A longitudinal study of the incidence of Avian Infectious Bronchitis in France using strain-specific haemagglutination inhibition tests and cluster analysis

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SUMMARY

The main objective of this study was to describe the Haemagglutination Inhibition (HI) titres observed in the field for the three most prevalent Avian Infectious Bronchitis (IB) strains in France (M41, 793B and QX) and to suggest rules of interpretation of these results, considering vaccine-induced antibodies and possible between-strains cross-reactions. A secondary objective was to describe the incidence of those strains.

A cohort study was carried out in a population of broilers with a lifespan of at least 45 days vaccinated only once, at the hatchery, with a live vaccine type H120. Two series of 10 blood samples were performed in each flock. The sera were analysed by HI for the three serotypes. A typology of the flock profiles, using Principal Components Analysis and Ascending Hierarchical Clustering was performed. The results of 69 flocks were available for analysis. The mean titres were the highest for serotype M41 at both sample points. The correlations between the titres of the various serotypes were significant, apart from the one between M41 and 793B at second sampling. Clustering allowed classifying the flocks in 5 clusters. We formulated and discussed the hypothesis that: clusters 1 and 2 contain vaccinated flocks not infected by a field virus, clusters 3 and 4 include vaccinated flocks challenged by a field virus of serotype M41 and cluster 5 includes vaccinated flocks challenged with a field virus of serotype 793B. From these HI results we concluded that the incidence rate of IBV infection in France could be estimated at 61% (CI95: 48-72), the serotype M41 representing 69% of cases.

Keywords: Avian Infectious Bronchitis, Broiler, haemagglutination inhibition tests, Cluster analysis

INTRODUCTION

Infectious bronchitis (IB) is one of the main diseases in chicken (Gallus gallus) due to its high contagiousness, its prevalence and its high economic impact [5]. The pathogen responsible for IB is a coronavirus, which is very variable due to mutations or recombinations, on account of its size and RNA genome [4, 11].

The diagnosis of infectious bronchitis may be facilitated using HI (haemagglutination inhibition), VN (virus neutralisation) and ELISA (enzyme-linked immunosorbent assay) on serum samples, or with PCR and sequencing of tracheal or cloacal swabs. The advantage of serological based diagnostics (HI, V1 and ELISA) is the historical aspect that they bring to the clinical case; the result measures the reaction of the bird to the disease causing agent over a period of time whereas direct diagnostic (PCR) requires the presence of the
Prevention of IB relies on vaccination, which is widely used in the field. However, the heterologous protection conferred by vaccination is very variable and often insufficient. In order to adjust the vaccination protocols, it is therefore necessary to know which field viruses are circulating. The various viral strains of IB can be classified according to serotype, genotype and protectotype. The different laboratory techniques allow the classification of strains according to serotype using virus neutralisation, or by genotype, using RT-PCR together with sequencing or a second specific-primer PCR, or, more rarely, using restriction fragment length polymorphism (RFLP) [5]. Furthermore, strains can be classified in protectotypes according to the protection spectra of the vaccines.

A genotype study of the IB virus in several Western European countries was carried out on broilers, layers and breeder flocks of Gallus gallus, from 2002 to 2006. The IB virus was identified by RT-PCR on tracheal swabs following clinical suspicion. Vaccinal and field strains could not be differentiated [16]. The three most prevalent strains in France were 793B (54% of the positive results), Massachusetts (Mass, 23%) and QX (12%). Strain 793B, to which the vaccine strains M41, 793B and QX can be linked [7]. The QX strain emerged in China in the late 1990s before appearing in Europe [1, 2, 17].

In this context, the main aim of this study was to describe the HI titres observed in the field for the three most prevalent strains in France and to suggest rules of interpretation of the HI results, considering vaccine-induced antibodies and possible between-strains cross-reactions. A secondary objective was to describe the incidence of the IB serotypes M41, 793B and QX in France in 2011.

**MATERIALS AND METHODS**

**STUDY DESIGN**

A cohort study was carried out in a broiler population vaccinated using a single vaccination against BI, and having a long enough lifespan to observe seroconversion.

The study population consisted of flocks of broilers with a lifespan of at least 45 days, with placement starting the first semester of 2011, receiving a single IB H120 vaccination at the hatchery. According to this criterion, two categories of flocks were included: “label rouge” type flocks (age at slaughter over 81 days) and “heavy” broiler type flocks (age at slaughter between 45 and 50 days).

The aim was to include 130 flocks, selected by cluster sampling. As a first step, 12 production organisations from all over France were selected and a number of flocks to be included was attributed to each of these organisations, according to their production potential. As a second step, flocks were chosen by the production organisations according to the date of placement and their geographical location. Flocks from the same organisation should be located at least 5km apart from each other. Presence of clinical signs indicative of infectious bronchitis was not a selection criterion.

**SAMPLING AND LABORATORY ANALYSIS**

Two series of 10 blood samples taken at least 2 weeks apart were performed in each flock. The first blood sample was taken at around 8 weeks of age for the “Label Rouge” flocks and at around 3 weeks of age for the “heavy” flocks. The blood samples were centrifuged and the serum was deep-frozen (-20°C). In case of emergence of clinical signs evocative of infectious bronchitis (respiratory problems, wet litter, retarded growth, heterogeneity), 10 tracheal swabs were taken within 7 days of the appearance of the signs. All samples were frozen and sent under frozen conditions to the IZS laboratory (Legnaro, Italy), were all samples were analysed.

The sera were analysed by HI for three serotypes (M41, 793B and QX) using the FLOCKSCREEn IB-HI kit (x-OvO Limited, Dumferline, United-Kingdom). The serum samples underwent serial halving dilution and were mixed with an equivalent amount of antigen. After incubation (30mn at 20°C), red blood cells from SPF chickens, prepared with an anticoagulant, were added. After incubation (40mn at 20°C), the results were read by inclination of the plaques and observation of the HI. The antibody titre of the serum sample, expressed in log10, corresponds to the highest serum dilution leading to complete inhibition. The highest tested titre is 12. The validity of results was ascertained by using a negative serum control sample that should not have a titre above or equal to 2 log10, and a positive serum control sample with a
titre that should be equal to the known titre plus or minus one dilution. The two sample series from the same flock were analysed simultaneously. For each flock, sample series and serotype, the arithmetic mean of the titre was calculated. For the rest of this article, the total of the six serological results for each flock (3 serologies x 2 sampling series) will be called "profile".

PCR and sequencing were only performed for the flocks for which there was suspicion of disease and in which tracheal swabs had been taken. Real-time generic PCR [3] was performed for IB. If this PCR was negative, the sample was considered negative for the IB coronavirus. If it was positive, sequencing was carried out after performing a nested RT-PCR. This nested RT-PCR is a two-step PCR with two pairs of different primers, with the second pair linking to the inside of the first amplicon. The first pair of primers was designed to attach to stable parts of the genome, and the second pair to amplify the genetic sequence needed for the sequence typing [6]. If the RT-PCR was positive, the hypervariable part of the genome of the S1 protein was sequenced and the sequence of amino acids was determined.

STATISTICAL METHODS

In the absence of a bimodal distribution of the results, which would have allowed defining an epidemiological cut-off value (Fig 2), a typology of the flock profiles was performed using clustering methods (Fig 1). This data-mining method allows grouping the flocks according to their resemblance without posing any prior hypothesis other than those used for the general study design (choice of flocks, age at sampling, sampling and testing).

As a first step, a principal components analysis (PCA) was carried out. PCA allows studying the overall variability of the dataset and creating new uncorrelated variables, summarising information that may well have a biological sense and that may contribute to differentiate flock groups. The study of the variability percentage explained by each of this new variables and their biological signification allow determining which of these new variables have to be retained for the rest of the analysis.

As a second step, ascending hierarchical clustering (HC) was performed. HC is based on the calculation of distances between individuals and then assembling them in groups according to proximity. The variables used in the HC to calculate the distances are the coordinates of the flocks on the retained axes by the PCA and we used Euclidian distances. The HC leads to a dendrogram used to define the optimal cluster number. This choice depends on the clarity of the separation between the dendrogram branches and on the number of flocks in each group.

As a third step, the means of the new variables created at the PCA step are calculated for each of the groups and, using the k-means method, each flock is reassigned to the group with the nearest mean. The representation of these groups on the PCA axes allows determining the discriminatory axes between groups. If certain axes turn out to be non-discriminatory, a new iteration of the analysis is performed.

**Figure 1:** Steps of the data analysis

**Figure 2:** Distribution of the mean titres of each flock according to the serotype and the time of sampling
Finally, as a fourth step, the distribution characteristics of the 6 mean serological titres within each group are described and the biological meaning of these groups is investigated.

All these analyses were performed using the R programme (version 2.13.1).

RESULTS

PARTICIPANTS

91 flocks of chickens were included in the study, 22 were lost to follow-up or excluded resulting in 69 flocks available for analysis. The causes of losing to follow-up and exclusion were the lack of performing one of the blood sample series (n=7), a sample preservation error (n=1) or a vaccination protocol other than a single vaccination at hatchery by spray with a H120 strain without booster (n=14). The 69 flocks included in the analysis were followed by 11 production organisations and were distributed over 16 departments of mainland France. 52 flocks were of the “Label Rouge” type and 17 of the “heavy” type.

The first series of blood sampling was carried out on an average of 57.2 days (SD=11.7 days) for the Label Rouge flocks and 30.1 days (SD=6.4) for the “heavy” flocks. The mean interval between the two blood sampling series was 20.1 days (SD=6.4) and was similar for both production types (test de Mann-Whitney, p=0.06). A total of 1,413 blood samples were taken, i.e. an average of 10.2 samples per series. Due to the presence of several samples with an insufficient quantity of serum, the number of results available for analysis was 1,402 for serotype M41, 1,408 for serotype 793B and 1,401 for serotype QX.

OUTCOME DATA

The distribution of mean titres observed for each serotype (793B, M41 and QX) and each sampling series (P1 and P2) are shown in Table I and Fig 2: Distribution of the mean titres of each flock according to the serotype and the time of sampling. The mean titres are higher for serotype M41 than for the two other serotypes. The correlations between the titres of the various serotypes are significant, apart from the one between M41 and 793B at second sampling. The strongest correlation was found between 793B and QX at second sampling (r =0.69, Table II).

CLASSIFICATION

Six new variables are created as outcome of the PCA step. The first variable tends to discriminate between flocks with high titres from those with low titres, independent of the serotype. This variable describes 48% of the observed variability. The second variable discriminates between flocks with high M41 titres from those with high 793B titres. This variable describes 20% of the observed variability. The third variable discriminates between flocks according to their difference in titres between the two sampling series. It describes 15% of the observed variability. The fourth variable discriminates according to the QX titre. It describes 11% of the observed variability. The two remaining variables describe a residual variability to which no biological meaning can be attributed. Only the first four variables have therefore been

<table>
<thead>
<tr>
<th></th>
<th>M41</th>
<th>S1</th>
<th>S2</th>
<th>793B</th>
<th>S1</th>
<th>S2</th>
<th>QX</th>
<th>S1</th>
<th>S2</th>
</tr>
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<td>2.7</td>
<td>2.0</td>
<td>2.8</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st Qu.</td>
<td>4.8</td>
<td>5.2</td>
<td>3.9</td>
<td>3.9</td>
<td>3.8</td>
<td>3.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>5.5</td>
<td>6.0</td>
<td>4.5</td>
<td>4.4</td>
<td>4.4</td>
<td>4.3</td>
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<td></td>
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<tr>
<td>Mean</td>
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<td>6.1</td>
<td>4.8</td>
<td>5.0</td>
<td>4.4</td>
<td>4.5</td>
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<tr>
<td>3rd Qu.</td>
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<tr>
<td>Max.</td>
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<td>9.1</td>
<td>7.8</td>
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<td>6.5</td>
<td>8.1</td>
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Table I: Distribution of the mean titres for the 3 serotypes and 2 sampling series (S1=first sampling, S2=second sampling, n=69 flocks)

<table>
<thead>
<tr>
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<th>First sampling</th>
<th>Second sampling</th>
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<tr>
<td></td>
<td>r</td>
<td>p</td>
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<td>M41 vs. 793B</td>
<td>0.33</td>
<td>&lt; 0.001</td>
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<tr>
<td>QX vs. 793B</td>
<td>0.49</td>
<td>&lt; 0.001</td>
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<tr>
<td>QX vs. M41</td>
<td>0.45</td>
<td>&lt; 0.001</td>
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</table>

Table II: Correlation between the mean titres and the three serotypes for the two sampling series.
INCIDENCE OF AVIAN INFECTIOUS BRONCHITIS IN FRANCE

HC allows assembling the flocks in 5 clusters (Fig 3).
In the end, after final assignment of each flock to a cluster using the k-means method, the clusters contain 9 to 18 flocks each (Table III, Fig 4). Clusters 1 and 2 show low titres, even though slightly more elevated for serotype M41 than for the two others. These two clusters are characterised by the evolution between the two sampling series: the trend is towards seronegativisation in group 1 and towards seropositivisation in group 2. Cluster 3 shows titres that are low and stable for 793B and QX, while they are high for M41. In this group, the M41 titres are the most distinct from the other serotypes (1.8 difference at first sampling, 2.0 at second sampling). Furthermore, the 793B and QX titres are stable between the two sampling series while there is a trend of an

for M41 and high titres for 793B and QX. The difference between M41 and 793B titres is not greater than in groups 1 and 2. Finally, cluster 5 includes the flocks for which the M41 titres are relatively low (mean titre only 0.5 higher than cluster 2) while the 793B titres are high.

DISTRIBUTION BETWEEN CLUSTERS ACCORDING TO PRODUCTION ORGANISATION

Analysis of the distribution of clusters within each of the production organisations shows differences between

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Obs</th>
<th>M41 P1</th>
<th>M41 P2</th>
<th>793B P1</th>
<th>793B P2</th>
<th>QX P1</th>
<th>QX P2</th>
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<tr>
<td>1</td>
<td>18</td>
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<td>5.0</td>
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<td>3.7</td>
<td>4.6</td>
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</tr>
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<td>2</td>
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<td>3.9</td>
<td>5.2</td>
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<tr>
<td>3</td>
<td>18</td>
<td>6.0</td>
<td>7.1</td>
<td>4.2</td>
<td>4.1</td>
<td>4.0</td>
<td>3.8</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>7.2</td>
<td>7.5</td>
<td>6.3</td>
<td>6.7</td>
<td>5.7</td>
<td>4.2</td>
</tr>
<tr>
<td>5</td>
<td>13</td>
<td>5.2</td>
<td>5.7</td>
<td>6.2</td>
<td>7.2</td>
<td>4.4</td>
<td>6.1</td>
</tr>
</tbody>
</table>

Table III: Mean titres of the 6 HI titres for each cluster

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<tr>
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</tr>
<tr>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
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<tr>
<td>4</td>
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<tr>
<td>5</td>
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</table>

Table IV: Distribution of clusters according to the production organisation and to the mean age at first sampling

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organisations (Table IV). Note that for organisation G, all flocks have been classified in group 1, and for organisation C, the majority of flocks have been attributed to group 4.

**PCR AND SEQUENCING**

In four of the 74 flocks (6%) included in the study, there was a clinical suspicion and tracheal swabs were taken. The four samples turned out to be PCR-positive and sequencing was possible in three cases. The three flocks for which sequencing was carried out were sampled between 51 and 65 days of age. The sequenced serotypes were H120, 4/91 vaccine vial and CR88 (Table V).

**DISCUSSION**

Statistical analysis allowed classifying the flocks of our observational field study into five groups with homogeneous characteristics. The biological significance of this classification may be discussed in light of the data by De Wit [15], the best experimental reference available to interpret the results. As opposed to numerous experimental studies performed using SPF birds, De Wit reported the results of an experimental challenge using commercial chicks with maternal antibodies. Furthermore, the vaccinated flocks of his study used a live H120 vaccine at D0, like the flocks in our study.

The unvaccinated, unchallenged flocks of the De Wit study showed mean HI titres of between 3.0 and 4.2. These titres are comparable to those observed in clusters 1, 2 and 3 for serotypes 793B and QX.

The vaccinated, unchallenged flocks of the De Wit study showed mean M41 titres between 3.5 and 5.3. These titres are comparable to those observed in our study in clusters 1 and 2 for serotype M41.

De Wit suggests using a titre of 7 as cut-off value if HI is used for serotyping. This titre level was reached on average by three clusters: cluster 3 (for serotype M41 at second sampling), cluster 4 (for serotype M41 at both samplings) and cluster 5 (for serotype 793B at second sampling)?

On the basis of these data, we can formulate the hypothesis that clusters 1 and 2 contain vaccinated flocks not infected by the field virus, that clusters 3 and 4 include vaccinated flocks challenged by a virus serotype M41, and that cluster 5 includes vaccinated flocks challenged with a virus serotype 793B. Fifteen out of the 18 flocks of cluster 1 had less than 40 days of age at first sampling. Therefore, their exposure time was shorter than for other flocks and they were sampled closer to vaccination. These two points are compatible with the fact that they had low titres with a trend to seronegativisation. Clusters 3, 4 and 5, which we considered infected by the field virus, include 42 out of the 69 flocks in our study. The incidence rate of IBV infection in France can therefore be estimated at 61% (CI95: 48-72). Serotype M41 would represent 69% of cases (cluster 3 and 4) and serotype 793B, 31% (cluster 5).

The sequencing results are compatible with this interpretation of classification. The flock for which a H120 virus (serologically related to the M41 strain) was sequenced, is part of cluster 3 and flocks for which virus 4/91 and CR88 (serologically related to the 793B strain) were sequenced, are part of cluster 5. However, the fact that there were only few sequencings possible is a limitation to our study. The systematic PCR analyses and sequencing, even in the absence of clinical signs, would have been preferable. However, this would only have been possible by taking blinded tracheal swabs at set dates, which would probably have led to a low success rate of virus identification. One solution could have been to prefer cloacal swabs due to the longer persistence of BI virus in the cloaca. Nevertheless, serological analyses were preferred as the objective was to perform a study in a general population, and antibodies are the memory of infection.

One of the difficulties of interpreting the HI results is the cross reaction between the different serotypes, which we tried to objectivise in our study by identifying significant correlations between the mean titres of the three serotypes. Indeed, the serotype specificity of HI is reduced in case of reinfection, in particular if the serotype is heterologous. Successive inoculations with the IB virus may induce antibodies that react during HI with serotypes to which the chicken had never been exposed [14]. The interpretation of these tests is therefore delicate in animals with a long lifespan (layers and

<table>
<thead>
<tr>
<th>Flock</th>
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<th>Age at sampling</th>
<th>Seq. length</th>
<th>Identities</th>
<th>Seq. length</th>
<th>Identities</th>
<th>Closest strain</th>
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<td>FGUE151</td>
<td>3</td>
<td>65</td>
<td>283</td>
<td>99%</td>
<td>92</td>
<td>97%</td>
<td>H120</td>
</tr>
<tr>
<td>FGIR166</td>
<td>5</td>
<td>51</td>
<td>343</td>
<td>100%</td>
<td>114</td>
<td>100%</td>
<td>4/91 Vaccine Vial</td>
</tr>
<tr>
<td>FLEV159</td>
<td>5</td>
<td>65</td>
<td>315</td>
<td>99%</td>
<td>103</td>
<td>98%</td>
<td>CR88</td>
</tr>
</tbody>
</table>

Table V: Strain identification results
breeders), having had numerous vaccinations. To reduce this inconvenience, our study was limited to broiler flocks that only received one single vaccination, with a live vaccine. In spite of this, cross reactions were observed, in particular in cluster 4 where the titres were very high for the M41 serotype and high for serotypes 793B and QX.

The typological analysis method used allowed interpretation of the results in spite of the existence of cross reactions. However, this method requires certain choices (number of axes, number of clusters) that have an impact on the final result; it is not an absolute classification. However, the fact that the obtained classification is coherent with the data of experimental infections [15], even though the bibliographical data were not used for the classification, is in favour of its relevance. Studies including more flocks and more birds in each flock are needed to confirm this classification.

It is difficult to know if these results can be used as an interpretation grid for other genetics or different sampling ages. Nevertheless, we propose that the results of this study may be used for the classification of a new profile from a flock vaccinated with H120 at the hatchery, even if there is only one sampling series. This classification could be done graphically by comparing the titres of the profile to be classified with that of the various clusters. In particular, a decision rule could be that, in case of animals vaccinated with H120, infection may be suspected by a 793B virus if the 793B titres are equivalent to the M41 titres. However, we cannot give any indication of thresholds to differentiate 793B from QX infection. This could either be due to a high level of cross reaction between those two serotypes, or to a low incidence of QX infection, which did not allow the statistical analysis to identify a “QX cluster”.

The study population was limited to broilers with a lifespan of at least 45 days, since it was necessary, to have animals with a sufficiently long lifespan to observe seroconversion in case of viral circulation, but also to have animals vaccinated at day-old to limit the cross reactions during HI analysis. For this reason, the layers were excluded, as these are multi-vaccinated, and the standard broilers were excluded as they have a very short lifespan, which makes the observation of seroconversion difficult. However, in view of the horizontal spread capacity of the virus [10] and the distribution of studied farms in the main French production regions, we consider that the population studied (broilers with a lifespan of at least 45 days) may be considered as a sentinel population and that the results of serological incidence can be extrapolated to all French farms rearing *Gallus gallus*. Finally, the clustering methodology is a tool which could be used in other production areas to assist to HI results interpretation.

**Funding**

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**References**


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