AHEAD IN
ANIMAL HEALTH

Retrospectieve screening van rundvee op aanwezigheid van antistoffen tegen hoog pathogene aviaire influenza

Eindrapportage (in Engels)

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Samenvatting

Introductie

In maart 2024 is het hoog pathogene aviaire influenza (HPAI) virus subtype H5N1 (HA clade 2.3.4.4b) gevonden bij melkkoeien in Texas, Verenigde Staten (VS). Vervolgonderzoek wees uit dat het virus binnen en tussen bedrijven is verspreid over meerdere staten van de VS. Daarnaast is er ook overdracht naar andere zoogdieren gezien, waaronder ook mensen. Het H5N1 virus komt ook voor in Europa, echter is het virus in Europa nog niet gemuteerd zoals in de VS. In Nederland is er een basismonitoringsprogramma, waardoor de verwachting was dat een grote HPAI uitbraak onder runderen in Nederland zou zijn gedetecteerd. Echter er is altijd een kans dat een beperkt aantal ongedetecteerde H5N1 infecties bij runderen hebben kunnen plaatsvinden, mede ook omdat er een aantal grote uitbraken zijn geweest in wilde en commercieel gehouden vogels in 2021-2023 in Nederland.

Jaarlijks worden er tienduizenden serummonsters van runderen getest voor verschillende pathogenen als onderdeel van verschillende monitoringsprogramma's bij Royal GD. Van deze monsters wordt elke week een random blok van 96 monsters verzameld en bewaard bij -20°C, welke op verzoek voor een retrospectieve screening kunnen worden gebruikt. Deze monsters gaven de mogelijkheid om te onderzoeken of runderen in Nederland in de periode 2022-2024 geïnfecteerd zijn geweest met HPAI virus. Het doel van dit onderzoek was te onderzoeken of er een indicatie is dat Nederlandse runderen in 2022-2024 mogelijk besmet waren geraakt met HPAI virus, door middel van het onderzoeken van aanwezigheid van antistoffen tegen AI H5/H7.

Materiaal en methode

Om aan te kunnen tonen dat de Nederlandse rundveepopulatie vrij is van HPAI virus, dienden tenminste 512 serummonsters onderzocht te worden, waarbij in geen enkel monster antistoffen werden aangetoond (uitgaande van een detectieprevalentie van 1% en met 99% betrouwbaarheid). Gegeven dat de beschikbare monsters niet volledig willekeurig en onafhankelijk waren, is besloten om minimaal het dubbele aantal monsters te onderzoeken. Er waren 11.200 ingevroren serummonsters aanwezig uit de periode januari 2022 tot februari 2024. Hoog-risico serummonsters werden geselecteerd op basis van de volgende criteria; 1) de metadata over het rund waarvan het monster was genomen moest compleet zijn met betrekking tot de leeftijd, geboorteland en locatie van het bedrijf waar het rund stond, 2) het rund waarvan het monster was genomen moest in Nederland zijn geboren, 3) het rund moest ten minste 2 jaar oud zijn op het moment dat het monster werd genomen en 4) het bedrijf waar het rund zich bevond moest zich bevinden in een tweecijferig postcodegebied waar HPAI H5 virus was gevonden in wilde vogels, zwerfkatten e/o pluimvee. Van de 11.200 beschikbare serummonsters, waren er 2.513 (22%) geclassificeerd als hoog-risico monsters. Doordat de serummonsters waren opgeslagen per 96 in blokken, en het selecteren van de individuele geselecteerde monsters/buizen tijdrovend en foutgevoelig zou zijn, werd besloten om blokken te selecteren waarvan ten minste 35% van de serummonsters aan alle bovengenoemde criteria voldeden, en dus konden worden geclassificeerd als hoog-risico monsters. Dit waren 23 blokken, met 2.206 serummonsters, waarvan er 1.062 aan alle criteria voldeden. De meeste monsters die niet voldeden aan de criteria, vielen af omdat het serummonster afkomstig was een dier dat jonger was dan 2 jaar.

Van de 2.206 monsters konden er 16 niet getest worden (omdat er bijvoorbeeld niet genoeg serum aanwezig was), waarvan er 11 hoog-risico monsters waren. De rest (2.190 monsters) werd getest met een Influenza A blocking ELISA. Positieve monsters zijn vervolgens naar Wageningen Bioveterinary Research (WBVR) gestuurd om daar te worden geconfirmeerd met de Luminex assay op aanwezigheid van antistoffen tegen H5/H7 en alle NA. Met de

Influenza A blocking ELISA wordt geen onderscheid gemaakt tussen de verschillende subtypes van Influenza A. De Luminex assay kan de HA (H5 en H7) en NA (N1 t/m N9) wel typeren.

Resultaten

Van de 2.190 geteste serummonsters, waren er waren er 1.051 die als hoog-risico monsters zijn geclassificeerd. De geteste monsters kwamen van dieren uit heel Nederland, echter niet uit alle tweecijferige postcodegebieden waar HPAI virus was gevonden in wilde vogels, zwerfkatten e/o pluimvee. Dit gold zowel voor alle geteste monsters, als voor hoog-risico monsters. De gebieden waar geen serummonster vandaan kwam waren gebieden met een lage rundveedichtheid. De 2.190 geteste serummonsters waren afkomstig van 367 bedrijven met een gemiddelde van 6 monsters per bedrijf (mediaan: 3).

Vier van de 2.190 geteste serummonsters (0.2%) reageerden positief in de Influenza A blocking ELISA. Deze monsters zouden ook vals-positief kunnen zijn, aangezien de specificiteit van de Influenza A blocking ELISA 99,8% is bij pluimvee. De vier monsters waren afkomstig van vier verschillende bedrijven verspreid over Nederland. Drie monsters waren ingestuurd in 2022 en het vierde monster was ingestuurd in 2023. Drie van de vier monsters waren hoog-risico monsters. Het vierde dier was jonger dan 2 jaar. Alle vier de monsters waren in de Luminex assay negatief. Dit geeft aan dat de runderen geen antistoffen hadden tegen HPAI H5 of H7 virus.

Conclusie

Ondanks dat er vier monsters positief waren in de Influenza A blocking ELISA, waren deze negatief voor antistoffen tegen aviair influenza virus subtype H5N1 (waar in de VS de runderen besmet mee zijn). Dit komt overeen met het feit dat we in Nederland in de monitoring de afgelopen jaren geen klinische verschijnselen hebben gezien, passend bij een HPAI virus infectie bij runderen. De conclusie van deze studie is dan ook dat er geen indicatie is voor een infectie met HPAI virus bij runderen in Nederland uitgaande van een detectieprevalentie van 1% en 99% betrouwbaarheid.

De gedetailleerde rapportage is in het vervolg van dit rapport terug te vinden. In overeenstemming met de opdrachtgever is deze in het Engels geschreven.

1 Introduction

Avian Influenza is a disease caused by an influenza A virus. The different subtypes of this virus can be divided into low and high pathogenicity variants. High pathogenic avian influenza (HPAI) virus specifically pose a threat to both wild birds and poultry globally. Especially the subtype H5N1 clade 2.3.4.4b, which is persisting in wild bird reservoirs in many parts of the world, is of great concern because of its potential spill-over into mammals, including humans. H5N1 infections have been reported in e.g. seals, foxes, mink and cats all over the word, but also in the Netherlands (e.g. Kopfleisch et al., 2007; Thiry et al., 2007; Marschall and Hartmann, 2008; Floyd et al., 2021; Rijks et al., 2021; Agüero et al., 2023; Bordes et al., 2023; Elsmo et al., 2023; Mirolo et al., 2023; Sillman et al., 2023).

In March 2024, the first case of HPAI virus subtype H5N1 of the HA clade 2.3.4.4b was detected in dairy cows in Texas, USA (Oguzie et al., 2024). Clinical signs included decreased feed intake and rumination time, abrupt drop in milk production, lethargy, mild respiratory signs, moderate fever, abnormal bowel movements, and colostrum-like yellow to brown-red milk colour with thick, sometimes curdled, consistency (Caserta et al., 2024; Oguzie et al., 2024). After the first detection in Texas, virus was detected in dairy cattle herds across multiple states in the USA, with spill-over to other mammals, including humans (Burrough et al., 2024; Caserta et al., 2024; USDA, 2024; Uyeki et al., 2024). Efficient cow-to-cow transmission of the virus, has been shown in the USA (Burrough et al., 2024; Caserta et al., 2024).

The situation regarding HPAI virus subtype H5N1 in the USA caused concerns in the Netherlands, even though the genotype of H5N1 clade 2.3.4.4b found in the USA has not been found yet in Europe. The Dutch monitoring and surveillance system for cattle health is very effective for detection of disease (Santman-Berends et al, 2016; Veldhuis et al. 2016; Vredenberg et al, 2023). Therefore, expectations would be that no large, clinical HPAI virus outbreak in cattle could go undetected. However, it is possible that a sporadic H5N1 infection in Dutch cattle might have occurred because of exposure due to the multiple outbreaks of HPAI virus in wild and commercial bird populations in the Netherlands between 2021-2023 (Wolters et al., 2024; WUR, 2023). At Royal GD (Deventer, The Netherlands), yearly ten thousands of serum samples of Dutch cattle are tested for various endemic pathogens as part of routine diagnostics in cattle herds. Of these samples, every week a random block with 96 serum samples are collected and stored at -20°C to enable the possibility for retrospective screening. This set of samples provided the opportunity to evaluate whether cattle in the Netherlands had been infected with HPAI virus. Therefore, the aim of this study was to retrospectively (2022-2024) screen Dutch dairy cattle for possible infections with HPAI virus using serological tests for the detection of AI H5/H7 antibodies.

2 Materials and Methods

2.1 Influenza A blocking ELISA

An Influenza A blocking ELISA (Idexx, Westbrook, USA) was used for the detection of antibodies to an avian influenza virus. This accredited test was already used in the Netherlands to detect influenza A antibodies in serum from poultry, wild birds, horses, minks and pigs. The sensitivity is estimated at 89-100% (data Royal GD) and the specificity at 99.8% in poultry. This blocking ELISA has been successfully used in the USA cattle cases, and has also been used by the German reference institute Friedrich-Loeffler-Institut (FLI) in a H5N1 challenge study in cattle (Kalthoff et al., 2008). To check on cross reactions with antibodies against the bovine Influenza D virus, 10 sera of Influenza D infected cattle with high Influenza D HAR titres were tested in the Influenza A blocking ELISA, all with negative results.

2.2 Selection of serum samples

From January 2022 to February 2024, 11,200 frozen cattle serum samples were available. These were anonymized for further analysis. To enable detection of 1% prevalence with 99% confidence using the sensitivity and specificity of the Influenza A blocking ELISA in poultry, 512 samples of these should be tested (WinEpi, 2024). Given that the sensitivity of the test is based on poultry and pig samples, and the test characteristics in cattle samples are unknown, it was decided to test at least the double amount of samples to ensure sufficient sensitivity. Testing more samples would also have enabled us to correct for repeated measures within herds, since the samples within a serum block were not independent from each other because often samples from multiple cattle in a herd is submitted and end up in the same block. Additionally, it was decided to select the potentially high-risk samples for this study. Samples were classified as high-risk samples based on the following criteria:

1. All information on the cow from which the sample was taken had to be available, i.e. age, place of birth and location where the herd was located.
2. The cow from which the sample was taken had to be born in the Netherlands.
3. The cow from which the sample was taken needed to be at least 2 years old at the moment of sampling, so it was likely that it has been on pasture during their life and has been lactating prior to sampling. Also, data from the USA suggested that older cows have a higher risk compared to young stock.
4. The cow needed to be housed in a two-digit postal code area where HPAI virus had been detected during our study period in in either wild birds, stray cats or poultry (Figure 1), based on data from the Faculty of Veterinary Medicine at Utrecht University, The Netherlands Food and Consumer Product Safety Authority (NVWA), and Wageningen Bioveterinary Research (WBVR).

Of the 11,200 stored serum samples 2,513 (22%) were classified as high-risk samples. The majority of the samples that were not high-risk samples originated from cattle younger than 2 years.

The serum samples were stored per set of 96 samples in serum blocks, and selecting individual serum samples was logistically challenging, and prone to selection errors. Therefore, it was decided to select complete serum blocks to be included in the study, if at least 35% of the serum samples in that serum block met the criteria to be classified as

high risk, and were therefore high-risk samples. This resulted in 23 serum blocks with 2,206 serum samples, of which 1,062 sera (48%) were high risk samples. Of these 2,206 samples 768 were collected in 2022 (of which 376 were high risk samples), 1,055 in 2023 (520 were high risk samples) and 383 in January and February of 2024 (166 were high risk samples).

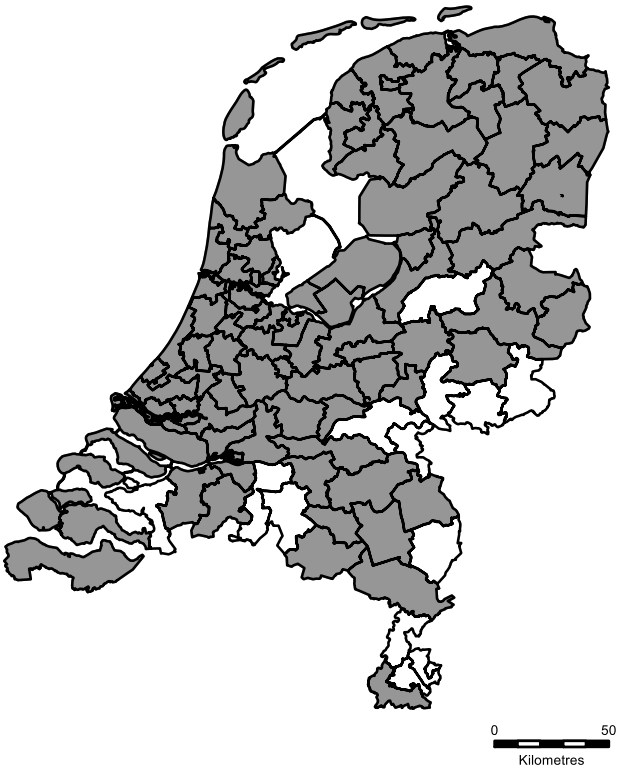

Figure 1. Two-digit postal code areas in the Netherlands where HPAI virus has been found in wild birds, stray cats or poultry during the period January 2022 – February 2024 shaded grey.

2.3 Influenza antibodies detection and subtyping

Of all 2,206 serum samples 2,190 were tested individually with the Influenza A blocking ELISA according to the manufacturer's protocol (Idexx, Westbrook, USA). The remaining 16 samples could not be tested, due to the fact that not enough serum was available. Samples were deemed to be positive with a S/N ratio (serum to negative ratio) below 0.5. The Influenza A blocking ELISA does not differentiate between the different subtypes of the influenza A virus. ELISA-positive serum samples (accompanied by the same number of ELISA-negative serum samples) were sent to WBVR for confirmation. Through the strategy of combining the Influenza A blocking ELISA and the test positive samples in the Luminex, the combined specificity of the tests is assumed to be 100%.

The confirmation test was a Luminex assay, a multiplex assay based on Luminex technology and previously described in Germeraad et al. (2019). The assay contains a subset of previously described recombinant proteins haemagglutinin and neuraminidase: H5 (n=5), H7 (n=3), N1 (n=3) and N2-N9 (n=1 for each). The recombinant proteins were coupled to colour-coded magnetic beads. Binding of serum antibodies to the antigens on the beads were detected by fluorescent secondary antibodies, and using the Luminex MAGPIX device, the different beads were identified. This allows subtyping of antibodies of H5, H7 and N1-N9.

3 Results

The 2,190 tested serum samples originated from cattle from 367 different herds with on average 6 samples per herd (median: 3). The interquartile range of the number of samples per herd was 1-5. In total 1,051 of the tested serum samples were classified as high-risk samples. These samples were from cattle from 142 different herds with on average 7 samples per herd (median: 2). Of the 1,139 serum samples that were not classified as high-risk samples, 888 were from cattle younger than 2 years (Figure 2), 170 were from cattle not born in the Netherlands, 63 were from cattle located in two-digit postal code areas where HPAI virus has not been found in wild birds, stray cats or poultry, and 18 had incomplete data. The herds from which the samples originated were distributed across the Netherlands, however not all two-digit postal code areas were represented (Figure 3A). This was the case for all the tested serum samples as well as the tested high-risk samples serum samples (Figure 3B). The number of serum samples tested per two-digit postal code area was in line with the cattle density in the Netherlands, and the two-digit postal code areas that were not represented were mainly areas with a low cattle density (Figure 3C).

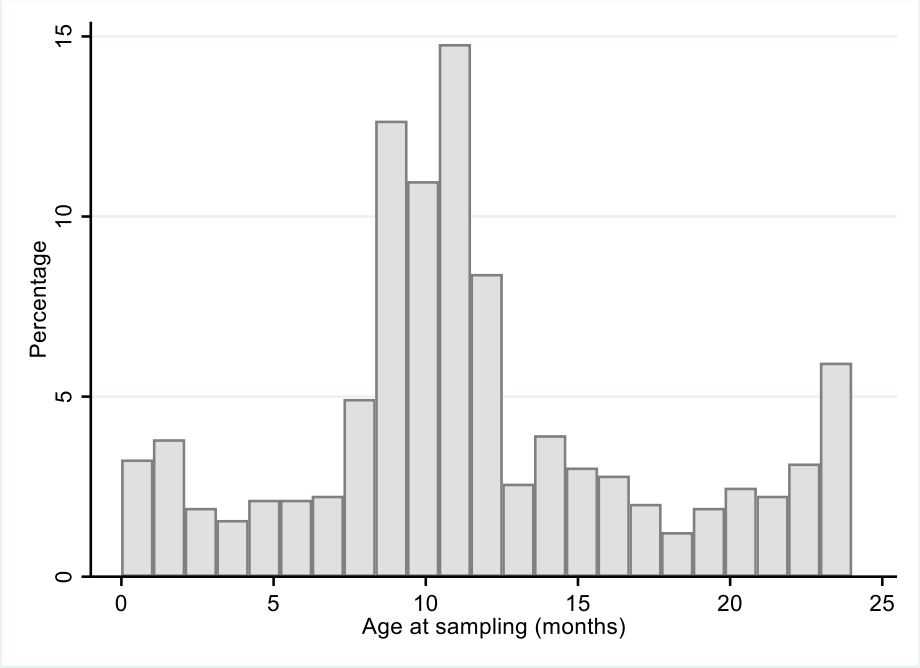

Figure 2. Age distribution of the cattle that were younger than 2 years at the moment the serum sample was taken, and were therefore not classified as high-risk samples in this study.

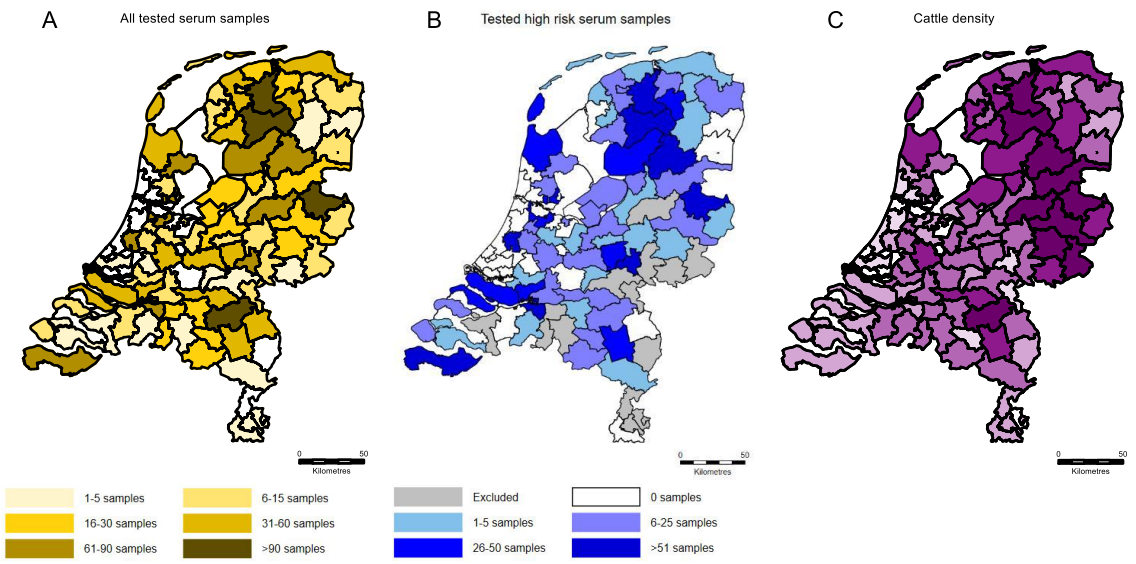

Figure 3. Number of tested serum samples (n=2,190) (A) and the number of tested serum samples that were classified as high-risk samples (n=1,051) (B), alongside the cattle density in the Netherlands (C) per two-digit postal code area.

Of the 2,190 tested serum samples, four samples (0.2%) were positive in the Influenza A blocking ELISA (Table 1), all from different serum blocks. Of the four seropositive serum samples, three were classified as high-risk samples while one serum sample was from cattle younger than 2 years old. The four samples were submitted in 2022 (n=3) or 2023 (n=1) and were collected from cattle from four different herds located in different areas in the Netherlands. Subsequently, these four ELISA positive serum samples, together with four random serum samples that were ELISA negative, were send to WBVR for confirmation. In the Luminex assay, all eight samples turned out to be negative (Table 1). This means that in none of the samples antibodies against H5, H7 and N1-N9 were found. This indicates that the four initial positive samples might have been false-positive in the Influenza A blocking ELISA, which fits with the 99.8% specificity as described in paragraph 2.1. The average S/N ratio of serum samples that were negative in the Influenza A blocking ELISA was 1.014 (median 1.013) with an interquartile range of 0.940-1.095.

Table 1. Results of the Influenza A antibody blocking ELISA (performed at Royal GD) and Luminex assay of the eight samples send for confirmation to Wageningen Bioveterinary Research (WBVR). Positive results (S/N ratio ≤ 0.5) are highlighted in bold.

Sample number	Influenza A blocking ELISA (Royal GD)		Luminex assay
	S/N ratio	Result	(WBVR)
1	**0.265**	Positive	Negative
2	**0.402**	Positive	Negative
3	**0.485**	Positive	Negative
4	**0.476**	Positive	Negative
5	1.114	Negative	Negative
6	1.135	Negative	Negative
7	1.052	Negative	Negative
8	1.090	Negative	Negative

4 Discussion and conclusion

Following an outbreak with the HPAI subtype H5N1-virus of clade 2.3.4.4b in cattle in the USA, we have investigated whether Dutch cattle have been infected with HPAI subtype H5N1. Even though four serum samples were initially found to be positive in the Influenza A blocking ELISA-test, it was concluded that these might have been false-positive in the Influenza A blocking ELISA, since none of them were positive in de Luminex assay and thus no antibodies against H5, H7 and NA have been found. This is in line with the fact that, there was no indication of clinical cases in cattle in the Netherlands in the past years in the national monitoring and surveillance system. Furthermore, our results are in line with a study in Germany where 1,400 bovine serum samples were tested for HPAI antibodies and 350 bulk milk samples for the virus, with no positive results (FLI, 2024).

The Influenza A blocking ELISA that was used was not validated for use in cattle samples. However, blocking ELISAs are generally suitable for testing different species. Moreover, in an experimental H5N1 challenge study performed by FLI, antibodies were detected post infection using the same ELISA (Kalthoff et al., 2008). The Influenza A blocking ELISA has been validated for poultry, and the specificity of 99.8% in this validation fits with the results that antibodies were initially found in four serum samples. This makes it likely that the results were actually false-positive.

The tested serum samples were not random selected given that the selection was based on convenience sampling of samples that veterinarians and farmers send to Royal GD voluntarily. There might be veterinarians and farmers in the Netherlands that never send samples from their herds to Royal GD, and could therefore never been included. Additionally, the tested serum samples were not completely independent because, within serum blocks multiple samples from one herd could be stored, and animals from the same herd might have been exposed to the same risk factors. However, since we have tested a larger number of samples than strictly necessary, we have compensated for these challenges. Furthermore, with an average of 6 samples per herd and a median of 3 samples per herd, there was not one herd where an extreme amount of samples originated from. Another reason why the tested serum samples were not random, is the fact that not all two-digit postal code areas where HPAI was found in wild birds, stray cats and poultry were represented. However, the areas of which no samples were available, were areas with low cattle density. The more dense cattle areas were well represented (Figure 3). Furthermore, the tested serum samples were not random because they were selected using a risk-based method to maximize the probability of detecting HPAI, if present.

In conclusion, there is no indication that HPAI H5N1 virus has circulated in cattle in the Netherlands in 2022-2024 given a design prevalence of 1% and 99% confidence.

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