ECRONICON OPEN ACCESS

EC MICROBIOLOGY
Research Article

# Antibody Discovery Using Japanese Quail: Towards New Anti-Infective Strategies

**Tiago Ochôa-Pires[1], Marguerita Rosa[1,2], João Laranjeira[1,2], Rafael Francisco[1,2], Diana Silva[1] and Ricardo S Vieira-Pires[1]***

[1]*Structural Biotechnology Group, Center for Neuroscience and Cell Biology (CNC), University of Coimbra, Portugal*

[2]*HBT - Saúde e Biotecnologia Lda, Coimbra, Portugal*

***Corresponding Author**: Ricardo S Vieira-Pires, Structural Biotechnology Group, Center for Neuroscience and Cell Biology (CNC), University of Coimbra, Portugal.

**Received:** March 21, 2020; **Published:** May 15, 2020

## Abstract

**Background:** Infections by microbial and viral pathogens can be efficiently tackled with antibody-based strategies. Avian hosts (e.g. chicken, goose, quail) have long attracted the attention of biopharmaceutical industries offering unique alternatives for antibody drug discovery. Indeed, egg laying birds enable generation of highly robust and specific antibodies, providing scalable, low-cost, high-yield production due to deposition of high concentration of IgY antibodies in their eggs.

**Aim:** In this work we developed a new technical and methodological setup to further promote antibody discovery using Japanese quail (*Coturnix japonica*).

**Methods:** We evaluated the performance of a novel modular housing system for egg laying quails, specifically designed for antibody discovery studies. We immunized quail layers with recombinant antigens and monitored the overall behavior and welfare of birds, their egg laying activity as well as egg IgY antibody producing capacity.

**Results:** The novel quail housing system meets all the requirements of the European Directive 2010/63 on animal welfare, including regulatory dimensions for quail experimentation, ease of bird handling, egg collection and sanitization. A standard 90-day immunization protocol was established using this system, and our results demonstrated a robust and reproducible pattern of egg laying activity and antigen-specific antibody production in eggs.

**Conclusion:** This work shows that quails can be efficiently used as hosts for antibody discovery and provides a novel avian housing setup and methodology to further explore this alternative strategy for development of biological antimicrobial and anti-infective drugs.

***Keywords:*** *Coturnix japonica; Animal Welfare; Housing System; Antimicrobials; Virulence Factors; Antibodies; Immunotherapies*

## Introduction

Multidrug resistance (MDR) of microbial and viral pathogens is a major global health threat for which innovative treatments are urgently needed [1]. Currently a large number of surface-exposed proteins, including channels, transporters and enzymes, are known to mediate pathogen survival, proliferation or host adhesion and invasion [2]. Promising therapeutic approaches are focused on the development of antibodies capable of targeting such virulence proteins and modulating the outcome of microbial and viral infections.

Antibodies have been extensively used in the development of advanced therapies to treat complex diseases, such as cancer and chronic inflammatory disorders [3]. Currently, six of the fifteen top selling biopharmaceutical drugs are monoclonal antibodies (mAbs), a class of biological drugs that effectively targets critical cell-surface proteins and modulates their functions [4,5]. Additionally, antibody therapy against bacterial pathogens is also emerging as a promising alternative to traditional drug therapies [6-10]. The main advantages of this approach include: 1) less susceptibility to known mechanisms of resistance; 2) binding specificity to pathogen virulence factors, reducing side-effects common in clinical patients with broad spectrum antibiotic treatment; 3) complementary or adjuvant effect over existing antibiotics, particularly important in treatments of multifactorial diseases; 4) versatility for fast target customization and ability to tackle undruggable protein regions, bypassing long antibiotic discovery periods. Of note, the fast turn-over of monoclonal antibody development out-competes the limited success of small molecule discovery, even if subsequent development into a safe therapeutic can be challenging.

Avian hosts (e.g., chicken, goose, quail, duck) enable generation of highly robust and specific antibodies, providing scalable, low-cost, high-yield production due to deposition of high concentrations of IgY antibodies in eggs [11,12] and thus have been attracting the attention of pharmaceutical industries. Moreover, generation of combinatorial antibody phage-display libraries from avian sources is simplified over mammalian systems given the unique organization of avian immunoglobulin genes, thus favoring screening and engineering strategies to develop target-specific monoclonal antibodies for therapy [13].

Quails have been extensively used as model animals in experimental and scientific studies of behavior and development, pharmacology, toxicology, genetics, growth, nutrition and physiology [14]. Among the different quail species commonly used, Japanese quail (*Coturnix japonica*) seems to be most favored, with a high number of behavioral and physiological data accumulating over the years [15]. Quails present a number of notable advantages when considered as alternative model hosts for immunization experiments and antibody development approaches. They have higher metabolic rates than chickens [16]; a short maturation period reaching adulthood, thus initiating laying activity, in about 6 - 8 weeks compared to chickens that take about 21 weeks to reach maturity. In addition, quails are also very robust and easy to manipulate in laboratory setups and their reduced size has greatly contributed to their adoption as experimental avian models [15].

The present work is part of major effort to setup and consolidate an inhouse Avian Technological Unit (Center for Neuroscience and Cell Biology, University of Coimbra, Portugal) to explore the full potential of avian hosts for antibody discovery. Herein, we present a novel modular housing system for egg-laying quails, specifically designed to enable immunization experiments with quail hosts, meeting the requirements of the European Directive 2010/63 on animal welfare [17]. This system allows the full exploitation of quails as an alternative avian host for polyclonal (pAb) and monoclonal (mAb) development. Finally, we briefly discuss how this novel setup is impacting our research and development of immunotherapies to combat infectious microbial and viral diseases.

## Materials and Methods

### Housing system for egg-laying quails

A novel modular housing system for egg laying quails was conceived and developed by our laboratory for the use of *Coturnix* hosts in experimental conditions according to the European Directive 2010/63 on animal welfare [17]. In particular, the system allows housing of quails during immunization experiments that lead to hyperimmune birds and generation of target specific antibodies; the later can easily be collected from quail eggs. System prototyping was done in collaboration with the company Ternox - Equipamentos Em Aço Inoxidável, Lda (Portugal), that also built the final system version. Further technical details of the system are described below. Of note, an international patent application was filed for this system [18].

### Immunization of birds

Thirty-five to forty days old female *Coturnix japonica* quails were acclimatized in flocks of 2 - 3 birds per cage. Birds were maintained with food and water *ad libitum*, at 20 - 25ºC and subjected to a photoperiod cycle of 16h light/8h dark for control of egg laying. After reaching maturity (6 - 8 weeks) female quails start a regular laying activity of about 1 egg per day per bird. At this stage the immunization protocol was initiated. Birds were routinely immunized with 25 - 100 µg of recombinant proteins (e.g. Yellow fluorescent protein, YFP; *Haloarcula marismortui* Bacteriorhodopsin, HmBRI; Figure 3), administered as emulsions (Complete or Incomplete Freund's Adjuvant, CFA/IFA) by intramuscular injection in the pectoral muscle. Four injections were typically performed at days 0, 15, 30 and 45 (Figure 3). All animal procedures were approved by the institutional Animal Welfare Body (Center for Neuroscience and Cell Biology, ORBEA Órgão Responsáveis pelo Bem-estar Animal).

### Protein antigens

Recombinant versions of Yellow fluorescent protein (YFP) and *Haloarcula marismortui* Bacteriorhodopsin (HmBRI) were obtained by standard expression and purification protocols adapted from [19,20]; highly pure samples (> 95%) were used for immunization procedures.

### Egg collection and processing

Eggs laid per cage were daily collected, allocated in storage boxes and rigorously catalogued. Our standard immunization protocol included continuous monitoring and collection of eggs for a period of 90 days. In a protocol using 3 birds, pools of 12 eggs corresponding to a 4-day laying period, were processed to separate the egg yolk from egg white; the final combined yolk volume was ~36 - 40 mL per each 4-day fraction. About 24 of these fractions were prepared during the full 90-day protocol. This allowed precise monitoring for the antigen specific response by ELISA (Figure 3B and 3C) during the full period.

### IgY antibody purification from egg yolk

Purification of total IgY polyclonal antibody from quail egg yolk was performed by standard methodologies [21] with minor changes. Three purification methods/steps are generally combined to reach high IgY purity grades: the water dilution method was used to obtain a water-soluble protein fraction (WSPF, < 40% purity); PEG precipitation (50 - 80% purity) and SEC - Size-exclusion chromatography (90 - 95% purity) (Figure 3A). An average of 1 mg at ~95% purity is obtained per 1 mL of quail egg yolk by a refined SEC approach.

### Antigen specific titer monitoring

Each of the 24 egg yolk fractions collected along a 90-day protocol, were processed by water dilution method to obtain water soluble protein fraction (WSPF) samples enriched in IgY (~40% purity) and free of lipid content. WSPF samples were analyzed by standard ELISA. Briefly, multi-well plates were coated with 0.5 µg of recombinant antigen and the WSPF tested in serial dilutions; the highest sample dilution with observed signal (titer) was plotted on a logarithmic scale as a function of time (Figure 3A and 3B).

## Results and Discussion

### A novel housing system for egg-laying quails

We have established and optimized a housing setup as well as number of methodologies for the use of quails for immunization experiments and antibody development. Indeed, until now the vast majority of avian IgY antibody development has been performed with chickens [21] and the use of quails for such purpose has been rather limited. During the development of this model, we identified a critical constraint to such an approach, namely the lack of adequate caging systems for housing and manipulation of egg-laying quails, meeting the requirements of Directive 2010/63/EU [17] on animal experimentation and the 3Rs principles [22]. Indeed the IgY Technology field, centered on exploiting birds as alternative antibody sources and egg yolk IgY antibody-based applications, has been historically committed to animal ethical and welfare aspects and was responsible for major advancements and refinements of avian experimental procedures [23]. This was a strong motivation to design and develop a novel housing system for egg laying quails: the goal was to combine optimal ethical and welfare features of quail housing with capabilities for efficient collection and harvest of laid eggs; these remain the ultimate biological samples to retrieve, since they are the source of high-value antibodies.

The housing system developed meets all the European guidelines for quails and is designed to ensure the expression of species-specific behaviors and ethical animal welfare. The system consists of a vertical stainless-steel rack with 4 levels (Figure 2A). Each level can harbor a single wide cage or two individual cages, allocating a total of 4-6 birds per level (Figure 2C and 2D). Transparent polycarbonate panels and doors build up the cage volume and ensure adequate light diffusion, which is critical for photoperiod control and ease of checking birds. The wide floor areas allow adequate environmental enrichment, ease of sanitation, egg collection and animal handling. The stainless-steel wire floor presents ideal grid dimensions to ensure bird comfort and low accumulation of droppings, that fall through the wire floor and are easily removed from metal trays below. These trays are also in an interlocking edge configuration specifically designed to avoid accumulations. The floor is designed to enable the collection of eggs from outside the cage as they roll down (Figure 2D and 2F) and in addition, the floor deck glides forward to enable non-stressful bird restraint (Figure 2B), facilitating staff work. Troughs can be filled from the outside and have a lid designed to avoid food waste and exposure to the exterior. The automatic water system rests attached to the facility wall and is independent of the main rack (Figure 2H), ensuring easy access for cleaning and maintenance of all components. A light emitting diode (LED) system is attached to the wall and ensures rigorous photoperiod control. All cage parts are removable and interchangeable for easy clean-up, sterilization and maintenance.

### Experimental setup and methodology to generate hyperimmune quails

An important point to emphasize is that the immunization of quails results in two main biological outcomes: 1) hyperimmune eggs, containing antigen-specific polyclonal IgY antibodies, that can be directly extracted and used for research, diagnostics or even therapeutic applications and 2) hyperimmune quails, harboring unique antibody repertoires as a result of B-cell clonal expansion and proliferation upon antigen exposure (Figure 1A). Together these ensure a wide potential of quail hosts for both polyclonal and monoclonal antibody development and thus an important complementary approach to new biopharmaceutical drug discovery.

Our current inhouse quail facility setup comprises 3 housing racks (Figure 2A), harboring a total of 24 individual cages (8 per rack), with typically 3 birds allocated per cage. Each cage is dedicated to an individual immunization experiment or protocol, resulting in a hyperimmune flock, producing antigen-specific antibodies of interest (Figure 3). Assuming an experimental turn-over of 2 - 3 months per protocol, our customization capacity with the current setup is of ~96 - 144 new antibodies per year. Notably, even if quail eggs present a lower IgY content (~10 - 30 mg/egg) than chickens, the current housing system ensures a versatile and welfare friendly setup for a competitive customization of avian antibodies. Moreover, combining multiple bird immunization capabilities with a non-invasive antibody

titer monitoring and recovery from laid eggs, makes the whole approach an attractive alternative to the more invasive antibody customization in mammals.

A schematic overview of the standard method used for the generation of quail hyperimmunity and recovery of their egg IgY antibodies, is presented in figure 3. The allocation of hens in social 2 - 3 bird flocks is optimal for the current setup, as group-housed hens present less stress behaviors and stable laying activity. In contrast, allocation and experimental procedures with a single hen per cage impacts the egg laying capacity and overall stress signals. The expected egg laying capacity of a 3-bird flock for example, should be ~3 egg per day (1 per hen); we typically launch immunization procedures when the group of 3 hens reaches an average > 2.5 egg per day. This implies a continuous daily harvest and cataloguing of eggs per cage/protocol (Figure 2G), namely the collection for 1 - 2 weeks prior to the start of immunization (Day 0); these egg samples are critical for analytical procedures, representing the pre-immune eggs with no antigen-specific titer. Daily egg collection is a critical procedure for egg-yolk IgY monitoring and recovery and a major advantage of using avian hosts. Unlike mammals, who are bleed at quite infrequent intervals, with eggs one can actually refine the antigen-specific titer monitoring per day and extract IgY from different time windows along the protocol, even after its conclusion. Nevertheless since our focus is customization of hyperimmune quails and small/medium scale production of antigen specific IgY polyclonal antibodies from eggs, we have established a standard approach that only monitors IgY titers from egg samples every 4-days; this turn out to be very accurate and reproducible, resulting in titer monitor curves that can easily be compared between protocols (Figure 3B). Our immunization schedule follows a standard approach; hens are subjected to 4 immunization events, one every 15 days (Figure 3A). We typically perform a 90-day protocol, meaning egg collection and titer monitoring of egg samples as described above, extend over a 90-day period. This as shown to be optimal for clearly distinguishing an antigen-specific high-titer window, extending from day 40 - 70 or even retaining a high signal after this (Figure 3B and 3C). Longer protocols typically result in a drop on specific antibody titer and require further immunization boosts if long term assays are needed.

Our results demonstrate that the design of the new quail housing system is very effective for maintaining birds and its combination with the immunization methodology described above allows high production of eggs and specific antibodies in a robust, accurate and reproducible manner.

## Anti-infective strategies against pathogenic microorganisms

With the emergence of multidrug resistance, every alternative to develop effective antimicrobial or anti-infective solutions needs to be considered and explored. Antibodies produced by avian hosts are an attractive alternative. Indeed, chicken egg-yolk IgY antibodies produced against tetanus toxin are a historical reference from the work of Felix Klemperer [24], who first showed the protective effect of these antibodies on mice treated with lethal dose of tetanus bouillon culture. Notably, this study is contemporary of the first works on mammalian serum-based therapies against both diphtheria and tetanus [25], by the 1901 Nobel Prize laureate Emil Adolf von Behring. Klemperer's work was undervalued until his legacy revival in the 90s with the consolidation of the IgY Technology field [21,23]. Currently, a vast number of IgY-based research, diagnostic and therapeutic applications has been developed against infections by bacteria, viruses, fungi and parasites [12,26].

A major research focus of our laboratory is on the use of IgY antibodies, both in polyclonal and monoclonal/recombinant formats, as molecular tools for characterization of virulence factors and pathways and ultimately as functional biotherapeutic candidates. The setup and methodology described above for quail antibody development allows a fast turn-over and cost-effective production of egg-yolk IgY polyclonal antibodies for direct biochemical, biophysical and functional assays; these enable the fast generation of solid preliminary results and observations. Moreover, the inherent generation of new quail immunological repertoires supports subsequent molecular cloning of phage-display libraries and ultimate antibody engineering towards more refined IgY monoclonal versions [27]. Notably, so far we have successfully produced quail antibodies against virulence targets of a number of bacteria, namely *Escherichia coli*, *Rickettsia conorii*, *Shigella flexneri*, *Vibrio cholerae*, *Vibrio anguillarum* and *Photobacterium damselae*. Virulence factors that mediate pathogen survival, proliferation or host adhesion and invasion, may include surface-exposed proteins, including channels, transporters and enzymes or adhesion molecules. We obtained robust results for the generation of antibodies targeting both soluble and membrane-embedded target proteins, as demonstrated in figure 3 for two canonical recombinant proteins.

Finally, another importantly advantage to consider when using avian IgY in pathogen studies is the fact that, unlike mammalian IgG, IgY does not bind to Protein A or G, or other known Ig-binging proteins, natively present at the surface of some microbials. This may be critical when exploring antibody targetability of virulence factors in a context/assay where the full microorganism might interfere with the functional outcome under study.

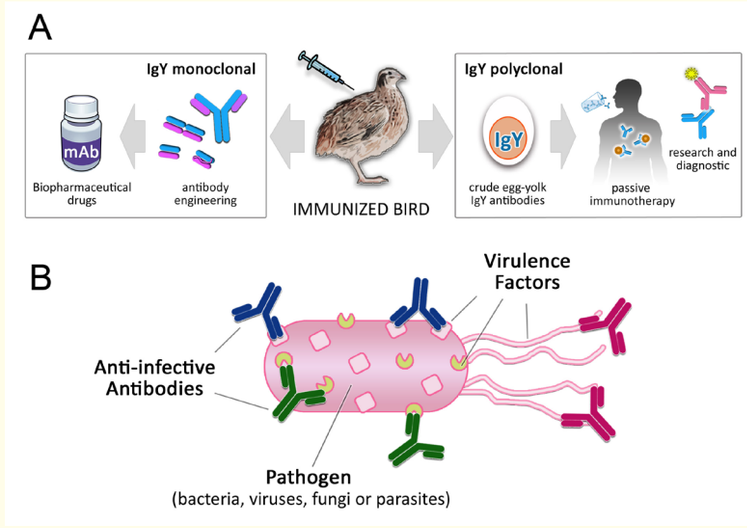


***Figure 1:*** *Potential of avian IgY antibodies in anti-infective strategies. A) Avian IgY antibodies can be obtained from immunized birds, either directly from crude egg-yolk extracts (IgY polyclonal) or from subsequent processing of antibody genetic repertoires (IgY monoclonal). B) These antibodies can be developed with robust specificity to efficiently target critical pathogen virulence factors such, as surface-exposed proteins. Ultimately these preformed antibodies may be used as active ingredients in anti-infective immunotherapies and functional cocktails (e.g. diet supplements).*


***Figure 2:*** *Modular housing system for egg-laying quails. Caging system for housing egg-laying quails in experimental conditions according to the European directives [17]. The system has been developed at the Centre for Neuroscience and Cell Biology (University Coimbra, Portugal) for dedicated studies on avian IgY antibody development and production, namely using Japanese quail (Coturnix japonica) as the selected avian host. A) Overall view of a 4-level housing rack, harboring up to two cages per level; B) Frontal, D) side and E) back views of a rack level, showing details of the wired-floor drawer and removable polycarbonate panels; F) Frontal egg-collecting section for easy monitoring and collection of laid eggs; G) Egg cataloguing box; H) LED light system for photoperiod control and I) water system with automatic refilling and harboring 4 water dispensers per level; both systems are independent from the main rack, resting attached to the facility wall. A supporting video with further technical details on the housing system can be found at [31]. Figure adapted from original [32] with permission.*

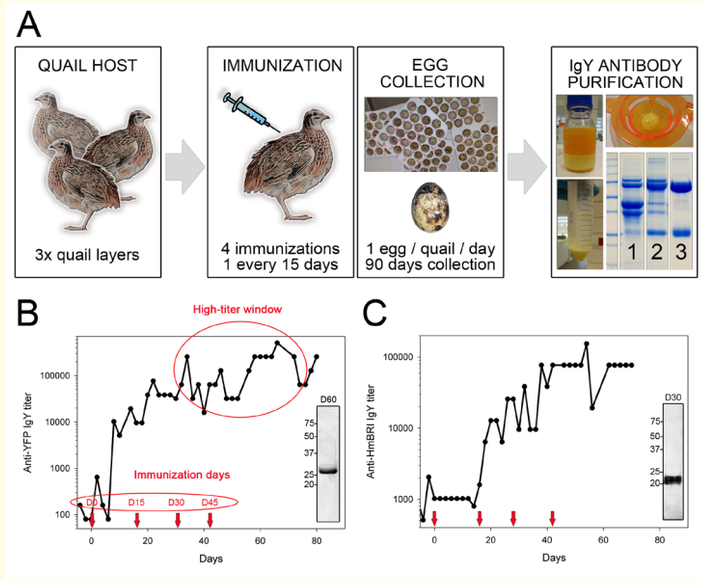


***Figure 3:*** *Overall methodology for generation of quail IgY antibodies. A) 3-bird flocks are immunized, the laid eggs are collected daily and processed to yolk samples. Inlet in the right panel shows an SDS-PAGE with IgY purification grades obtained by different methods 1) Water-dilution, 2) PEG precipitation and 3) Size-exclusion chromatography; for reference, upper and lower protein bands in 3 correspond respectively to IgY heavy chain and light chains. B and C) Analysis of antigen specific titer by ELISA of two independent immunization protocols using as antigen B) a soluble recombinant protein, Yellow fluorescent protein (YFP) and C) a membrane-embedded recombinant protein, Haloarcula marismortui Bacteriorhodopsin (HmBRI), demonstrates the versatile potential of developing quail IgY antibodies against major protein topologies. Titer analysis of 24 yolk samples (black data points), along 90-day protocols is shown (yolk sample dilution, titer vs days). Immunization events at days 0, 15, 30 and 45 are shown by red arrows; a reference high titer window (~days 40 - 70) is depicted with a red circle. Inlets show western blot analysis performed using yolk sample at given timepoint.*

## Conclusion

In this study we presented a novel housing system for egg laying quails, developed according to Directive 2010/63/EU on animal experimentation and the 3Rs principles. This system provides unique technical capabilities that allow the use of egg laying quails for IgY antibody customization. We developed standard immunization and egg processing methodologies for this housing setup and showed that quails presented a highly robust and consistent egg laying and antibody production capacity. The approach enables fast turn-over and cost-effective production of IgY polyclonal antibodies as well as generation of new avian immunological repertoires for subsequent molecular cloning of antibody phage-display libraries. Indeed, the potential of quail immune repertoires for mAb development remains largely underexplored, given the fact the methodology applied for chickens cannot be directly translated (unpublished). In addition, *Coturnix japonica* genome has only been available since 2013 [28] and with limited annotation of immunoglobulin encoding genes. Nevertheless, quails remain a promising avian host for monoclonal antibody development. Moreover, with the advance of powerful genome editing tools prompting research and development of biological drugs (e.g. therapeutic proteins, monoclonal antibodies) with egg-laying birds [29], quails also regain interest as they allow the robust generation of transgenic birds [30]. We believe our housing system can also be critical in this area of research, as well as in others including avian genetics, physiology, behavior and production.

Finally, we are convinced that quails can be used to explore novel antimicrobials and anti-infective strategies against different pathogens. Our setup enables the acceleration of the study of antibody targetability of different pathogen virulence factors, thus contributing to the development of research, diagnostics and ultimately therapeutic solutions to fight infections mediated by bacteria, viruses, fungi or parasites.

Overall, the technical and methodological setup presented will open new possibilities to explore quails as sources of antibodies for research, diagnostics and therapy, providing a complementary approach to new biopharmaceutical drug discovery.

## Funding

This work was supported by Portuguese national funds via FCT (Foundation for Science and Technology) and by ESF (European Social Fund) through POPH (Human Potential Operational Programme) under grant IF/00123/2013 and ERDF (European Regional Development Fund) through COMPETE 2020 (Operational Programme for Competitiveness and Internationalization) under the projects BYDRUG (POCI-01-0145-FEDER-030550) and CNC.IBILI (refs. POCI-01-0145-FEDER-007440 and UID/NEU/04539/2019). M. Rosa was supported by Santander Universidades Scholarships Program. The housing system was funded through the program QREN - Programa Operacional Regional do Centro 2007-2013 with the support of Mais Centro and the European Union through the project "CNC Biotech - Investigação em Biotecnologia e Capacitação do Sector Empresarial".

## Conflicts of Interest

The Center for Neuroscience and Cell Biology (University Coimbra, Portugal) is the sole applicant of the International PCT application "Modular Bird Cage" (PCT/IB2017/054766), for the housing system herein presented. HBT - Saúde e Biotecnologia Lda (Coimbra, Portugal) is a private company developing protein and antibody products for research and diagnostic purposes. R. S. Vieira-Pires declares a minor research funding (< 2%) by HBT. M. Rosa, J. Laranjeira and R. Francisco developed commercial products, under internship programs within HBT.

## Acknowledgements

The authors would like to acknowledge Patricia M. Morgan for constructive feedback on this manuscript.

## Bibliography

1. LB Rice. "Emerging issues in the management of infections caused by multidrug-resistant gram-negative bacteria". *Cleveland Clinic Journal of Medicine* 74.4 (2007): S12-S20.

2. A Casadevall. "Passive antibody administration (immediate immunity) as a specific defense against biological weapons". *Emerging Infectious Diseases* 8.8 (2002): 833-842.

3. DC Wraith. "The Future of Immunotherapy: A 20-Year Perspective". *Frontiers in Immunology* 8 (2017): 1668.

4. A DiGiandomenico., *et al*. "Identification of broadly protective human antibodies to Pseudomonas aeruginosa exopolysaccharide Psl by phenotypic screening". *Journal of Experimental Medicine* 209.7 (2012): 1273-1287.

5. G Walsh. "Biopharmaceutical benchmarks 2010". *Nature Biotechnology* 28.9 (2018): 917-924.

6. C Saylor., *et al*. "Monoclonal antibody-based therapies for microbial diseases". *Vaccine* 27.6 (2009): G38-G46.

7. L Pirofski and A Casadevall. "Immunomodulators as an antimicrobial tool". *Current Opinion in Microbiology* 9.5 (2006): 489-495.

8. L Czaplewski., *et al*. "Alternatives to antibiotics - a pipeline portfolio review". *The Lancet Infectious Diseases* 3099.15 (2016): 1-13.

9. JD Berry and RG Gaudet. "Antibodies in infectious diseases: Polyclonals, monoclonals and niche biotechnology". *New Biotechnology* 28.5 (2011): 489-501.

10. S Péchiné., *et al*. "Emerging monoclonal antibodies against Clostridium difficile infection". *Expert Opinion on Biological Therapy* 17.4 (2017): 415-427.

11. E Spillner., *et al*. "Avian IgY antibodies and their recombinant equivalents in research, diagnostics and therapy". *Biologicals* 40.5 (2012): 313-322.

12. J Kovacs-Nolan and Y Mine. "Egg yolk antibodies for passive immunity". *The Annual Review of Food Science and Technology* 3 (2012): 163-182.

13. J Andris-Widhopf., *et al*. "Methods for the generation of chicken monoclonal antibody fragments by phage display". *The Journal of Immunological Methods* 242.1-2 (2000): 159-181.

14. J Baer., *et al*. "Chapter 22 - Japanese Quail as a Laboratory Animal Model, Third Edit". Elsevier Inc (2015).

15. D Huss., *et al*. "Japanese quail (Coturnix japonica) as a laboratory animal model". *Lab Anim* (NY) 37.11 (2008): 513-519.

16. JJ Filho., *et al*. "Energy requirement for maintenance and gain for two genotypes of quails housed in different breeding rearing systems". *Revista Brasileira de Zootecnia* 40.11 (2011): 2415-2422.

17. The European Parliament and The Council of the and European Union, Directive 2010/63/Eu of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes (2010).

18. RS Vieira-Pires., *et al*. "Modular Bird Cage" (2016).

19. A Rekas., *et al*. "Crystal structure of venus, a yellow fluorescent protein with improved maturation and reduced environmental sensitivity". *Journal of Biological Chemistry* 277.52 (2002): 50573-50578.

20. MF Hsu., *et al*. "Using Haloarcula marismortui Bacteriorhodopsin as a Fusion Tag for Enhancing and Visible Expression of Integral Membrane Proteins in Escherichia coli". *PLoS One* 8.2 (2013).

21. CS Rüdiger Schade., *et al.* "Production and Application: IgY-Technology". Springer Lab Manual (2001).

22. WMS Russell and RL Burch. "The principles of humane experimental technique". England: London, Methuen (1959).

23. R Schade., *et al.* "The Production of Avian (Egg Yolk) Antibodies: IgY". Altern. to Lab. Anim 24 (1996): 925-934.

24. F Klemperer. "Über natürliche Immunität und ihre Verwertung für die Immunisierungstherapie". *Arch. für Exp. Pathol. und Pharmakologie* 31 (1893): 356-382.

25. NobelPrize.org, "Emil von Behring – Nobel Lecture". *Nobel Media AB* (2020).

26. D Thirumalai., *et al.* "Chicken egg yolk antibody (IgY) as diagnostics and therapeutics in parasitic infections – A review". *International Journal of Biological Macromolecules* 136 (2019): 755-763.

27. C Harley and RS Vieira-Pires. "Antibody fragment technology and avian IgY antibodies: a powerful combination". *Drug Target Revivew* 3 (2016): 4-8.

28. R Kawahara-Miki., *et al.* "Next-generation sequencing reveals genomic features in the Japanese quail". *Genomics* 101.6 (2013): 345-353.

29. A Pharmaceuticals. "FDA Approves Kanuma (Sebelipase Alfa)" (2015).

30. BB Scott and C Lois. "Generation of tissue-specific transgenic birds with lentiviral vectors". *Proceedings of the National Academy of Sciences of the United States of America* 102.45 (2005): 16443-16447.

31. RS Vieira-Pires. "Technological Platform for the development of Egg-based Biopharmaceuticals". *Center for Neurosciences and Cell Biology* (2017).

32. C Pinto and RS Vieira-Pires. "Avian biotech research: a new housing system for egg laying quails". *Press Office, University of Coimbra, Portugal* (2018).

**Volume 16 Issue 6 June 2020**