Presence and Pathogenic Relevance of Antibodies to Clustered Acetylcholine Receptor in Ocular and Generalized Myasthenia Gravis

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**Background:** Clustered acetylcholine receptor antibodies (clustered AChR-Abs) have been detected in a proportion of patients with previously “seronegative” (SN) generalized myasthenia gravis (GMG), but their presence in patients with ocular MG (OMG) and their pathogenicity in vivo are unknown.

**Objective:** To test the presence of clustered AChR-Abs and their pathophysiologic properties in patients with SNMG.

**Design:** Screening and diagnostic tests.

**Setting:** Regional specialist myasthenia center and clinical laboratory.

**Patients:** Serum samples from 16 patients with SN and OMG were tested for binding to clustered AChRs. Results from 28 further SN patients (14 OMG) were correlated with their single fiber electromyography values.

**Main Outcome Measures:** Presence, complement-fixation capacity, correlation with neurophysiologic changes, and in vivo pathogenicity of clustered AChR-Abs.

**Results:** Up to 50% of patients with previous SN-OMG had complement-fixing IgG1 clustered AChR-Abs. IgG binding (n=28) and complement deposition (n=21) each correlated with the mean consecutive difference (jitter) on single-fiber electromyography. Injection of purified IgG from 2 patients with clustered AChR-Abs into wild-type or complement regulator–deficient mice reduced miniature end plate potential amplitudes to an extent similar to that found with AChR-Abs, and complement was deposited at the end plates. A trend was noted toward an increase in the number of packets of acetylcholine released (quantal content).

**Conclusions:** A proportion of patients with SN-GMG or OMG have clustered AChR-Abs that correlate with their electrophysiologic features. Clustered AChR-Abs can passively transfer disease to mice, demonstrating their pathogenicity, and the mechanisms seem similar to those of patients with typical AChR-Abs.


**MYASTHENIA GRAVIS (MG) is characterized by fatigable muscle weakness and responds to immunosuppressive therapy or plasma exchange. In 80% to 85% of patients with generalized MG (GMG), antibodies are directed against the acetylcholine receptor (AChR-Abs), but a small proportion of patients (5%-8%) have antibodies against muscle-specific kinase (MuSK-Abs). The remaining patients (approximately 10%) have been referred to as having “seronegative” (SN) MG. Many patients with MG are first seen with ocular symptoms and later develop generalized symptoms. However, a subgroup of patients does not generalize and is referred to as having ocular MG (OMG). Patients with OMG are less likely to be positive for AChR-Abs (approximately 50%). This leaves a much higher proportion of patients with OMG who are SN and whose diagnosis may be difficult. The MuSK-Abs are infrequent in OMG.

We recently developed a sensitive cell-based immunofluorescence assay that can detect AChR-Abs in approximately 60% of patients with SN-GMG. The assay is based on binding of immunoglobulin G (IgG) to AChRs expressed in a cell line and clustered by the intracellular scaffolding protein rapsyn. This method enables Abs that**

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bind only to high densities of AChRs (eg, those present at the neuromuscular junction) to be detected in vitro, and herein we call them clustered AChR-Abs; the patients have clustered AChR-MG. We asked whether clustered AChR-Abs are also present in some patients with OMG and whether complement-dependent mechanisms are likely to be important in their disease, as they are in typical MG. We correlated their complement-fixing ability with single-fiber electromyography (SFEMG) findings and demonstrated evidence for their pathogenicity in a passive transfer mouse model.

**IMMUNOCYTOFLUORESCENCE FOR COMPLEMENT ACTIVATION IN VITRO**

Complement activation was detected by identification of C3b or membrane attack complex on the transfected HEK cell surface after appropriate sensitization, as previously published. To increase detection, the intrinsic complement regulators on the HEK cell surface were first blocked using anti-CD55 and anti-CD59 blocking Abs. All the slides were stored at 4°C, protected from light exposure, and examined 16 to 24 hours later using a fluorescence microscope (Carl Zeiss, Ltd). The binding of the Abs or of the complement deposition was scored using the semiquantitative method validated previously on a scale from 0 (no binding) to 4 (very strong binding). Photographs were taken using a Zeiss fluorescence microscope and Openlab software (Openlab; PerkinElmer), with constant exposure times for all slides in any given experiment.

**FLOW CYTOMETRY**

Flow cytometry was used to confirm and quantify Ab binding (see the study by Leite et al). Briefly, cells transfected with AChR subunits and rapsyn without the enhanced green fluorescence protein tag were trypsinized and incubated with serum samples at 1:20 dilution in suspension. The IgG binding was detected using fluorescein-labeled anti–human IgG. The median fluorescence of IgG-bound (green) cells in the test samples was compared with that of cells incubated without any serum and that of cells with control serum incubation.

**PASSIVE TRANSFER TO MICE**

To identify the most appropriate patients, plasma exchange samples of several patients with SNMG were first screened for binding either to mouse neuromuscular junctions (in mouse leg muscle sections) or to HEK cells transfected with mouse AChR subunits clustered with rapsyn. Four plasma samples were identified that gave the best staining in both of these techniques (data not shown). Two of these samples were available in sufficient quantities for IgG purification and are referred to as SNMG1 and SNMG2. The IgG was purified using protein G sepharose.

The initial experiments were conducted in wild-type C57BL/6 mice, but to try to increase complement-mediated damage, mice lacking the genes Dafl/Dafl2 and CD59a/CD59b, which code for the complement regulatory proteins CD55 and CD59 and which have been shown to be more susceptible to complement-mediated autoimmunity, were used. The mice were from Harlan Olac Ltd but had been produced at Cardiff University using techniques described previously. To obtain Dafl–/CD59a–/ Dafl1–/CD59a– mice were cross-bred to obtain Dafl1–/CD59a– double knockouts. Reverse transcriptase–polymerase chain reaction was performed on the mouse ear clips to confirm the knockout status, as previously described (data not shown).

All the animal groups were injected intraperitoneally with corresponding amounts of IgG (10-50 mg/d for 3-5 days) for each experiment. They were assessed daily for weight loss, weakness (using ability to hang on to an inverted screen for at least 10 minutes), and general behavioral changes. Twenty-four hours after the last injection, mice were humanely killed. Phrenic nerve–hemidiaphragm preparations were used for ex vivo electrophysiologic studies.

**EX Vivo Electrophysiologic Studies**

Electrophysiologic studies were performed on mouse phrenic nerve–hemidiaphragm preparation as previously described.
Nerve-evoked end plate potentials (EPPs) and spontaneous miniature EPPs (MEPPs) were recorded intracellularly using conventional borosilicate glass electrodes and were filtered at 1 kHz. Up to 20 MEPPs followed by up to 20 EPPs (stimulated at 1 Hz) were recorded per end plate for later offline analysis. Recordings from many end plates were accumulated over 1 hour. The quantal content (number of packets of ACh released) was calculated by the ratio of the amplitude of EPP (corrected for nonlinear summation) to that of the corresponding MEPP.

IMMUNOFLUORESCENCE OF MOUSE NEUROMUSCULAR JUNCTION

To assess the intensity of AChR staining and the area of the postsynaptic apparatus, 1 hemidiaphragm of each animal was stretched and pinned down onto a Sylgard block (Dow Corning, Ltd) and was incubated with Alexa Fluor 594 (Invitrogen)–labeled α-bungarotoxin, 1:2500 dilution, for 30 minutes and protected from light. The preparation was washed thrice with Kreb solution, fixed in 3% formaldehyde, washed overnight at 4°C, and mounted on slides with fluorescence mounting me-

dia. The end plates were visualized using a Zeiss fluorescence microscope. Representative pictures from 20 to 30 end plates were taken using the Openlab software, and the area was measured using microscopy automation and image analysis software (MetaMorph; Molecular Devices LLC) by carefully drawing around each end plate with sharply defined margins and measuring the area using color thresholding.

Leg muscles were snap frozen in isopentane and were cryosectioned for visualizing IgG and complement deposition at the end plates. The cryosections were warmed gradually to room temperature and were fixed in 4% paraformaldehyde (Sigma-Aldrich Corp) (pH 7.4), blocked in combination of 2% fetal calf serum and 5% normal goat serum (Dako) in phosphate-buffered saline solution for 30 minutes, and incubated in the same solution with rabbit anti–human IgG (green-labeled, Alexa Fluor 488; 1:200 dilution) and α-bungarotoxin (red-labeled, Alexa Fluor 594; 1:5000 dilution), which stains the AChRs in the postsynaptic membrane. For C3b deposition, fixed and blocked sections were incubated with rabbit anti–human C3b (1:500 dilution) for 30 minutes. This was visualized using goat anti–rabbit IgG (green-labeled, Alexa Fluor 488; 1:200 di-

Figure 1. Cell-based assay using clustered acetylcholine receptors (AChRs) in myasthenia gravis (MG). A, Immunocytofluorescence of ε-AChR–, γ-AChR–, and muscle-specific kinase (MuSK)–transfected human embryonic kidney cells showing the presence of low-affinity AChR antibodies (Abs) in previously “seronegative” (SN) patients with ocular MG (OMG). The patient with MuSK-Abs (OMG2) detected using the cell-based assay also had ACHR-Abs (original magnification, x1000). EGFP indicates enhanced green fluorescence protein. B, The cell-based assay scores for the OMG and generalized MG (GMG) groups of SN patients were similar (unpaired t test, $P = .43$). C, Quantification by fluorescence-activated cell sorting of a representative patient (SN-OMG14). The background fluorescence is assessed using nonstained cells and the regions gated. Cells in the M1 region are considered to have background fluorescence, and those in the M2 region are thought to have specific immunoglobulin G (IgG) binding. D, Comparison of flow cytometry quantification of IgG binding in SN and AChR-positive OMG. There was a significant difference in IgG binding by the serum of SN and AChR-positive patients compared with that of controls (analysis of variance with Bonferroni multiple comparisons).
lution) with simultaneous α-bungarotoxin, as described previously herein. Secondary antibody incubation was performed for 45 minutes, protected from light. After further washing, sections were mounted and observed using a Zeiss fluorescence microscope.

RESULTS

CLUSTERED AChR-Abs IN PATIENTS WITH SN-OMG

Eight of 16 patients with SN-OMG (50%) had clustered adult AChR-Abs, similar to those found previously in patients with GMG.7 Examples are shown in Figure 1A in which some AChR-Ab–positive OMG and SN-OMG samples can be seen to bind strongly to clustered adult AChRs, whereas the healthy individual’s serum does not. In contrast to most patients with OMG studied, SN-OMG15 also bound to clustered fetal AChRs. Seronegative OMG2 did not bind to AChRs (data not shown) but did bind to MuSK when it was expressed on HEK cells (Figure 1A). Overall, the binding scores of the SN-OMG sera were not different from those of the SN-MGM samples tested for comparison (Figure 1B).

The results were confirmed using fluorescence-activated cell sorting. Figure 1C shows the fluorescence-activated cell-sorting profiles of a control, SN-OMG14, and an AChR-Ab–positive patient. The serum of patients with SN-OMG binds almost as strongly as the AChR-Ab–positive serum. Overall, the mean (SD) percentage of cells with specific IgG staining was 43.49% (21.97%) (n=12) for the SN-OMG group and 67.28% (11.25%) (n=4) for representative patients with AChR-OMG; both were different from the controls (16.2 [8.8], P<.001, r²=0.41) (Figure 1D, analysis of variance with the Bonferroni multiple comparison posttest).

CLUSTERED AChR-Abs ARE OF THE IgG1 SUBCLASS AND ARE CAPABLE OF ACTIVATING THE COMPLEMENT SYSTEM IN VITRO

The clustered AChR-Abs were predominantly the IgG1 subclass (data not shown) and were capable of activating complement in vitro (Figure 2). Activation of the terminal membrane attack complex C9neo was apparently a little stronger than that of the earlier component C3b. However, to demonstrate complement deposition by the SN-OMG samples, we needed to block the complement regulator system on the HEK cell surface with antibodies to CD59 and DAF. Under these conditions, 10 of 16 patients with SN-OMG were positive for activation of complement, suggesting marginally increased sensitivity compared with the IgG assay.

CLINICAL AND NEUROPHYSIOLOGIC CORRELATES OF CLUSTERED AChR-Abs

We then turned to the 97 patients with MG with routine AChR-Ab titers and SFEMG findings from the same day. Findings from SFEMG in the orbicularis oculi muscles were significantly abnormal in approximately half the patients with OMG and were not different from those in patients with predominantly limb or bulbar symptoms (Figure 3A); overall, a significant correlation was noted between AChR-Ab titers and jitter values (n=97, P<.001, r²=0.41) (Figure 3B), as partially reported previously.3 Seventeen of 71 mean consecutive difference values were normal in patients who had positive AChR-
Abs, and, conversely, 8 of 26 mean consecutive difference values were raised in AChR-Abs–negative patients. Testing the 28 available SNMG serum samples, only a modest correlation was noted between clustered AChR-Ab scores and jitter values (\(r^2=0.20\), \(P=0.02\)) (Figure 3C), but the correlation between the mean consecutive differences and the C3b binding scores was significant (\(r^2=0.81\), \(P<0.001\), \(n=21\)) (Figure 3D).

PASSIVE TRANSFER OF CLUSTERED AChR-Abs PRODUCES EXPERIMENTAL MG IN ANIMAL MODELS

As large volumes of plasma were unavailable from patients with OMG, for passive transfer we used IgG purified from 2 patients with SN-GMG. SNMG1 was a man with GMG symptoms since age 18 years, seen first at the University of Oxford at age 43 years. He had a normal scan of his thymus and responded well to immunosuppression and plasma exchange. His plasma exchange fluid (AChR-Ab and MuSK-Ab negative) consistently scored 2 on the clustered AChR-Ab assay. SNMG2 was a 56-year-old woman with severe generalized symptoms, onset at age 39 years, and always negative for AChR-Abs and MuSK-Abs. She responded well to immunosuppressive therapy and had several courses of plasma exchange with good benefit. Her plasma exchange fluid scored 3 on the clustered AChR-Ab assay.

The initial unblinded experiments involved injection of 10 mg/d of IgG for 5 days into wild-type C57BL/6 mice using IgG from SNMG1 (3 mice), controls (4 mice), and pooled AChR-MG (4 mice). No phenotypic changes (ie, weight loss, weakness, or behavioral changes) were seen in any of the 3 groups. However, there was a significant reduction in MEPP amplitudes in the SNMG and AChR-MG groups compared with control-injected mice (Table), with no differences in MEPP frequencies, EPP amplitudes, or quantal contents. We then injected IgG, 50 mg/d, for 3 days into 4 mice in each coded group (AChR-MG, SNMG2, and controls). Again, weakness was not observed, but a significant reduction was seen in MEPP amplitudes and AChR expression at the end plates in the 2 test groups compared with control results (Table).

PASSIVE TRANSFER TO COMPLEMENT REGULATOR–DEFICIENT MICE

To try to enhance the effect of the Abs, which deposited complement on clustered AChRs (Figure 2), we injected IgG, 50 mg/d, for 5 days into each coded group (AChR-MG, SNMG2, and controls). Again, weakness was not observed, but a significant reduction was seen in MEPP amplitudes and AChR expression at the end plates in the 2 test groups compared with control results (Table).

### Table. Electrophysiologic Results in Passive Transfer of SNMG and AChR-MG IgG to Mice

<table>
<thead>
<tr>
<th>Passive Transfer</th>
<th>Control</th>
<th>SNMG1</th>
<th>SNMG2</th>
<th>AChR-MG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice immunized, No.</td>
<td>4</td>
<td>3</td>
<td>NA</td>
<td>4</td>
</tr>
<tr>
<td>MEPP, mean (SEM), mV</td>
<td>1.30 (0.04)</td>
<td>0.96 (0.05)^{a}</td>
<td>NA</td>
<td>1.10 (0.05)^{b}</td>
</tr>
<tr>
<td>EPP, mean (SEM), mV</td>
<td>23.81 (0.98)</td>
<td>30.09 (3.5)</td>
<td>NA</td>
<td>26.01 (0.94)</td>
</tr>
<tr>
<td>Quantal content, mean (SEM)</td>
<td>27.12 (2.1)</td>
<td>35.71 (5.5)</td>
<td>NA</td>
<td>33.81 (2.1)</td>
</tr>
<tr>
<td>MEPP frequency, mean (SEM), Hz</td>
<td>1.13 (0.07)</td>
<td>1.54 (0.20)</td>
<td>NA</td>
<td>1.23 (0.08)</td>
</tr>
<tr>
<td>AChR above threshold, mean (SEM), µm²</td>
<td>273.5 (12.9)</td>
<td>261.4 (8.2)</td>
<td>NA</td>
<td>165.9 (6.8)^{a}</td>
</tr>
<tr>
<td>MEPP, mean (SEM), mV</td>
<td>1.19 (0.05)</td>
<td>0.97 (0.03)^{b}</td>
<td>1.04 (0.04)^{c}</td>
<td></td>
</tr>
<tr>
<td>EPP, mean (SEM), mV</td>
<td>27.68 (1.1)</td>
<td>NA</td>
<td>23.20 (0.86)^{b}</td>
<td>23.69 (1.0)^{c}</td>
</tr>
<tr>
<td>Quantal content, mean (SEM)</td>
<td>30.11 (2.1)</td>
<td>NA</td>
<td>31.33 (2.2)</td>
<td>34.07 (1.7)</td>
</tr>
<tr>
<td>MEPP frequency, mean (SEM), Hz</td>
<td>1.06 (0.06)</td>
<td>NA</td>
<td>1.11 (0.10)</td>
<td>1.2 (0.10)</td>
</tr>
<tr>
<td>AChR above threshold, mean (SEM), µm²</td>
<td>358.7 (13.9)</td>
<td>NA</td>
<td>236.8 (7.2)^{a}</td>
<td>223.6 (7.9)^{a}</td>
</tr>
<tr>
<td>MEPP, mean (SEM), mV</td>
<td>1.180 (0.05)</td>
<td>0.714 (0.05)^{a}</td>
<td>0.860 (0.03)^{a}</td>
<td>0.844 (0.03)^{a}</td>
</tr>
<tr>
<td>EPP, mean (SEM), mV</td>
<td>28.36 (1.1)</td>
<td>23.47 (1.6)</td>
<td>24.76 (0.8)</td>
<td>24.19 (0.9)</td>
</tr>
<tr>
<td>Quantal content, mean (SEM)</td>
<td>35.44 (1.7)</td>
<td>40.45 (3.2)</td>
<td>43.47 (4.3)</td>
<td>41.16 (2.2)</td>
</tr>
<tr>
<td>MEPP frequency, mean (SEM), Hz</td>
<td>1.73 (0.17)</td>
<td>1.40 (0.10)</td>
<td>1.20 (0.12)</td>
<td>1.47 (0.27)</td>
</tr>
<tr>
<td>AChR above threshold, mean (SEM), µm²</td>
<td>346.4 (8.8)</td>
<td>228.1 (6.6)^{a}</td>
<td>238.2 (5.9)^{a}</td>
<td>211.0 (4.4)^{a}</td>
</tr>
</tbody>
</table>

Abbreviations: AChR, acetylcholine receptor; EPP, end plate potential; IgG, immunoglobulin G; MEPP, miniature end plate potential; MG, myasthenia gravis; NA, not applicable; SN, seronegative.

^{a} P < .001 vs the control data.

^{b} P < .01.

^{c} P < .05.
to be greater than those in the control IgG–injected group (analysis of variance, $P = .23$) (Figure 4 and Table). When the end plate area was measured using Alexa Fluor 594–bungarotoxin labeling, there was substantial reduction in the expression of AChRs at the neuromuscular end plates in AChR-MG and SNMG mice compared with control end plates and complement deposition at the AChR-MG IgG–injected and SNMG IgG–injected end plates (Table and Figure 5).

Clustered AChR-Abs, initially termed low-affinity AChR-Abs, were first reported in patients with GMG who were SN for typical AChR-Abs or MuSK-Abs. Herein, we show that these Abs are also present in a substantial proportion of patients with previously SN-OMG and that they also have the potential to deposit complement on the cell surface. The IgG binding and complement deposition correlated with increased jitter on SFEMG, suggesting the likely relevance of these antibodies to the electrophysiologic defects. To support this hypothesis, we show that IgG preparations from 2 patients, positive only for clustered AChR-Abs, could passively transfer the typical defect of MG, reduced MEPP amplitudes, when injected into mice. This was associated with deposition of complement, although the results were not obviously enhanced in the complement regulator–deficient mice. Overall, the results were not different between the typical AChR-Abs and the clustered AChR-Abs, suggesting that the clustered AChR-Abs are also pathogenic and act by similar mechanisms.

Other researchers have suggested that the relative lack of intrinsic complement regulators and the differential expression of fetal AChRs in the extraocular muscles may contribute to the susceptibility of these muscles to autoimmune attack in MG. We found that most clustered AChR-Abs in OMG were directed against the adult form of the AChR and did not bind the fetal form, contrasting with most patients with AChR-Ab–positive GMG, who usually bind both, but agree that complement fixation is likely to be an important pathogenic mechanism in patients with OMG.
Although IgG binding to clustered AChRs and complement activation were shown in vitro, the pathogenic role of these Abs could be confirmed only by electrophysiologic experiments in injected mice. These experiments demonstrated reduced MEPP amplitudes, the pathophysiologic hallmark of MG. Because an intact and fresh diaphragm was essential for electrophysiologic experiments, the muscles were not perfused to remove any nonspecific background from the blood vessels, and there was a high IgG background in the muscles. This finding limited demonstration of IgG deposition at the end plates or analysis of the complement deposition. Further experiments could be performed to demonstrate these findings more quantitatively.

Much earlier attempts to transfer SNMG to mice showed neurophysiologic changes similar to those of MG,12-14 but retrospective analysis showed that most of these patients had MuSK-Abs. Recent passive and active induction of disease in MuSK-MG have demonstrated a loss of AChRs and reduced MEPP amplitudes,15 not different from those shown herein, and compared the results with AChR-MG. The AChR-Ab passive transfer or active immunization, but not the equivalent MuSK-Ab experiments, produced a modest increase in quantal contents, which is thought to be a compensatory mechanism, described previously in patients with MG and in mice immunized against AChR.16 Although this increase was not significant herein, all the mean quantal contents were higher in SNMG IgG– and AChR IgG–injected mice than in controls, contrasting with the results in MuSK experiments12 and further supporting a similar pathogenic role for typical AChR-Abs and clustered AChR-Abs.

Recent studies have demonstrated a role for novel complement C5 inhibitors to ameliorate disease processes in passive transfer MG models,17 and experiments using the rat anti–mouse muscle AChR-Ab McAb-3 found significantly worse phenotypic changes in complement regulator knockout mice. We hoped, therefore, that we would enhance the changes in complement regulator–deficient mice. However, although we found a reduction in the size of thebungarotoxin–labeled end plates, there was no apparent morphologic disruption, and the electrophysiologic defects were not consistently enhanced when either AChR-Abs or clustered AChR-Abs were injected into mice lacking an efficient complement regulator system. This finding suggests that the electrophysiologic defects were due to antigenic modulation or functional inhibition rather than to complement-induced damage at the end plates.

Because some researchers believe that supplementing a source of human complement is important to demonstrate phenotypic changes in mouse autoantibody–mediated disease models,18,19 it would be interesting to do this in future experiments to try to confirm the role of complement–mediated mechanisms that are clearly important in humans.

Limited previous studies of the correlation of SFEMG abnormalities with clinical severity20 have concluded that the neurophysiologic changes correlate more closely with clinical severity than with antibody titers, and, in general, it is difficult to predict severity using antibody titers alone except in individual patients.21-24 Herein, in a large group of patients with MG, including SNMG, studied at the time of SFEMG, we found a correlation between jitter values and AChR-Ab titers and clustered AChR-Abs in patients with SNMG. The correlation of complement–fixing capacity with neuromuscular jitter was greater, again supporting the likely importance of the complement system in the pathogenesis of AChR-Ab–positive MG. It would be interesting to perform the complement assays prospectively in a group of patients with MG along with their SFEMGs to confirm these findings.

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Study concept and design: Jacob, Webster, Hilton-Jones, and Vincent. Acquisition of data: Jacob, Viegas, Leite, Webster, Cossins, Kennett, Hilton-Jones, and Vincent. Analysis and interpretation of data: Jacob, Viegas, Leite, Webster, Cossins, Kennett, Morgan, and Vincent. Drafting of the manuscript: Jacob and Vincent. Critical revision of the manuscript for important intellectual content: Jacob, Viegas, Leite, Webster, Cossins, Kennett, Morgan, and Vincent. Statistical analysis: Jacob and Webster. Obtained funding: Jacob and Vincent. Administrative, technical, and material support: Jacob, Viegas, Leite, Cossins, and Morgan. Study supervision: Leite, Webster, Kennett, Hilton-Jones, Morgan, and Vincent.

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REFERENCES


