Molecular diagnosis of cow's milk allergy

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Purpose of review

To identify and discuss studies on the molecular diagnosis of cow's milk allergy (CMA) with a view to update allergists since a general review of the methodology in 2006.

Recent findings

Seven basic research studies reporting the use of component-resolved diagnostics in CMA were found. All studies were on children positively reacting to a formal challenge with cow's milk. Six studies used natural allergens and three used recombinant milk proteins. Microarray platforms were customized and, thus, differed across studies. Three studies assessed the association between molecular-scale patterns and different presentations of the condition, that is the association between anaphylaxis, gastrointestinal symptoms and other severe phenotypes and the pattern of protein sensitization. Two studies assessed the association between positive oral food challenge and the persistence of milk allergy over time. Protein profiling could be useful to indicate appropriate specific immunotherapy.

Summary

Accurate diagnosis of CMA is challenging and essential. The determination of the immunoglobulin E (IgE)-mediated response to sequenced and characterized allergens may be more useful in predicting the presence and severity of clinical allergy than the currently used skin or blood tests performed with whole extracts. However, as component recognition pattern heterogeneity is observed in different areas, further clinical studies are essential to correlate useful molecular diagnostics and biological markers with disease and patient profiles. Until such markers are found and validated in different age groups, oral food challenge remains the reference standard for the diagnosis of CMA.

Keywords

casein, cow's milk, component-resolved diagnosis, Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA), molecular diagnosis

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Introduction

The burden of cow's milk allergy (CMA) is increasing and this condition, peaking during early childhood, is now widespread. CMA is an adverse clinical reaction mediated by immunological mechanisms, the most common of which is mediated by IgE and in which binding between immunoglobulins and the corresponding antigens results in loss of tolerance [1,2]. The professional work-up includes in-vivo and in-vitro tests as well as a diagnostic dietary elimination to determine a cause–effect relationship between cow's milk and symptoms is a positive oral food challenge carried out under double-blinded conditions in a secured setting and interpreted by an allergist [3–5]. However, the double-blind, placebo-controlled food challenge is difficult to perform and interpret; hence, the quest for the holy grail of allergy research, an alternative or improved diagnostics [6–11]. Currently, the state-of the-art diagnostic pathway is a consensus to avoid oral food challenge with milk only in the precise circumstances in which 'it is not considered a requirement for making a diagnosis of immunoglobulin E (IgE)-mediated CMA'. This occurs in patients with a high pretest probability of CMA when either skin prick tests or specific IgE (sIgE) determinations would be positive or in patients with a low pretest probability of CMA when either skin prick tests or sIgE determinations would be negative [2].

A breakthrough was achieved when it became apparent that these complex diagnostic procedures could be speeded up by technological enhancement. Miniaturization and high-throughput platforms have been reviewed elsewhere [12,13] and represent the new frontier of allergy research. Clinical applications are just around the corner with personalized allergy medicine becoming a reality as clinical validation progresses [14,15].

In this review, we update clinicians on the molecular diagnosis of CMA literature of the past 5 years [16].

Physical–chemical characterization of cow's milk allergens

The proteins of the caseins and whey protein fractions of cow's milk are listed in Table 1 [17]. Each fraction contains five major components [17,18]. Although 80% of total protein is contained in the casein fraction, whey proteins are less dominant. The only protein not present in human milk is β-lactoglobulin (β-LG).

For the purposes of molecular diagnosis, each cow's milk protein is referred to according to its allergen nomenclature, an international code containing a sequence of three letters/space/one letter/space/one number. The first three letters are the first three letters of the genus, followed by the first letter of the species (thus, *Bos d* for *Bos domesticus* [19,20]) and a number indicating the order of allergen identification.

α-Lactalbumin (α-LA, *Bos d* 4), a functional subunit of whey lactose synthase, plays a controversial role in milk sensitization (http://www.uniprot.org/uniprot/P00711&format=html) [21], but up to 80% of allergic individuals react to it [22], perhaps because diagnostic methods tend to vary too. β-LG, the most abundant whey protein, is absent from human milk (http://www.uniprot.org/uniprot/P02754&format=html), but the percentage of allergic individuals reacting to this protein ranges between 13 and 76% [2]. BSA (*Bos d* 6) is involved in CMA and in beef allergy (http://www.uniprot.org/uniprot/P02769&format=html) [23,24]. It correlates with the cardinal clinical features of lip edema, urticaria, cough and rhinitis in children allergic to beef and is probably of the greatest diagnostic relevance [25]. Individuals allergic to cow's milk with a positive reaction to BSA range between 0 and 88%, whereas clinical reactions occur among up to 20% of these patients [26].

The bovine immunoglobulins (designated under the monicker of *Bos d* 7) are present in blood, tissue fluids and many secretions. Bovine IgGs seldom cause clinical symptoms in CMA.

The composite allergen *Bos d* 8 (which consists of α_s1-caseins, α_s2-caseins, β-caseins and κ-caseins) exhibit limited sequential homology. In spite of this, polysensitization to many casein fractions is usually observed, perhaps due to cross-sensitization through some common or closely related epitopes. Sensitization is particularly frequent against α-casein (100%) and κ-casein (91.7%) [27]. Several studies have identified α_s1-casein as a major allergen inducing strong immediate or delayed allergic reactions [28]. Although α_s1-casein represents a class I food allergen, it was found to contain both conformational and sequential IgE epitopes [29].

Key points

- Molecular methods of diagnosis do not afford greater precision than specific immunoglobulin E determinations.
- Platforms based on recombinant isoforms of allergen need to be correlated with well defined caseloads.
- Epitope mapping will ensure greater precision of future lab-on-a-chip point-of-care diagnostics.
- In order to avoid overdiagnosis, clinical and bioinformatic inputs are still necessary.

Molecular diagnosis of cow's milk allergen

Theoretically, the main breakthrough introduced by biotechnologies at molecular level in diagnostic medicine is represented by the precise engineering of epitopic sequences identified as immunodominant by in-vivo or clinical studies. Nothing, therefore, but technological advances themselves – which require validation and calibration – stand in the way of validated recombinant isoform on allergy chips for diagnostic purposes. Allergen homologs are also useful as markers for cross-reactivity,

Table 1 Cow's milk proteins

Fraction	Protein	Allergen name	Concentration (g/l)	Total proteins (%)	Molecular weight (kDa)	Amino acids	pI
Caseins			~30	80			
	α_{s1}-casein		12–15	29	23.6	199	4.9–5.0
	α_{s2}-casein		3–4	8	25.2	207	5.2–5.4
	β-casein	*Bos d* 8	9–11	27	24.0	209	5.1–5.4
	γ_1-casein				20.6	180	5.5
	γ_2-casein		1–2	6	11.8	104	6.4
	γ_3-casein				11.6	102	5.8
	κ-casein		3–4	10	19.0	169	5.4–5.6
Serum proteins			~5.0	20			
	α-lactalbumin	*Bos d* 4	1–1.5	5	14.2	123	4.8
	β-lactoglobulin	*Bos d* 5	3–4	10	18.3	162	5.3
	Immunoglobulin	*Bos d* 7	0.6–1.0	3	160.0	–	–
	BSA	*Bos d* 6	0.1–0.4	1	67.0	583	4.9–5.1
	Lactoferrin	–	0.09	Traces	800.0	703	8.7

Bos D, *Bos domesticus*. Reproduced from [17].

but this is hardly relevant in CMA in which major allergens, primary sensitizers and their conformational counterparts are already well known [17,30].

Of the seven studies [31,32,33••,34•,35••,36•,37••] that have so far made use of these novel diagnostic technologies, basic research and proof of principle studies dominate and only two [33••,37••] are comparable with standard clinical diagnostic methods Thus, a full comparison between the standard allergy work-up of clinical history, skin prick test (SPT) and IgE determinations carried out by an allergist and an experimental setting, including molecular technologies, remains to be investigated under double-blinded conditions of oral food challenges in CMA.

The proof of principle in favor of a library of allergens for microarrays has been provided by Cerecedo *et al.* [36•] who confirmed the utility of casein sensitization for diagnostic purposes and identified β-casein amino acids 52–74, β-LB amino acids 58–77 and κ-casein amino acids 34–53 as best markers of CMA on the basis of their performance in their caseload. In this study, a differential recognition pattern between challenge-defined tolerant and allergic patients has been identified by peptide microarray-based immunoassay on the basis of a statistical association with the reactive group of a whole array of newly sequenced epitopes binding IgE and IgG4 and recognized by more than 75% of patients in their reactive group. α_s1-Caseins, α_s2-caseins and β-caseins were recognized by 75% of patients who did not tolerate milk, whereas the 58–77 amino acid linear sequence of β-casein, β-LG and κ-casein were significantly associated with 81.3% of the 16 reactive sera at Fisher's exact test. This study represents a preliminary, but needed, step toward improving our exploration of the allergens, defining a more precise cow's milk allergic patient profile. However – and this is actually an advantage – it gears us toward test utilities more adapted at identifying patients who will tolerate cow's milk. The gap (25–18.3 in percentage points) between percentages makes this assay already a screening instrument in the allergist's toolbox. In this study, children were sensitized to 10 milk rule-out epitopes. Only one of these was already known as a potential marker of prognosis [38].

Hochwallner *et al.* [34•] provided evidence of the validity of IgE reactivity profiling and confirmed that recombinant allergens were of equivalent potency in terms of recognition of natural caseins, although α-LA was their best marker allergen. Theirs was the first in-silico immunoassay of red blood cells (RBL) cells expressing the human Fcε receptor for the establishment and calibration of an allergen chip using recombinant milk allergens. This study provided useful information for the IgE reactivity profiling with recombinant peptides. In this study, recombinant caseins were as potent as natural caseins in recognizing sera from CMA patients.

Further studies validating the epitope mapping abilities of microarrays were Lin and Sampson [35••] who established the principle of large-scale epitope mapping to confirm caseins and β-LB allergens and Wang *et al.* [32] who found that IgE epitope diversity on microarray corresponded to clinical phenotype (persistent allergy correlates with higher epitope diversity on their platform), thereby confirming peptide microarrays equivalence with spot membrane technology. This latter study is also the first food challenge – confirmed tolerance study at molecular level, in which different, hypothesized phenotypes of tolerance correlate with informative epitopes of caseins and β-LB.

Of further clinical relevance, Gaudin *et al.* [31] established the principle that the combination of customized microarrays with fluorescence detection confirmed the diagnostic utility of casein and unexpectedly found that bovine lactoferrin was a significant marker of CMA.

Ott *et al.* [37••] were the first to investigate the clinical performance characteristics of a component-based allergen microarray with respect to the outcome of the oral food challenge in suspected CMA. Using the commercially available allergen microarray assay Immuno Solid phase Allergen Chip (ISAC, VBC Genomics Bioscience Research, Vienna, Austria), no advantage over the usual diagnostic tests, that is skin prick test and extract-based specific IgE binding measured by ImmunoCAP (Phadia AB, Uppsala, Sweden), was found by this comparative study of methodologies. Evaluating natural allergens (*Bos d* 4, 5, 6 and 8), no single allergenic component was found to be superior at discriminating between clinically irrelevant sensitization and genuine CMA. The attempt to sum the performance characteristics of the single allergens on the platform also failed to yield significant clinical findings. The authors concluded that ISAC was able to give a picture of the repertoire of clinically relevant cow's milk proteins.

D'Urbano *et al.* [33••] established the principle of the negative predictive value (NPV) of microarrays. In a study measuring the specific IgE level of cow's milk allergens with microarray technology vs. SPT, IgE and oral food challenge, 104 children, 58 of whom underwent open challenge, were assessed. This study measured the area under the curve (AUC) of a microarray technology plotted against ImmunoCAP at receiver operating characteristic (ROC) analysis. The authors used an allergen microarray platform similar to Ott *et al.*, customizing their chip to contain natural *Bos d* 4, *Bos d* 5.0102 (β-LB A), *Bos d* 5.0101 (β-LB B), *Bos d* 6, *Bos d* 7, *Bos d* 8 (casein), *Bos d* 8-α S1(α-casein), *Bos d* 8-β (β-casein), *Bos d* 8-κ

(κ-casein) and *Bos d* lactoferrin. The best performing diagnostic component was *Bos d* 8, which showed the largest AUCs at ROC analysis. These results were not significantly different from those obtained using ImmunoCAP technology. Using 95% clinical decision points, *Bos d* 8 yielded a NPV of 78% vs. a 57% NPV with ImmunoCAP. Thirty-two of 58 patients (or 55% of oral food challenges) were positive and clinical decision points could not be calculated for rarely occurring IgE. The sum of cow's milk components on the platform did not yield increased rule-in power over the performance characteristics of single components. With ImmnoCAP determinations for cow's milk and casein, AUC averaging 0.9% at ROC analysis, the performance of microarrays for *Bos d* 8 was equivalent at 0.876. The sequential use of the two methodologies, which was also evaluated at ROC analysis, yielded only a minor improvement in work-up performance. More unexpectedly, when clinical decision points are used instead of cut-offs for cow's milk and *Bos d* 8, which are much higher than the 0.35 kUI/l manufacturer-set lower detection level usually deemed clinically significant for positivity at ImmunoCAP IgE determination, they yielded a highly specific 96% and were unexpectedly poorly sensitive at 41% with a confidence interval too wide for goodness-of-fit. The specificity of the microarrays (which are not limited by the lower detection threshold of IgE determinations) is high, but does not translate into an acceptable NPV to make this technology a reliable instrument of rule-out screening in the setting of CMA.

The kits used in these studies were eventually made available under the trademark of ImmunoCAP-ISAC (VBC Genomics; Phadia). One hundred and three native/recombinant component allergens from 43 allergen sources are represented in the platform, and include naturally sourced isoforms of the allergens *Bos d* 4, *Bos d* 5, *Bos d* 6, *Bos d* 8, and *Bos d* lactoferrin. The two main advantages are that it assesses simultaneously specific IgE to different components and requires thin amounts of serum, which is relevant in pediatrics.

Role of patient profiling in the diagnosis of cow's milk allergen

In IgE-mediated allergy, circulating IgE recognize specific conformational and linear molecular structures (epitopes) on the allergenic proteins and cause clinical reactions. These advances allow the more accurate drawing of a patient profile picture in ever greater detail, but with the proviso that these profiles be matched to clinical studies defining the confines of these clinical settings. Thus, it is possible that different epitopes are associated with severity and duration of the disease [34•,36•]. Although those with severe systemic reactions showed stronger IgE reactivity to more components, IgE reactivity testing did not allow distinguishing persons without symptoms from patients with severe and gastrointestinal symptoms.

Role of patient profiling in the prognosis of cow's milk allergen

Several studies that investigated the IgE-binding epitopes of different milk allergens (including α_s1-caseins, α_s2-caseins, β-caseins and κ-caseins, α-LB and β-LB) have compared epitope recognition patterns between patients under 3 years of age who were likely to outgrow their milk allergy and had low levels of specific IgE and older patients with persistent milk allergy and high levels of milk-specific IgE antibodies. All these studies show that most older patients recognize a much greater number of IgE-binding epitopes than younger ones, suggesting an association between recognizing certain epitopes and clinical symptoms of CMA. In persistent disease, casein sensitization [39] and the presence of IgE against linear epitopes [40] have been demonstrated. Studies evaluating milk IgE-binding epitopes not only associate high epitope diversity with long-lasting allergy but also demonstrate that sequential epitopes recognized by IgE antibodies in older patients with persistent allergy differ from those found among younger children likely to outgrow CMA. Certain epitope-specific IgE antibodies are present from a very early age in patients who later developed persistent disease. Therefore, these 'informative' epitopes may be useful as biomarkers of persistence. The development of tolerance to cow's milk allergens is associated with a reduction of allergen-specific IgE levels and a reduction of IgE recognition of such sequential epitopes [41]. Therefore, an evaluation of linear epitopes related to allergic reactions using a peptide microarray could help us understand likely clinical outcomes [35••]. Although ImmunoCAP-ISAC includes a natural isoform of *Bos d* 8, it is now possible to engineer recombinant casein peptides and their derivates for developing new diagnostics [42]. Their clinical application to the field of prognosis remains to be assessed. Using an older panel of microarrayed proteins, an association with disease persistence was found in 2005 [43]. The problem remains, however, that no accuracy study has so far been published to ground these associations into clinical practice.

Potential role of molecular diagnosis for cow's milk allergen immunotherapy

Is there a role for molecular diagnostic methods in oral immunotherapy for CMA? Despite the paucity of the literature except under the heading of clinical hypotheses, the concept of identifying patients and clarifying indication for immunotherapy has gained weight in recent years. In a nonpeer-reviewed abstract, patients who had undergone a desensitization protocol for CMA

were evaluated using a microarray for the parallel analysis of IgE and IgG4 binding to β-LG and caseins. According to this report, successfully desensitized patients show a decrease in IgEbinding and an increase in IgG4 binding, whereas in the unsuccessful group, the opposite trend was noted [44]. This phenomenon had earlier been observed among similar caseloads and these data have been recently confirmed under double-blinded experimental conditions [45]. The proof of concept of a use of recombinant allergens for immunotherapy (IT) has only been validated in two studies, reviewed by Pauli and Malling, and regard sub-cutaneous immunotherapy (SCIT) for asthma and allergic rhinitis. Thus, it is premature to make recommendations on new immunotherapeutic strategies for CMA similar to those that have been developed on the basis of recombinant allergens for respiratory allergies [46,47].

Conclusion

Altogether, the studies reviewed argue in favor of molecular diagnostics in the context of CMA. The scaling down of diagnostic technology to microchip size will enable allergy medicine to move toward unprecedented standards of care, diagnostic and prognostic detail and personalized treatment. In the future, we will be able to describe patients with CMA from their whey and casein allergen sensitization profiles and map their sensitizing epitopes with a degree of detail that will take testing from the lab to point-of-care settings. These potential breakthroughs should be taken in stride; however, as the degree of precision already achieved is creating such a wealth of information (especially regarding the conformation of cow's milk allergens) that data management is increasingly complex and clinically relevant material of difficult interpretation. We are still waiting for bedside applications of cow's milk allergen molecular information. Personalization is not necessarily synonymous with simplification. There will always be a need for the allergy specialist-cum-bioinformatician. A foreseeable risk, therefore, is that of overdiagnosing rather than misdiagnosing CMA and this can only be allayed by the earnest call that we urge on our fellow allergists: investigate and publish in this new frontier of allergy medicine.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 271).

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