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# Multi-Barcode for Detection of Avian Bacterial and Viral Pathogens: A Review

Hamong Suharsono[1], Nengah I. K. Besung[2], Bayu K. Mahardika[3], Ida B. K. Suardana[4], Gusti A. Suartini[1], Nyoman I. Suartha[5], Xiaole Qi[6] and Gusti N. Mahardika[3,4]*

[1]Biochemistry Laboratory, Faculty of Veterinary Medicine, Udayana University of Bali, Jl. PB Sudirman, Denpasar, Indonesia
[2]Microbiology Laboratory, Faculty of Veterinary Medicine, Udayana University of Bali, Jl. PB Sudirman, Denpasar, Indonesia
[3]The Animal Biomedical and Molecular Biology Laboratory, Udayana University of Bali, Jl. Sesetan-Markisa #6, Denpasar 80226, Indonesia
[4]Virology Laboratory, Faculty of Veterinary Medicine, Udayana University of Bali, Jl. PB Sudirman, Denpasar, Indonesia
[5]Internal Medicine Laboratory, Faculty of Veterinary Medicine, Udayana University of Bali, Jl. PB Sudirman, Denpasar, Indonesia
[6]Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, State Key Laboratory of Veterinary Biotechnology, 678 Haping Road, Xiangfang District, Harbin, 150069, P.R. China
***Corresponding author:** gnmahardika@unud.ac.id

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## ABSTRACT

The global poultry industry constantly faces the threat of various bacterial and viral diseases, some of which are underreported, especially in developing countries. Even when official reports are available, the scope of the problem and its impact are unknown. Testing for individual pathogens is costly and tedious and may lead to inappropriate management, especially in the case of immunosuppressive diseases. Next-generation sequencing (NGS) provides an opportunity to discover all the events leading to clinical diseases. NGS for bacteria is well established, but this is not the case for viral agents. Because of their many variants, it is impossible to establish barcodes to detect all viruses. Therefore, we propose to establish a multi-barcode system to detect all bacteria and viruses in clinical avian specimens. The libraries can be generated with a one-step reverse transcription–PCR system for bacteria and RNA and DNA viruses, after priming with a single bacterial primer barcode and primer sets for all viruses or targeted viral genes of interest.

**Key words:** Avian Disease, Bacteria, NGS, Multi-Barcode, Virus

## INTRODUCTION

Various viral and bacterial avian pathogens cause diseases of Office International des Epizooties (OIE)-listed and beyond (FAO-OIE 2017). Unlisted pathogens must also be considered because they are known to cause significant economic losses. The presence of pathogens is usually underreported in developing countries (Conan et al. 2012; Perry et al. 2013; Shittu et al. 2016; McElwain and Thumbi 2017; Brown et al. 2018). Although such pathogens can lead to clinical diseases and mortality, some can cause only mild or subclinical infections, and others cause cryptic subclinical infections, allowing the clinical manifestations of co-infecting pathogens to dominate (Blackall 1999; Butt et al. 2022). Such complex situations present challenges for pathogen detection. The time-consuming and costly detection of individual pathogens causes uncertainty in the management of poultry health and may also lead to misdiagnoses because the underlying infection is not detected, particularly in cases of immunosuppression (Blackall 1999; Butt et al. 2022; Mehnaz et al. 2023).

Next-generation sequencing (NGS) provides an opportunity to detect all the events that contribute to clinical disease. The current technology involves a high-throughput system that can detect all the genetic material in a specimen (Behjati and Tarpey 2013). The NGS has been proven to be an important tool for confronting virus

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pandemics (Quer et al. 2022). The application of NGS to bacteria is almost straightforward because an almost completely conserved gene or gene fragment has been identified for all bacterial classes and genera. However, the application of NGS to virus detection is still problematic because a highly conserved gene or gene fragment has not been identified for all viral families (Moser et al. 2016). The use of random primers may detect all viruses, but the host background sequences will dominate the hits, whereas any viral sequence will occur in very low quantities, as it has been demonstrated in various experiments (Marston et al. 2013; Perlejewski et al. 2015; Schlaberg et al. 2017). Primer independent approach has been proposed in a review to study avian virus diversity (Kapgate et al. 2015).

Here, we review the methods used to overcome this problem by using multiple primer sets that amplify specific bacterial and viral targets as libraries for NGS.

**Avian Diseases and Their Economic Impacts**

The global chicken industry constantly faces the threat of various bacterial and viral diseases. The OIE has reported these as OIE-listed diseases and other important diseases (FAO-OIE 2017). The listed bacterial diseases are fowl cholera, chlamydiosis, fowl typhoid, mycoplasmosis, pullorum disease, and avian tuberculosis, whereas those of viral origin are avian influenza (AI), Newcastle disease (ND), infectious bronchitis (IB), infectious laryngotracheitis (ILT), Marek's disease, and infectious bursal disease (IBD). Other unlisted pathogens should also be considered because they have been shown to cause significant economic losses. That includes fowl pox, reovirus, avian adenoviruses, chicken infectious anemia (CIA), coryza, and colibacillosis. Important avian adenoviruses cause egg drop syndrome (EDS) and inclusion body hepatitis (IBH) (Hess 2000; Wibowo et al. 2019; Liu et al. 2021; Tchoupou-Tchoupou et al. 2022; Du et al. 2023; Abdel-Alim et al. 2023). Some of these agents also have zoonotic potential, such as avian chlamydiosis, avian tuberculosis, fowl typhoid, pullorum disease, AI, and ND.

A large body of evidence shows that AIV frequently causes epizootic, epidemic, and pandemic, which makes it constantly of high interest. The genome of the virus provides the first challenge to its detection. There are many subtypes of the virus based on variations in the hemagglutinin (HA) and neuraminidase (NA) gene sequences (COMMITTEE 1980). Eighteen HA and nine NA subtypes have been identified (Wu et al. 2014; Sutton et al. 2017). Among the subtypes, some H5 and H7 subtypes belong to the highly pathogenic avian influenza viruses (HPAIV) (FAO 2014). At the end of 2016, an AIV of H9N2 subtype was introduced into and spread throughout Indonesia, causing massive economic losses (Jonas et al. 2018). Its co-circulation with AIV-H5N1 (Mahardika et al. 2016) requires rigid surveillance. The AIV-H9N2 has been shown to contribute to the generation of new strains of AIV (Yu et al. 2015), which may have significances for animal and human health. The introduction of AIV-H5N1 and -H9N2 has made it clear that other subtypes must be monitored in Indonesia. The HPAIV H7N9, which is endemic in Asia (Gilbert et al. 2014; Qi et al. 2014) might also spread to Indonesia.

Huge economic losses have been reported, arising from mortality, reduced egg and meat production, and immunosuppression. These economic losses have been attributed to mycoplasma (Zhu et al. 2018), IBV (van Beurden et al. 2018), AI-H9N2 (Yang et al. 2017), colibacillosis (Lau et al. 2012), coryza (Blackall 1999), and much more.

**Existing Diagnostic Protocols and NGS**

Conventionally, the diagnostic procedure for any infection is used to confirm the tentative diagnosis, which is made based on clinical signs, anatomical pathology, and epizootiology. As described above, the clinical signs of many diseases are often inconclusive, unless pathognomonic signs are prominent. Reduced egg production, for example, can be caused by many infections. The immunosuppressive effects of some infections might allow infections by other pathogens, so the predisposing agent is hidden and unsuspected. Vaccination may blur the clinical signs, as in the case of partial immunity, when the infection presents milder symptoms with lower mortality. Testing for individual pathogens to confirm a differential diagnosis is laborious, costly, and time-consuming.

The latest NGS technology allows the simultaneous detection of various genetic materials in a specimen (Plyusnin et al. 2020). However, the performance of various platforms varies. Three platforms of NGS marketed in 2011 are Ion Torrent's PGM, Pacific Biosciences' RS, and the Illumina MiSeq (Quail et al. 2012). In a comparison of the Illumina and Ion Torrent platforms, other groups (Salipante et al. 2014) found that the latest produced higher error rates with premature sequence truncation. Another group of researchers used Escherichia coli strain DH1 to analyze the quality of sequencing results of the Roche Genome Sequencer FLX System (FLX), Illumina Genome Analyzer (GA), and Applied Biosystems SOLiD system (SOLiD) (Suzuki et al. 2011). They found that the SOLiD produced the largest fraction of data, which could not be aligned to the reference sequence; the GA produced the lowest sequence accuracy; and the FLX produced the smallest sequence coverage (Suzuki et al. 2011).

Other evidence shows that the performance of various platforms varies. In detecting Human immunodeficiency virus 1 receptors, Raymond et al. (2017) found that the Illumina platform could be used to detect minor receptor variants. When applied to the detection of blood pathogens, Frey et al. (. 2014) showed that the Roche-454 Titanium platform could detect the lowest dengue virus titers. For bacterial samples, only the MiSeq platform was reported to be able to provide unambiguous sequencing read. Other comparisons showed that the Roche-454 Titanium performed the best in producing longer reads; the MiSeq in depth and breadth of coverage; and the Ion Torrent in sequencing speed (Frey et al. 2014).

**DNA Barcoding**

**Under this Topic add more Literature as Much Published Material is Available**

To detect multiple pathogenic agents simultaneously, as proposed in this manuscript, a DNA barcoding approach seems most suitable. DNA barcoding is a short

DNA sequence to identify family, genus, or species of organism (Hebert et al. 2003; Tanabe and Toju 2013).

For bacteria, the 16S ribosomal RNA (16S rRNA) fragment is mostly recommended as a barcode (Tanabe and Toju 2013), together with other fragments such as rpoB, dnaK, gyrB, and recA (Ramesh et al. 2021). The gene 16S rRNA poses nine variable regions located between conserved regions (Panek et al. 2018). The V3 and VP variable regions are commonly used in the genus or species classification in various species (Klindworth et al. 2013; Panek et al. 2018). This fragment should be appropriate for the detection of bacteria in poultry.

When we accessed the internationally published articles available in PubMed on July 9, 2019 using the EndNote software, and selected only detection of agent genetic material, there were only 15 papers with the keywords "NGS avian pathogen" or "NGS avian disease" or "NGS poultry pathogen" or "NGS poultry disease". However, none dealt with the detection of multiple disease agents in poultry. Instead, the papers dealt with individual agents, such as Newcastle disease (Cattoli et al. 2011; Butt et al. 2019; Lebdah et al. 2022; Akhtar et al. 2023), Marek's (Maceachern et al. 2011), influenza and poxvirus (Croville et al. 2012; Croville et al. 2018), avulavirus-1 (Tal et al. 2019), infectious bronchitis virus (van Borm et al. 2021), Avian Metapneumovirus Kariithi et al. 2022), duck herpesvirus (Damir et al. 2023), and enteric viruses (Day and Zsak 2013).

Application of NGS has been applied to detect and predict the virulence of NDV, which includes a platform to subtract the host sequences (Butt et al. 2018). A similar platform was used to describe the ability to obtain almost complete genomes on 28 avian paramyxoviruses (APMV), four AIV, and two IBV in a single run (Dimitrov et al. 2017). In another report, Sequence-Independent-Single-Primer-Amplification (SISPA) was able to detect and identify avian RNA viruses from specimens of high virus titers (Chrzastek et al. 2017). Such an approach might not be optimum, as the virus titer in the specimen can be extremely low.

Experience in the application of NGS to human virology should facilitate its application to avian diseases. cDNA libraries are usually generated with reverse transcription (RT)–PCR or PCR, using targeted primer sets for influenza virus (Croville et al. 2012; Zou et al. 2016), Seoul virus (Kim et al. 2018), herpes simplex virus (Fujii et al. 2018), rhinovirus (Greninger et al. 2017a), parainfluenza (Greninger et al. 2017b). Those again were engaged for individual virus families or species. Non-targeted NGS application for poultry viruses has been applied (Parris et al. 2022). The protocol must include a non-target RNA depletion strategy to increase its sensitivity (Parris et a. 2022).

Primer crowding followed by an enrichment step seems to be a suitable way to detect viral pathogens in poultry specimens. The primer crowd was applied to sequence Epstein-Barr Virus (EBV), in which 59 or 60 primer sets were used in a single PCR (Kwok et al. 2012; Simbiri et al. 2015). In a review of the sequencing of the EBV genome, Kwok and Chiang (Kwok and Chiang 2016) evaluated the benefits of PCR enrichment. With PCR enrichment, it was possible to detect the EBV genome in tumor biopsies, although only around 0.01% of the total reads were mapped to EBV. In poultry, the metagenomics approach has been applied in poultry farms in China and could simultaneously detect major viruses infecting farms (Qiu et al. 2019).

**Future Direction**

In the short future, automatic chain termination Sanger's method will still find its use. This method is still being improved by various scientists. With the decreasing cost and increasing efficiency, NGS has been applied in clinical and public health laboratory practice (Besser et al. 2018). In other words, NGS is ready to move from research to a (human) clinical setting (CADTH 2014), although it is not problem-free (Perlejewski et al. 2020). We think that we are not far away from seeing its application in animal clinical settings.

Instead of detecting single to very few agents we previously discussed, we believe multi-barcoding to detect multiple agents is promising to be applied in poultry health management. This is also valid for other kinds of animal husbandry. Multi-barcoding of avian pathogens should extend our knowledge of the presence of diseases in the chicken industry and their biology. All pathogens should be detected simultaneously, so a complete picture of the disease status of a farm can be determined. All avian pathogens might contribute to economic losses and compromise the provision of protein resources for human consumption throughout the world. NGS provides an opportunity to meet this challenge because it allows us to detect all the genetic material in a specimen simultaneously. This approach will provide invaluable data on the genetic variation and spread of certain pathogens.

Due to the nature of high throughput and the cost, the NGS approach might be better engaged for the management of farms. In other words, it does not fit to be used as a diagnostic tool. The result will be a strong base for better farm management. This will lead to a scientifically sound understanding of the pathogenesis and possible involvement of many pathogens that lead to health disturbance. Genetic variation will also benefit the vaccine design and zoonoses control.

The number of bacterial and viral avian pathogens exceeds 20. These include OIE-listed and non-OIE-listed organisms. In some cases, the clinical signs of these pathogens are indistinguishable. Moreover, clinical signs might be attributable to certain pathogens while those of others are hidden, although they are the predisposing factors that lead to superinfection. This is particularly valid for immunosuppressive agents such as IBDV, CIAV, and AIV-H9N2. For the latest, immunosuppressive effects have been attributed to AIV-H9N2 (Qiang and Youxiang 2011), although clinical infections also occur (Jonas et al. 2018).

Barcodes are available for bacterial detection. Metagenomic studies of bacteria are frequently accomplished by investigating the 16S rRNA gene. Illumina has marketed a 16S metagenomic barcode aiming at the 16S rRNA V3 and V4 regions based on the study by Klindworth et al. (2013b). Illumina adapter sequences are added to the 5'-end of the primer set. The addition of these non-target sequences to primer sets of various virus gene fragments should work well. This adapter can be used to enrich the DNA library.

The future application of NGS for the detection of many viruses in poultry is bright. The hope is huge, including in the study of wildlife. With NGS, it is possible to detect many viruses associated with Antarctic wildlife (Smeele et al. 2018). However, the application of NGS to the detection of many viruses remains challenging. So far, no specific sequence can be attributed to all existing viruses. Primer-free amplification, in which all DNA in the sample is sequenced, has been used for library preparation. Using this approach, viral sequences that can be mapped to any virus sequence might constitute only a tiny proportion of the reads. When the technique was used to detect EBV, only 0.01% of the total reads were mapped to the EBV genome. The best option seems to be the PCR amplification of all the viral pathogens in a sample. When EBV was detected with up to 60 primer sets, 90% of the reads generated could be mapped to the reference sequence (Kwok et al. 2012; Simbiri et al. 2015). The application of random primers might detect all viruses, but the background host sequences will dominate the hits and any viral sequences will occur in small low quantities.

The modification of 16S rRNA metabarcoding with gene-specific viral primers is a plausible option. Illumina adapter can be added to the 5'-end of the primers. Viral target genes occur as single gene fragments, except for AIV. In that case, the subtyping of AIV is usually preferred, because only some subtypes of AIV exist in a specific region. When analyzing this virus, segment-specific HA and NA sequences of AIV-H5N1 and AIV-H9N2 or other subtypes of interest should be included in the primer mixture. 16S rRNA metabarcoding allows sequence reads of up to 600 bp (including the adapter). We believe that this length is sufficient to annotate a specific sequence to a particular species and subtype.

The NGS-Metabarcoding with primer crowding approach might also be extended to important poultry parasitic diseases which have economic as well as zoonotic impacts. The NGS has been applied to identify individual parasites such as blastocysts (Higuera et al. 2021; Maloney et al. 2021), *Histomonas meleagridis* (Palmieri et al. 2021), and Eimeria spp. (Hauck et al. 2019), and Listeria monocytogenes (Sioutas et al. 2023), as examples.

A graphical presentation of proposed steps in the development of NGS for avian bacterial and viral diseases is presented in Fig. 1. In step 1, a database of various virus families is downloaded from GenBank. The primer pair is selected from each family, and illumina overhang forward (OF) and backward (OB) are added at the 5'-end of the respective primer (www.illumina.com). The primer sets are available upon request. The illumina 16S-RNA primers for bacterial detection are proposed based on Klindworth et al. (2013) are chosen. In step 2, all bacterial and viral RNA and/or DNA from one flock are isolated with a DNA-RNA isolation kit and amplified with primers designed in Step 1 using RT-PCR one-step amplification kit. In step 3, first step PCR products are isolated and amplified using PCR with OF and OB primers. In step 4, the second step PCR product is isolated and subjected to further library prep protocol for indexing, normalization, and other steps prior to application into the NGS machine.

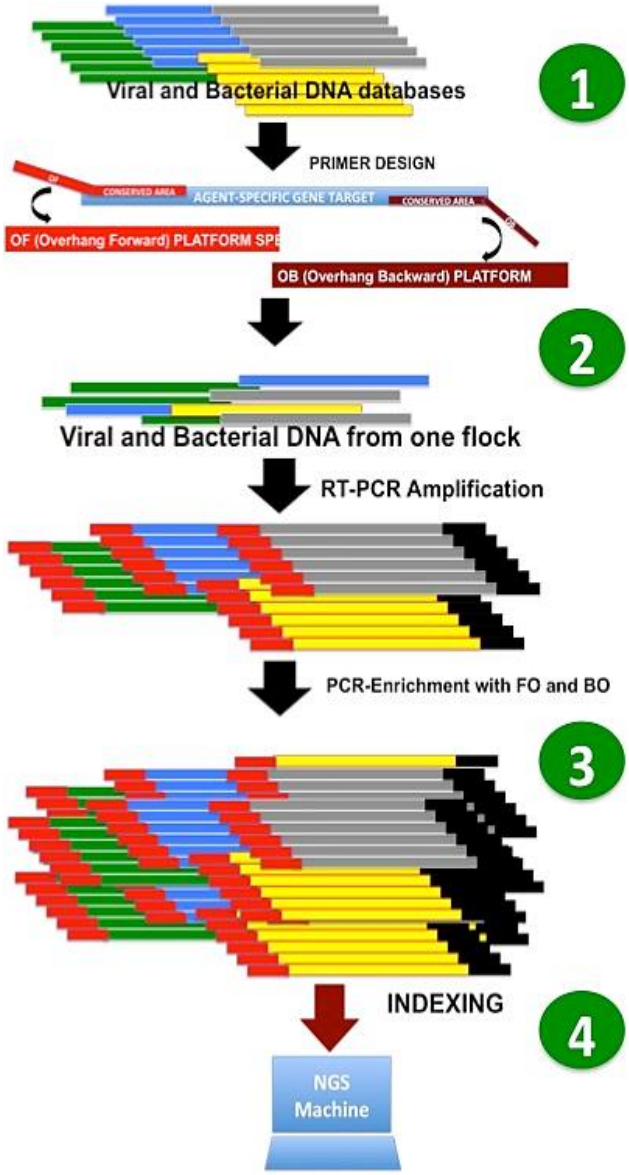


**Fig. 1:** Graphical presentation of proposed steps in the development of NGS for avian bacterial and viral diseases. Step 1: A database of various virus families is downloaded from GenBank. Primer pair is selected from each family, and Illumina overhang forward (OF) and backward (OB) are added at 5'-end of respective primer (www.illumina.com). The primer sets are available upon request. The illumina 16S-RNA primers for bacterial detection are proposed based on Klindworth et al. (2013) are chosen. Step 2: all bacterial and viral RNA and/or DNA from one flock are isolated with a DNA-RNA isolation kit and amplified with primers designed in Step 1 using RT-PCR one-step amplification kit. Step 3: First-step PCR products are isolated and amplified using PCR with OF and OB primers. Step 4: In the second step PCR product is isolated and subjected to further library prep protocol for indexing, normalization, and other steps prior to application into the NGS machine (Hess et al. 2020).

Modified from Malla et al. (2018), Plyusnin et al. (2020), and Garfias-Gallegos et al. (2022), the simplified bioinformatics pipeline flowchart in the application of NGS in poultry farms using targeted and crowded primer sets is shown in Fig. 2. The raw data from the machine will undergo quality checks to remove short and low-

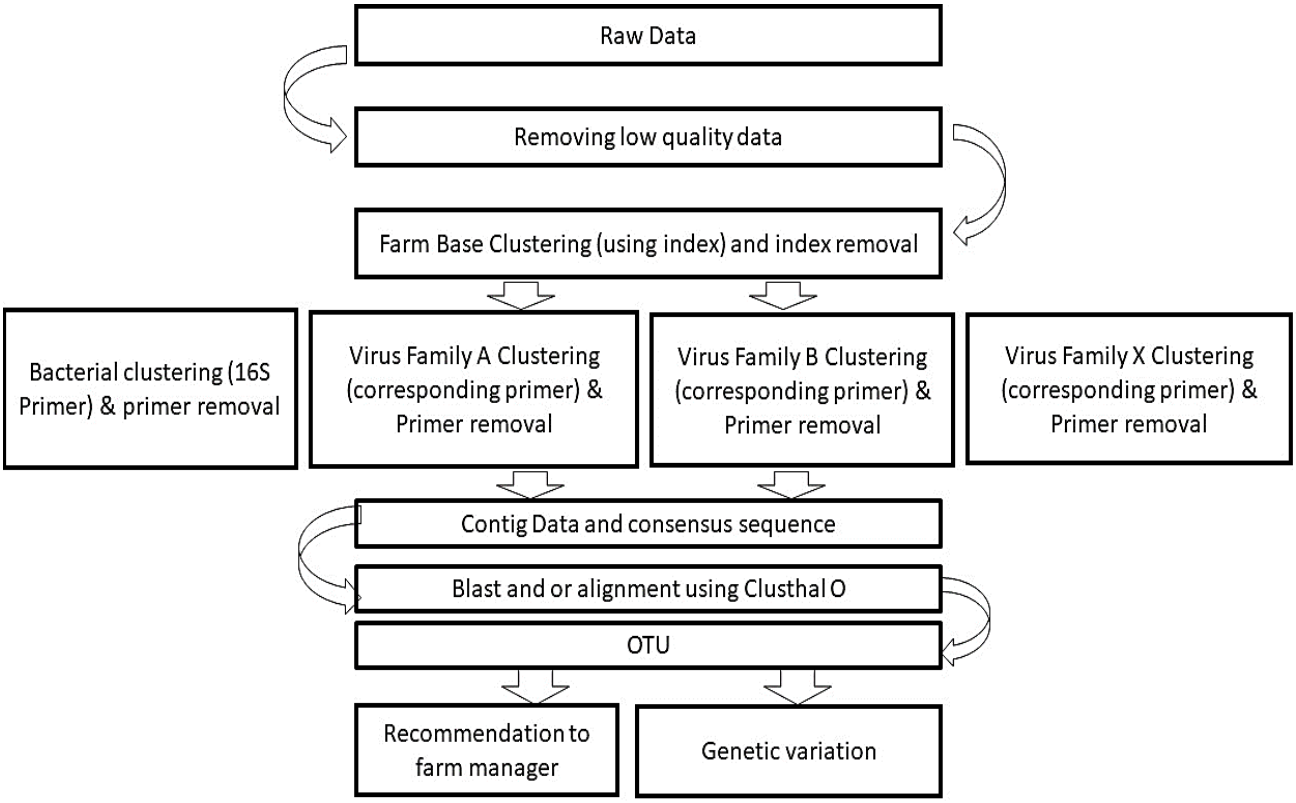


**Fig. 2:** Simplified Bioinformatics Pipeline in the Application of NGS in poultry farm using targeted and crowded primer sets. The raw data from the machine will undergo a quality check to remove short and low-quality reads. The data will then be clustered into respective farms by calling the respective index sequence before the index is removed. Clean data will thereafter be sub-clustered into bacteria and virus families by calling the respective primers before the primers are trimmed. Contig data or consensus sequences will be generated and subjected to BLAST search or eventually aligned using Clustal Omega to produce clustered sequences. The output will be Operational Taxonomic units (OTUs). The end products will be a recommendation to the farm manager for farm improvement as well genetic variation of each OTU. The flowchart is modified from Malla et al. (2018), Plyusnin et al. (2020), and Garfias-Gallegos et al. (2022). The software for each step has been described in those references.

quality reads. The data will then cluster into respective farms by calling the respective index sequence before the index will be removed. Clean data will thereafter be sub-clustered into bacteria and virus families by calling the respective primers before the primers are trimmed. Contig data or consensus sequences will be generated and subjected to BLAST search or eventually aligned using Clustal Omega to produce clustered sequences. We might have to make data partition before conducting Clustal Omega. The output will be Operational Taxonomic units (OTUs). If needed, OTU verification might be needed as exemplified by Qiu et al. (2019). The end products will be a recommendation to the farm manager for farm improvement as well genetic variation of each OTU. Further research questions might ascend such as the need for vaccine improvement, the pathogenicity of novel bacteria, and many others. The software for each step has been described (Malla et al. 2018; Plyusnin et al. 2020; Garfias-Gallegos et al. 2022).

The implementation of this protocol is still challenging. PCR is usually conducted with a specific concentration of oligonucleotide primers, and excessive amounts of primers could disturb the polymerase reaction (Lorenz 2012). Overall cost can be feasible. The test should be done with samples from hundred farms. As time is needed to collect samples from various farms, this approach is suitable for long-term farm health management.

## Conclusion

In conclusion, multi-barcoding for bacterial and viral pathogens should benefit the chicken-farming industry throughout the world. It will allow the detection of all possible disease agents, so a complete picture of the disease status of a region can be drawn, and subsequent management strategies put in place accordingly. The challenge of excessive oligonucleotides should be anticipated, and its resolution will allow the full potential of NGS to be exploited.

## Declaration of Competing Interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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**Abbreviations**

| | |
|---|---|
| AI: | Avian influenza |
| CIA: | Chicken infectious anemia |
| DNA: | Deoxyribonucleic acid |
| EBV: | Epstein-Barr Virus |
| EDS: | Egg drop syndrome |
| HA: | Hemagglutinin |
| HPAI: | How pathogenic avian influenza |
| IB: | Infectious bronchitis |
| IBD: | Infectious Bursal Disease |
| IBH: | Inclusion body hepatitis |
| IL: | Infectious Laryngotracheitis |
| LPAI: | Low pathogenic avian influenza virus |
| MD: | Marek's Disease |
| NA: | Neuraminidase |
| ND: | Newcastle disease |
| NGS: | Next Generation Sequencing |
| OB: | Overhang backward |
| OF: | Overhang forward |
| OIE: | *Office International des Epizooties* |
| PCR: | Polymerase chain reaction |
| RNA: | Ribonucleic acid |
| RT-PCR: | Reverse transcriptase-PCR |
| SISPA: | Sequence-Independent-Single-Primer-Amplification |
| stLFR: | Single-tube long fragment read |

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