High-throughput sequencing as a potential tool in the quality control of infectious bronchitis vaccines

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Abstract

Introduction: In Europe, veterinary vaccines are strictly controlled by the Official Medicines Control Laboratories (OMCLs) of the General European OMCL Network, coordinated by the European Directorate for the Quality of Medicines & HealthCare. Despite a meticulous verification programme for immunological veterinary medicinal products (IVMPs), the products' genomic composition has not yet been subject to evaluation in veterinary pharmacy. **Material and Methods:** A study was carried out on Poland's poultry vaccines containing the infectious bronchitis virus which have the greatest market penetration. Three batches of three different vaccines were high-throughput sequenced and analysed for genomic composition, frequency of variants and the phylogeny of the strains. **Results:** The main genetic component of each vaccine was infectious bronchitis coronavirus. The identity of the vaccine strain types was confirmed to be consistent with the manufacturer's declaration (793B, Mass and QX). Most variants were identified for the same nucleotide positions in all three batches of each vaccine, demonstrating the homogeneity of the samples, while unique variants specific to single batches were rare. **Conclusion:** High-throughput sequencing (HTS) is an effective alternative poultry IVMP quality control tool for OMCLs. This technique allows in-depth characterisation of a vaccine strain and assessment of its conformance to the manufacturer's declaration. Importantly, HTS brings new cognitive value to IVMP quality control, because it makes monitoring the level of revertants possible. However, before it could be introduced into routine quality control, a thorough analysis and characteristics of the IVMP to be evaluated is needed from the medical product's manufacturer.

Keywords: high-throughput sequencing (HTS), vaccine, infectious bronchitis virus (IBV), Official Medicines Control Laboratory (OMCL).

Introduction

Modern achievements in the field of vaccinology and molecular biology set the direction for organisations responsible for protecting public health around the world. Innovative solutions have improved 21st-century vaccination far beyond what the first vaccines' creators could offer under the limits of their research capabilities. Vaccinology and molecular biology are undergoing enormous development nowadays, and the combined use of both makes it possible to develop new vaccines and new methods to monitor them. One such technology that currently enables this progress is high-throughput sequencing (HTS). It enables the reading and transferring into a digital record of massive amounts of genetic information stored in the form of DNA or RNA (in the case of RNA viruses) (21). High-throughput sequencing is used in many disciplines, such as human medicine (*e.g.* in the development of innovative drugs, clinical biopsy trials and oncological research), the food industry (*e.g.* in the analysis of microbiological contamination), veterinary medicine (*e.g.* in epidemiological investigations, resistance monitoring and characterisation of microorganisms) or forensic analyses (32).

Despite such a wide application of HTS, it is still not used in the quality control of immunological veterinary medicinal products (IVMPs). In Europe, IVMPs are currently under the strict control of Official Medicines Control Laboratories (OMCLs), which comprise the General European OMCL Network (GEON) and are coordinated by the European Directorate for the Quality of Medicines & HealthCare (EDQM) under the Council of Europe. This Directorate also works alongside the European Medicines Agency and the World Health Organization (WHO). All these institutions have one common goal – protecting public health in Europe and worldwide (28). A veterinary network has also been established within the GEON –

the Veterinary Batch Release Network (VBRN). Like the OMCLs in the GEON network concerned with human medical products, having the supervision and monitoring of the quality of medicinal products both before and after marketing as their main task, the OMCLs in the VBRN carry out the supervision and monitoring of IVMPs. The only Polish veterinary OMCL is the National Veterinary Research Institute (NVRI) in Puławy (22), which is the institute where the current investigation was conducted.

According to EDQM data, over 80% of active pharmaceutical substances used on the European market were manufactured in countries outside the EU and the United States (13). For the full assurance of European users of products containing these substances that their quality is good, the EDQM has developed a special quality control programme for medicinal products, creating a system of mandatory certificates awarded to manufacturers in accordance with the standards of the European Pharmacopoeia. It is a guarantee for both national authorities and buyers (doctors, patients and farm owners) that these products have come through quality control. Under Article 128 of Regulation 2019/6 of the European Parliament and the Council (15), OMCLs issue two types of IVMP quality-control certificates – the official control authority batch release (OCABR) and the official batch protocol review (OBPR). This article in the regulation allows a Member State to request IVMP control documentation from the marketing authorisation holder confirming that the quality-control tests have been carried out by the holder using the methods specified in the marketing authorisation. Based on the review of this documentation, an OMCL issues an OBPR certificate (15). However, selected IVMPs are included in a shortlist for which further testing by OMCLs is required. A restricted list of justified tests for OMCLs has been agreed with associated guidelines for these laboratories, pursuant to Article 128 (14). For such an immunological veterinary medicinal product to be placed on the market, it must be OCABR certified (15). According to the Mutual Recognition Procedure, an OCABR or an OBPR issued by any OMCL must be recognised by all OMCLs from other Member States.

In Poland, the vaccines most commonly introduced to the market are IVMPs against infectious bronchitis (IB), containing the infectious bronchitis virus (IBV) (10). This virus is the first coronavirus that was discovered long before human alpha and beta coronaviruses. It is an enveloped virus with a round or pleomorphic shape and a diameter of about 120 nm. On its surface there are projections about 20 nm long made of the spike protein (S), which is a determinant of IBV tropism (25, 42). In addition to the spike protein, virions consist of the envelope (E), membrane (M) and nucleocapsid proteins (N), the latter of which additionally contains the genome in the form of single-stranded RNA. The virus genome is approximately 27.6 kb in size and contains several open reading frames (ORFs) (11, 25, 36).

Regardless of the tropism of the virus (the respiratory, excretory, digestive or reproductive system), the main route of entry of the infectious agent into the body is the respiratory system. According to data from the World Atlas of Animal Diseases (quantitative analysis of global data on animal health, 2006–2009), in 2006–2009, IB was the second-most-common disease causing heavy losses in the poultry industry, between highly and low-pathogenic avian influenza (46). Immunoprophylaxis plays an important role in preventing the disease. Vaccines containing IBV must receive a pre-market batch control OBPR certificate issued by an OMCL in order to be introduced to the Polish market. In addition, these vaccines are also subject to annual post-market surveillance monitoring (23).

So far, IVMP quality control has been carried out exclusively using the methods of classical virology and/or microbiology and physical chemistry. The use of HTS gives insight into previously uncontrolled quality parameters extending to the structure of the genomes of the microorganisms contained in vaccine vials. The aim of this study was to apply HTS to examine the genomic composition of the most frequently marketed poultry vaccines in Poland, which are those against IB.

Material and Methods

IB vaccines. Three commercially available IBV vaccines were used in the study, each represented by three biological replicates from different batches. These vaccines were labelled V-01, V-02 and V-03. All were live attenuated, with doses of 1,000 (V-01/01, V-01/03 and V-02/02) or 5,000 (V-01/02, V-02/01, V-02/03, V-03/01, V-03/02, V-03/03). The lyophilised vaccines were stored under refrigerated conditions (2°C–8°C) and protected from light. Prior to use, they were reconstituted at an ambient temperature of 15°C–25°C, and once reconstituted they were used within two hours, following the manufacturers' guidelines.

RNA extraction. A Maxwell RSC simplyRNA Tissue Kit (Promega, Madison, WI, USA) was used for RNA extraction. The amount of material for extraction was standardised and set as six lyophilised beads from a blister. The obtained RNA was subjected to quality control as described below.

Reverse transcription and double-stranded (ds) DNA synthesis. The reverse transcription reaction was performed using the SuperScript IV First-Strand Synthesis kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) in a Bioer LifeTouch thermal cycler (Bioer Technology Co, Hangzhou, China). In a test tube, 22 µL of isolated RNA was combined with a reaction mixture consisting of 2 µL of random hexamers [d(N)6] (50 ng/µL; Invitrogen) and 2 µL of dNTPs (10 mM; Invitrogen). Subsequent sample incubations were performed according to the time-temperature profile described by the manufacturer. The second strand of dsDNA was synthesised using 1 µL of DNA

polymerase I (Klenow Fragment, New England Biolabs, Ipswich, MA, USA). The mixture was gently mixed, incubated and then stored at 4°C. The total volume of the reaction mixture was 43 µL.

Purification of dsDNA. The obtained dsDNA was cleaned using paramagnetic AMPure microbeads (Beckman Coulter, Brea, CA, USA) to remove the remains of the reaction mixture, in accordance with the manufacturer's guidelines. The dsDNA was mixed with the microbeads in a volume ratio of 1:1.5. Elution was performed in 25 µL of tris-HCl buffer.

RNA and DNA quality control. The quality of the extracted RNA and dsDNA was thoroughly assessed. Three parameters were measured: the concentration of the obtained nucleic acids, their purity, and the DNA's integrity. The purity was checked using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). In turn, nucleic acid concentration was measured using a Qubit 3.0 fluorimeter (Thermo Fisher Scientific, Wilmington, DE, USA). The integrity of the DNA was assessed by capillary electrophoresis using a Fragment Analyzer (Agilent Technologies, Ames, IA, USA). A DNF-488 High Sensitivity Genomic DNA Analysis Kit (Agilent Technologies) was used.

Library preparation. Sequencing libraries were prepared with the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA) with dual indexing, using the Biomek i5 automated workstation (Beckman Coulter). The fragments comprising the obtained libraries were subjected to quality control to check their concentration, the quality of the purification process and their length. For this purpose, the following were performed: capillary electrophoresis (on the Fragment Analyzer system using the DNF-473 Standard Sensitivity NGS Fragment Analysis Kit (1–6,000 base pairs (bp)); Agilent Technologies) and fluorimetric measurement of concentration relative to the standard (using the Qubit 3.0 fluorimeter).

HTS sequencing. Sequencing was performed on a MiSeq sequencer (Illumina) using 2 × 300 bp V3 kits (Illumina).

Bioinformatics analysis. Free, publicly available, non-commercial bioinformatics tools were used to analyse the resulting raw reads. Reads were filtered and trimmed with Trimmomatic software version 0.40 (5). Using the BBDuk tool, positive filtering of reads was performed for 43 IBV sequences downloaded from the NCBI database (Supplementary Table 1) (6). *De novo* assembly was performed from the reads after selection with the SPAdes tool version 3.15.4 (4). The genetic composition of the vaccines was checked with the following tools: Burrows–Wheeler Aligner (BWA) version 0.7.17 (30), Kraken2 and Bracken (http://ccb.jhu.edu/software/kraken/) (45). Analyses of fastq files and then fasta were performed using BLAST version 2.12.0 (33). The analysis of the frequency of IBV variants was performed using the freebayes tool version 1.3.2 with the R package (20, 39). The analysis of variants type was performed using vcf-annotator (version vcf-annotator.py 0.5; https://github.com/rpetit3/vcf-annotator). Phylogenetic trees for the IBV contained in the vaccines were constructed in the Iqtree program (35) using the maximum-likelihood tree algorithm, with the number of BV repeats (bootstrap value) set at 1,000. The tree was then visualised *via* iTOL version 5 (29). Characterisation of the whole-genome sequences of all vaccine batches for their similarity to each other was performed using the Clustal Omega tool version 1.2.1 (41).

Results

The lengths of the IBV sequences obtained from individual batches of each vaccine V-01, V-02 and V-03 were over 27,400 nucleotides. A high number of reads (1,181,940–5,090,925) were obtained and the number of assembled contigs (47–121) and the coverage (99–100%) were determined (Supplementary Tables 2, 3 and 4).

Analyses of the vaccines' genetic composition. The results obtained using the Kraken2 and BWA bioinformatic tools coincided in the proportions of avian coronavirus and eukaryote reads, but they differed in the classification of the eukaryotic genome (Table 1).

Table 1. The results of the analysis of the genetic composition of three commercial poultry infectious bronchitis vaccines carried out using the Kraken2 and Burrows–Wheeler Aligner (BWA) tools

Vaccine	Batch	Number of reads	BWA		Kraken2		
			% of reads mapped to the IBV genome	% of reads mapped to the *Gallus gallus* genome	% of reads mapped to the avian coronavirus genome	% of reads mapped to eukaryotic genome	% of reads mapped to bacterial genome
V-01	01	3,911,175	86	14	73	13	15
	02	3,260,251	84	16	70	15	14
	03	3,971,161	89	10	75	10	15
V-02	01	4,172,990	85	14	83	8	9
	02	4,487,555	84	15	81	8	11
	03	5,090,925	85	14	83	7	10
V-03	01	4,712,611	68	31	58	23	19
	02	4,193,830	75	23	63	16	21
	03	1,181,940	51	48	43	39	18

Table 2. The vcf-annotator tool results of viral variant type analyses of commercial poultry infectious bronchitis vaccines sequenced with high throughput

Vaccine	Batch	Total variants				Unique variants			
		Total number of variants	Type of changes			Number of unique variants	Type of changes		
			Synonymous	Non-synonymous	Unknown		Synonymous	Non-synonymous	Unknown
V-01	01	35	8	22	5	7	2	2	3
	02	38	7	22	9	10	1	2	7
	03	35	7	21	7	7	1	1	5
V-02	01	7	1	4	2	0	0	0	0
	02	8	1	4	3	1	0	0	1
	03	11	1	4	6	4	0	0	4
V-03	01	3	1	0	2	0	0	0	0
	02	8	0	1	7	5	0	0	5
	03	5	0	2	3	2	0	1	1

According to Kraken2, between 70% (batch No. V-01/02) and 75% (V-01/03) of the reads from the V-01 vaccine were assigned to avian coronaviruses, depending on the replicate or batch. Bacterial genomes constituted between 14% (V-01/02) and 15% (V-01/01 and V-01/03) of this vaccine. In addition, the presence of eukaryotic genomes was noted at between 10% and 15% depending on the batch (Table 1). The eukaryotic genome was classified as the human genome according to Kraken2 database. However, more in-depth mapping *via* the BWA tool showed on average 86% of the reads in each batch of V-01 to be the IBV genome, while the remainder (13% on average) to be the chicken *Gallus gallus* genome.

For the V-02 vaccine in turn, the Kraken2 tool attributed avian coronavirus identity to between 81% (V-02/02) and 83% (V-02/01 and V-02/03) of the reads. Bacterial genomes was noted for between 9% (V-02/01) and 11% (V-02/02) of reads and eukaryotic genomes constituted between 7% and 8% in the vaccine (Table 1). And again, analysis with the BWA tool showed that on average 85% of the reads in each batch of V-02 belonged to the IBV genome, while the remaining reads belonged to the *Gallus gallus* genome (14% on average).

The lowest percentages of reads categorised as avian coronavirus were obtained for all three batches of the V-03 vaccine, and this was true for both tools used, Kraken2 (43%–63%) and BWA (51%–75%). In turn, the numbers of reads of other organisms were the highest (between 37%–57% and 23%–48% with the Kraken2 and BWA tools, respectively) (Table 1).

Phylogenetic analysis (identification of vaccine strains). For the phylogenetic analysis of the whole IBV genome identified in the V-01, V-02 and V-03 vaccines, the 11 IBV strains most frequently contained in vaccines were downloaded from GenBank as references (Supplementary Table 5). Four of these strains belonged to the GI-1 lineage (Mass strains), and the V-02 vaccine tested in this study shared the same phylogenetic branch with them (Fig. 1). The V-01 vaccine was on the same branch of the phylogenetic tree as the reference 4/91 strain (the GI-13 lineage and 793B strains). The virus genomes contained in all three batches of the V-03 vaccine

showed the highest homology with the L1148 strain, which belonged to the GI-19 lineage and QX strains.

Occurrence of IBV variants in vaccines. The consensus sequence of the viral strain contained in each batch of a given vaccine was determined from the sequencing data (taking into account the median depth of coverage before and after the removal of identical reads). An identical consensus was obtained for all batches of a given vaccine. These measures allowed the detection of genetic variants of the virus with a frequency of presence above 5% of the variant-allele frequency (VAF) cut-off. Most variants were identified for the same nucleotide positions in all three batches, demonstrating the homogeneity of the samples, while unique variants specific to single batches were rare.

Some variants were identified in the V-01 vaccine, totalling 35, 38 and 35 for the consecutive batches 01, 02 and 03, of which 7, 10 and 7 were unique, respectively (Table 2). Non-synonymous unique variants were identified within the spike protein (in batch 01 at position 21,645 as a T → C mutation) and in the region of polyprotein 1a (in batches 01 and 03 at position 2,986 as a G → T mutation, and in batch 02 at positions 11,289 and 11,292 as an A → T mutation). The only identified unique changes in the nucleocapsid region were exclusively synonymous (in batches 1 and 3). Moreover, in batches 01 and 02, an additional nucleotide insertion was also identified at position 22,233 as G → GA mutations in both within the structure of the S gene. The VAF for these insertions was low at 6% and 5% for batches 01 and 02, respectively, but was within the adopted criterion.

Significantly fewer virus variants were detected in the V-02 vaccine: 7, 8 and 11, of which 0, 1 and 4 were unique to the respective batches 01, 02 and 03. All of them were classified as changes of unknown type (Table 2). All identified unique variants in batches 02 and 03 were in the 3' untranslated region (UTR) which does not code (Fig. 3). In the third batch, a deletion was also identified at position 27,180 (TA → T) with a VAF of 18%.

The virus in the V-03 vaccine was the most genetically stable, presenting only 3, 8 and 5 variants, of which 0, 5 and 2 were unique to the respective batches 01, 02

and 03 (Table 2). Only one non-synonymous unique variant was identified, occurring only in V-03/03 within the 1a polyprotein region (at position 5,754 as an A → C mutation) (Fig. 4). The remaining unique variants (five variants in batch V-03/02 and one in batch V-03/03) were of an unknown type and included an insertion within the spike protein region in batch V-03/02 (at position 23,069 as a C → CA mutation) with a low VAF of 5.3%.
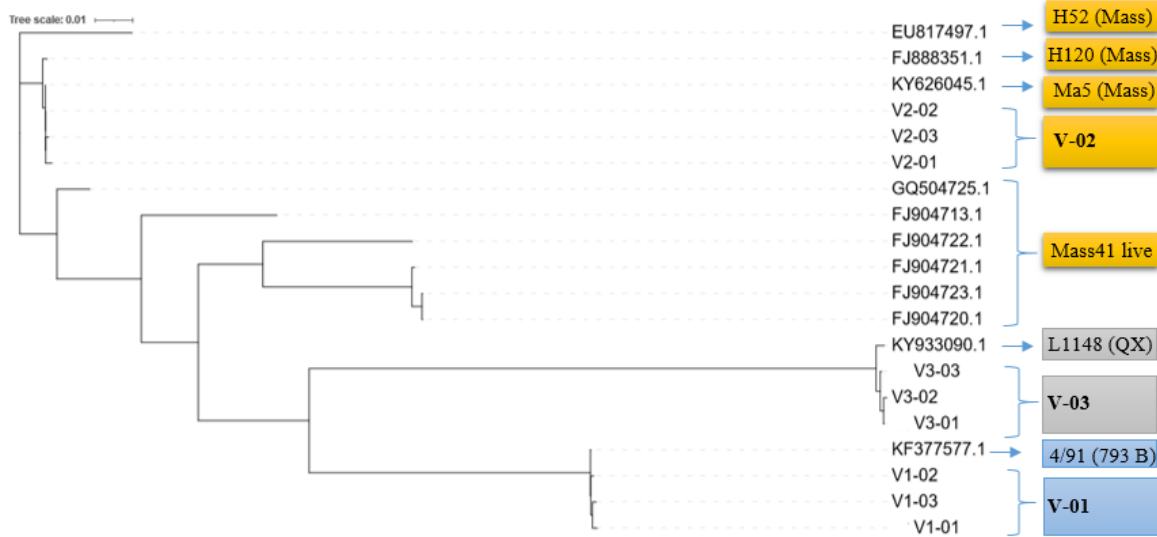

Fig. 1. Phylogenetic tree of the whole viral genome identified by high-throughput sequencing in three batches of three commercial poultry infectious bronchitis vaccines
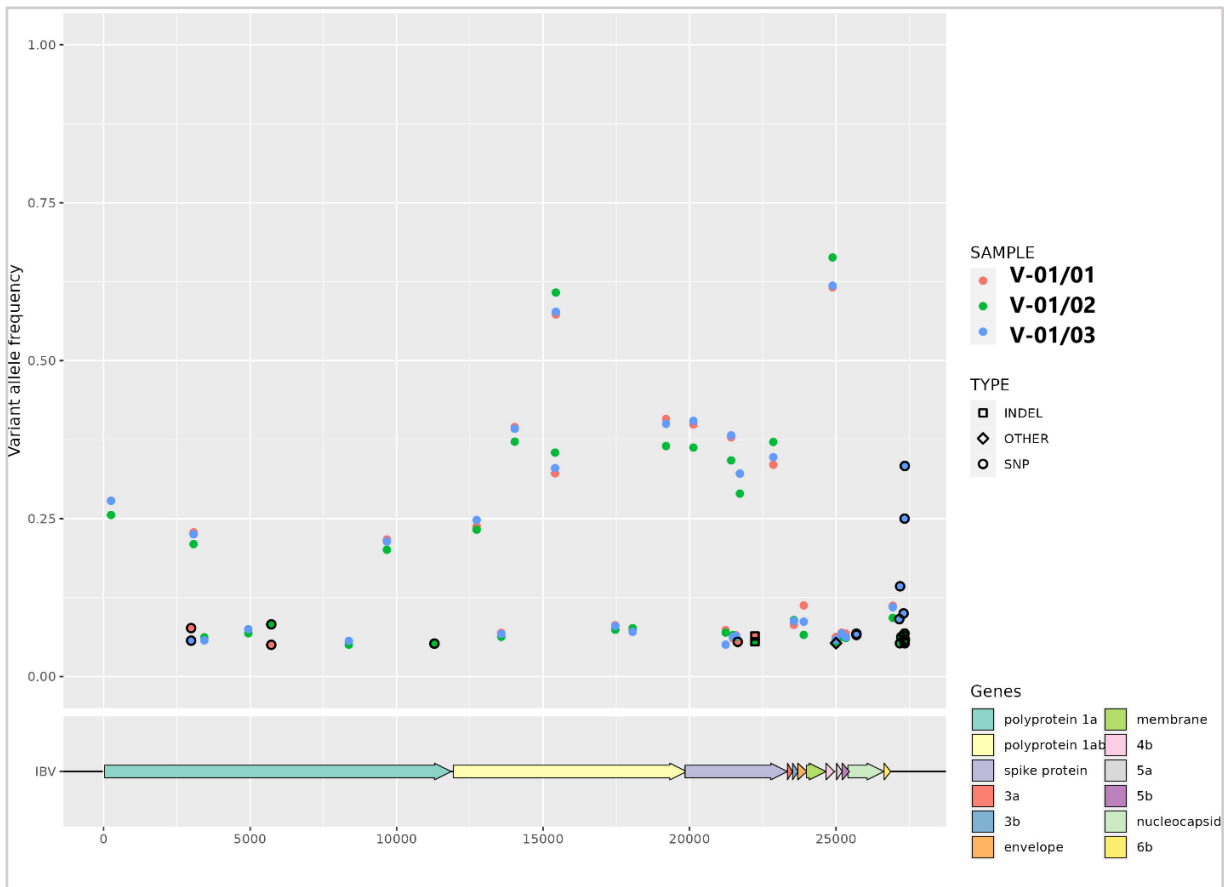

Fig. 2. Frequency of occurrence of infectious bronchitis virus variants in different batches of the V-01 vaccine, in relation to the position of individual nucleotides in the genome

Fig. 3. Frequency of occurrence of infectious bronchitis virus variants in different batches of the V-02 vaccine, in relation to the position of individual nucleotides in the genome

Fig. 4. Frequency of occurrence of infectious bronchitis virus variants in different batches of the V-03 vaccine, in relation to the position of individual nucleotides in the genome
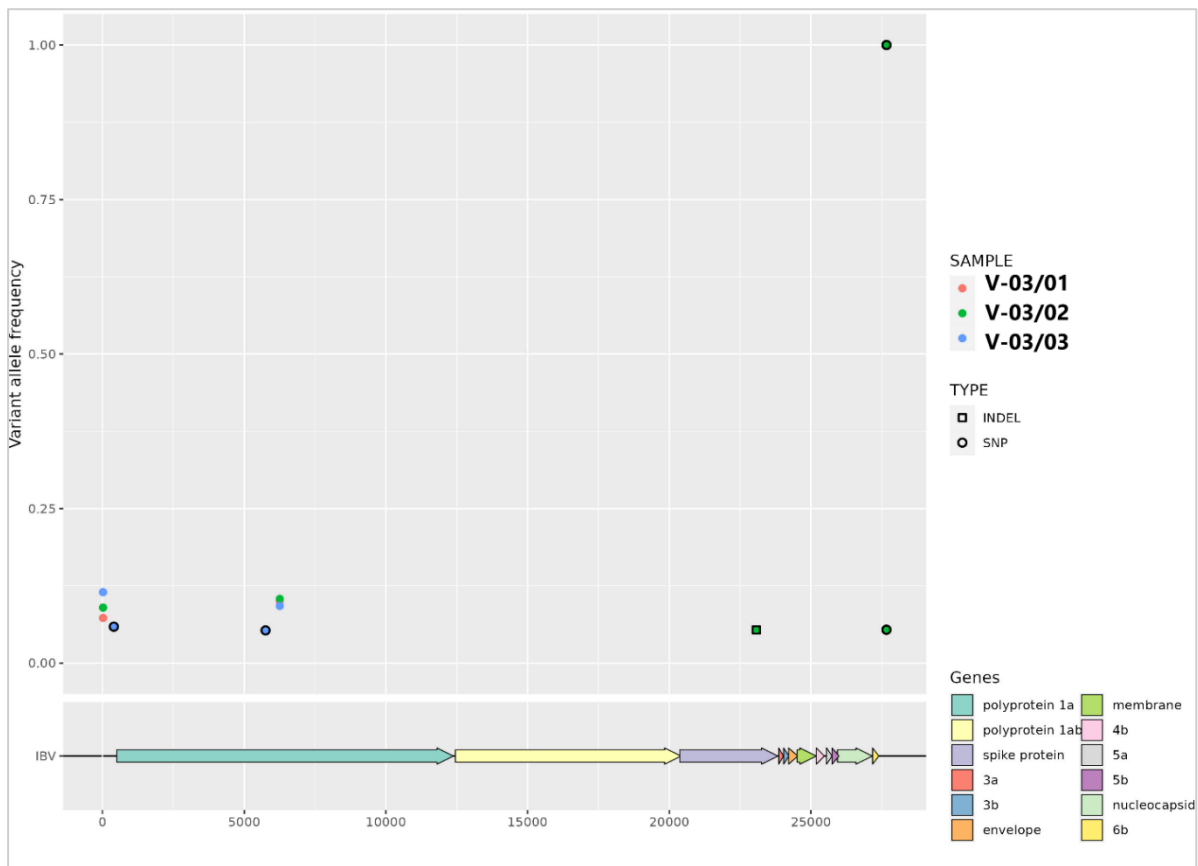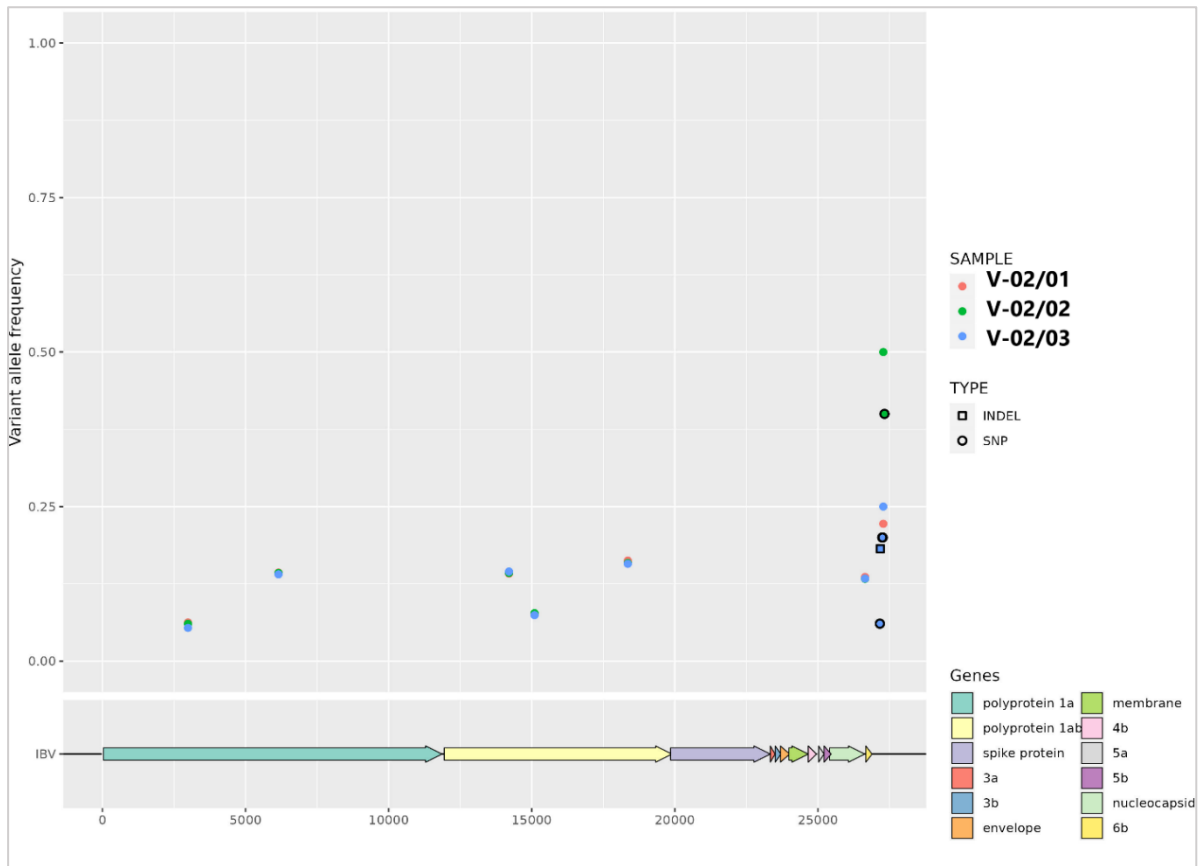
Discussion

Poland has been the leader in the production of poultry meat and eggs in the European Union for over 17 years. According to data provided by Eurostat, in September 2024, Polish poultry production accounted for 21% of EU production (18). Preventive vaccination is one of the most important factors in maintaining the good health of poultry flocks. Therefore, strict quality control of vaccines is crucial, as it guarantees the safety of immunotherapy, and is strongly promoted by the EDQM (26). In Europe, their quality is controlled officially by the OMCL network of laboratories, including the National Veterinary Research Institute in Puławy. The Polish OMCL laboratory conducts monitoring tests for IVMPs on both the domestic and European markets. All these tests are carried out using physicochemical and biological techniques in methods certified by the EDQM. The importance of quality control by OMCLs was highlighted by the Paul-Ehrlich-Institut, a German OMCL laboratory. Out of 5,850 human influenza vaccine batches analysed in various OMCLs in Europe in 2006–2016, 13 (0.22% of all analysed batches) were revealed to be out of specification, which resulted in their withdrawal from the market. The discrepancies mainly concerned incorrect results of vaccine potency and pH tests, but also included some sterility results. This analysis clearly confirmed the value of government vaccine control, without which batches of inferior quality would have been administered to patients (28). An important element of the EDQM policy is raising the safety standards of introduced immunological preparations by implementing modern analytical methods for their testing. It seems that the evaluation of such preparations, especially those containing live attenuated pathogens, should also include their genetic composition and molecular characteristics. Such tests are slowly being introduced as part of the quality control of human vaccines (2). This work is a pilot study on the application of high-throughput sequencing methods in the quality control of veterinary vaccines.

Previous analysis of the IVMPs batch control database of the NVRI showed that the most commonly introduced poultry vaccines in Poland are those against infectious bronchitis (10). Such a high demand for vaccines against IB reflects the scale of poultry production in Poland, but also the risks associated with the occurrence of the clinical form of this disease, which affects poultry production economics. Infectious bronchitis virus, being a *Gammacoronavirus* subgenus species, is genetically highly variable. This shapes its biological properties, ranging from its pathogenicity, immunogenicity, and adaptiveness to new hosts, to its potential to be the agent of diseases which emerge with previously unknown clinical courses. In Poland, the presence of several IBV genotypes/lineages – Mass (GI-1), 793B (GI-13), QX (GI-19), Var2 (GI-23), D1466 (GII-1), D181 (GII-2) and IB80 (GVIII-1) was

confirmed over several years (11, 12). Currently, there are 27 live attenuated and 10 inactivated vaccines against IB approved for marketing in Poland (37). The live vaccines include five different genotypes: GI-1, GI-12, GI-13, GI-19 and GI-23. The genotype GI-12, contrastingly, occurs only in the form of a bivalent vaccine together with the GI-1 type strain. From all available vaccines against IB, three were selected for HTS which contained IBV strains from the GI-1, GI-13 and GI-19 lineages. The compositions of the vaccines are covered by patents and are the intellectual properties of the manufacturers. The idea of the project was to apply the possibilities offered by the HTS technology to the quality control of various IB vaccines, rather than to a specific preparations, so the vaccines' trade names were anonymised with acronyms.

The genetic compositions of all three batches of a given vaccine were identical, but in percentage terms they differed from the others. Avian coronavirus reads predominated in each of them, although the most reads being in the V-02 vaccine (81–83%), the least in V-03 (43–63%), and an intermediate amount in V-01 (70–75%). Similarly, the number of reads for organisms from other taxonomic groups also varied. The highest number of reads classified as eukaryotes were in the V-03 vaccine (16–39%), and the lowest in V-02 (7–8%). The number of reads classified as bacterial was similar the highest being in V-03 (18–21%) and the lowest in the V-02 vaccine (9–11%). It should be noted that at the beginning of the analyses, only the Kraken2 tool was used, which identified the eukaryote reads as human. Therefore, additional mapping was carried out using the BWA tool which allowed the assignment of eukaryote reads to the species *Gallus gallus*. The Kraken2 tool uses k-mers, the profile of which is almost identical for chickens and humans – the species share more than 60% of the genome. Furthermore, the tool's database does not contain chicken sequences, resulting in reads being assigned to the next most similar organism in the database, *i.e.* humans. A similar problem with the Kraken2 tool was described previously by Asif *et al.* (3). They used Kraken2 to taxonomically classify fowlpox virus isolated directly from the skin tissues of a laying flock, and assigned 48–56% of reads to the human genome. Additional basic local alignment search tool testing revealed that these reads belonged to the *Gallus gallus* genome, which is missing from the Kraken2 database (3). Moreover, while Kraken2 generally classified the dominant reads as avian coronavirus, BWA indicated a particular species: infectious bronchitis virus. The BWA tool, with proven higher sensitivity than Kraken2, turned out to be the better choice for use in studies of the genomic composition of vaccines.

The exact process of vaccine production is a trade secret covered by a patent. However, it is known that in the case of live attenuated vaccines against IB, the viral material is propagated on specific-pathogen-free chicken embryos, *i.e.* in fertilised eggs from laying hens free from selected pathogens (25). The presence of chicken

DNA may be a consequence of the vaccine production process. It should be remembered that one of the basic criteria for allowing a vaccine to be placed on the market is its sterility: without meeting this condition as attested to by a European OMCL, a vaccine cannot be put on sale (16). According to the recommendations of the European Pharmacopoeia, one of the quality control requirements for live IB vaccines which manufacturers must comply with is analysis of the presence of bacteria and fungi (17). The guidelines clearly specify that the vaccine cannot contain more than 1 microorganism per dose. This does not therefore totally exclude the possible presence of such microorganisms, albeit a minimal one. The possibility of introducing bacteria into the vaccine together with the embryonic fluid should be excluded. Firstly, they would be alive, secondly, having the SPF status eliminates such a possibility. The presence of host genome artefacts in the vaccine at levels of 7–39% seems not to be much. Therefore, the results for the virus content obtained in the work indicated a high degree of vaccine purity and good efficiency of the methodology used, which resulted in high coverage of the virus genome, allowing for reliable analyses. In addition, a relatively comparable distribution of individual taxonomic groups in a given vaccine was obtained. The composition of the metagenome of the V-03/03 vaccine batch clearly differed from the other two batches, in which coronavirus/eukaryote reads were 43/39 compared to 58-63/23-16. If they reflect any change at the production stage or are the result of the testing methods is a matter which needs to be examined further. Closer cooperation with the manufacturers is needed if HTS control of vaccines is to be implemented in the future. In the batches of the other two vaccines, the composition of the metagenome did not show such differences.

Whole-genome phylogenetic analysis enabled the identification of the genotype and lineage of IBV strains contained in the vaccine vial. The obtained full-consensus sequences of the strains contained in the V-01 vaccines were identified as "infectious bronchitis virus strain 4-91 vaccine", in the V-02 vaccine vials as "Ma5 Massachusetts type vaccine strain" and in the V-03 vials as "vaccine strain derived from virulent progenitor strain 1148-A, genotype QX". The homogeneity of the strain population was also assessed. The highest number of virus variants was detected in the V-01 vaccine of the GI-13 (793B) strain. In these vaccines, unique virus variants with nucleotide changes in the replicase 1a and the S and N genes as well as in the 3'UTR were identified. The lowest number of virus variants were present in the V-03 vaccine of the GI-19 lineage (QX) of IBV. In these vaccines only seven unique variants were identified with changed nucleotides within the replicase 1a and S genes and the 5'UTR and 3'UTR non-coding regions. The V-02 vaccine of the GI-1 (Mass) IBV lineage contained unique variants only in the 3'UTR and only in batches 02 and 03. No unique variants were identified in batch 01. The presence of variants with nucleotide changes within the UTR was identified for all

vaccines: in the 3'UTR for V-01, V-02 and V-03 but also in the 5'UTR for V-03. The mutations with the highest VAF values (at 25–100%) were indeed located in both untranslated regions, but it seems that their presence at these sites was due to the imperfection of the sequencing method itself. The closer to the 5'UTR or 3'UTR, the lower was the recorded coverage. Wang et al. (44) proved that the HTS platform has significantly lower coverage and poorer single-nucleotide polymorphism–calling performance in the 5'-UTR regions of the genome, and theorised that HTS experiments targeting non-coding regions should have higher sequencing depth than the normal genomic region. In the present study, no tests were conducted that focused on the accuracy of UTR sequencing; therefore, most of the variants identified in this study in noncoding regions are likely to be insignificant. It is assumed that the 5'UTR and 3'UTR regions are characterised by rather low variability in the IBV genome. For this reason, they are even used by many diagnostic laboratories to detect IBV in field samples (7). On the other hand, the presence of subpopulations of vaccine viruses differing in the structure of the 3'UTR fragment has been reported previously. In Australia, several vaccines are used to immunise chickens against IB (38). One of the earliest, introduced to that market in the 1960s, was the VicS vaccine. Because post-vaccination reactions occurred in the respiratory system in young chickens, it was rarely used to immunise such birds. However, it was commonly used to vaccinate older hens – commercial and reproductive layers. This vaccine transpired to contain two dominant subpopulations of viruses that differed in the presence of a 40-nucleotide insert in the 3'UTR structure and in their pathogenicity. The vaccine virus with a deletion in the 3'UTR fragment multiplied less easily in the birds and induced weaker histopathological changes than those of the virus with the full 3'UTR fragment (24). Our analysis also revealed a number of variants differing in nucleotide sequence in the replicase gene (vaccines V-01, V-02 and V-03) as well as within the S gene (vaccines V-01 and V-02). Changes in these structures may affect features such as replication efficiency, and thus could affect the pathogenicity of the virus (34, 43, 47).

A similar study on the diversity of vaccine strains has also been conducted in Brazil (40). For many years, the only vaccines against IB approved for use in that country were those based on the Mass (GI-1) strains. These included vaccines containing the H120 strain (from the early 1980s) and the Ma5 strain (introduced in the 1990s) produced by Brazilian pharmaceutical companies. The results of the study showed the existence of subpopulations of the H120 strain differing in the S1 gene sequence in three positions, which may significantly affect their antigenicity. The field strain CU/1/2014 identified in Egypt appeared to be a revertant of the H120 vaccine strain which had acquired higher virulence as a result of a gradual accumulation of point mutations. The H120 vaccine strain, which had been

used for a long time to control IB in Egypt, was likely to have been genetically unstable and to have caused outbreaks in some situations (1). It cannot be excluded that a pathogenic variant was already present in the vaccine and became dominant in the field because of unknown selection pressure, causing disease in vaccinated chickens. The presence of different variants of the vaccine strain is thus to be regarded as an undesirable feature, and a greater homogeneity of the strains contained in the vial should be sought. Reversion to virulence of vaccine virus strains has been described in both veterinary and human medicine. Subpopulations of avian metapneumovirus present in vaccines have been shown to induce respiratory disease in vaccinated turkeys (8, 19, 31). This has also been described as a human vaccine effect, but their return to virulence is rigorously monitored. During the production of the attenuated oral poliovirus vaccine (OPV), which consists of three serotypes of the Sabin strain, in some cases a mutation occurs at a specific site on the Sabin 3 strain genome which is directly related to neurovirulence. If the proportion of such revertants among all strains exceeds the limit of 0.9%, then the vaccine batch does not pass the quality control test and is not approved for use. Therefore, an important part of the quality control of this preparation is monitoring its genetic composition. This is routinely checked by quantitative testing of revertants in batches of live virus vaccines using the marker-assisted precise restriction-enzyme cleavage (MAPREC) test. This analysis is highly sensitive and reliable, but requires the use of radioisotopes, which complicates its use in quality-control laboratories. In close cooperation with the WHO, a French OMCL laboratory reported the implementation of an HTS-based test as an alternative method to MAPREC (2). Eight laboratories subsequently tested 11 OPV vaccines using HTS and MARPEC. This comparison confirmed that HTS is an equivalent method to MAPREC, and additionally guarantees shortened working time and eliminates the need to use radioactive isotopes (9).

The HTS technique offers unique possibilities in vaccine quality testing – the approach is direct (a digital record of all genetic information present in the sample is made), and it enables retrospective analyses through data archiving. The question is, why has this technology not been widely used in quality control so far? There seem to be two main reasons for this state of affairs. One of them is the amount of HTS research. However, while the cost of HTS can be substantial, each year we see that it is more affordable and popular. This is due to increased availability and optimisation of method capacity. The prognosis seems to confirm this trend. Furthermore, when we consider the cost of HTS research, we must also consider the opportunity cost in potential economic losses due to vaccine inactivity or the introduction of wild-type viruses into national and EU poultry production. These adverse outcomes could have a significant impact on both vaccine producers and poultry farmers, particularly in Poland, given the scale of the country's poultry industry. In this context, the cost of a single HTS analysis per batch appears more justifiable. This approach would guarantee the widespread availability of bioinformatic tools exploiting HTS to control vaccine quality. However, the second and main reason for not using HTS in IVMPs quality control so far seems to be the lack of guidelines for its use from the controlling bodies. That is why the prospect of quality testing using HTS as an alternative method is currently even more promising, as it coincides with the work on the implementation of a new chapter of the European Pharmacopoeia fully devoted to HTS technology and laying down guidelines regarding method validation (27): 2.6.41 "High-throughput sequencing for the detection of extraneous agents in biological products".

Conclusion

High-throughput sequencing is an effective alternative poultry IVMPs quality control tool for implementation by OMCLs. This technique makes possible the in-depth characterisation of vaccine strains, and, above all, the assessment of their conformity to the manufacturer's declaration. Importantly, HTS brings new cognitive value to IVMPs quality control because it allows monitoring of the level of revertants. However, there are some needs which should be met by policy setters such as the EDQM in cooperation with OMCLs but also by the manufacturers of vaccines: the implementation of genomic testing guidelines, the creation of dedicated bioinformatics tools with strictly defined analysis parameters, the conduct of proficiency testing and the determination of the genomes of reference organisms. The present study constitutes the basis for these activities and for HTS application to more vaccine groups.

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