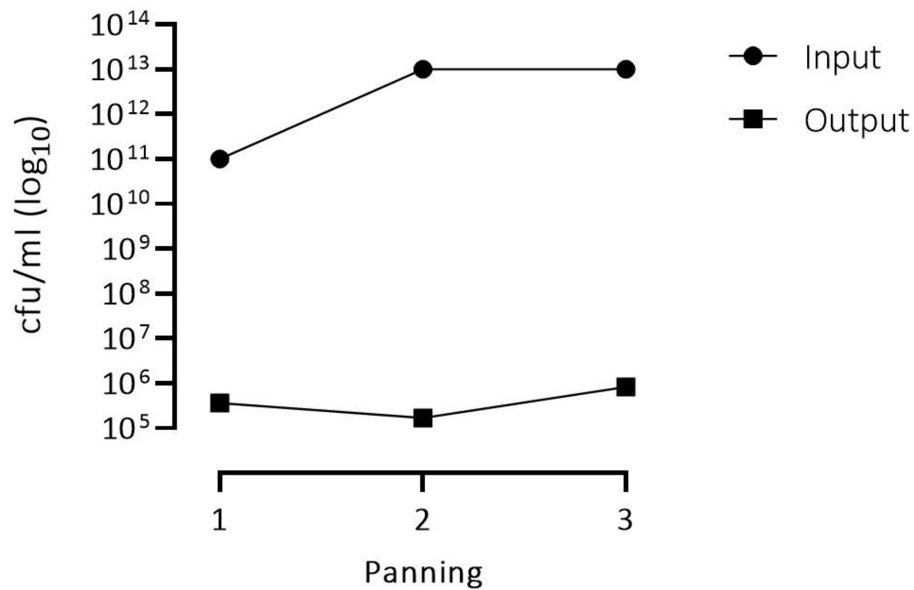

Figure 2: Development of phage display input and output titres for each panning round (cfu/ml).

Following each panning round, individual clones were picked and propagated on 96-well plates by ELISA assay to determine binding capacity of individual clones to EHV-1. An expected increase of mean OD values of all clones per panning round was observable (Figure 3). Mean OD in panning round one was 0.05634 (n=472), slightly less than mean OD in panning round two (0.08614; n=435). This almost doubled (1.8x) in panning round three, mean OD being 0.1584 (n=348).

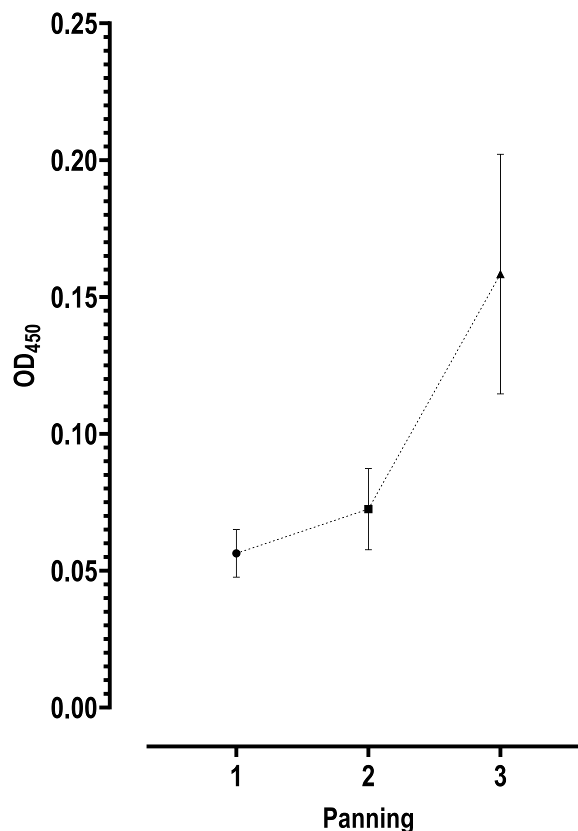

Figure 3: Mean OD values including SD during phage display panning rounds.

After panning round three, 11 clones displayed OD signals higher than 0.25 (Figure S4) and were further investigated by Colony PCR. It revealed one clone to harbour a scFv insert of expected size (~800 bp; Figure S5) plus approximately 200 bp accounting for PCR primers R1/R2. Remaining clones appeared to be 300 to 600 bp approximately (Figure S5).

The clone scFv-3H2 was cultivated overnight, plasmid DNA was extracted and Sanger sequenced. The sequence of scFv-3H2 (GenBank accession number MW082606) in Figure 4 shows the scFv ORF, including g3 signal sequence, equine variable region of the heavy chain, the linker to the light chain variable region, and the E-Tag sequence and amber stop codon. CDR regions contain both, hydrophobic and hydrophilic amino acid residues, yet hydrophilic residues predominate as desired in antibody sequences. The ORF includes antibody-specific Cys23 and substitutions of conserved amino acid residues Trp41, Leu89, Cys104 to Glycine, Methionine and Tyrosine, all the while containing Tryptophan, Leucine and Cysteine, merely

in different positions. A single nucleotide polymorphism (SNP) occurs in the linker sequence: the circled nucleotide should be Guanine instead of Adenine, which leads to an amino acid exchange; Glycine was substituted by Serine. Figure 5 shows a 3D prediction model of scFv-3H2 which portrays a molecule with CDR-regions lying close to each other on the surface and CDR-H3 lying in the center of the molecule. CDR-H2 (Pt 0,48) and CDR-L1 (Pt 0.48) are most likely to interact with the antigen according to proABC-2 (14).

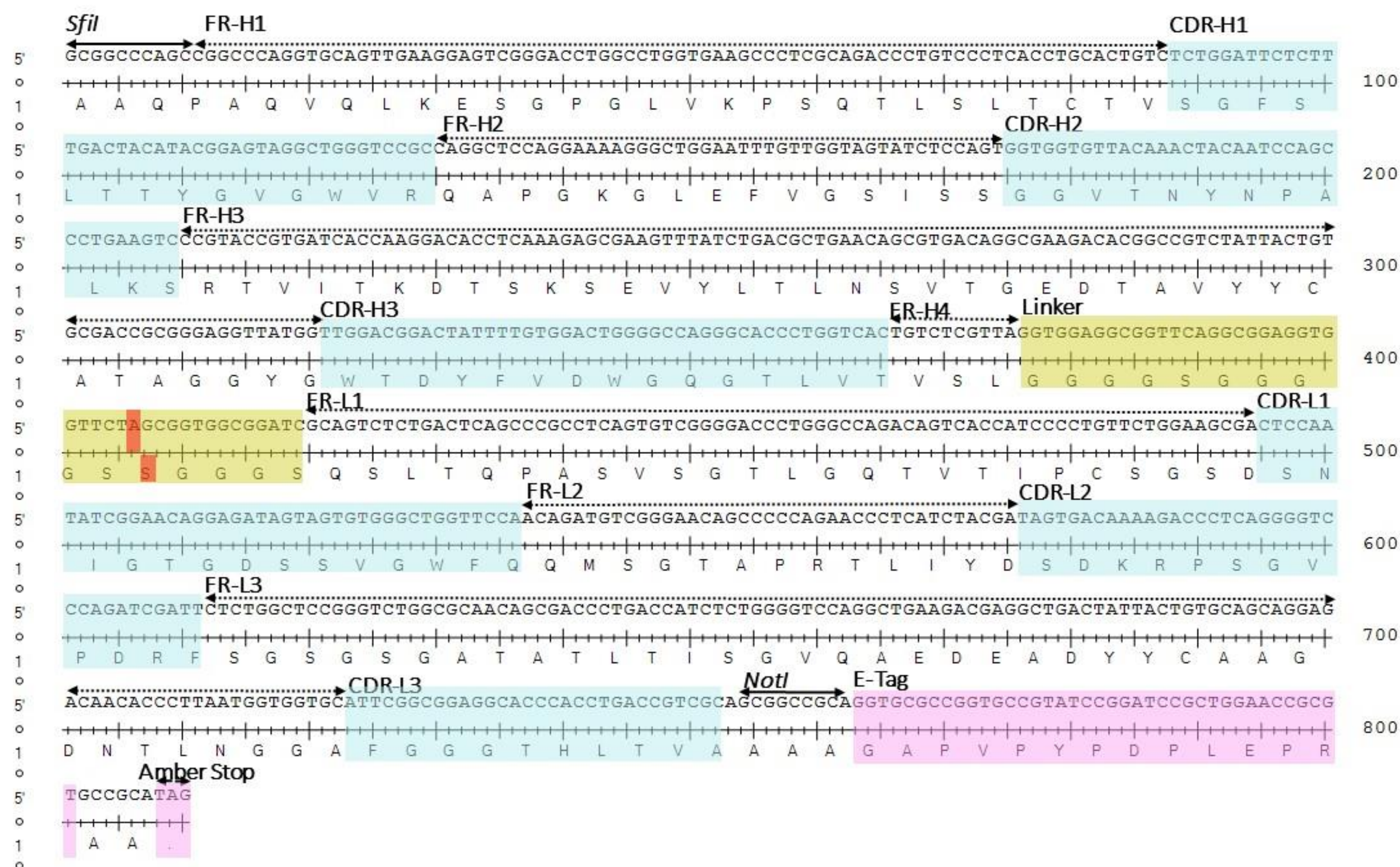

Figure 4: Nucleotide and amino acid sequence of scFv-3H2. Marked are CDRs (light blue), the $(G_4S)_3$, linker sequence (yellow) and the E-Tag and amber stop codon sequences of phagemid vector pCANTAB 5E (purple). Circled nucleotide and amino acid indicate a SNP.

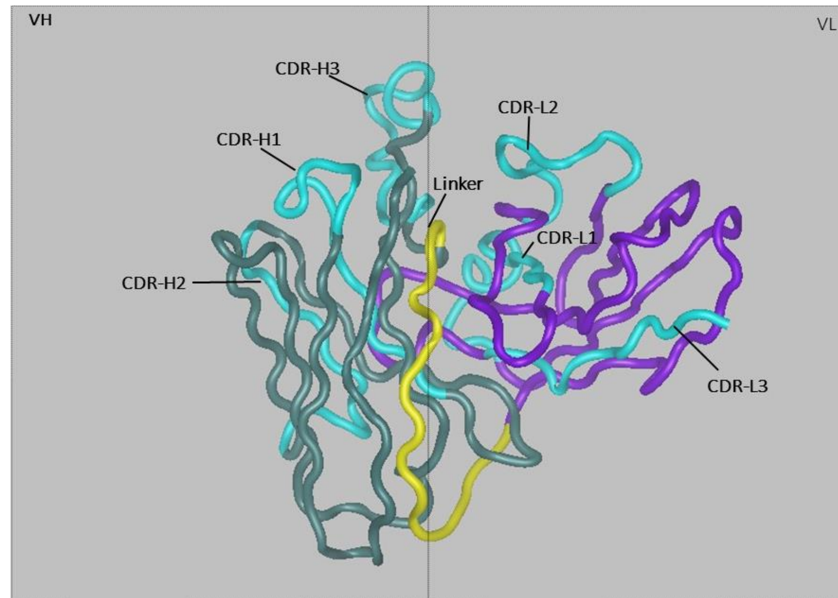

Figure 5: 3D prediction model of scFv-3H2. Green - heavy chain variable domain. Purple - light chain variable domain. Light blue – CDR. Yellow - $(G_4S)_3$ peptide linker.

Antibody purification

The scFv-3H2 was purified from the HB2151 *E. coli* culture peri-plasmic fraction as well as supernatant by affinity chromatography using the ÄKTA™ start chromatography system using an anti-E-Tag HiTrap column with a flow rate of 1 ml/min. The scFv was found to accumulate in the *E. coli* periplasm and was also released into the supernatant (Figure 6). After purification and dialysis, all fractions were pooled and Bradford assay yielded concentrations of 66,78 µg/ml.

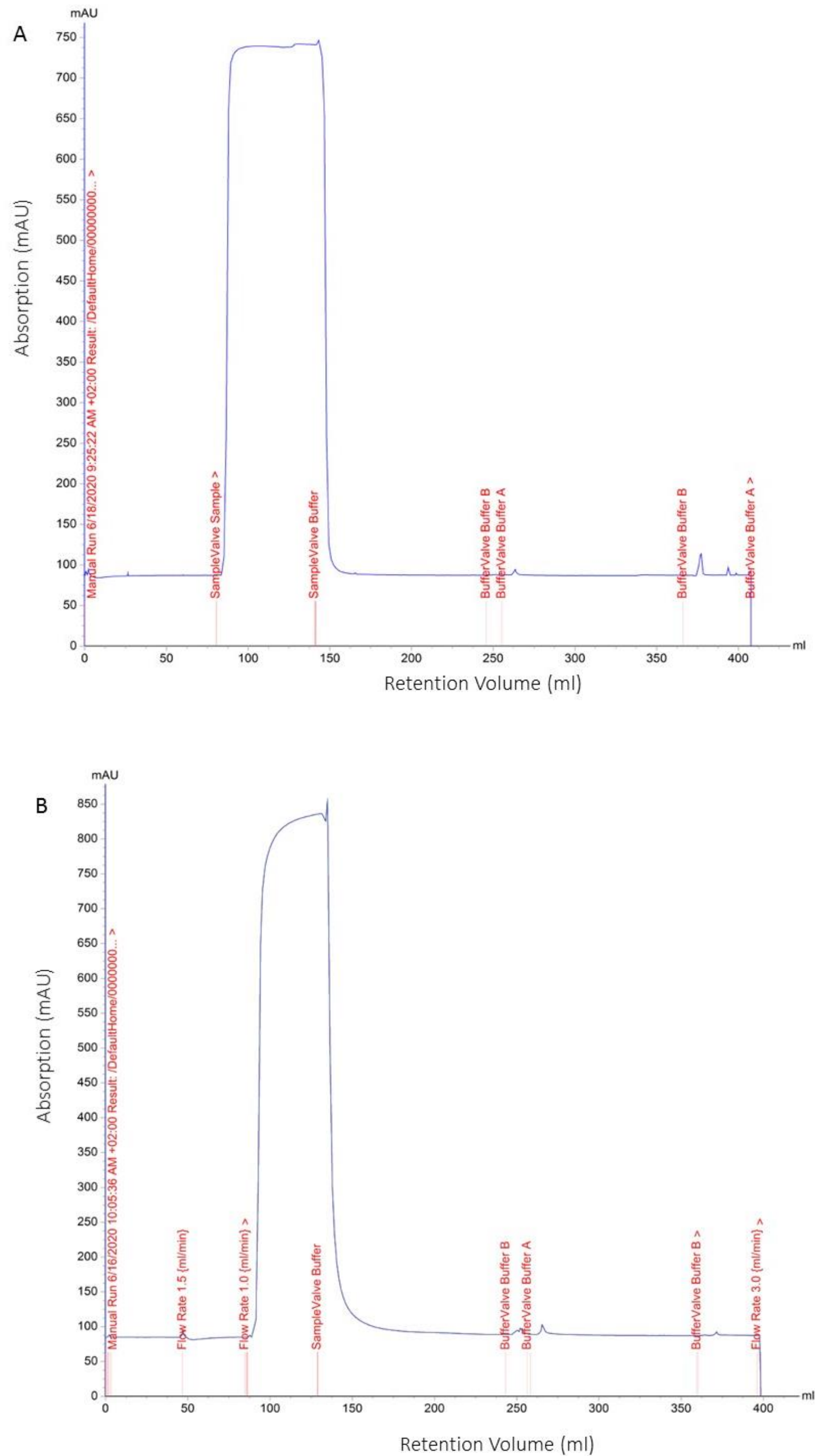

Figure 6: Chromatogram profile of the purification of scFv-3H2 from peri-plasmic (A) and supernatant (B) fractions *E.coli* cell culture. Chromatographic column: ÄKTA^{TM} start chromatography system using an anti-E-Tag HiTrap column with a flow rate of 1 ml/min.

Antibody characterisation

ELISA assay was performed verifying reactivity of purified scFv-3H2 to EHV-1. As shown in Figure 7, scFv-3H2 (red) was not comparable to the PC mAb 6B11 (blue) in its reactivity to EHV-1. The positive control also reacted on wells that were not coated with EHV-1 dilution. In comparison to remaining results of this template within the assay, this result can be deemed an outlier.

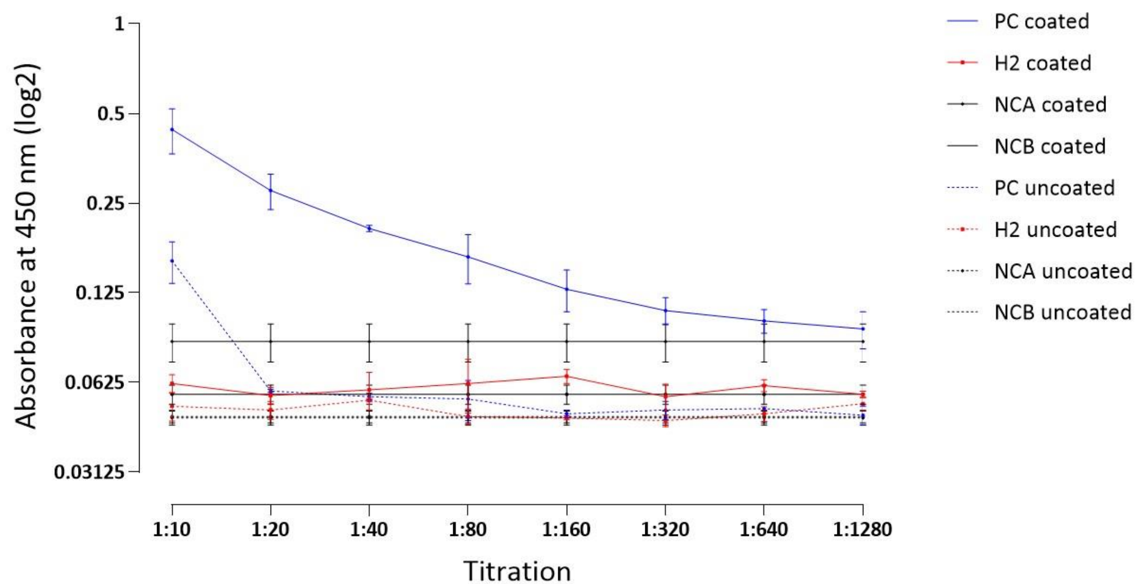

Figure 7: ELISA assay of scFv-3H2 binding to EHV-1. Titration of positive control and scFv-3H2 (log2) on a 96-well plate partly coated with 2 µg/ml purified EHV-1, partly not coated with antigen. NCA –negative control A: no template, α-E-Tag-HRP. NCB- negative control B: no template, no detection antibody.

ScFv-3H2 was analysed by electrophoresis on 12% SDS-PAGE gels (Figure 8) to assess expression efficiency. Induction with IPTG was proven successful since cell culture pre-induction medium contained low concentrations of proteins and post-induction cell culture medium expresses high concentrations of proteins. Typical molecular weight of scFv fragments is 27 kDa. A band at approximately 27 kDa was detected primarily in the unpurified periplasmic

fraction, but a faint band at 27 kDa was also noticeable in the unpurified supernatant. Concentrations of protein in eluted fractions are too low for detection by SDS-PAGE analysis.

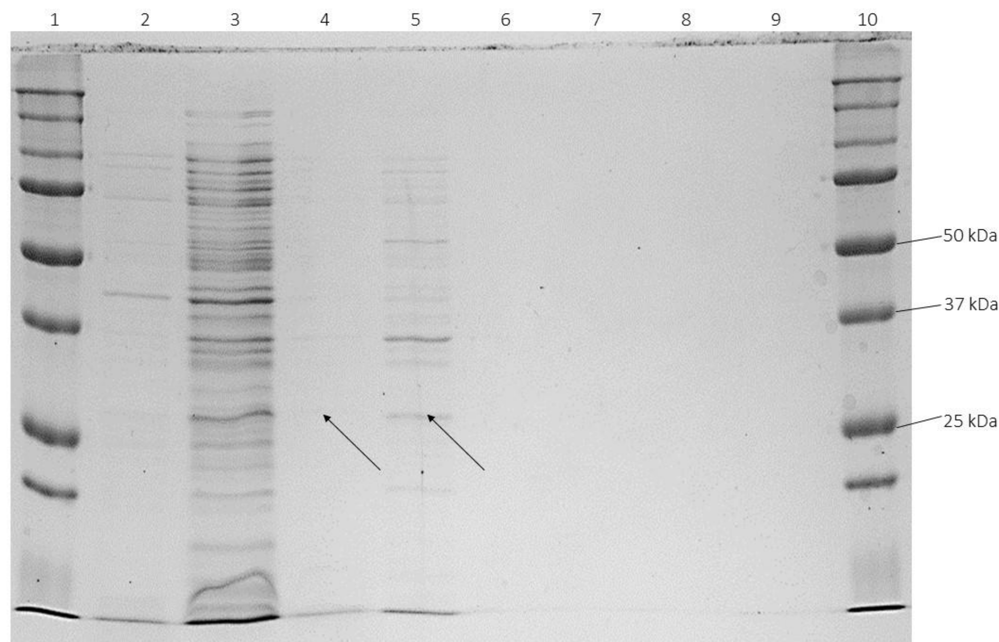

Figure 8: 12% SDS - PAGE analysis of scFv-3H2. Lane 1 & 10: Precision Plus Protein Standard. Lane 2: Bacterial culture medium pre-induction, Lane 3: Bacterial culture medium post-induction, Lane 4: Unpurified supernatant, Lane 5: Unpurified peri-plasm, Lane 6 – 9: eluted fractions.

The target protein scFv-3H2 was further characterised with EHV-1 under reducing conditions in western blot analysis. For comparison, unpurified supernatant and purified dialysed scFv-3H2 were used on two separate blots both loaded with differing concentrations of EHV-1 in designated lanes (2 µg/ml, 5 µg/ml and 10 µg/ml). Neither supernatant nor purified antibody showed a reaction in western blotting under given conditions (Figure 9).

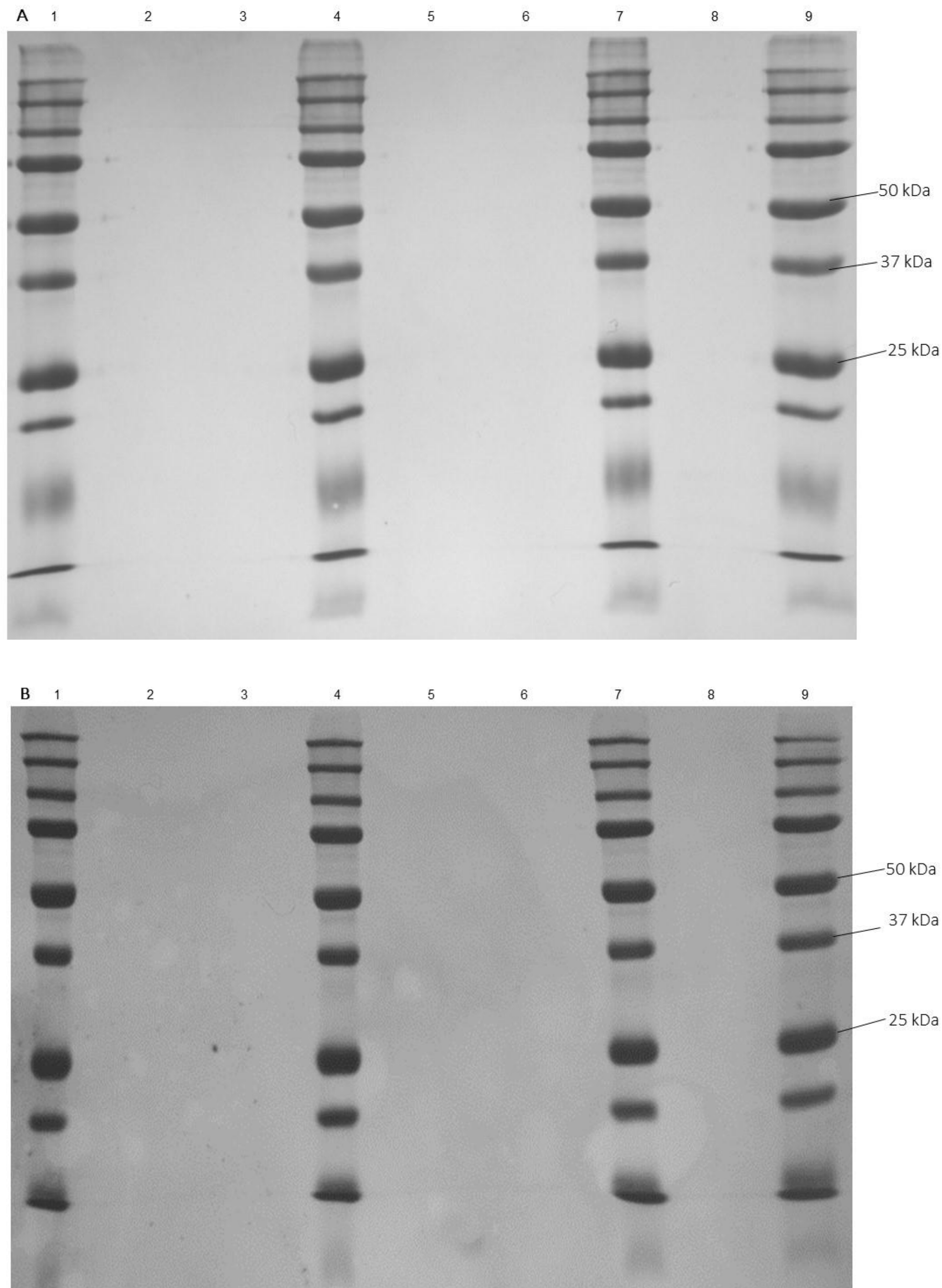

Figure 9: Western blot analysis of precipitated supernatant of scFv-3H2 (A) and dialysed scFv-3H2 (B). Differing concentrations of EHV-1 were loaded onto a 12% SDS gel and blotted on a nitrocellulose membrane. Antibody aliquots were incubated on the membrane before α-E-Tag conjugated detection antibodies were added. Lanes 1, 4, 7, 9: Precision Plus Protein Standard. Lane 2: EHV-1 (2 µg/ml). Lanes 3, 6: empty. Lane 5: EHV-1 (5 µg/ml). Lane 8: EHV-1 (10 µg/ml).

4.2.5 Discussion

Our proof-of-principle study successfully generated an equine antibody phage display library and isolated a promising scFv antibody candidate using phage display technology. The immunized equine antibody library in the scFv format provided the basis for this study (15). Immunized libraries have the advantage of containing antibody fragments that are derived from B-lymphocytes of already immunised individuals and have therefore undergone *in vivo* affinity maturation towards the desired antigen (16). The donor animal serving as a source of immunoglobulin genes for the construction of the library had previously been immunised against EHV-1 along with EHV-4, Tetanus and Influenza and was therefore a promising source of isolating an EHV-1-specific antibody. Prior to biopanning on EHV-1, a plasma sample of the donor animal was tested in an ELISA to confirm presence of EHV-1 antibodies (Figure S3). Their presence was confirmed, yet assays determining neutralising activity of these antibodies and excluding binding to background would have added to a thorough investigation of the nature of origin immunoglobulins.

Since the likelihood of selecting a highly affine antibody fragment from this library is enhanced, library sizes ranging from $1x10^6$ to $1x10^8$ individual clones are deemed plentiful for the isolation of a specific antibody fragment (16, 17). The library used contained $4.8x10^8$ individual clones, which was declared sufficient for our purposes. Individual scFv clones were ligated into phagemid vector pCANTAB 5E, which contains the phage gene pIII, required for infection. Phagemid vectors are a popular vector system, preferred over phage vectors: in a phage vector system, every phage would carry three to five copies of the antibody fragments on its surface (multivalent display) (18). Assembly of a multivalent phage display library would risk creating background noise within the phage library with incomplete scFv fragments. In the phagemid vector system, each phage contains only one copy of the antibody fragment on its surface (monovalent display) (18). When phagemid vector systems are utilized, as it is the case in this study, a phage carrying a mutation affecting phage coat protein pIII, are used for phage

library assembly to improve antibody display and selection (18). This mutated phage, the hyperphage M13K07ΔpIII, carries a gene III deletion, which leads to a multivalent display in combination with a phagemid (18-21). This combination allows the generation of a larger phage library as a basis for selection purposes. We chose to apply this combination: the equine immunized library was ligated into a phagemid vector and enabled packaging as many scFv fragments into the phage display library by using the hyperphage in the first round and increasing stringency in the subsequent rounds by employing the helper phage M13K07. We infected the library (15) with hyper phages and yielded 7x 10^{11} cfu/ml (data not shown) after precipitation. This result corresponds with studies that also packaged recombinant antibodies into hyper phages yielding $1x10^{11}$ to $2.5x10^{11}$ cfu/ml, selecting human antibodies (21, 22). For panning purposes a defined amount of phages was exposed to antigen. This amount ranged from $1x10^{11}$ to $1x10^{13}$ cfu/ml which was in coherence with other studies (23, 24). Throughout panning rounds an increase of phage output is expected since eluted antigen specific scFv-phage are amplified (25, 26). In this study, a decrease rather than an increase was observed after the second panning round, which is a common observation and most likely due to a change from hyper- to helper phage (26, 27). M13K07 helper phages were used for infection in panning round two and three for a monovalent display and to increase stringency of the panning process as recommended by many other protocols (28-30). Phage output of panning round three increased, reflecting the expected amplification and selection process of phage display technology and corresponds with similar studies where phage output titres ranged from 10^4 to 10^6 cfu/ml (23-26, 31, 32).

While the phage output titre does not record a steep increase, the mean OD values of all tested clones increases with every panning, reflecting enhanced binding abilities of scFv fragments. Mean OD values range from 0.0156 after the first panning round to 0.158 in the third panning round, indicating a 10-fold increase. The values on their own appear low, but the enrichment factor of 10 speaks for appropriate and successful selection strategy and is reflective

of selecting binders with higher affinities every round. Other studies have obtained merely a doubling of mean absorbance (24). Low OD values might indicate that selected antibody fragments are not specific to EHV-1 since the risk remains that sucrose-gradient purified virus aliquot is not entirely free from cell debris and binding might have occurred to contaminants of the aliquot.

Our selection strategies of decreasing coating conditions and increasing washing steps are in coherence with previous studies, who employed the same strategies reaching similar results (20, 24, 33). Selection stringency conditions can be considered successful when the negative control within the ELISA assays is 10 to 20-fold lower than the positive control as suggested in a current protocol (33). In the study on hand, the absorbance of negative control was on average 10-fold lower than the positive control, leaving no doubt that selection stringency strategies were implemented correctly.

Since suggestion in the current literature are highly controversial, one study suggested to declare clones worth further investigation when absorbance was 5-fold higher than the negative control (24), others consider clones for further evaluation when absorbance is 2.5-fold higher than the negative control (34). In the present study, no clone reached an absorbance of 5-fold higher than the negative control, but clones of the third round reached absorbance 2.5-fold higher than the negative control. Those were considered for further evaluation. This decision was further supported by findings of Sengupta et al. (35), who deemed clones noteworthy when absorbance was above 0.2. Application of a non-equine-specific antigen as additional negative control would have contributed to distinction of EHV-1-positive and-negative clones. We decided to evaluate all clones from panning round three which yielded absorbance above 0.25 in a colony PCR to detect full-length antibody fragments.

Similar to human (28) and macaque (30) scFv fragments, equine full-length scFv antibody fragments were expected to be approximately 800 bp in size. When visualised on an

agarose gel after colony PCR, a band at 1 kb should be expected taking PCR primer restriction sites into consideration. One clone, scFv-3H2, reaching an absorbance of 0.254, proofed to contain a full-length insert and was therefore considered for further characterisation by sequencing. Remaining clones reached absorbance of above 0.25 as well, however, they did not contain full-length scFv inserts since sizes ranged from 300 to 600 bp. The occurrence of defective clones is not uncommon (28, 36, 37). When constructing a recombinant antibody library, its quality is continuously assessed by colony PCR after each transformation reaction. It is recommended to assemble the complete library if at least 80 % of inserts contain full-length inserts (28). This library was assembled from sublibrary aliquots containing 80 % to 100 % full-length inserts. While this presents a high likelihood of isolating functioning, full-length inserts, this leaves room for packaging of possibly defective clones or empty vectors into the phage library (36). These may retain some antigen binding abilities as well as ampicillin resistance obtained from the phagemid vector and thus continue to be amplified during panning rounds. This could lead to background noise in terms of resource management in growth media, preventing or blocking antigen binding possibilities and may grow as satellite colonies on densely grown agar plates (36). During selection of colonies, no discrimination between defective and non-defective clones is possible as no marker (e.g. blue-white-screening) other than ampicillin resistance was used. Clones derived their ampicillin resistance from the vector, hence it is possible for empty vectors or defective clones to grow in spite of the presence of ampicillin. Defective clones showing bands of approximately 350 bp can be deemed to depict insert-less vectors according to Kuegler et al. (28). Clones of approximately 600 bp were assumed to contain incomplete scFv fragments and were not considered for further evaluation.

Sequencing of scFv-3H2 confirmed that it contains a full-length insert consisting of a VH domain connected by a peptide linker $(Gly_4Ser)_3$ to a VL domain and was successfully ligated into phagemid vector pCANTAB 5E. Its size of 743bp corresponds with abovementioned findings. Sequence analysis revealed predominantly hydrophilic amino acid

residues, which is known to enhance flexibility (38). Serine, Glycine and Tyrosine are key amino acid residues in antibody sequences and ideally abundantly present. This was the case for scFv-3H2. The key residue Cys23 was present, yet Trp41, Leu89 and Cys104 were substituted by Glycine, Methionine and Tyrosine, some of which bear similar biochemical characteristics with the original key residues. One irregular nucleotide base exchange occurred in the linker sequence which resulted in glycine being exchanged by serine. Sequencing results were assessed thoroughly for overlapping peaks or interruptions to exclude contamination or erroneous interpretation, which could have influenced the translation of bases. No interruptions or traces of contamination were observed (Figure S5).

Faulty linker sequences are thought to affect proper protein folding and rendering antibody proteins functionless (39). A 3D model of scFv-3H2 confirmed CDRs to lie in their expected positions, with the CDR-H3 lying at the center of the molecule as expected. Highest probability to interact with the antigen was obtained for CDR-H2 (Pt 0,48) and CDR-L1 (Pt 0.48) (14). CDRs remained close to each other on the antibodies surface and folded into a compact molecule. This leaves to assume that the base exchange in the linker did not affect antibody properties of scFv-3H2 and a characterisation of this antibody is worthwhile. It must be kept in mind however, that Phyre^2, the 3D prediction software, is limited in its capacities to predict accurate models. Amino acid sequences as the sole source, as it was in this study, may not show point mutations to have an effect on 3D representations. Further 3D models are sculpted on known, representative molecules rather than true structure of the antibody and whether or not the molecule is truly compact and stable is best assessed by noticing presence of cysteine residues within CDRs, particularly CDR-3 (40). Despite its seemingly compact structure scFv-3H2 unfortunately lacks the cysteine residues, along with mutations within the linker sequence. Both observations should be kept in mind during downstream applications in regards to molecule stability as they can have an effect on the antibody fragments binding ability, expression and stability (39).

scFv-3H2 was propagated and purified by several centrifugal processes to isolate periplasmic fractions from supernatant of *E. coli* cell cultures at the end of the 23 hr induction. Purification of periplasmic and supernatant fractions via ÄKTA chromatography revealed peaks in both, periplasmic and supernatant fractions, which is surprising, since scFv are usually expressed within the periplasmic fraction. This result is however in accordance with the results obtained by electrophoresis on 12% SDS-PAGE gels. It confirmed the majority of scFv expression to occur within the periplasmic fraction, even though a faint band at 27 kDa is detectable within the supernatant fraction as well. Studies report that an escape into the supernatant is not uncommon (41). Since its concentration is low and the band at 27 kDa faint, it could also be argued that it might be a protein expressed by *E.coli* rather than indicative for the scFv fragment. From thereinafter supernatant fraction and peri-plasmic fraction were pooled for further characterisation and maximisation of scFv output, which is a customary procedure (41).

For further downstream application fulfilling characterisation purposes, protein concentrations ranging from 0.2 to 0.8 mg/ml are recommended (42-44). Higher concentrations would result in scFv aggregation and lower concentrations could be representative of scFv inclusion bodies, insoluble scFv molecules (42-44). ScFv-3H2 yielded 0.06678 mg/ml and is well below abovementioned thresholds. It is reported that scFv yields from *E. coli* production systems vary often (43). This is thought to be in conjunction with IPTG concentration during induction since merely 5 µM per L have been reported sufficient to induce soluble scFv molecules rather than forming inclusion bodies (43). In this study, 2mM per L were used for induction purposes, which might have caused the production of inclusion bodies as well. Inclusion bodies can be solubilized by centrifugation and refolding procedures such as urea treatment; unfortunately, we did not apply this treatment to recover scFv molecules. Incubation temperature during induction is also considered to have an effect on protein yield (43). While we incubated the culture at 30°C for 23 hrs, other studies incubated at 25°C and 37°C for 16

hours which resulted in differing yields (43). While *E. coli* production systems are reliable and robust production systems, given these observations, we recommend to perform IPTG titrations and temperature adjustments prior to future soluble scFv production to prevent formation of inclusion bodies.

Given this low protein concentration, it is no surprise that purified scFv-3H2 failed to react in further assessments of binding activity by ELISA and Western Blot analysis when exposed to the antigen EHV-1. In ELISA assay, its binding specificities were not comparable to the full size mAb 6B11 positive control and did not seem to retain any of the binding properties of the full IgG molecule it was derived from. No binding activity is noticeable in Western Blot analysis either, yet no PC to confirm presence of EHV-1 proteins was applied to membranes either; hence it cannot be excluded that lack of binding activity of scFv-3H2 is not due to problems with viral coating. Furthermore, given its low concentration, preparing a dilution series and beginning with a 1:10 dilution was not advantageous. Additionally, even though panning occurred against EHV-1, due to a high genetic and antigenic similarity to EHV-4, there is a small chance that the selected scFv might exhibit better binding affinities to EHV-4 instead of EHV-1 (45, 46). Further assessments by e.g. ELISA would elucidate whether or not cross-reactivity are of concern in this study. Furthermore, the base exchange within the linker sequence might have had an effect on the scFv-3H2 properties after all (39).

4.2.6 Conclusion

We selected an anti-EHV-1 scFv via three rounds of phage display biopanning from an immunized equine scFv library using EHV-1 as target antigen (47). The selected antibody was expressed in *E. coli*, pre-dominantly within the peri-plasmic fraction, however not at sufficient concentrations for further characterisation by downstream applications. In future, we recommend to assess whether concentration and binding affinity could be enhanced by error-

prone PCR, mutation strains, chain shuffling or DNA shuffling during the library assembly stage (47) or to perform IPTG titrations and temperature adjustments prior to future soluble scFv production to prevent formation of inclusion bodies during protein expression (43). Our results lay the groundwork for further investigation of recombinant equine antibodies for application in equine veterinary therapeutic or diagnostic agents (47).

4.2.7 References (Vancouver)

1. Loving NS. All Horse Systems Go: The Horse Owner's Full-color Veterinary Care and Conditioning Resource for Modern Performance, Sport, and Pleasure Horses: Trafalgar Square Books; 2014.

2. Stokol T, Soboll Hussey G. Editorial: Current Research in Equid Herpesvirus Type-1 (EHV-1). Frontiers in Veterinary Science. 2020;6(492).

3. Pusterla N, Hatch K, Crossley B, Wademan C, Barnum S, Flynn K. Equine herpesvirus-1 genotype did not significantly affect clinical signs and disease outcome in 65 horses diagnosed with equine herpesvirus-1 myeloencephalopathy. The Veterinary Journal. 2020;255:105407.

4. Oladunni FS, Horohov DW, Chambers TM. EHV-1: A Constant Threat to the Horse Industry. Front Microbiol. 2019;10(2668).

5. Lunn D, Davis-Poynter N, Flaminio M, Horohov D, Osterrieder K, Pusterla N, et al. Equine herpesvirus-1 consensus statement. Journal of Veterinary Internal Medicine. 2009;23(3):450-61.

6. Maxwell LK, Bentz BG, Gilliam LL, Ritchey JW, Pusterla N, Eberle R, et al. Efficacy of the early administration of valacyclovir hydrochloride for the treatment of neuropathogenic equine herpesvirus type-1 infection in horses. American journal of veterinary research. 2017;78(10):1126-39.

7. Tallmadge RL, Žygelytė E, Van de Walle GR, Kristie TM, Felippe MJB. effect of a histone Demethylase inhibitor on equine herpesvirus-1 activity In Vitro. Frontiers in veterinary science. 2018;5:34.

8. Frenzel A, Kügler J, Helmsing S, Meier D, Schirrmann T, Hust M, et al. Designing Human Antibodies by Phage Display. Transfusion Medicine and Hemotherapy. 2017;44(5):312-8.

9. Solemani Zadeh A, Grässer A, Dinter H, Hermes M, Schindowski K. Efficient Construction and Effective Screening of Synthetic Domain Antibody Libraries. Methods and protocols. 2019;2(1):17.

10. Crivianu-Gaita V, Thompson M. Aptamers, antibody scFv, and antibody Fab' fragments: An overview and comparison of three of the most versatile biosensor biorecognition elements. Biosensors and Bioelectronics. 2016;85:32-45.

11. Lou J, Marzari R, Verzillo V, Ferrero F, Pak D, Sheng M, et al. Antibodies in haystacks: how selection strategy influences the outcome of selection from molecular diversity libraries. Journal of immunological methods. 2001;253(1-2):233-42.

12. Czerny C-PB, Caroline. Purification of EHV-1 specific mAb 6B11. 2013.

13. Czerny C-P, Eichhorn W. Characterization of monoclonal and polyclonal antibodies to bovine enteric coronavirus: establishment of an efficient ELISA for antigen detection in feces. Vet Microbiol. 1989;20(2):111-22.

14. Ambrosetti F, Olsen TH, Olimpieri PP, Jiménez-García B, Milanetti E, Marcatilli P, et al. proABC-2: PRediction of AntiBody contacts v2 and its application to information-driven docking. Bioinformatics. 2020.

15. Pilger FI, Ahsendorf, H. P., Knauf, S., Schwarz, A., Walther, S., Czerny, C.-P., Diesterbeck U. S., Fechner, K. PCR primer for the construction of an equine immunoglobulin library in the single-chain fragment variable (scFv) format. 2020.

16. Benhar I. Design of synthetic antibody libraries. Expert opinion on biological therapy. 2007;7(5):763-79.

17. Breitling F, Dübel S. Rekombinante Antikörper: Spektrum Akademischer Verlag Heidelberg Berlin; 1997.

18. Ponsel D, Neugebauer J, Ladetzki-Baehs K, Tissot K. High affinity, developability and functional size: the holy grail of combinatorial antibody library generation. Molecules. 2011;16(5):3675-700.

19. Breitling F, Dübel S, Seehaus T, Klewinghaus I, Little M. A surface expression vector for antibody screening. Gene. 1991;104(2):147-53.

20. Rondot S, Koch J, Breitling F, Dübel S. A helper phage to improve single-chain antibody presentation in phage display. Nature biotechnology. 2001;19(1):75-8.

21. Heitner T, Satozawa N, Mclean K, Vogel D, Cobb RR, Liu B, et al. Obligate multivalent recognition of cell surface tomoregulin following selection from a multivalent phage antibody library. Journal of biomolecular screening. 2006;11(8):985-95.

22. Hust M, Meyer T, Voedisch B, Rülker T, Thie H, El-Ghezal A, et al. A human scFv antibody generation pipeline for proteome research. Journal of Biotechnology. 2011;152(4):159-70.

23. Pansri P, Jaruseranee N, Rangnoi K, Kristensen P, Yamabhai M. A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. BMC Biotechnology. 2009;9(1):6.

24. Lakzaei M, Rasaee MJ, Fazaeli AA, Aminian M. A comparison of three strategies for biopanning of phage-scFv library against diphtheria toxin. Journal of Cellular Physiology. 2019;234(6):9486-94.

25. Kuhn P, Fühner V, Unkauf T, Moreira GMSG, Frenzel A, Miethe S, et al. Recombinant antibodies for diagnostics and therapy against pathogens and toxins generated by phage display. PROTEOMICS – Clinical Applications. 2016;10(9-10):922-48.

26. Frenzel A, Kugler J, Wilke S, Schirrmann T, Hust M. Construction of human antibody gene libraries and selection of antibodies by phage display. Methods in molecular biology (Clifton, NJ). 2014;1060:215-43.

27. Russo G, Meier D, Helmsing S, Wenzel E, Oberle F, Frenzel A, et al. Parallelized antibody selection in microtiter plates. Phage Display: Springer; 2018. p. 273-84.

28. Kügler J. TF, Frenzel A., Hust M. Construction of Human Immune and Naive scFv Libraries. . 2018. In: Phage Display Methods in Molecular Biology [Internet]. New York, NY: Humana Press.

29. Diesterbeck US. Construction of Bovine Immunoglobulin Libraries in the Single-Chain Fragment Variable (scFv) Format. Methods in molecular biology (Clifton, NJ). 2018;1701:113-31.

30. Avril A, Miethe S, Hust M, Pelat T. Construction of macaque immune-libraries. Phage Display: Springer; 2018. p. 83-112.

31. Ch'ng ACW, Hamidon NHB, Konthur Z, Lim TS. Magnetic nanoparticle-based semi-automated panning for high-throughput antibody selection. Phage Display: Springer; 2018. p. 301-19.

32. Hust M, Maiss E, Jacobsen HJ, Reinard T. The production of a genus-specific recombinant antibody (scFv) using a recombinant potyvirus protease. J Virol Methods. 2002;106(2):225-33.

33. Lee CM, Iorno N, Sierro F, Christ D. Selection of human antibody fragments by phage display. Nature protocols. 2007;2(11):3001.

34. Wang G, Huynh K, Barhate R, Liu J, Tam P, Rodrigues W, et al. Validation of a New Recombinant Antibody Fragment (rFab)-Based Homogeneous Enzyme Immunoassay for the Highly Specific Detection of 6-Acetylmorphine in Urine. J Anal Toxicol. 2015;39(9):726-33.

35. Sengupta PP, Rudramurthy GR, Ligi M, Jacob SS, Rahman H, Roy P. Development and evaluation of recombinant antigen and monoclonal antibody based competition ELISA for the sero- surveillance of surra in animals. J Immunol Methods. 2018;460:87-92.

36. Yang J, Chen R, Wei J, Zhang F, Zhang Y, Jia L, et al. Production and characterization of a recombinant single-chain antibody against Hantaan virus envelop glycoprotein. Appl Microbiol Biotechnol. 2010;86(4):1067-75.

37. Yamanaka HI, Kirii Y, Ohmoto H. An improved phage display antibody cloning system using newly designed PCR primers optimized for Pfu DNA polymerase. The Journal of Biochemistry. 1995;117(6):1218-27.

38. Walther S, Czerny C-P, Diesterbeck US. Exceptionally long CDR3H are not isotype restricted in bovine immunoglobulins. Plos One. 2013;8(5):e64234-e.

39. van Rosmalen M, Krom M, Merkx M. Tuning the Flexibility of Glycine-Serine Linkers To Allow Rational Design of Multidomain Proteins. Biochemistry. 2017;56(50):6565-74.

40. Kelley LL, Momany C. Generation of a phagemid mouse recombinant antibody fragment library by multisite-directed mutagenesis. Biotechniques. 2003;35(4):750-2, 4, 6 passim.

41. S.M. K. High-Level Periplasmic Expression and Purification of scFvs. In: (eds) AR, editor. Antibody Phage Display Methods in Molecular Biology™. 178. Humana Press.: Humana Press.; 2002.

42. WoÈrn A, PluÈckthun A. Stability engineering of antibody single-chain Fv fragments. J Mol Biol. 2001;305(5):989-1010.

43. Miller KD, Weaver-Feldhaus J, Gray SA, Siegel RW, Feldhaus MJ. Production, purification, and characterization of human scFv antibodies expressed in Saccharomyces cerevisiae, Pichia pastoris, and Escherichia coli. Protein Expres Purif. 2005;42(2):255-67.

44. Cnudde T, Lakhrif Z, Bourgoin J, Boursin F, Horiot C, Henriquet C, et al. Exploration and Modulation of Antibody Fragment Biophysical Properties by Replacing the Framework Region Sequences. Antibodies. 2020;9(2):9.

45. Telford E, Watson MS, Perry J, Cullinane AA, Davison AJ. The DNA sequence of equine herpesvirus-4. J Gen Virol. 1998;79(5):1197-203.

46. Ma G, Azab W, Osterrieder N. Equine herpesviruses type 1 (EHV-1) and 4 (EHV-4)--masters of co-evolution and a constant threat to equids and beyond. Vet Microbiol. 2013;167(1-2):123-34.

47. Wei J, Liu Y, Yang S, Xu J, Kong H, Han B, et al. Screening of single-chain variable fragments against TSP50 from a phage display antibody library and their expression as soluble proteins. Journal of biomolecular screening. 2006;11(5):546-52.

5 GENERAL DISCUSSION

The aim of this thesis was the construction of an equine-specific recombinant antibody library, which was accomplished successfully, to make up for a shortage thereof. A further aim, was the establishment of phage display technology as a suitable display method for isolation of equine scFv fragments from the newly constructed library, which was accomplished. The library was screened against EHV-1, since it is one of the most prevalent and detrimental pathogens affecting horse populations worldwide. The overall quality of the newly constructed library is evaluated best by considering certain key features: sequence diversity within the library, ease of generation of desired antibody fragment format and the format itself in regards to structural stability and key antibody features and suitability of the chosen display method (1).

In the natural host, antibody sequence diversity is generated during B-cell proliferation in the presence of antigen, whereby the immunoglobulin locus undergoes SHM (1). Horses in particular are hypothesized to develop the greater part of antibody sequence diversity in that particular manner (2). In the course of generating large antibody libraries, efforts are made to mimic SHM, yet initially sequence diversity is solely dependent on naturally occurring diversity (1, 3). It is determined according to the number of individual clones within a library, which equates with the size of a library (1, 3-5). The immunized library constructed in this study, has an absolute size of $4.8x10^8$ individual clones, whereby absolute size has to be differentiated from functional size: while the absolute size is an estimation of all individual clones available in the library, the functional size refers to all individual clones encoding full-length scFv antibodies (1, 3-5). Hence, the functional size is more meaningful than the absolute size since a high functional size indicates high occurrence of individual clones encoding full-length scFv antibody sequences, thereby high sequence diversity (1). Nevertheless, the probability of identifying individual clones encoding full-length scFv antibody sequences increases with

absolute library size (1). Functional size of the library on hand, was determined by colony PCR and 80 % of individual clones were observed to contain full-length scFv antibody fragments. The remaining 20 % are considered defective clones. Sequence diversity was further confirmed by fingerprint analysis as multiple repetitions of restriction patterns were absent. The sequence diversity of the equine library can therefore be considered high and is comparable to other studies constructing immunized libraries (6, 7), yet a broader coverage of allelic sequence diversity and elimination of defective clones could be pursued in future studies (1).

One approach to improve sequence diversity is non-targeted diversification which includes error-prone PCR, use of mutator *E. coli* strains or chain shuffling (8, 9). All of these methods are utilized to introduce mutations, thereby randomly inserting sequence diversity into antibody sequences (8). Several studies have successfully increased library sizes up to 100-fold with these techniques (9). Yet, non-targeted diversification has also had disadvantageous effects as frame shift mutants occurred frequently and lead to non-functional proteins during diversification procedures; additionally, CDR regions, crucial antigen binding regions, were predominantly affected by deleterious mutations (1). The approach of targeted diversification within these regions could prevent deleterious mutations and contribute to sequence diversification (1, 8, 9). Not only would it increase functional library sizes, hence sequence diversity but also affinity of individual antibody clones, since it is reported that functional library size and individual affinities correlate (10). Methods enabling targeted diversification include the generation of synthetic antibody libraries introducing oligonucleotides already at the library generation level, trinucleotide mutagenesis, CDR-walking and CDR-cassettes (1). All methods could increase functional library size by inserting diversity by substitution of only few amino acids (4 aa to 6aa) or entirely replacing CDR regions *in silico,* thereby controlling CDR regions and avoiding unwanted amino acids and stop codons (11-13).

Further considerations could be the application of vector systems containing a positive selection marker for protein integrity such as ß – lactamase as selection for in-frame, full size protein expression (1). In this case, simply clones unaffected by frameshift or stop codons would survive protein expression and respective antibiotic selection (1).

In humans, recombinant antibody and library research is thus far, that it is known which VH framework is frequently found in human antibodies and combines well with all light chains, expresses well in bacteria and displays well on phages (1), which would be something to aim for in equine immunoglobulin research. Investigations have already observed preferential gene segment usage in both equine heavy as well as light chains (14-16). Out of 50 VH gene segments, four were found expressed frequently and two were recurring in every clone assessed in their studies (15). Preferential use of certain heavy chain joining and diversity gene segments was reported as well (15, 16). This information could be used in the *in silico* design of equine antibodies and when establishing a single VH framework frequently found in horses to combine with a range of light chains and subsequently expresses well in microbial production systems as mentioned above.

Beside sequence diversity, ease of generation of desired antibody fragment format and the format itself play a crucial role during library construction as it influences antibody expression and lastly drug tolerance (1). ScFv fragments are a popular format due to their favourable expression properties in periplasm of *E. coli* cells and their manipulability within this system to enhance selection of specific, high affinity binders (1, 4, 5). In this dissertation, comprehensive primer sets facilitated the generation of equine scFv by amplifying variable antibody domains of heavy and light chains and fusion thereof by SOE-PCR. Sequences of exemplary antibody sequence HL1 (Manuscript I, Figure 6) and candidate scFv-3H2 (Manuscript II, Figure 4) both revealed g3 signal sequence of phagemid vector pCANTAB 5E as well as variable regions of equine antibody heavy and light chains in one reading frame.

Presence of conserved key amino acid residues in antibodies, was confirmed, yet only Cys23 was at the desired position. Trp, Leu and the second Cys residue were present abundantly, yet substituted by other residues at key positions.

In theory, the fusion of two antibody chains into one gene, allows the expression of equal amounts of both chains guaranteed as one homogenous protein in amounts and concentrations sufficient for diagnostic and therapeutic purposes (4, 5). This is in conflict with the results on hand, as the selected scFv-3H2 was expressed at low concentrations. After antibody purification via ÄKTA chromatography, Bradford assay yielded a concentration of 0.06678 mg/ml, which is below recommended concentrations of 0.2 to 0.8 mg/ml for characterisation purposes (17-19). This result could be due to aggregation and formation of inclusion bodies (17-19) or due to the faulty linker sequence, as a point mutation was observed and affected linker sequences are considered to affect proper protein folding and therefore binding capacities (20). Additionally, scFv-3H2 lacks stabilising Cys bridges within CDRs, rendering the molecule potentially instable.

This result is not uncommon as in numerous other studies scFv antibody fragments, have proven to be expressed at frequently fluctuating levels within bacterial systems, be only moderately stable and showed a tendency to form multimers or aggregates (17-19). Given these findings, human recombinant antibody therapeutics, which had initially been developed in the scFv format, were re-combined with Fc regions of either the target species or the original species to enhance binding kinetics (18, 19, 21). This approach of re-combining scFv into mono-, bi- or multivalent scFv-Fc or Fab complexes occurs frequently since the Fc region renders the fragments into molecules with higher structural stability, reduced aggregation, higher monomeric proportions and a reduced likelihood of impairment of function (1). Thus, these complexes have replaced scFv in more recent libraries (1).

Another alternative to scFv present sdAbs: this format fulfils the requirements of high soluble expression in *E. coli* periplasm, contain long CDR3 regions and facilitate antigen binding due to their exposed CDR structures (1). The missing Fc part however limits sdAbs half-life *in vivo*, which could be prevented through e.g. PEGylation increasing molecule size thereby preventing premature kidney clearance (1). This kind of modification is costly and might outweigh the advantages gained by cheap bacterial expression (1). Small recombinant antibodies in general miss Fc's effector functions and are only utilizable for applications that do not require such functions (1).

Given our findings and multiple reports (17-19, 21-24), one could consider re-combining scFv-3H2 into a scFv-Fc complex prior to expression. This format has proven effective against other equine viral infections: a study by Ruelker et al (22) isolated a human-like scFv from a non-human primate scFv antibody library to find a therapeutic agent against VEEV. The anti-VEEV-scFv was re-cloned into a bivalent scFv-Fc complex to increase stability and was successful in inducing protective immunity against VEEV in mice (22). Huelseweh et al (23) isolated four scFv fragments from two immunized macaque libraries to isolate antibody therapeutics against WEEV for human use. Isolated scFv fragments were also reformatted into scFv-Fc complexes and three out of four showed neutralizing activity against WEEV (23). Considering these results, the scFv fragment generated in this study, provides a valuable starting point for future re-combination and development of equine antibody therapeutics. A more straightforward recommendation is hard to come by as none of currently approved, recombinant antibody scFv therapeutics neither human nor veterinary, have been raised against viral agents.

We have determined that the newly constructed equine, immunized antibody library provided a high diversity of antibody sequences in the versatile scFv format. This leaves to evaluate the suitability of phage display technology to isolate high affinity binders from this library: phage display technology has proven the most robust technology over the past 30 years

and the most frequently used display method in numerous studies (6, 25, 26). In this study, it proved suitable as well since it facilitated isolation of an equine antibody fragment, scFv-3H2, after three rounds of biopanning. Successfully implemented biopanning rounds are characterised by an increase of mean OD values, which is deemed reflective of selection of antibody candidates with higher binding affinities to the antigen of interest (27). In previous publications, mean absorbance of selected clones, have doubled throughout panning rounds (28), which is in accordance with mean absorbance of scFv clones in this dissertation as clones in round three had mean absorbance three-fold higher than in round one. Furthermore, an interplay of phage input and output titre ensures correctly implemented selection strategy of phage display technology (29), which was observable in the experiment on hand. The selected clone scFv-3H2 yielded an absorbance of 0.254, which is one of the highest absorbance yielded in this study, yet it could be improved and reach absorbance similar to scFv phage clones isolated in a study by Kirsch et al (27), which reached OD values ranging from approximately 0.6 to 2.1. Their results must be compared to our result with caution as they use scFv phage clones rather than soluble scFv as produced in our study. Some antibody fragments bind only as antibody phage particles and might lead to false positives (27).

Absorbance is also strongly influenced by the antigen used (30): we utilised sucrose-gradient purified EHV-1 as the antigen of interest and applied it to all downstream applications. Purification of viral capsids from herpesvirus infected cells appeared successful as a clear band was detectable and extractable after centrifugation. Recent protocols (31) suggest herpesvirus purification using PBS buffer, however our experiments in advance of phage display have resulted in clearer and cleaner bands using TRIS buffer and subsequent ELISA confirmed binding between EHV-1 and an EHV-1-specific mAb. Sucrose-gradient purified EHV-1 quality was deemed sufficient to apply within subsequent procedures. However, purification process bears the risk of not being entirely free of cell debris and as the possibility of selecting antibodies binding to viral background rather than the antigen is possible. Further functional

testing (e.g. titration in cell culture or western blotting) of purified virus prior to downstream applications, would have elucidated the viral infectivity and virus integrity further.

Alternative display methods could be considered with the aim of selecting binders with higher affinities: ribosome display would be an alternative as it omits laborious transformation procedures, which otherwise may lead to deleterious mutations and frameshifts, all the while generating libraries of sizes up to 10^{12} individual members (1). Ribosomal display method merely requires mild elution ingredients to elute high-affinity binders, which is not possible in technologies that rely on elution based on disruption of antibody-antigen interaction such as phage display (1). On the other hand, it is technically more challenging than phage display and solely scFv fragments can be displayed which would excluded re-cloned formats e.g. the scFv-Fc (1). Yeast display can be excluded as alternative approach to isolate antibody fragments of smaller sizes since it is only applicable to full length IgG (1). A more frequently used approach is the abovementioned isolation of smaller antibody fragments from immunized libraries via phage display and re-combining the candidates into scFv-Fc as applied for the approved and frequently utilized therapeutic recombinant antibody Belimumab (Benlysta) (32). The two veterinary preparations have also been adjusted by recombination for better drug tolerance within the target species (33, 34).

In regards to fighting EHV-1 infection, recombinant equine antibodies generated in our experiments may provide biological reagents aiding researchers in the near future to further elucidate viral proteins and antigenic epitopes involved in viral infectivity, replication, latency and host immune response as well as ultimately aid in impairment of these processes involved in viral pathogenesis (35).

5.1 Conclusion & Outlook

Horses are and will be exposed to a variety of pathogens throughout their lives, EHV-1 being one of the most prevalent, infectious and detrimental ones. Current prevention and therapeutic agents, both on the market and in research, do not prevent viremia and latent infection. Recombinant antibody therapeutics offer new options studying and fighting viral transmission.

This dissertation presented the construction of recombinant equine antibodies in a highly diverse equine, immunized antibody library in the verstaile scFv format. A primer set specifically designed to amplify equine antibody variable regions, including linker sequences to enable fusion of antibody chains into the scFv format, was successful. Moreover, it enabled the ligation into a phagemid vector for subsequent phage library assembly. Phage display technology proofed suitable as a display method to isolate equine scFv from the newly constructed library, yet modifications to viral purification, and soluble scFv expression protocols should be considered. Most authors in the field of antibody generation and the results of this dissertation confirm the general consensus that there is more than one good solution for designing antibody libaries and each method should be adjusted to carefully (1).

In future, recombinant antibody therapeutics will be of high interest as alternative to eradicate “hardy” pathogens, present an alternative treatment to antibiotics, where antibiotic resistance has played a role and treat cancerous tissue (36). Professionals will aim for standardised and economical production of antibody therpeutics and diagnostics with outstanding pharmacokinetics and sufficient half-life periods within the patient. The results of this dissertation will contribute and lay the groundworks for further investigation of recombinant equine antibodies for application in equine veterinary therapeutic or diagnostic agents and contribute to veterinary and human health

5.2 References (Vancouver)

1. Ponsel D, Neugebauer J, Ladetzki-Baehs K, Tissot K. High affinity, developability and functional size: the holy grail of combinatorial antibody library generation. Molecules. 2011;16(5):3675-700.

2. Wagner B. Immunoglobulins and immunoglobulin genes of the horse. Developmental & Comparative Immunology. 2006;30(1-2):155-64.

3. Mondon P, Dubreuil O, Bouayadi K, Kharrat H. Human antibody libraries: a race to engineer and explore a larger diversity. Front Biosci. 2008;13(1):1117-29.

4. Ahmad ZA, Yeap SK, Ali AM, Ho WY, Alitheen NBM, Hamid M. scFv Antibody: Principles and Clinical Application. Clinical & Developmental Immunology. 2012.

5. Breitling F, Dübel S. Rekombinante Antikörper: Spektrum Akademischer Verlag Heidelberg Berlin; 1997.

6. Nian S, Wu T, Ye Y, Wang X, Xu W, Yuan Q. Development and identification of fully human scFv-Fcs against Staphylococcus aureus. BMC Immunol. 2016;17(1):8-.

7. Li Y, Cockburn W, Kilpatrick JB, Whitelam GC. High affinity ScFvs from a single rabbit immunized with multiple haptens. Biochem Biophys Res Commun. 2000;268(2):398-404.

8. Hawkins RE, Russell SJ, Winter G. Selection of phage antibodies by binding affinity: mimicking affinity maturation. J Mol Biol. 1992;226(3):889-96.

9. Irving RA, Kortt AA, Hudson PJ. Affinity maturation of recombinant antibodies using E. coli mutator cells. Immunotechnology. 1996;2(2):127-43.

10. Eaton BE, Gold L, Zichi DA. Let's get specific: the relationship between specificity and affinity. Chemistry & biology. 1995;2(10):633-8.

11. Virnekäs B, Ge L, Plückthun A, Schneider KC, Wellnhofer Gn, Moroney SE. Trinucleotide phosphoramidites: ideal reagents for the synthesis of mixed oligonucleotides for random mutagenesis. Nucleic Acids Res. 1994;22(25):5600.

12. Prassler J, Steidl S, Urlinger S. In vitro affinity maturation of HuCAL antibodies: complementarity determining region exchange and RapMAT technology. Immunotherapy. 2009;1(4):571-83.

13. Steidl S, Ratsch O, Brocks B, Dürr M, Thomassen-Wolf E. In vitro affinity maturation of human GM-CSF antibodies by targeted CDR-diversification. Molecular immunology. 2008;46(1):135-44.

14. Sun Y, Wang C, Wang Y, Zhang T, Ren L, Hu X, et al. A comprehensive analysis of germline and expressed immunoglobulin repertoire in the horse. Dev Comp Immunol. 2010;34(9):1009-20.

15. Walther S, Rusitzka TV, Diesterbeck US, Czerny C-P. Equine immunoglobulins and organization of immunoglobulin genes. Developmental & Comparative Immunology. 2015;53(2):303-19.

16. Tallmadge RL, McLaughlin K, Secor E, Ruano D, Matychak MB, Flaminio MJB. Expression of essential B cell genes and immunoglobulin isotypes suggests active development and gene recombination during equine gestation. Developmental & Comparative Immunology. 2009;33(9):1027-38.

17. WoÈrn A, PluÈckthun A. Stability engineering of antibody single-chain Fv fragments. J Mol Biol. 2001;305(5):989-1010.

18. Miller KD, Weaver-Feldhaus J, Gray SA, Siegel RW, Feldhaus MJ. Production, purification, and characterization of human scFv antibodies expressed in Saccharomyces cerevisiae, Pichia pastoris, and Escherichia coli. Protein Expres Purif. 2005;42(2):255-67.

19. Cnudde T, Lakhrif Z, Bourgoin J, Boursin F, Horiot C, Henriquet C, et al. Exploration and Modulation of Antibody Fragment Biophysical Properties by Replacing the Framework Region Sequences. Antibodies. 2020;9(2):9.

20. van Rosmalen M, Krom M, Merkx M. Tuning the Flexibility of Glycine-Serine Linkers To Allow Rational Design of Multidomain Proteins. Biochemistry. 2017;56(50):6565-74.

21. Baker DE. Adalimumab: human recombinant immunoglobulin g1 anti-tumor necrosis factor monoclonal antibody. Rev Gastroenterol Disord. 2004;4(4):196-210.

22. Rülker T, Voß L, Thullier P, O' Brien LM, Pelat T, Perkins SD, et al. Isolation and Characterisation of a Human-Like Antibody Fragment (scFv) That Inactivates VEEV In Vitro and In Vivo. Plos One. 2012;7(5):e37242.

23. Hulseweh B, Rulker T, Pelat T, Langermann C, Frenzel A, Schirrmann T, et al. Human-like antibodies neutralizing Western equine encephalitis virus. MAbs. 2014;6(3):718-27.

24. Carlsson F, Trilling M, Perez F, Ohlin M. A dimerized single-chain variable fragment system for the assessment of neutralizing activity of phage display-selected antibody fragments specific for cytomegalovirus. Journal of immunological methods. 2012;376(1-2):69-78.

25. Kügler J. TF, Frenzel A., Hust M. Construction of Human Immune and Naive scFv Libraries. . 2018. In: Phage Display Methods in Molecular Biology [Internet]. New York, NY: Humana Press.

26. Williams MN, Freshour G, Darvill AG, Albersheim P, Hahn MG. An antibody Fab selected from a recombinant phage display library detects deesterified pectic polysaccharide rhamnogalacturonan II in plant cells. Plant Cell. 1996;8(4):673-85.

27. Kirsch MI, Hulseweh B, Nacke C, Rulker T, Schirrmann T, Marschall HJ, et al. Development of human antibody fragments using antibody phage display for the detection and diagnosis of Venezuelan equine encephalitis virus (VEEV). BMC Biotechnol. 2008;8:66.

28. Lakzaei M, Rasaee MJ, Fazaeli AA, Aminian M. A comparison of three strategies for biopanning of phage-scFv library against diphtheria toxin. Journal of Cellular Physiology. 2019;234(6):9486-94.

29. Miersch S, Sidhu S. Synthetic antibodies: concepts, potential and practical considerations. Methods. 2012;57(4):486-98.

30. Lee CM, Iorno N, Sierro F, Christ D. Selection of human antibody fragments by phage display. Nature protocols. 2007;2(11):3001.

31. Dai X, Zhou ZH. Purification of Herpesvirus Virions and Capsids. Bio Protoc. 2014;4(15):e1193.

32. Nixon AE, Sexton DJ, Ladner RC, editors. Drugs derived from phage display: from candidate identification to clinical practice. MAbs; 2014: Taylor & Francis.

33. Michels GM, Walsh KF, Kryda KA, Mahabir SP, Walters RR, Hoevers JD, et al. A blinded, randomized, placebo-controlled trial of the safety of lokivetmab (ZTS-00103289), a caninized anti-canine IL-31 monoclonal antibody in client-owned dogs with atopic dermatitis. Veterinary Dermatology. 2016;27(6):505-e136.

34. Bustamante-Córdova L, Melgoza-González EA, Hernández J. Recombinant antibodies in veterinary medicine: an update. Frontiers in Veterinary Science. 2018;5:175.

35. Stokol T, Soboll Hussey G. Editorial: Current Research in Equid Herpesvirus Type-1 (EHV-1). Frontiers in Veterinary Science. 2020;6(492).

36. Kaufmann SH. Emil von Behring: translational medicine at the dawn of immunology. Nature Reviews Immunology. 2017;17(6):341.

6 ZUSAMMENFASSUNG & SUMMARY

Zusammenfassung

Pferde sind einer Vielzahl von Krankheitserregern und Krankheiten ausgesetzt. Das Equine Herpesvirus Typ 1 (EHV-1) ist einer der am häufigsten vorkommenden viralen Krankheitserreger, die Pferde infizieren. Rund 60 % der Pferdepopulation weltweit sind mit diesem Virus infiziert. Die Symptome einer EHV-1-Infektion reichen von einer leichten Atemwegserkrankung bis zu einer möglicherweise tödlich verlaufenden neonatalen Pneumonie, einem Trächtigkeitsabbruch während des letzten Trimesters oder der schweren neurologischen Erkrankung der Equine Myeloencephalopathie (EHM). Es ist nicht nur ein hoch ansteckendes Virus, vor allem aber stellen Tiere, die nur latent infiziert sind, eine besondere Gefahr der Infektion dar. Virusinfektionen im latenten Status können jederzeit reaktiviert werden und schwere Krankheiten verursachen sowie zur weiteren Übertragung unter Pferden beitragen. Gegenwärtige Präventionsmethoden bestehen aus Impfregimen, Anpassungen der Pferdehaltung oder symptomatischer Behandlung. Impfregime zielen darauf ab, Virämie zu beseitigen und nachfolgende Krankheiten zu verhindern. Es hat sich jedoch gezeigt, dass diese Methoden die Dauer der Virämie nicht verändert oder vor latenten Infektionen schützt. Therapeutika gegen EHV-1-Infektionen wurden zu diesem Zeitpunkt nur *in vitro* getestet, und ihre Wirksamkeit hat sich als noch nicht bahnbrechend erwiesen. Man ist sich einig, dass der Schutz gegen EHV-1, pferdespezifische, neutralisierende Antikörper erfordert. Ein moderner Ansatz zur Lösung dieses Problems ist die Erzeugung rekombinanter Antikörper in Antikörperbibliotheken. Rekombinante Antikörper werden synthetisch hergestellt und gemäß einem bestimmten Wirt oder Antigen manipuliert. Infolgedessen können diese Antikörper als vorbeugende oder therapeutische Arzneimittel verwendet werden. Meine Dissertation zielt darauf ab, rekombinante equine Antikörper im single-chain-Fragment-variable Format (scFv) in einer Antikörperbibliothek zu konstruieren, da bisher keine derartigen rekombinanten

Antikörpertherapeutika für Pferde existieren. Als Qualitätsnachweis der Bibliothek, wird diese Bibliothek auf scFv Antikörper gegen EHV-1 untersucht.

Equine scFv-Antikörper wurden unter Verwendung eines Primer-Sets konstruiert, das zur Amplifikation der gesamten genomischen Immunglobulin-Diversität von Equiden entwickelt wurde. Die Primer und die PCR-Bedingungen wurden vor der scFv-Konstruktion getestet und optimiert. Zur Primer-Etablierung und scFv-Konstruktion wurden PBMCs eines Wallachs isoliert, der zuvor gegen EHV-1 immunisiert wurde. Die RNA wurde extrahiert und in cDNA transkribiert. Variable Antikörperregionen von schweren und leichten Antikörperketten wurden separat amplifiziert und während einer SOE-PCR durch eine Linkersequenz verbunden, wodurch scFv-Sequenzen erzeugt wurden. Zunächst wurde durch Klonieren und Sequenzieren bestätigt, dass alle PCR-Produkte die gewünschten Sequenzen enthielten und dann mit der Bibliotheksassemblierung fortgefahren. scFv-Sequenzen wurden in einen Phagemidvektor ligiert und in phagenkompetente *E.coli*-Zellen transformiert. Dieser Prozess der Ligation und Transformation wurde mehrfach wiederholt, bis eine Bibliotheksdiversität von $4{,}8 \times 10^8$ einzelner Klone erreicht wurde.

Die Bibliothek wurde mittels der Phagendisplay-Methode auf einen anti-EHV-1-scFv-Antikörper untersucht. Während der Phagendisplay-Methode werden scFv-Sequenzen der Bibliothek in Bakteriophagen eingebaut. Diese Bakteriophagen exprimieren die scFv-Sequenzen sowohl im Genotyp als auch im Phänotyp. Alle Bakteriophagen werden daraufhin dem Antigen (EHV-1) ausgesetzt, und diejenigen Bakteriophagen, die phänotypisch scFv-Sequenzen exprimieren, die an EHV-1 binden, werden eluiert. Die Aussetzung der Phagen gegenüber dem Antigen erfolgt während der sogenannten Biopanning-Runden. Während jeder Biopanning-Runde werden die Bedingungen, z.B. die Konzentration des Antigens oder die Waschschritte stringenter eingestellt, um schwach bindende scFv-Sequenzen von stark bindenden scFv-Sequenzen zu trennen. Mit Hilfe von ELISA werden stark bindende scFv-Sequenzen ermittelt, wenn das OD-Signal des scFv fünfmal höher ist als das OD-Signal der

Negativkontrolle. Zur Vorbereitung der Phagendisplay-Technik wurde das Antigen EHV-1 durch Saccharosegradientenzentrifugation aufgereinigt. Die erzeugte Antikörperbibliothek wurde in M13KO7-Bakteriophagen verpackt und während drei Biopanning-Runden, EHV-1 ausgesetzt. Nach der dritten Runde wurde ein spezifischer scFv-Antikörper isoliert. Der scFv-3H2 zeigt eine starke Bindung und Sequenzierung ergab, dass es ein vielversprechender scFv-Antikörperkandidat ist, der weiter charakterisiert wird, um das pharmakokinetische Potenzial für zukünftige therapeutische Anwendungen zu beweisen.

Meine Dissertation trägt zur Entwicklung neuartiger, alternativer Lösungen zur Vorbeugung und Behandlung von Krankheitserregern und Krankheiten bei Pferden bei. Die scFv-Antikörperbibliothek für Pferde bietet einen Ausgangspunkt für die breitere Etablierung von rekombinanten Antikörpern und Phagendisplay in der Entwicklung von Pferde- und Tierarzneimitteln zur Verbesserung der Tiergesundheit und des Tierschutzes.

Summary

Horses are exposed to a large variety of pathogens and diseases. Equine herpesvirus Type 1 (EHV-1) is one of the most prevalent viral pathogens infecting horses. Approximately 60 % of the horse population worldwide are infected with this virus. EHV-1 infection symptoms range from mild respiratory disease to potentially fatal neonatal pneumonia, abortion during late gestation or the severe neurological disease equine myeloencephalopathy (EHM). Not only is it a highly contagious virus but the most critical form of transmission are the silent shedders once the virus has undergone latency. Viral infection can be reactivated at any time and cause severe diseases and contribute to transmission among horses. Current prevention methods consist of vaccination regimes, horse husbandry adjustments or symptomatic treatment. Vaccination programs aim to eliminate viremia and prevent subsequent diseases. However, these methods have not proven to alter duration of viremia (1, 2) nor protect against latent infection. Therapeutics against EHV-1 infection have only been trialled *in vitro* at this point and their efficacy has not been proven to be ground breaking. It is suggested that protection and treatment will require equine specific neutralizing antibodies. A modern approach to resolve this issue, is the generation of recombinant antibodies in antibody libraries. Recombinant antibodies are produced synthetically and manipulated according to a specific host or antigen. As a result, these antibodies can be used as preventative or therapeutic drugs. My dissertation aims to construct recombinant equine antibodies in the single-chain-Fragment-variable format (scFv) in an antibody library since to date, no such recombinant antibody therapeutics exist for horses. As proof-of-principle, this library was screened against EHV-1.

Equine scFv antibodies were constructed by using a primer set designed to amplify the whole equine genomic immunoglobulin diversity. Primers were tested and PCR conditions optimised before scFv construction. For primer establishment and scFv construction, PBMCs were isolated from a gelding that has been immunized against EHV-1. RNA was extracted and

reversely transcribed into cDNA. Variable antibody regions of antibody heavy and light chains were amplified separately and connected during SOE PCR by a linker sequence, thereby generating scFv sequences. In preparation of library assembly, all PCR products were confirmed to contain desired sequences by cloning and sequencing. ScFv sequences were ligated into a phagemid vector and transformed into phage-competent *E. coli* cells. This process of ligation and transformation was repeated until a library diversity of 4.8×10^8 individual clones was reached.

The library was screened for an anti-EHV-1 scFv antibody by phage display technique. During the phage display technique scFv sequences of the library are incorporated into bacteriophages. These bacteriophages will express the scFv sequences on their genotype as well as on their phenotype. All bacteriophages are exposed to the antigen of interest (EHV-1) and those bacteriophages that are phenotypically expressing scFv sequences binding to EHV-1 are eluted. The exposure to antigen occurs during biopanning rounds. Throughout each panning rounds, conditions such as concentration of antigen or washing steps, are adjusted for more stringency, aiming to separate weak binding scFv sequences from strong binding scFv sequences. ELISA will determine strong binders, when OD signals are five times higher than OD signals of the negative control. Prior to the screening, the antigen of interest, EHV-1 was purified by sucrose gradient centrifugation. The generated antibody library was packaged into M13KO7 bacteriophages and exposed to EHV-1 during three rounds of biopanning. After the third round, a specific scFv antibody was isolated. The scFv-3H2 shows strong binding ability and sequencing revealed it to be a promising scFv antibody candidate, which will be characterised further to prove pharmacokinetic potential for future therapeutic application.

My thesis contributes to the development of novel, alternative solutions in the prevention and treatment of equine pathogens and diseases. The equine scFv antibody library presents a starting point for the broader establishment of recombinant, equine antibodies and phage

display method in equine and veterinary drug development improving animal health and welfare.

7 APPENDIX

7.1 Authors contributions

7.1.1 Manuscript I: PCR primer for the construction of an equine immunoglobulin library in the single-chain fragment variable (scFv) format

Franziska I. Pilger, Henrike P. Ahsendorf, Sascha Knauf, Anja Schwarz, Stefanie Walther, Claus-Peter Czerny, Ulrike S. Diesterbeck, Kim Fechner

Task	Authors
Conceptualization	**FP**, CPC
Supervision	CPC, KF
Investigation and Methodology	**FP**, UD, HA, AS, SW
Evaluation and preparation of results	**FP**
Writing Original Draft	**FP**, KF
Review and Editing	All authors

7.1.2 Manuscript II: Isolating a high-affinity, equine, single-chain antibody fragment (scFv) from an equine phage display library neutralizing Equine Herpesvirus-1

Franziska I. Pilger, Dorothee Arthaus, Caroline Bierschenk, Sebastian Schimkowiak

Task	Authors
Conceptualization	**FP**, CPC, KF
Supervision	KF
Investigation and Methodology	**FP**, DA, CB, SS
Evaluation and preparation of results	**FP**
Writing Original Draft	**FP**
Review and Editing	All authors

7.2 Supplementary files

7.2.1 Manuscript I: PCR primer for the construction of an equine immunoglobulin library in the single-chain fragment variable (scFv) format

Table S1: Sequences used for primer design based on published equine immunoglobulin isotype IgG, subtypes IgG1-7, sequences and database entries. Using BLAST software, the database was screened for equine heavy and light chain sequences of the variable, joining, and first constant immunoglobulin gene segments.

Primer set	Gene segment region	Accession number	Reference
PCR1Hpool_FOR	Immunoglobulin heavy chain V-D-J	HM176092	Sun et al. (2010)
PCR2Hpool_REV		HM175940	Sun et al. (2010)
		HM175963	Sun et al. (2010)
		HM176085	Sun et al. (2010)
		HM175938	Sun et al. (2010)
		HM176014	Sun et al. (2010)
		HM176050	Sun et al. (2010)
		HM175992	Sun et al. (2010)
		L81155	Schrenzel et al. (1997)
		HQ403634	Tallmadge et al. (2003)
		HM175920	Sun et al. (2010)
		HM176023	Sun et al. (2010)
		VH4	Sun et al. (2010)
		VH5	Sun et al. (2010)
		HM175979	Sun et al. (2010)
		HM176009	Sun et al. (2010)
		HM175900	Sun et al. (2010)
		HM176049	Sun et al. (2010)
		HM175919	Sun et al. (2010)

		HM175894	Sun et al. (2010)
		HM176021	Sun et al. (2010)
		HM176027	Sun et al. (2010)
		HM175968	Sun et al. (2010)
		HM175991	Sun et al. (2010)
		pVH18	Sun et al. (2010)
		VH10	Sun et al. (2010)
		pVH20	Sun et al. (2010)
		VH11	Sun et al. (2010)
		HQ403618	Tallmadge et al. (2003)
		VH3	Sun et al. (2010)
		VH9	Sun et al. (2010)
		VH13	Sun et al. (2010)
		HM175915	Sun et al. (2010)
		VH_ORF2	Sun et al. (2010)
		pVH34	Sun et al. (2010)
		pVH26	Sun et al. (2010)
		pVH10	Sun et al. (2010)
		VH_ORF1	Sun et al. (2010)
		pVH27	Sun et al. (2010)
		pVH1	Sun et al. (2010)
		VH_ORF3	Sun et al. (2010)
		VH6	Sun et al. (2010)
		pVH19	Sun et al. (2010)
		pVH6	Sun et al. (2010)
		VH_ORF6	Sun et al. (2010)
		pVH24	Sun et al. (2010)
		pVH31	Sun et al. (2010)

		pVH30	Sun et al. (2010)
		pVH3	Sun et al. (2010)
		pVH32	Sun et al. (2010)
		VH8	Sun et al. (2010)
		VH7	Sun et al. (2010)
		JH1	Sun et al. (2010)
		JH2	Sun et al. (2010)
		HM175973	Sun et al. (2010)
		HM175987	Sun et al. (2010)
		HM175895	Sun et al. (2010)
		HM175889	Sun et al. (2010)
		HM176039	Sun et al. (2010)
		HM175984	Sun et al. (2010)
		HM176051	Sun et al. (2010)
		HM176052	Sun et al. (2010)
		HM175991	Sun et al. (2010)
		HM175969	Sun et al. (2010)
		HM175959	Sun et al. (2010)
		HM175957	Sun et al. (2010)
		HM176042	Sun et al. (2010)
		DQ125416	Almagro et al. (2006)
		L81157	Schrenzel et al. (1997)
		HM176028	Sun et al. (2010)
		HM176004	Sun et al. (2010)
		HM176011	Sun et al. (2010)
		HM175921	Sun et al. (2010)

Primer set	Gene segment region	Accession number	Reference
PCR1Hpool_REV	Immunoglobulin heavy chain constant region	GSP2	txid9796
Primer set	Gene segment region	Accession number	Reference
PCR1Lpool_FOR	Immunoglobulin lambda light chain V-J	HM176279	Sun et al. (2010)
PCR2Lpool_FOR		HM176280	Sun et al. (2010)
PCR2Lpool_REV		HM176281	Sun et al. (2010)
		HM176282	Sun et al. (2010)
		HM176283	Sun et al. (2010)
		HM176284	Sun et al. (2010)
		HM176285	Sun et al. (2010)
		HM176286	Sun et al. (2010)
		HM176287	Sun et al. (2010)
		HM176288	Sun et al. (2010)
		HM176289	Sun et al. (2010)
		HM176290	Sun et al. (2010)
		HM176291	Sun et al. (2010)
		HM176292	Sun et al. (2010)
		HM176293	Sun et al. (2010)
		HM176294	Sun et al. (2010)
		HM176295	Sun et al. (2010)
		HM176296	Sun et al. (2010)
		HM176297	Sun et al. (2010)
		HM176298	Sun et al. (2010)
		HM176299	Sun et al. (2010)
		HM176300	Sun et al. (2010)
		HM176301	Sun et al. (2010)

		HM176302	Sun et al. (2010)
		HM176303	Sun et al. (2010)
		HM176304	Sun et al. (2010)
		HM176305	Sun et al. (2010)
		HM176306	Sun et al. (2010)
		HM176307	Sun et al. (2010)
		HM176308	Sun et al. (2010)
		HM176309	Sun et al. (2010)
		HM176310	Sun et al. (2010)
		HM176311	Sun et al. (2010)
		HM176312	Sun et al. (2010)
		HM176313	Sun et al. (2010)
		HM176314	Sun et al. (2010)
		HM176315	Sun et al. (2010)
		HM176316	Sun et al. (2010)
		HM176317	Sun et al. (2010)
		HM176318	Sun et al. (2010)
		HM176319	Sun et al. (2010)
		HM176320	Sun et al. (2010)
		HM176321	Sun et al. (2010)
		HM176322	Sun et al. (2010)
		HM176323	Sun et al. (2010)
		HM176324	Sun et al. (2010)
		HM176325	Sun et al. (2010)
		HM176326	Sun et al. (2010)
		HM176327	Sun et al. (2010)
		HM176328	Sun et al. (2010)
		HM176329	Sun et al. (2010)

		HM176330	Sun et al. (2010)
		HM176331	Sun et al. (2010)
		HM176332	Sun et al. (2010)
		HM176333	Sun et al. (2010)
		HM176334	Sun et al. (2010)
		HM176335	Sun et al. (2010)
		HM176336	Sun et al. (2010)
		HM176337	Sun et al. (2010)
		HM176338	Sun et al. (2010)
		HM176339	Sun et al. (2010)
		HM176340	Sun et al. (2010)
		HM176341	Sun et al. (2010)
		HM176342	Sun et al. (2010)
		HM176343	Sun et al. (2010)
		HM176344	Sun et al. (2010)
		HM176345	Sun et al. (2010)
		HM176346	Sun et al. (2010)
		HM176347	Sun et al. (2010)
		HM176348	Sun et al. (2010)
		HM176349	Sun et al. (2010)
		HM176350	Sun et al. (2010)
		HM176351	Sun et al. (2010)
		HM176352	Sun et al. (2010)
		HM176353	Sun et al. (2010)
		HM176354	Sun et al. (2010)
		HM176355	Sun et al. (2010)
		HM176356	Sun et al. (2010)
		HM176357	Sun et al. (2010)

		HM176358	Sun et al. (2010)
		HM176359	Sun et al. (2010)
		HM176360	Sun et al. (2010)
		HM176361	Sun et al. (2010)
		HM176362	Sun et al. (2010)
		HM176363	Sun et al. (2010)
		HM176364	Sun et al. (2010)
		HM176365	Sun et al. (2010)
		HM176366	Sun et al. (2010)
		HM176367	Sun et al. (2010)
		HM176368	Sun et al. (2010)
		HM176369	Sun et al. (2010)
		HM176370	Sun et al. (2010)
		HM176371	Sun et al. (2010)
		HM176372	Sun et al. (2010)
		HM176373	Sun et al. (2010)
		HM176374	Sun et al. (2010)
		HM176375	Sun et al. (2010)
		HM176376	Sun et al. (2010)
		HM176377	Sun et al. (2010)
		HM176378	Sun et al. (2010)
		HM176379	Sun et al. (2010)
		HM176380	Sun et al. (2010)
		HM176381	Sun et al. (2010)
		HM176382	Sun et al. (2010)
		HM176383	Sun et al. (2010)
		HM176384	Sun et al. (2010)
		HM176385	Sun et al. (2010)

		HM176386	Sun et al. (2010)
		HM176387	Sun et al. (2010)
		HM176388	Sun et al. (2010)
		HM176389	Sun et al. (2010)
		HM176390	Sun et al. (2010)
		HM176391	Sun et al. (2010)
		HM176392	Sun et al. (2010)
		HM176393	Sun et al. (2010)
		HM176394	Sun et al. (2010)
		HM176395	Sun et al. (2010)
		HM176396	Sun et al. (2010)
		HM176397	Sun et al. (2010)
		HM176398	Sun et al. (2010)
		HM176399	Sun et al. (2010)
		HM176400	Sun et al. (2010)
		HM176401	Sun et al. (2010)
		HM176402	Sun et al. (2010)
		HM176403	Sun et al. (2010)
		HM176404	Sun et al. (2010)
		HM176405	Sun et al. (2010)
		HM176406	Sun et al. (2010)
		HM176407	Sun et al. (2010)
		HM176408	Sun et al. (2010)
		HM176409	Sun et al. (2010)
		HM176410	Sun et al. (2010)
		HM176411	Sun et al. (2010)
		HM176412	Sun et al. (2010)
		HM176413	Sun et al. (2010)

		HM176414	Sun et al. (2010)
		HM176415	Sun et al. (2010)
		HM176416	Sun et al. (2010)
		HM176418	Sun et al. (2010)
		HM176419	Sun et al. (2010)
		HM176420	Sun et al. (2010)
		HM176421	Sun et al. (2010)
		HM176422	Sun et al. (2010)
		HM176424	Sun et al. (2010)
		HM176425	Sun et al. (2010)
		HM176426	Sun et al. (2010)
		HM176427	Sun et al. (2010)
		HM176428	Sun et al. (2010)
		HM176429	Sun et al. (2010)
		HM176430	Sun et al. (2010)
		HM176431	Sun et al. (2010)
		HM176432	Sun et al. (2010)
		HM176433	Sun et al. (2010)
		HM176434	Sun et al. (2010)
		HM176435	Sun et al. (2010)
		HM176436	Sun et al. (2010)
		HM176437	Sun et al. (2010)
		HM176438	Sun et al. (2010)
		HM176439	Sun et al. (2010)
		HM176440	Sun et al. (2010)
		HM176441	Sun et al. (2010)
		HM176442	Sun et al. (2010)
		HM176443	Sun et al. (2010)

		HM176444	Sun et al. (2010)
		HM176445	Sun et al. (2010)
		HM176446	Sun et al. (2010)
		HM176447	Sun et al. (2010)
		HM176448	Sun et al. (2010)
		HM176449	Sun et al. (2010)
		HM176450	Sun et al. (2010)
		HM176451	Sun et al. (2010)
		HM176452	Sun et al. (2010)
		HM176453	Sun et al. (2010)
		HM176454	Sun et al. (2010)
		HM176455	Sun et al. (2010)
		HM176456	Sun et al. (2010)
		HM176457	Sun et al. (2010)
		HM176458	Sun et al. (2010)
		HM176459	Sun et al. (2010)
		HM176460	Sun et al. (2010)
		HM176461	Sun et al. (2010)
		HM176462	Sun et al. (2010)
		HM176463	Sun et al. (2010)
		HM176464	Sun et al. (2010)
		HM176465	Sun et al. (2010)
		HM176466	Sun et al. (2010)
		HM176467	Sun et al. (2010)
		HM176468	Sun et al. (2010)
		HM176469	Sun et al. (2010)
		HM176470	Sun et al. (2010)
		HM176471	Sun et al. (2010)

		HM176472	Sun et al. (2010)
		HM176473	Sun et al. (2010)
		HM176474	Sun et al. (2010)
		HM176475	Sun et al. (2010)
		HM176476	Sun et al. (2010)
		HM176477	Sun et al. (2010)
		HM176478	Sun et al. (2010)
		HM176479	Sun et al. (2010)
		HM176480	Sun et al. (2010)
		HM176481	Sun et al. (2010)
		HM176482	Sun et al. (2010)
		HM176483	Sun et al. (2010)
		L07562	Home et al
Primer set	Gene segment region	Accession number	Reference
PCR1Lpool_REV	Immunoglobulin lambda light chain constant region	L07563	Home, Ford, Gibson (1992)
		L07564	Home, Ford, Gibson (1992)
		L07565	Home, Ford, Gibson (1992)
		L07566	Home, Ford, Gibson (1992)
		L07567	Home, Ford, Gibson (1992)
		L07568	Home, Ford, Gibson (1992)
		L07569	Home, Ford, Gibson (1992)
		L07570	Home, Ford, Gibson (1992)
		L07571	Home, Ford, Gibson (1992)
		XM_001488388	txid9796
		XM_001492561	txid9796
		XM_001494029	txid9796

		XM_001502913	txid9796
		XM_001915395	txid9796
		XM_001915405	txid9796
		XM_003365445	txid9796
		XM_003365475	txid9796
		XM_003365486	txid9796
		XM_003365656	txid9796
		XM_003365694	txid9796
Primer set	**Gene segment region**	**Accession number**	**Reference**
PCR1Kpool-FOR	Immunoglobulin kappa light chain V-J	HM176254	Sun et al. (2010)
PCR2Kpool_FOR		HM176261	Sun et al. (2010)
PCR2Kpool_REV		HM176246	Sun et al. (2010)
		HM176245	Sun et al. (2010)
		HM176262	Sun et al. (2010)
		VkORF1s	Sun et al. (2010)
		HM176228	Sun et al. (2010)
		X75611	Ford et al. (1994)
		HM175128	Sun et al. (2010)
		HM176266	Sun et al. (2010)
		HM176123	Sun et al. (2010)
		HM176226	Sun et al. (2010)
		HM176229	Sun et al. (2010)
		HM176268	Sun et al. (2010)
		HM176238	Sun et al. (2010)
		HM176165	Sun et al. (2010)
		X75612	Ford et al. (1994)
		HM176196	Sun et al. (2010)

		HM176140	Sun et al. (2010)
		HM176161	Sun et al. (2010)
		HM176233	Sun et al. (2010)
		HM176273	Sun et al. (2010)
		HM176137	Sun et al. (2010)
		Vk2Suns	Sun et al. (2010)
		Vk7Suns	Sun et al. (2010)
		XM_003362935	Wade et al. (2009)
		XM_003362936	Wade et al. (2009)
		XM_003362938	Wade et al. (2009)
		XM_003362952	Wade et al. (2009)
		VkORF2s	Sun et al. (2010)
		HM176207	Sun et al. (2010)
		HM176163	Sun et al. (2010)
		HM176209	Sun et al. (2010)
		HM176213	Sun et al. (2010)
		HM176214	Sun et al. (2010)
		HM176275	Sun et al. (2010)
		HM176208	Sun et al. (2010)
		HM176211	Sun et al. (2010)
		HM176156	Sun et al. (2010)
		HM176216	Sun et al. (2010)
		HM176217	Sun et al. (2010)
		HM176274	Sun et al. (2010)
		Vk13Suns	Sun et al. (2010)
		Vk15Suns	Sun et al. (2010)
		HM176124	Sun et al. (2010)
		HM176184	Sun et al. (2010)

		HM176174	Sun et al. (2010)
		HM176179	Sun et al. (2010)
		HM176097	Sun et al. (2010)
		HM176248	Sun et al. (2010)
		Vk1Suns	Sun et al. (2010)
		Vk19Suns	Sun et al. (2010)
		HM176394	Sun et al. (2010)
		HM176410	Sun et al. (2010)
		HM176200	Sun et al. (2010)
		Vk6Suns	Sun et al. (2010)
		Vk8Suns	Sun et al. (2010)
		HM176152	Sun et al. (2010)
		HM176204	Sun et al. (2010)
		HM176271	Sun et al. (2010)
		HM176149	Sun et al. (2010)
		HM176139	Sun et al. (2010)
		HM176201	Sun et al. (2010)
		HM176205	Sun et al. (2010)
		HM176102	Sun et al. (2010)
		HM176160	Sun et al. (2010)
		HM176190	Sun et al. (2010)
		XM_003362934	Wade et al. (2009)
		HM176155	Sun et al. (2010)
		HM176276	Sun et al. (2010)
		XM_001916665	Wade et al. (2009)
		HM176147	Sun et al. (2010)
		HM176133	Sun et al. (2010)
		HM176232	Sun et al. (2010)

		HM176182	Sun et al. (2010)
		HM176253	Sun et al. (2010)
		HM176259	Sun et al. (2010)
		Jk4	Sun et al. (2010)
		HM176106	Sun et al. (2010)
		HM176179	Sun et al. (2010)
		HM176194	Sun et al. (2010)
		HM176165	Sun et al. (2010)
		HM176251	Sun et al. (2010)
		HM176229	Sun et al. (2010)
		Jk1	Sun et al. (2010)
		HM176126	Sun et al. (2010)
		HM176139	Sun et al. (2010)
		HM176093	Sun et al. (2010)
		HM176101	Sun et al. (2010)
		HM176145	Sun et al. (2010)
		Jk3	Sun et al. (2010)
		HM176103	Sun et al. (2010)
		HM176102	Sun et al. (2010)
		HM176129	Sun et al. (2010)
		HM176125	Sun et al. (2010)
		Jk2	Sun et al. (2010)
		Jk5	Sun et al. (2010)
Primer set	Gene segment region	Accession number	Reference
PCR1Kpool_REV	Immunoglobulin kappa light chain constant region	HM176158	Sun et al. (2010)

		HM176159	Sun et al. (2010)
		HM176160	Sun et al. (2010)
		HM176161	Sun et al. (2010)
		HM176162	Sun et al. (2010)
		HM176163	Sun et al. (2010)
		HM176164	Sun et al. (2010)
		HM176165	Sun et al. (2010)
		HM176166	Sun et al. (2010)
		HM176167	Sun et al. (2010)
		HM176168	Sun et al. (2010)
		HM176169	Sun et al. (2010)
		HM176170	Sun et al. (2010)
		HM176171	Sun et al. (2010)
		HM176175	Sun et al. (2010)
		HM176176	Sun et al. (2010)
		HM176177	Sun et al. (2010)
		HM176178	Sun et al. (2010)
		HM176181	Sun et al. (2010)
		HM176182	Sun et al. (2010)
		HM176183	Sun et al. (2010)
		HM176184	Sun et al. (2010)
		HM176185	Sun et al. (2010)
		HM176187	Sun et al. (2010)
		HM176188	Sun et al. (2010)
		HM176189	Sun et al. (2010)
		HM176190	Sun et al. (2010)
		HM176192	Sun et al. (2010)
		HM176193	Sun et al. (2010)

		HM176194	Sun et al. (2010)
		HM176195	Sun et al. (2010)
		HM176196	Sun et al. (2010)
		HM176197	Sun et al. (2010)
		HM176198	Sun et al. (2010)
		HM176200	Sun et al. (2010)
		HM176201	Sun et al. (2010)
		HM176202	Sun et al. (2010)
		HM176203	Sun et al. (2010)
		HM176204	Sun et al. (2010)
		HM176205	Sun et al. (2010)
		HM176206	Sun et al. (2010)
		HM176207	Sun et al. (2010)
		HM176209	Sun et al. (2010)
		HM176212	Sun et al. (2010)
		HM176214	Sun et al. (2010)
		HM176215	Sun et al. (2010)
		HM176216	Sun et al. (2010)
		HM176217	Sun et al. (2010)
		HM176218	Sun et al. (2010)
		HM176219	Sun et al. (2010)
		HM176220	Sun et al. (2010)
		HM176221	Sun et al. (2010)
		HM176222	Sun et al. (2010)
		HM176223	Sun et al. (2010)
		HM176226	Sun et al. (2010)
		HM176227	Sun et al. (2010)
		HM176228	Sun et al. (2010)

		HM176229	Sun et al. (2010)
		HM176230	Sun et al. (2010)
		HM176231	Sun et al. (2010)
		HM176232	Sun et al. (2010)
		HM176235	Sun et al. (2010)
		HM176236	Sun et al. (2010)
		HM176238	Sun et al. (2010)
		HM176239	Sun et al. (2010)
		HM176240	Sun et al. (2010)
		HM176241	Sun et al. (2010)
		HM176242	Sun et al. (2010)
		HM176244	Sun et al. (2010)
		HM176245	Sun et al. (2010)
		HM176246	Sun et al. (2010)
		HM176247	Sun et al. (2010)
		HM176248	Sun et al. (2010)
		HM176249	Sun et al. (2010)
		HM176250	Sun et al. (2010)
		HM176251	Sun et al. (2010)
		HM176252	Sun et al. (2010)
		HM176253	Sun et al. (2010)
		HM176255	Sun et al. (2010)
		HM176258	Sun et al. (2010)
		HM176259	Sun et al. (2010)
		HM176260	Sun et al. (2010)
		HM176262	Sun et al. (2010)
		HM176263	Sun et al. (2010)
		HM176264	Sun et al. (2010)

		HM176265	Sun et al. (2010)
		HM176266	Sun et al. (2010)
		HM176267	Sun et al. (2010)
		HM176268	Sun et al. (2010)
		HM176269	Sun et al. (2010)
		HM176270	Sun et al. (2010)
		HM176273	Sun et al. (2010)
		HM176274	Sun et al. (2010)
		HM176275	Sun et al. (2010)
		HM176277	Sun et al. (2010)
		HM176278	Sun et al. (2010)
		X75612	Ford, Home, Gibson (1994)
		X75613	Ford, Home, Gibson (1994)
		XM_003362951	txid9796
		XM_003362952	txid9796

Protocol S1: Modifications to restriction enzyme digestion of scFv DNA and vector DNA with *Sfi*I and *Not*I

The restriction digestion master mix contained 1 µl *Not*I (New England Biolabs GmbH, Frankfurt, Germany), 5 µl CutSmart Buffer, (New England Biolabs GmbH, Frankfurt, Germany) 1 µg of template DNA and was adjusted to the final volume of 50 µl with HPLC water. The mixture was incubated at 50°C over night. The following day, 5 µl CutSmart Buffer were added as well as 1 µl of *Sfi*I (New England Biolabs GmbH, Frankfurt, Germany). This mixture was incubated over night at 37°C and inactivated the next day at 65°C for 20 minutes. Calf intestine phosphatase (CIP; New England Biolabs GmbH, Frankfurt, Germany) was used to dephosphorylate the vector DNA to prevent re-ligation by adding 1,5 µl CIP to the digested vector mix and incubating it for 60 minutes at 37°C.

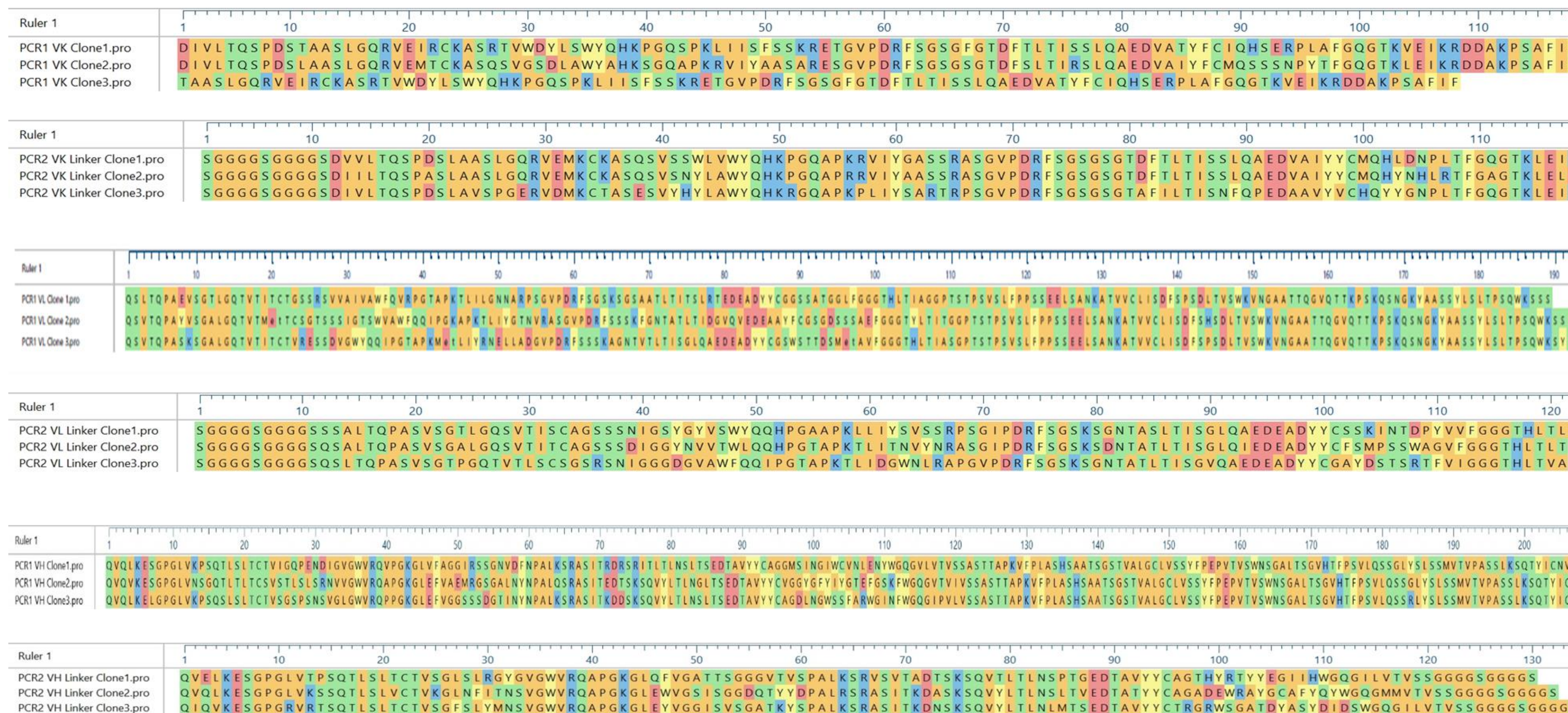

Figure S1: Amino acid sequence alignments of three clones per antibody chain per PCR.

7.2.2 Manuscript II: Isolating a high-affinity, equine, single-chain antibody fragment (scFv) from an equine phage display library neutralizing Equine Herpesvirus-1

Protocol S1: Confirmation of EHV-1 binding capacity after purification via ELISA.

Forty-eight wells of a 96-well ELISA plates (C96 Maxisorp NUNC-Immuno Plate, Thermo Fisher Scientific Inc., Waltham, USA) were coated with 1 µg/ml (Plate 1) and 2 µg/ml (Plate 2) of EHV-1 at 37°C for 4 hours. Remaining 48 wells were not coated. The plates were washed three times with PBS+0.1 % T and blocked with 300 µl blocking solution (2 % SMP, 10 % FCS in PBS+0.1 % T) at 37°C for 2 hours. The positive control equine monoclonal antibody mAb 6B11 (12) was added in duplicates and diluted (log2) from row A to H on both, the coated as well as the un-coated half of the plate. The negative control, consisting of a monoclonal antibody (mAb 5B4/2F2 (13)) was applied equally. Remaining columns received blocking solution. After one hour of incubation at 37°C, all wells received α-mouse-horse-radish peroxidase (HRP; 1:2000 in 2% SMP in PBS+0.1 % T; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and plates were incubated at 37°C for one hour. The plate was washed 10 times with PBS+0.1 % T, followed by the addition of 100 µl TMB substrate (IDEXX GmbH, Ludwigsburg, Germany). After 20 minutes incubation at RT in the dark, 50 µl HCl (IDEXX GmbH, Ludwigsburg, Germany) stop solution was added and OD was measured with the Sunrise microplate reader (Tecan, Maennedorf, Switzerland) at 450 nm.

Result:

ELISA assay confirmed sucrose purified EHV-1 allows binding of the positive control and the course of dilution ran smoothly. It did not react on uncoated wells. The negative control did not bind well to EHV-1 on both plates and neither on coated nor uncoated wells.

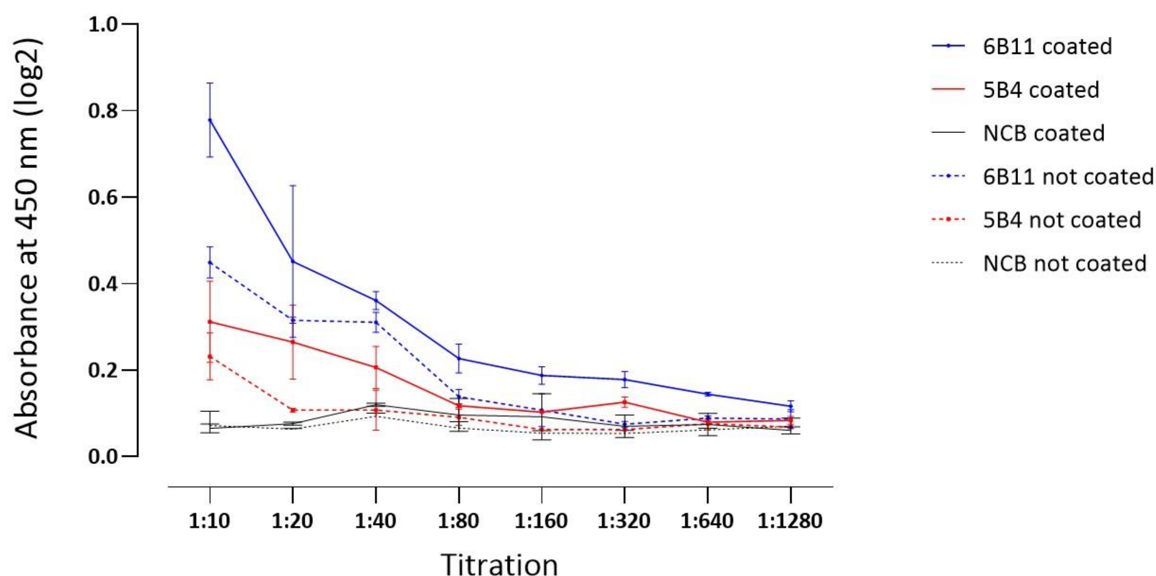

Figure S1: The course of mean absorbance (OD_{450}) values of the dilution series (log2) of the positive and negative control on EHV-1 coated (1 µg/ml EHV-1) as well as on uncoated wells to examine binding kinetics of sucrose purified EHV-1 after first viral purification.

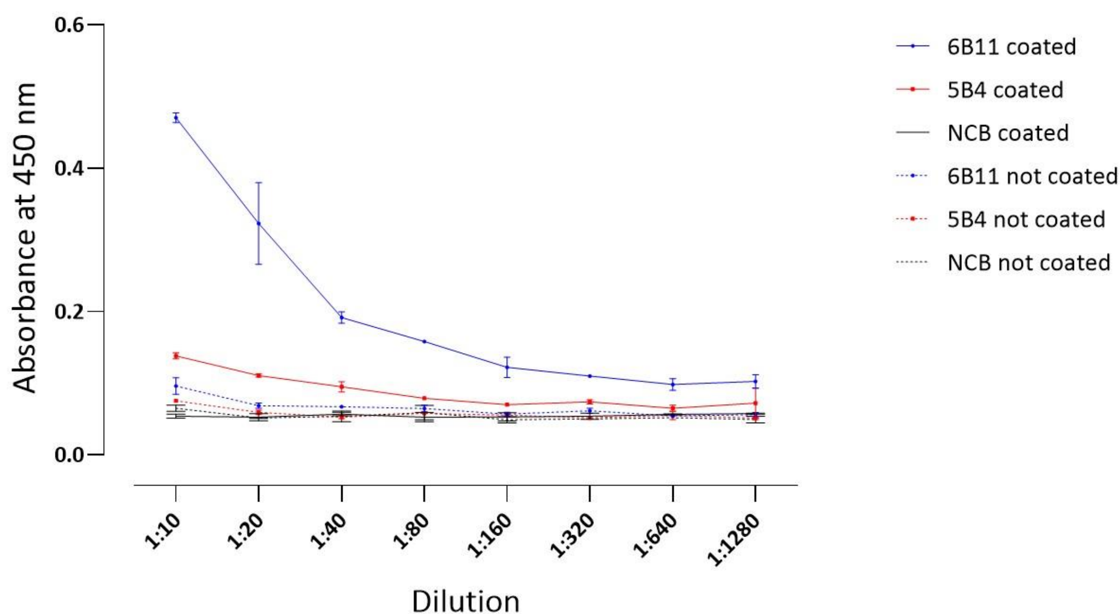

Figure S2: The course of mean absorbance (OD_{450}) values of the dilution series (log2) of the PC and NC on EHV-1 coated (2 µg/ml EHV-1) as well as on uncoated wells to examine binding kinetics of sucrose purified EHV-1 after second viral purification.

Protocol S2: Confirmation of presence of anti-EHV-1 immunoglobulins within plasma of donor horse William, which the equine immunised library is based on.

Forty-eight wells of a 96-well ELISA plates (C96 Maxisorp NUNC-Immuno Plate, Thermo Fisher Scientific Inc., Waltham, USA) were coated with 1 µg/ml EHV-1 at 37°C for 4 hours. The plates were washed three times with PBS+0.1 % T and blocked with 300 µl blocking solution (2 % SMP, 10 % FCS in PBS+0.1 % T) at 37°C for 2 hours. 20µl plasma sample was added to column A1-C1 and diluted (log2) until column 12. Row D was dedicated for the negative control and did not receive any sample. After one hour of incubation at 37°C, all wells received α-horse-radish peroxidase (HRP; 1:2000 in 2% SMP in PBS+0.1 % T; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and incubated at 37°C for one hour. The plate was washed 10 times with PBS+0.1 % T, followed by the addition of 100 µl TMB substrate (IDEXX GmbH, Ludwigsburg, Germany). After 20 minutes incubation at RT in the dark, 50 µl HCl (IDEXX GmbH, Ludwigsburg, Germany) stop solution was added and OD was measured with the Sunrise microplate reader (Tecan, Maennedorf, Switzerland) at 450 nm.

Result:

ELISA confirmed the presence of antibodies against EHV-1. As shown in Figure S3, plasma sample of the immunized gelding yielded high reactivity to EHV-1, while negative control only yielded low OD values as expected.

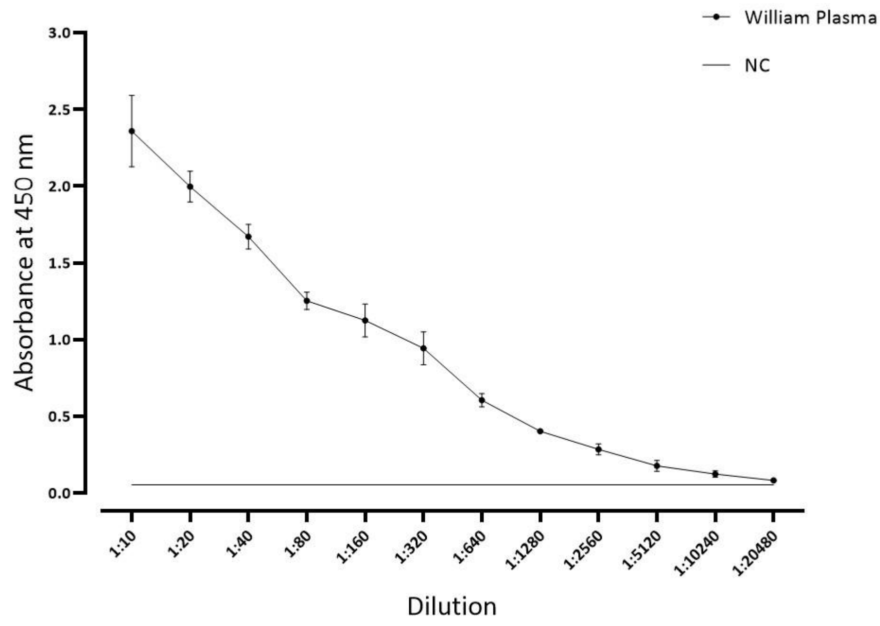

Figure S3: OD values of antibodies against EHV-1 in plasma sample of immunized gelding.

Protocol S3: ELISA protocol to detect binding affinity of randomly selected scFv colonies after biopanning.

Wells of 96-well ELISA plates (C96 Maxisorp NUNC-Immuno Plate, Thermo Fisher Scientific Inc., Waltham, USA) were coated with 2 µg/ml EHV-1 at 37°C for 4 hours. The plates were washed three times with PBS+0.1 % T and blocked with 300 µl blocking solution (2 % SMP, 10 % FCS in PBS+0.1 % T) at 37°C for 2 hours. Wells were emptied and 50µl of blocking solution were added to every well but well A1. It received 100 µl of blocking solution and the positive control consisting of an EHV-1 specific equine monoclonal antibody (Mab 6B11) was diluted (log2) until well D1. Wells E1 to H1 received solely blocking solution to determine the background. The remaining wells were incubated with 50 µl of the induced scFv supernatants. Samples and controls incubated for 2 hours at RT, before washing plates five times with PBS+0.1 % T. The detection antibodies α-mouse horse radish peroxidase (HRP; 1:2000 in 2% SMP in PBS+0.1 % T; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for the positive control and α-E-Tag HRP (1:2000 in 2% SMP in PBS+ 0.1 % T, abcam, Cambridge, UK) were added to wells and incubated at 37°C for one hour. Wells E1 to H1 received blocking solution. Following incubation, wells were washed ten times with PBS+0.1 % T, followed by the addition of 100 µl TMB substrate (IDEXX GmbH, Ludwigsburg, Germany). After 20 minutes incubation at RT in the dark, 50 µl HCl (IDEXX GmbH, Ludwigsburg, Germany) stop solution was added and OD was measured with the Sunrise microplate reader (Tecan, Maennedorf, Switzerland) at 450 nm.

Results:

The following graph (Figure S4) shows OD values of 87 scFv fragments out of 348 clones after panning round three as an example of selection procedures.

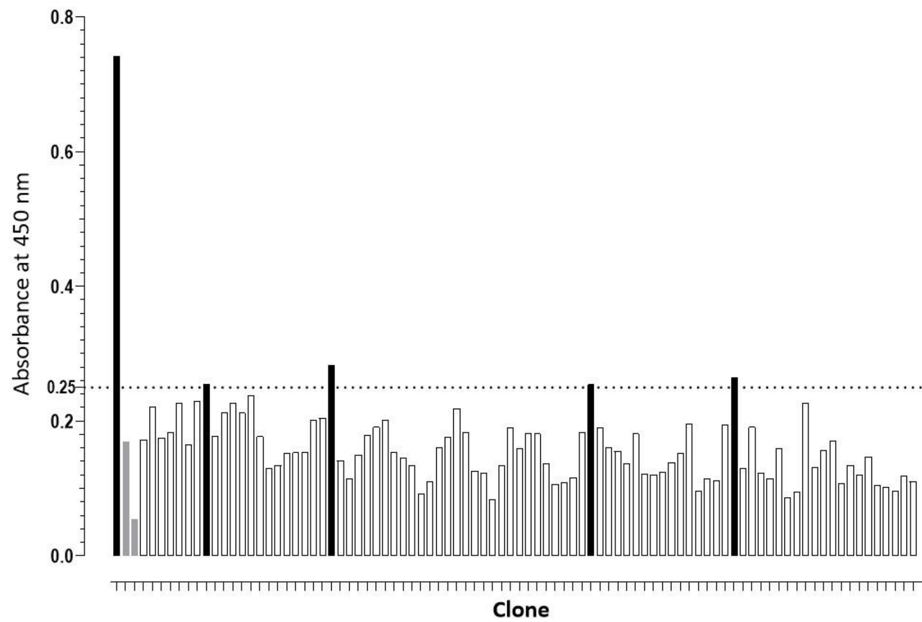

Figure S4: OD values after ELISA assay of individual clones on a 96-well plate after panning round 3 as an example of selection procedures. PC – positive control equine mAb 6B11 (first black column). NCA- negative control A: no template, α-E-Tag-HRP (grey). NCB- negative control B: no template, no detection antibody (grey). Black columns - OD values >0.25. White columns – OD values <0.25.

Protocol S4: Colony PCR of scFvs exhibiting OD values above 0.25 after biopanning.

A 20 µl master mix contained 2 µl of scFv supernatant, 2 µl 10x Standard Reaction Buffer with $MgCl_2$ (Biotools B&M Labs S.A., Madrid, Spain), 10 µM primer R1 (5´-CCA TGA TTA CGC CAA GCT TTG GAG CC-3´), 10 µM primer R2 (3´-CCT TTC TGC TGT TT GAA ATC TAG C-5`), 10 mM dNTPs (Carl Roth GmbH&Co.KG, Karlsruhe, Germany), 1 U/µl DNA polymerase (Biotools B&M Labs S.A., Madrid, Spain) and adjusted to the final volume with HPLC water (Carl Roth GmbH&Co.KG, Karlsruhe, Germany). PCR was run in a T3000 cycler (Biometra, Goettingen, Germany) under the following conditions: 94°C for 10 minutes, 30 cycles at 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, followed by 72°C for 15 minutes. Amplification products were visualized on 1% agarose gels (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Clones of approximately 1000 bp were considered for Sanger sequencing with primers R1 and R2 (Microsynth SeqLab, Goettingen, Germany).

Results:

After panning round three, 11 clones displayed OD signals higher than 0.25 and were further investigated in a Colony PCR. It revealed one clone to harbour a scFv insert of expected size (~800 bp; Figure S5) plus approximately 200 bp accounting for sequencing primers R1/R2. Remaining clones appeared to be 300 to 600 bp approximately (Figure S5).

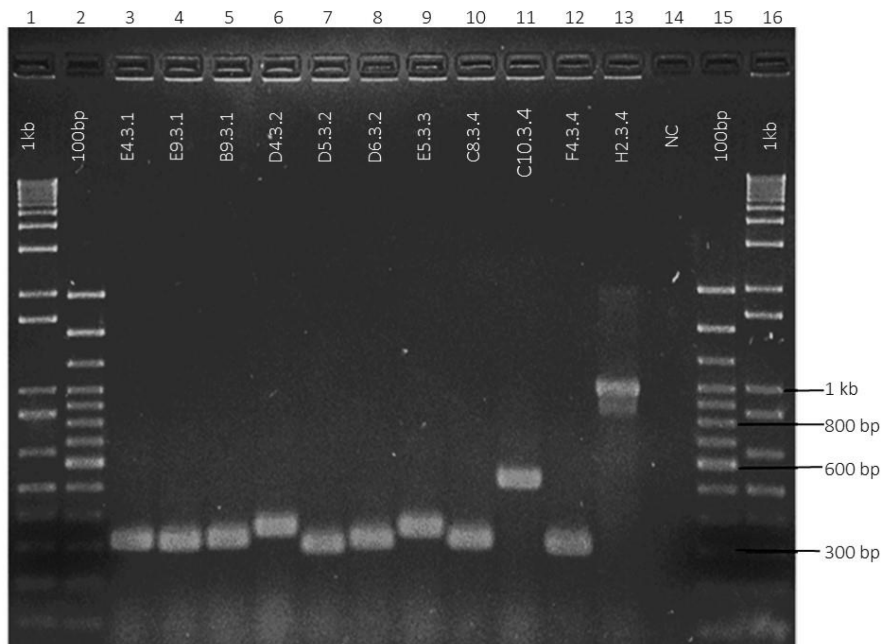

Figure S5: Colony PCR of scFv clones with OD values above 0.25 after panning round 3 on a 1 % agarose gel. Lane 1 & 16: 1 kb ladder. Lane 2 & 15: 100 bp ladder. Lane 14: negative control (HPLC). Lane 3 to 13: individual clones; names correspond with position in 96-well plate, panning round and number of plate.

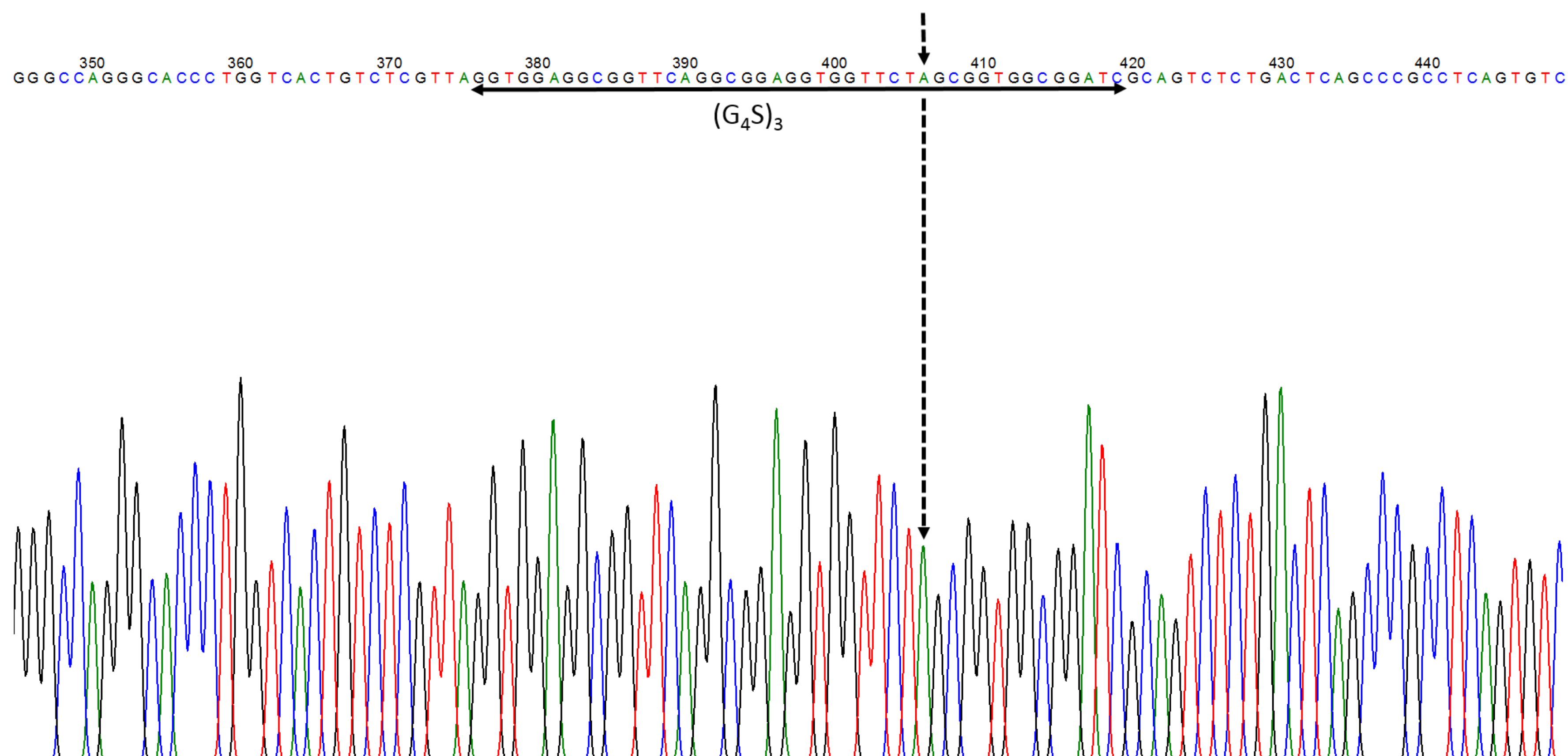

Figure S6: Trace data of Sanger sequencing results showing the linker sequence of scFv-3H2. Black arrows indicating the base exchange resulting in one amino acid substitution.

7.3 Curriculum vitae

7.4 Acknowledgement

I would like to take this last chapter of my thesis as a chance to thank and acknowledge everyone who has supported me along the way.

I would like to thank **Prof. Dr. Czerny** for giving me the opportunity to pursue my doctoral degree at the Division of Animal Hygiene and his supervision. I would like to thank **Prof. Dr. Tetens** for taking on the role as secondary supervisor after **Prof. Dr. Knorr**'s pre-mature death and accepting the role as primary supervisor, after Prof. Dr. Czerny's passed away. Thank you for a trusting and reliable cooperation. I would like to thank **Prof. Dr. König von Borstel** for connecting me to the Division of Animal Hygiene and continuously being part of my supervisory committee. A further member of my supervisory committee is **Dr. Kim Fechner**, who I would like to thank for her scientific and personal support on site. I would like to thank **Sascha Knauf (PhD)** for his feedback and filling in as third referee. I would also like to thank **Dr. Ulrike Diesterbeck** for her feedback on the manuscripts.

Thank you to **everyone at the Division of Animal Hygiene**, who was part of my time at the division over the last few years. I would particularly like to point out **Caroline Bierschenk**, **Sebastian Schimkowiak** and **Martin Seeländer,** who have supported me greatly in the laboratory.

I would also like to mention **Jonas Kissenkötter**, **Henrike Ahsendorf**, **Sören Hansen**, **Nico Dreymann** and **Dorothee Arthaus,** with whom I had the pleasure to establish great collegial relationships with. Thanks to all my friends near and far, who have always encouraged me.

Finally, I would like to thank **my parents, my brother, my sister-in-law and my nieces** for always having my back. Last but not least, I would like to thank my four-legged supporters **Kleo** and **Fiete**.

8 DECLARATION

I, hereby, declare that this Ph.D. dissertation has not been presented to any other examination body either in its present or a similar form.

Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

X

Franziska Pilger

I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorized aid.

X

Franziska Pilger