

## Hypothesis

## Bifidobacterium lipoteichoic acid and false ELISA reactivity in aspergillus antigen detection

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**A major difficulty with the detection of circulating galactomannan, a cell-wall polysaccharide released by *Aspergillus* sp during growth, in the serodiagnosis of invasive aspergillosis is the occurrence of false-positive ELISA results, especially in neonates and infants. On the basis of molecule similarity, we postulate that a lipoteichoic acid of *Bifidobacterium* sp can act as epitope for the monoclonal antibody used in the ELISA. The neonatal gut is heavily colonised with *Bifidobacterium* sp and these bacteria or their lipoteichoic acid might cause ELISA reactivity with serum after translocation because of immaturity of the intestinal mucosa. If our hypothesis is correct, we might find a method to discriminate between false-positive and true-positive ELISA results and thereby prevent unnecessary pre-emptive treatment of patients.**

Invasive aspergillosis is a fungal infection that is frequently fatal in immunocompromised patients, including those treated for haematological malignant diseases, those treated with high-dose corticosteroids, and transplant patients. Despite the development and registration of new potent antifungal agents, the survival rate remains less than 50%. The overall poor outcome is due partly to the difficulty in establishing the diagnosis at an early stage of infection. Presenting signs and symptoms are non-specific and sensitivity of fungal cultures is low. Characteristic lesions might be present on high-resolution CT of the chest, but these findings are not specific for aspergillus infection.

In view of the limitations of these techniques, non-culture-based methods were developed that detect circulating markers released by the fungus.<sup>1</sup> Several PCR-based techniques have been developed, including real-time PCR, which enable detection of circulating fungal DNA at an early stage of infection. Several investigators have reported excellent results with prospective testing of serial blood samples in bone-marrow-transplant recipients. However, widespread use of PCR-based methods is restricted by the lack of standard techniques. Alternatively, the aspergillus antigen galactomannan can be detected in body fluids of patients with invasive aspergillosis. A sandwich ELISA is commercially available (*Platelia aspergillus*, BioRad, Marnes-la-Coquette, France) which has a detection limit of 1 µg/L galactomannan in serum.<sup>2,3</sup> In prospective studies, circulating galactomannan has been detected with high sensitivity and specificity, and at an early stage of infection in patients with haematological malignant disease,

especially when serial serum samples are tested.<sup>4,5</sup> This assay is now widely used as a diagnostic tool in high-risk patients in centres throughout the world.

### Galactomannan

Galactomannan is a cell-wall polysaccharide that is released by *Aspergillus* sp during growth. The antigen is heat-stable and probably widely distributed throughout nature, given the widespread nature of *Aspergillus* sp. Galactomannan has been detected in beverages such as tea and milk, in foods including pasta, rice, and pepper, and even in several antibiotics.<sup>6</sup> The molecule structure of galactomannan, derived from in-vitro culture supernatants, as proposed by Latgé and colleagues,<sup>7</sup> consists of a linear mannan core with side chains of an average of five galactofuranosyl residues (figure). Four of these β (1–5)-linked residues present the epitope that binds a rat IgM monoclonal antibody (EB-A2) used in the ELISA kit.<sup>3</sup> One galactomannan molecule contains more than ten of these galactofuran epitopes.<sup>7</sup> The antigen can be released by the fungus as a pure polysaccharide of about 21 kDa, as part of fungal exocellular glycoproteins of 35–94 kDa, or as part of a lipopeptidogalactomannan of more than 100 kDa.<sup>7</sup> However, the exact nature of the in-vivo circulating galactomannan-bearing antigens in serum samples of infected patients is unknown. Since galactomannan is immunogenic, it might be bound to antibodies to galactomannan or other human proteins.

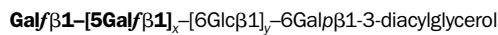
Despite the fact that the ELISA has become an important tool in diagnosing invasive aspergillosis, all studies to date have reported false-positive reactivity in serum from patients without this disorder. False-positive reactivity varies between 5% in adults and as much as 83% in neonates.<sup>8–10</sup> Although false-positive reactivity is frequently limited to one serum sample, persistent ELISA reactivity in neonates is seen in consecutive serum samples.<sup>8,9</sup> Several theories have been proposed to explain these results. One explanation is gastrointestinal translocation of fungal galactomannan from contaminated food.<sup>6</sup> During growth and harvesting, moulds that originate from soil, water, and air generally contaminate the surface of fruit, vegetables, and cereals. The heat-stable fungal galactomannans are not destroyed during production processes and are consequently found in human faeces. Molecules with a size similar to aspergillus galactomannan are not absorbed in appreciable amounts

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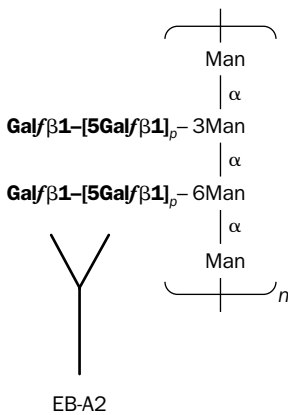
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A



B



**Proposed structures of lipoteichoic acid from *B bifidum* sp *pennsylvanicum*<sup>16</sup> (A) and galactomannan from *A fumigatus*<sup>7</sup> (B)**  
 $x=6-9$ .  $y=8-15$ .  $p>3$ .  $n\geq 10$ . Galf=galactofuranosyl, Galp=galactopyranosyl. Glc=glucose. Man=mannose. Binding site of EB-A2 antibody is galactofuran side chain, given in bold.

in intact intestinal mucosa.<sup>11</sup> However, molecules as large as 45 kDa might translocate in patients with immature intestinal mucosa or with reduced integrity of the intestinal-mucosal barrier due to cytotoxic chemotherapy, and the amount of translocated faecal antigen seems to be sufficient to cause detectable antigenaemia.<sup>6</sup> This theory is supported by the finding of ELISA reactivity with dietary components and by cases in which antigenaemia was correlated with consumption of contaminated food or drinks.<sup>12,13</sup> Furthermore, false-positive reactivity was absent in healthy adult donors, thereby supporting a role for the integrity of the mucosal barrier. However, serum from patients who do not eat during the episode of severe mucositis might have positive ELISA results. Moreover, antigenaemia is expected to be variable when related to cross-reacting components in patients' diets, whereas neonates especially have persistent antigenaemia.

An alternative explanation is cross reactivity of the IgM monoclonal antibody with other molecules present in serum, but this theory could not be confirmed with blood products and antigens from bacteria that cause bacteraemia.<sup>14</sup> Cross-reactivity of the EB-A2 monoclonal antibody with galactomannan from penicillium or paecilomyces has been reported in vitro, but these moulds very rarely cause invasive infection in human beings.<sup>14</sup>

### Hypothesis

We postulate that an alternative source of the target epitope, which is recognised by the EB-A2 monoclonal antibody, is present in the gut and causes false-positive serum reactivity by bacterial translocation—ie, the transmucosal passage of viable and non-viable microbes and their by-products.<sup>15</sup> A search of published studies showed that a membrane-associated molecule of *Bifidobacterium bifidum* sp *pennsylvanicum* mimics the epitope recognised by EB-A2.<sup>16</sup> This lipoteichoic acid, which is exposed to the bacterial cell surface or may even be excreted, contains a terminal linear polysaccharide of more than seven galactofuranosyl residues (figure). Since four of these  $\beta$  (1-5)-linked residues present the epitope for the EB-A2 monoclonal antibody, reactivity with the sandwich ELISA can be expected. Also other

*Bifidobacterium* sp have a similar lipoteichoic acid.<sup>17</sup> *Bifidobacterium* sp are common members of the gastrointestinal microflora of human beings, comprising up to 3% of the total faecal microflora of adults and forming up to 91% and 75% of the total intestinal microflora in breastfed and milk formula-fed infants, respectively.<sup>18</sup> Because of their possible probiotic effects, *Bifidobacterium* sp are widely used as food additives. The propensity for transmucosal passage of bacteria or antigens in preterm neonates is increased and, in experimental studies, translocation of *Bifidobacterium* sp has occurred because of a reduced integrity of the gastrointestinal mucosa during sepsis and on exogenous administration of high doses of bifidobacteria.<sup>19,20</sup>

The high load of *Bifidobacterium* sp in the gut of neonates corresponds with the high number of neonates that show false-positive reactivity with serum. Furthermore, gastrointestinal colonisation can act as a continuous source of lipoteichoic acid, which is consistent with the observation of persistent serum ELISA reactivity in neonates.

### Testing the hypothesis

To test our hypothesis, cross reactivity of the monoclonal antibody EB-A2 with the lipoteichoic acid can be seen in vitro by testing reference strains of bifidobacterium, cell fragments, or culture filtrates for reactivity in the ELISA. Preliminary experiments with a suspension of bifidobacterium cells scraped from agar plates show positive reactivity. Faecal samples from neonates could be investigated for the presence of *Bifidobacterium* sp by PCR and culture. ELISA reactivity of the strains recovered from the stool would confirm the presence of reactive lipoteichoic-acid epitopes in the intestine of the target patient population. Translocation of lipoteichoic acid from the intestine to the blood could be demonstrated in an experimental model. For instance, feeding radioactive labelled lipoteichoic acid to neonatal rabbits and subsequent detection of radioactivity outside the intestine would indicate translocation. Finally, false-positive serum samples obtained from neonates could be analysed for the structure of the cross-reacting molecule. To gain an insight into the structure of this molecule, antibodies to the mannan core of galactomannan or to epitopes, other than galactofuran, in lipoteichoic acid would be required. Since all studies to date aimed at characterising the galactomannan molecule have been done with culture supernatants, the form of galactomannan present in the blood remains unclear. The fraction of antigen bound to protein is unknown, as is the variability of the structure of the galactomannan molecule produced by aspergillus during infection. The number of galactofuran epitopes could vary dependent on the local conditions at the site of infection. Several techniques would allow analysis of the molecule structure of the antigen that causes false reactivity in serum samples, including affinity chromatography with immobilised lectins and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. These techniques are, however, confounded by the very low concentration of antigen in patients—in the range of  $\mu\text{g/L}$ —and the low volumes of blood that can be obtained from neonates.

Although the kinetics of galactomannan and cross-reactive epitopes in patients at risk for invasive aspergillosis are not well understood, the cause of false reactivity is probably multifactorial. With more insight, a distinction might be made between true positives, biological false positives through detection of galactomannan not representing invasive aspergillosis,

and false positives caused by lipoteichoic acid, thereby improving the diagnostic value of antigen detection in the early diagnosis of invasive aspergillosis.

*Conflict of interest statement*

None declared.

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