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'Don't blame it all on antibodies – the need for exhaustive characterisation, appropriate handling, and addressing the issues that affect specificity'

Highlights

- Antibodies are used to determine biomedical, environmental and food analytes.
- Recombinant antibodies offer greater opportunities for use/characterisation.
- Quality-control is critical for specificity testing.
- New analytical technologies make greater characterisation feasible.
- Poor antibody performance may be due in part to inappropriate usage.
- This review highlights these issues and suggests ways to successfully address them.

Abstract

Recently there have been numerous very thought-provoking reports describing many issues relating to the overall quality of antibodies that are currently available. It appears that multiple major clinical and other analytical studies suffer from lack of reproducibility and this has been associated, to a significant degree, with the lack of specificity of the antibodies used and the lack of adequate controls/testing to ensure that non-specific binding to irrelevant antigens was not occurring. This appears to be a major issue and it is vital that it is addressed as a matter of urgency. However, not all the problems can be ascribed to antibodies *per se* as inappropriate storage/handling/use in different analytical approaches and platforms is also of major significance. It was stated that antibodies need to be fully characterised with the entire sequence defined and published or available and this is now facilitated through the use of recombinant antibodies. This review critically evaluates these issues and suggests a way forward to address them.

Introduction

Antibodies are very widely used for a host of diagnostic, prognostic and therapeutic applications and represent a major focus for biotechnology and new pharmaceutical developments [1-6]. A number of very significant and thought-provoking articles highlighting the many issues identified with the overall quality of antibodies have been published indicating that many important clinical and other studies suffer from lack of reproducibility [7-14]. This was attributed, to a large degree, to the lack of specificity of the antibodies and the lack of adequate controls to ensure that non-specific binding to a plethora of irrelevant antigens was not occurring. In many cases this appears to be a very significant issue and must be addressed as a matter of urgency.

The use of highly defined recombinant antibodies would also be very beneficial to improve the current situation [15]. However, it is also the fact that antibodies are often incorrectly or inappropriately used and this can also be a significant factor in the generation of poor results even

when the antibody has the required performance characteristics. In this paper we seek to address these issues highlighting the need for stringency in the choice, characterisation and applications of antibodies in order to generate meaningful and accurate results. The importance of often overlooked issues will be described, practical approaches will be listed and case studies will be used to demonstrate vital factors that should be considered.

In 2015, in a letter to Nature, leading antibody experts called for a radical overhaul of the available antibodies and their usage due to the presence of profoundly worrying inabilities to reproduce the results of clinical trials, the associated waste of time and resources and the consequent implications for patient therapies and welfare [7]. Over many years a number of papers have highlighted these and other deficiencies, yet the issues still persist and while potential remedies have been cited, the costs of addressing them, or perhaps their implications, still leaves a gaping hole that is being avoided rather than filled [9, 12-14, 16, 17].

Immunoassays and associated issues

The development of antibody-based-assays was a huge step forward in terms of the existing laborious analytical methods used to determine a variety of different targets. Initially polyclonal antibody preparations derived from serum from animal hosts immunised with the target antigen were used with the aim of generating high levels of specific antibody [8, 12-14]. The resulting serum was often used directly or subjected to relatively crude purification steps involving salt precipitation. More recently different chromatographic methods have been applied to remove major protein contaminants or antigen-based affinity chromatography was also utilised to ensure greater purity. These methods and approaches are well documented. However, by their very nature polyclonal antibodies will contain a number of different antibodies to the target antigen and generally additional antibodies to non-target antigens. Such antibodies are now in general use in many immunoassays where they are often used as the labelled secondary antibody in, for example, an enzyme-linked immunosorbent assay (ELISA). The basic principle of an ELISA is shown in Figure 1. Many secondary antibodies are generated by immunising a host with a non-host antibody (e.g. immunisation of a goat with a mouse antibody such as IgG). The secondary labelled antibody is used to demonstrate or quantitate the degree of binding of the primary antibody, and implementation of this strategy greatly increases the sensitivity of the assay and the secondary antibody can be used with many different mouse primary antibodies to target antigens. Many of the issues that may arise from immunoassay-based approaches are highlighted in Table 1 and all of these must be addressed if this type of assay is selected [13, 14, 17].

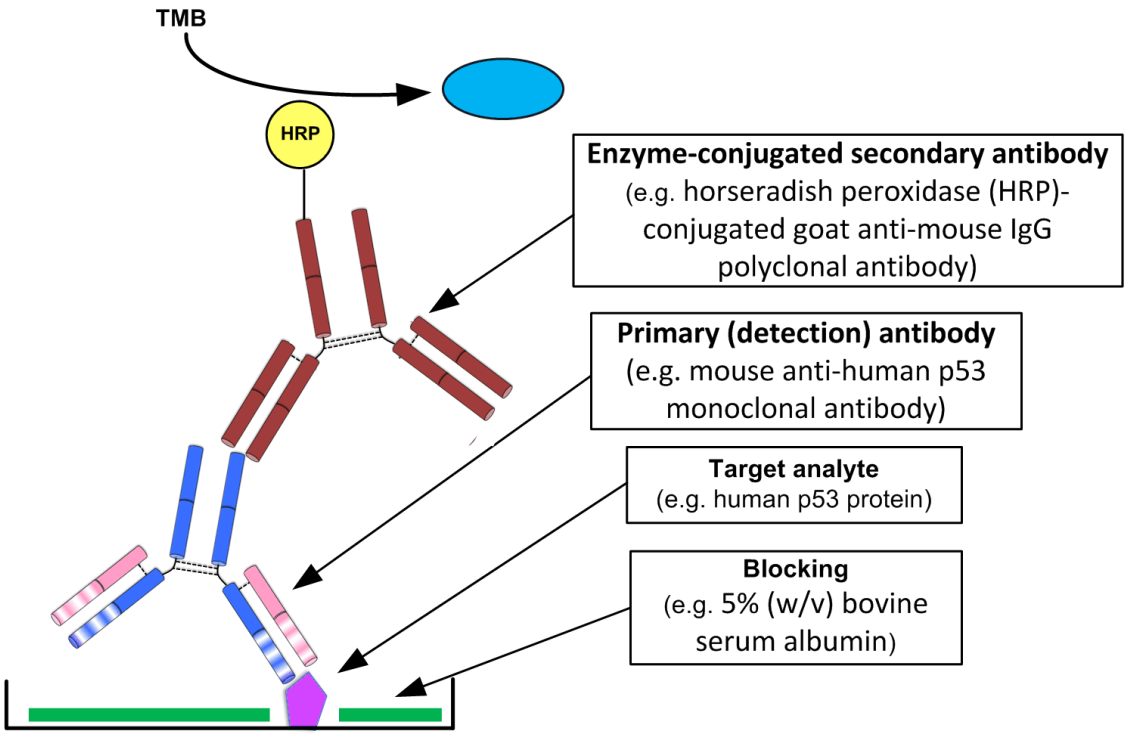

Figure 1: Basic principal of an indirect ELISA: An ELISA begins with a coating step, in which the target analyte is adsorbed onto a 96-well polystyrene plate (e.g. plate coated with human p53 protein). This is followed by a blocking step in which all unbound sites are coated with a blocking agent (e.g. 5% (w/v) bovine serum albumin). The plate is then incubated with the primary (detection) antibody (e.g. mouse anti-human p53 monoclonal antibody (mAb). Next the plate is incubated with an enzyme-conjugated secondary antibody (e.g. horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG polyclonal antibody (pAb)) that binds to the primary antibody. Finally a substrate (e.g. 3,3',5,5'-tetramethylbenzidine (TMB)) is added, producing a colourimetric signal that can be read to determine the quantity of analyte present.

Table 1: Issues to be addressed with the selection and use of polyclonal antibodies

	Issues to be addressed
Specificity	What is the specificity of the antibody and how exactly was this confirmed?
Secondary antibody	Was the secondary antibody tested against many different antigens?
Purification procedure	Is the antibody purified; if so how, and could the isolation procedure effect the antibodies properties (e.g. specificity, stability, aggregation)?
Anti-antibodies	In the case of anti-antibodies is the species specificity defined and is it appropriate?
Adequate information	Is there adequate information available in the catalogue or literature to support the choice of antibody? This refers to full information on performance rather than frequent usage.
Batch-to-batch variation	Is there sufficient material available as batch-to-batch variation may be a major issue?

Antibody binding and specificity

The immune system is a highly integrated multi-component system that is very active and effective in defence against disease. Antibodies are key elements of this system and they have been effectively exploited to generate multiple assay systems and are extensively used commercially with many therapeutic applications [18, 19]. The basic structure of an antibody molecule and a general structure of the antigen binding site, as exemplified by IgG, are shown in Figure 2. The general antibody structure consists of 2 light and 2 heavy chains linked by disulphide bonds with the binding site consisting of 3 complementarity determining regions (CDRs) in the light and heavy chains that are predominantly involved in binding to the antigen. There are also framework regions that provide a supporting scaffold for these CDRs and are important for their localisation to facilitate binding specificity. The CDRs may vary in their importance in terms of binding between different antigens and it is the variation in their amino acid composition that effectively plays a key role in attributing specificity. However, other regions of the antibody may also be important in determining the conformation of the antibody and the availability of the binding site. This includes the constant regions, presence/absence of glycosylation, and, especially for recombinant and other antibodies, the overall format including linkages between chains and degree of multimerisation. The binding of antigens to antibodies involves non-covalent interactions such as Van der Waal's forces, ionic and hydrophobic interactions. Studies have shown that the CDRH3 region may be very important for binding some antigens but the predominance of different areas may be very much dependent on the nature of the antigen (e.g. it will be different for small molecules than for large proteins). The structure of the binding site may vary depending on the antibody type, the nature of the antibody structure and its origin, as many different species have their own inherent variations [2, 3, 20-23]. The 'goodness-of-fit' between the antibody's binding region and the target antigen is referred to as the antibody affinity and for a good antibody the affinity is in the nanomolar range.

In addition to IgG, there are four other classes of antibodies, namely IgM, IgA, IgE and IgD, in mammals, all with their own structural variations and inherent properties. For example, IgM is pentameric, consisting of five basic linked units, and is the first antibody class generated following immunisation. Its pentameric nature enables it to be effective in aggregation of antigens and this property is often exploited in agglutination assays. IgY is the predominant antibody form found in the eggs of chickens. It is very stable and possesses additional constant regions in the heavy chains. There are also significant variations in the number of disulphide linkages found in the antibodies of different species.

While polyclonal antibodies were originally used in immunoassays the advent of monoclonal antibody technology, whereby clones of B lymphocytes (the cells within the body responsible for antibody production) could be isolated and used to generate antibodies to a specific targeted antigen, did much to improve antibody quality. However, monoclonal antibodies also need to be extensively characterised and their specificity well validated.

Increasingly, antibody-derived fragments and recombinant antibodies are used in diagnostics and therapies. For example, the use of Fab fragments (as shown in Fig. 2), derived by proteolytic treatment or by recombinant approaches, eliminates issues associated with the Fc portion of the antibody that sometimes interfere with assays (e.g. due to binding of serum proteins such as complement). Genetic engineering has provided a range of antibody-based fragments composed

mainly of the binding regions of antibodies, such as single chain fragment variable (scFv) consisting of V_H and V_L regions linked together by a glycine-serine linker or a single chain antibody (scAb) which has an additional constant region. The latter may aid stability and provide additional sites for labelling. The structure of a scFv and a scAb are shown in Figure 2. Recombinant antibodies offer major advantages due to the capacity to be able to engineer them to maximise their specificity and sensitivity, enhance stability through structural modifications, incorporate tags, labels or elements that facilitate purification, develop novel reporter systems/labels to generate unique assays and new approaches to optimise antibody orientation, thus maximising performance.

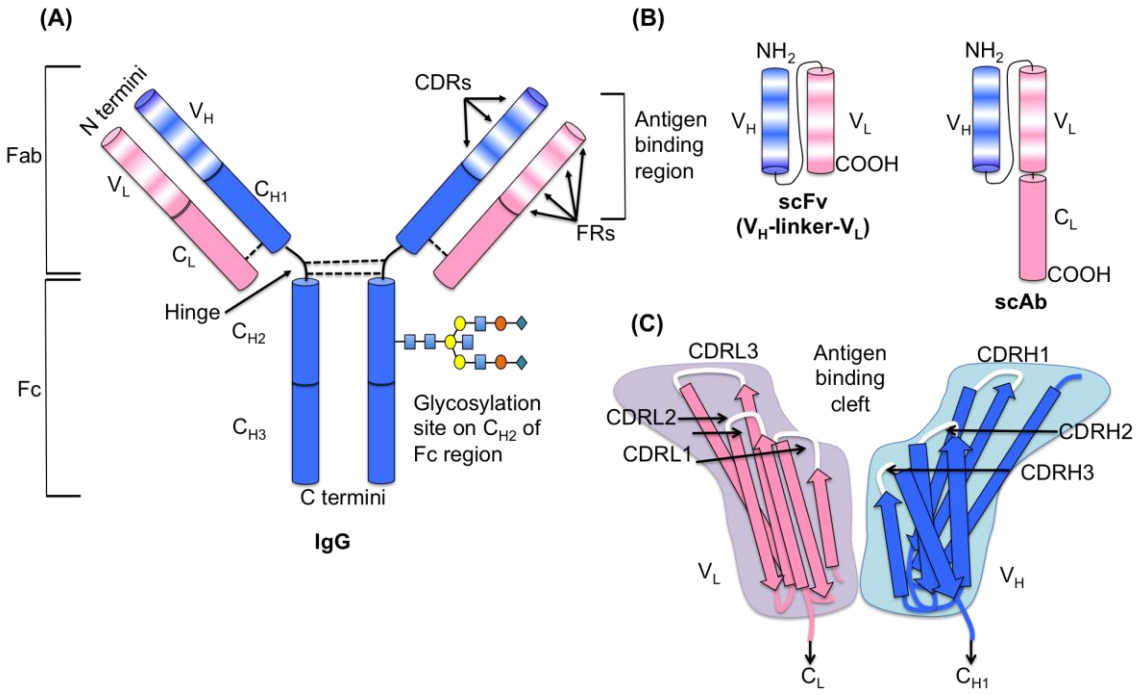

Figure 2: Antibody structure: (A) A typical IgG molecule is composed of four polypeptide chains, two heavy chains (shown in blue) and two light chains (shown in pink). They are linked together by disulphide bonds indicated by dashed black lines. The heavy chain is composed of one variable heavy (V_H) and three constant (C_{H1}, C_{H2} and C_{H3}) domains, the light chain is composed of one variable light (V_L) and one constant light (C_L) domain. The substructure of IgG can be divided into the antigen-binding fragment (Fab) and the crystallisable fragment (Fc) which are connected by an amino acid hinge region. (B) Examples of recombinant antibody fragments created using genetic engineering techniques include the scFv (consisting of a V_H and a V_L domain connected by a glycine-serine linker) and a scAb (composed of a C_L domain connected to the V_L of a scFv). (C) The IgG has two antigen binding regions each composed of one V_L and one V_H domain. Each variable region is composed of three complementarity determining regions (CDRs) (CDRH1, CDRH2 and CDRH3 pertaining to the heavy chain and CDRL1, CDRL2 and CDRL3 from the light chain) (shown in white), with each CDR flanked by structurally conserved framework regions (FR). A highly-conserved glycosylation site on the C_{H2} region of the Fc domain is important for maintaining antibody activity.

Individuals have the ability to generate very many antibodies against potential pathogens and this is a key facet of their effectiveness. In addition, antibodies also play a role in a range of other events mediated mainly via the constant regions located well away from their binding sites. While these

regions are vital to their physiological role they can be the source of interference when antibodies are exploited in different types of immunoassays.

It is also important to consider the way in which antibodies are designed to encounter and interact with target antigens. This usually occurs when the antibodies are either mobile in solution or are localised on the membrane of B-cells. Thus, they have considerable flexibility in accessing the antigen and are correctly orientated with the antigen binding sites available for binding. When in solution or on fluid membranes there is capacity to move to change the density of antibodies per unit area, thus enhancing the possibilities for unhindered binding. Natural antibodies generally have two or more arms with binding sites (depending on the class of antibody). Binding of an antigen to one arm of the antibody can enhance binding by the second arm. In addition, the flexibility within the structure to rotate, owing to the hinge or other similar structures (depending on species), are also important factors in assisting binding. However, when antibodies are immobilised either chemically or by adsorption there is high probability that the binding capacity of many antibodies may be reduced [24, 25], as illustrated in Figure 3. It has been suggested that when antibodies are adsorbed on to many surfaces (e.g. on an immunoassay plate) a high percentage of the antibodies may be effectively inactive due to denaturation, incorrect orientation, blockage of binding sites due to proximity effects and inaccessibility to antigen (e.g. with large proteinaceous antigens, and effects of pH, salt concentrations, temperature, stability and matrix interferents) [24, 26]. The result of such factors, even with highly specific antibodies, may be significant and the environmental effects may also impinge on binding specificity and on sensitivity (an extensive list of assay interferents are detailed in Table 2). While it is obvious that this may affect polyclonal antibody preparations, the presence of multiple antibodies with varying specificities and properties may actually be less of an issue, due to the lack of homogeneity, but it may be more operationally significant for monoclonal and recombinant antibodies that interact with a single target epitope. Generally these factors can be partially allowed for by the incorporation of standards and adequate controls but it does not always lead to good reproducibility and the capacity to generate comparable results in many different laboratories/platforms. Many assays also involve a series of incubations and washing steps and these may also lead to further problems as individual operators often have their own interpretations and approaches. Following adsorption, leaching of antibodies from surfaces may occur during assay washing steps. Given the differences in antibody affinities it is not surprising that individual assays need to be carefully optimised [27]. Thus, it is advisable that any elements that can be significantly affected by operators should ideally be automated to ensure reproducibility.

Table 2: List of commonly associated assay interferents

Interferents	Associated issues
Endogenous protein	Presence of endogenous label (e.g. peroxidase in horse serum) that can lead to erroneous signals
Cross-reactors	Cross-reactivity effects due to shared or overlapping epitopes or lack of specificity of antibody
Storage containers	Issues with sample containers (adsorption of antibodies/analytes to containers; interferences from inherent chemicals in the composition/material of the tubes; effects of anti-coagulants or other chemicals or ions)
Matrix interference	Matrix interference effects due to non-specific binding by non-relevant proteins or lipids
Stability	Lack of stability of sample analyte or assay reagents
Enzymes	Effects of enzymes causing decomposition of analyte (e.g. proteases)
Contaminants	Effects of microbial contaminants; can decompose relevant analytes or generate additional analytes
Haemolysis	Effects of haemoglobin in sample (*in vivo* or *in vitro* release of haemoglobin from erythrocytes) can interfere with the assay
Non-specific binding	Presence of specific/non-specific binders (e.g. of proteins, hormones or other targets)
Auto-antibodies	Auto-antibodies are produced by the immune system against the body's own proteins. These can bind to the analyte under study, blocking recognition by the assay antibody, thus leading to incorrect level determinations
Anti-antibodies	Antibodies present in serum that bind to other antibodies (e.g. antibody components in an assay)
Hook effect	Hook effect can occur when excess analyte is present beyond the dynamic range of the method, leading to incorrect values being reported
Chemical treatments	Destruction of antigen during sample processing (due to chemical treatments such as fixation, presence of salts; significant pH changes; temperature)
Physical damage	Aggregation and precipitation can damage the reagents (analyte or antibody)
Specific to assay	Presence of component of assay format in tissue (e.g. biotin)

Covalent attachment of antibodies to surfaces is widely used and can improve performance, though orientation may still be an issue and some covalently attached antibodies may be bound in such a manner as to inactivate or occlude their binding sites, as illustrated in Figure 3. Covalent attachment will improve the stability of the attachment and this should improve overall performance but the method used needs to be judiciously chosen [28].

To maximise functionality, antibodies need to be correctly orientated and this can be achieved in a number of ways including the use of various tags that facilitate immobilisation of the antibody via non-antigen binding regions such as the Fc region of an intact antibody [25]. Biotinylation of antibodies is often used, and the antibodies are then immobilised via the use of avidin, or ideally streptavidin or neutravidin, attached to the assay platform surface. However, with non-directed chemical linkage there is always the danger that antibody-antigen binding can be affected and this may also be an issue when antibodies are labelled [29, 30]. The advent of recombinant antibodies has allowed the incorporation of a range of tags and site specific modulations (e.g. to facilitate addition of biotin) that aid immobilisation and correct orientation and have been used to develop highly effective and reproducible assays.

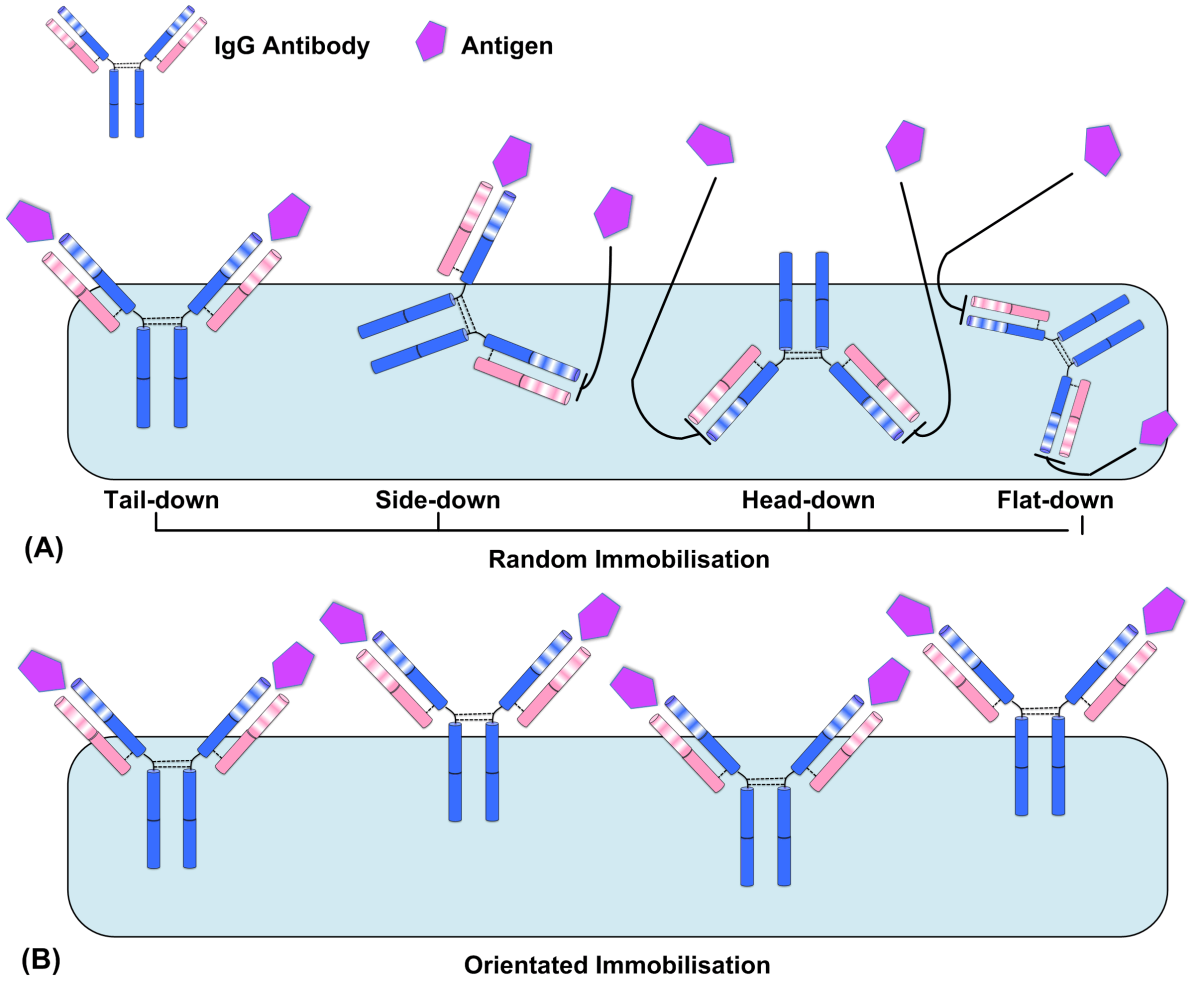

Figure 3: Antibody orientation: Antibodies can be immobilised in a random or orientated fashion. (A) Antibodies immobilised in a random fashion either covalently or by adsorption may bind in such a manner as to inactivate or occlude their antigen binding sites. Antibodies can bind in a tail-down, side-down, head-down and flat-down orientation, as depicted. (B) Site-directed immobilisations that facilitate immobilisation of the antibody via non-antigen binding regions can significantly improve immunoassay sensitivity. There are three conventionally used site-directed antibody immobilisation techniques in immunoassay design; immobilising antibodies on the assay surface via affinity interactions with a pre-formed layer of the Fc binding proteins, (e.g. protein A, protein G, Fc region-specific antibodies or various recombinant proteins), attaching chemically or genetically engineered antibody fragments to the assay surface via the sulfhydryl groups present in the hinge region and antibody immobilisation via an oxidised oligosaccharide moiety present in the Fc region of the antibody.

Exhaustive evaluation of performance of immunoassays (e.g. in serum or in histochemistry) [27, 31], serves to highlight factors that can significantly affect immunoassay performance. Currently many assays use both proteins and detergents to minimise non-specific binding of reagents or interferents. Proteins used include bovine serum albumin (BSA), ovalbumin (OVA), casein, fish proteins, milk proteins and other poorly defined materials. Many of these, while reducing non-specific binding, may have other attendant problems leading to lack of reproducibility. It should also be noted that tubes and other containers used for the preparation of standards or samples can themselves pose problems with binding leading to inaccurate concentration determinations. There are many other factors associated with samples that need to be considered. For example in sera the presence of human anti-mouse antibodies (HAMA) is well known and can cause issues with assays

leading to erroneous results. However, this may be the tip of the iceberg. There are many diseases where antibodies are generated to proteins present in serum [32-37]. While this would be expected in autoimmune diseases such as Grave's disease, the sera of patients with cancer (e.g. prostate, colorectal) often possess antibodies to a variety of different proteins that may be overexpressed in cancer [35-37]. Such antibodies could bind to and occlude epitopes on target antigens leading to underestimations when analysed by immunoassay.

Some case studies as exemplars of complications in immunoassays

A very interesting example of the complications that may exist can be illustrated with a review of the issues associated with the determination of Troponin I (TnI), a marker of cardiac disease of particular significance in acute myocardial infarction (AMI) [38-40]. To determine the levels of TnI accurately in serum may require the use of multiple antibodies e.g. one antibody may act to capture TnI from serum and two or more additional antibodies, that identify other sites on the captured TnI are necessary to accurately determine the actual levels present The reasons for the need for multiple antibodies are listed in Table 3. Given that this is the case with troponin, it is highly likely that other antigens may also have similar issues.

Table 3: Factors affecting the assay of Cardiac troponin I

Cardiac troponin I assay complications
Shared peptide regions with skeletal muscle troponin leading to cross-reactivity
Failure of antibody to recognise TnI when complexed with troponin C (TnC) due to occlusion of the binding epitope
Lack of stability of part of TnI due to proteolytic activity leading to loss of epitope regions
Phosphorylation of protein leading to changes in conformation and occlusion of antigenic sites
Presence of auto-antibodies to selected regions of TnI
Interference from rheumatoid factor

As a result of these factors TnI is normally assayed using at least 4 antibodies to capture and correctly measure the levels present. It may also be the case that the use of more than 4 antibodies may actually be necessary to accurately determine TnI levels [36, 37]. This case study clearly illustrates the complexity involved in the effective application of some immunoassays. There is now a major focus on developing high sensitivity assays for TnI and other troponins in the pg/mL or pg/L ranges to generate results that could radically improve patient diagnosis in AMI.

The presence of antibodies with general specificity, so called heterophile antibodies, is well documented and known as a source on interference in assays [34]. The presence of such antibodies is hardly surprising given the role of the immune response as a surveillance mechanism. It is also highly probable that there may be multiple shared epitopes across a range of antigens due to homogeneities in amino acid sequences or due to the topography of the epitopes, from a three

dimensional and charge perspective, as encountered by the antibody. The term 'sticky' antibody has also been used and though poorly defined it often refers to the propensity of some antibodies to bind to surfaces and this can become a significant issue, for example, in microfluidics-based devices where adequate surface treatment is essential to prevent this happening [41]. Inappropriate care in procedures used in antibody isolation can also lead to polyreactivity [42].

Determination of Prostate specific antigen (PSA)

Measurement of PSA was for many years used extensively for detection of prostate cancer and still has applicability in monitoring disease progression [43]. However, PSA assays used as screening tests for prostate cancer and also in the selection process of patients for therapy, may have been inappropriately used and this is undergoing intense scrutiny [44]. Other markers and PSA forms, including, free, complex and associated peptides and/or their ratios are now deemed more effective in certain situations. It has also been suggested that monitoring of fractions of PSA and/or glycosylation at much lower levels than currently thought to be clinically relevant might also be more beneficial [44]. The situation described here highlights the need for the appropriate selection of biomarkers for disease detection and it is increasingly being realised that the use of multiple biomarkers may be necessary to increase sensitivity and specificity to the level necessary for effective clinical utilisation. It also highlights the need for integrated use of highly selective antibodies or appropriate binders (e.g. lectins) to allow adequate diagnosis.

Are we going about antibody development if the best way?

The current approach for the production of many antibodies appears to focus on the achievement of high specificity/sensitivity without paying sufficient regard to the actual test or platform that will be used. The literature has many descriptions of antibodies that work well on some systems but not on others (e.g. work in ELISA and on blots but not in immunochemistry or other applications) [11, 16]. Perhaps the 'whys and wherefores' need to be systematically investigated so that we can understand the underlying issues and design the antibody production strategy to address them. The current approach is very much 'try it and see' which wastes considerable amounts of money and time. An established approach is to incorporate the eventual platform-of-use at a very early stage in the antibody screening process and this certainly works but it would be very valuable if a deeper understanding of what antibody characteristics underpin utility were fully understood [40]. All too often the antibody is selected without adequate prior knowledge of its potential applicability in a particular situation. With the recognition of the need for defined antibodies, and the availability of tools to refine the structure and associated characteristics, we suggest that the issues could be adequately addressed resulting in far better reagents.

Bound or blocked antigens

A high percentage of molecules in circulation are bound, either to carrier proteins or as part of complexes arising due to their normal physical state or aggregation. Many hormones are bound to carrier proteins in the blood and drugs are conjugated, due to well established, and possibly less well established mechanisms, and this may also lead to lack of recognition [44]. In the case of toxins (e.g. microcystins and mycotoxins) it is clear that there are an ever expanding number of derivatives/metabolites that may or may not be detected by current assays. So the key approach may be to have cocktails of matched fully characterised antibodies with the requisite specificity to cover the target species and their derivatives.

Are we naïve in relation to epitope availability?

It is very evident from extensive glycomics-based studies that expressed proteins may have a number of different carbohydrate side chains and that some may vary during the life-time of the protein leading to speculation that carbohydrate-associated epitopes may be affected. Carbohydrates in proteins have many functions including protection against proteolytic degradation and it is probable that tumour cells and parasites also use carbohydrate-based structural alterations to effect/reduce antigenicity and host surveillance. It is also a fact that parasites have mechanisms to cleave antibodies thus rendering them ineffective in initiating an immune response [45]. Could such phenomena also be interfering with the efficacy of antibodies used in assays?

Storage

Each antibody has its own optimum storage conditions that must be adhered to in order for the antibody to retain its functional activity. Therefore, the ideal storage temperature and pH need to be determined. A decision also needs to be made on whether or not to add cryoprotectants such as glycerol. The presence of cryoprotectants in an antibody preparation can greatly extend shelf-life. However, their presence can also interfere with many assays and tests. Glycerol (10%, v/v) is often used as a very effective cryoprotectant but its presence also interferes with many tests such as the BCA assay and the Biuret test. Sodium azide is also a very effective preservative due to its role in inhibiting the growth of contaminants, such as bacteria or fungi. However, sodium azide is very toxic and also inhibits HRP, which is a typical label in secondary antibodies. Therefore, storage conditions and storage components need to be carefully considered.

An informed strategy to improve antibody selection and use

Having outlined all the key issues it is vital is to develop a strategy to address them and thereby lead to greatly improved antibody selection and usage to prevent the current great wastage of time and resources and to remove the very significant frustrations involved. Table 4 lists key steps that are considered necessary.

Table 4: Suggested steps to test antibody utility and improve usage

Steps	Considerations
Publication of antibody usage	Check antibody use in the literature and take note of user evaluations – there are several websites and groups that have recorded and are recording outcomes of antibody usage (e.g. www.pabmabs.com).
Sufficient validation	Insist on getting as much validation information as possible from the supplier. In many cases what is given is not sufficient. Mass spec and knockdown/knockout approaches (where the target analyte is eliminated i.e. no longer expressed on the cell surface and subsequent testing demonstrates elimination of antibody binding) are valuable in validating binding specificity.
Antibody source?	If the original source is listed contact the researchers involved in the generation of the antibody. Often valuable usage data is not published and many labs have a store of additional information on their antibodies.
Determine affinity	Take the necessary time to check data on affinity and how this was determined; some of the information in the literature can be questionable.
Citing antibody usage in literature	When citing information on an antibody you have used include as much detail as possible including the batch number.
Question secondary antibodies	Don't stop with the primary antibody. Secondary antibodies can be major causes of erroneous results due to lack of specificity.
Sequence information	Knowing the sequence of the antibody can be useful but it is not sufficient to define operational specificity.
Cross-reactivities	If possible the antibody should be tested against arrays with multiple proteins etc. Shared epitopes are a potential problem particularly if working in complex matrices such as blood.
Handle with care	Handle the antibody carefully – many false results may be self-inflicted through usage of inappropriate, temperatures, pHs, salt concentrations and storage conditions. Antibody preparations are prone to microbial contamination, protease activity and aggregation.

Future approach

While the problems outlined relate mainly to antibodies incorporated into immunoassays it is highly probable that comparable rigorous approaches need to be applied with therapeutic antibodies, antibody-derived therapeutic conjugates and other antibody-derived moieties. Such antibody constructs and their uses have recently been reviewed [46]. Equally, care with specificity and use in assay development would also be relevant for a range of binders e.g. aptamers or affimers.

It is clear that antibodies have provided major advances in diagnostics and therapeutics but there is a very significant need to increase stringency in their selection and characterisation. While this may be costly it is necessary to prevent lost expenditure of time and resources and will be beneficial for research strategies aimed at improving health, environmental and food quality. A recent paper in Nature Methods [47] has clearly set out practical steps that should be taken in order to define antibody specificity and for antibody validation. Adaption of their recommendations and the approaches outlined in this review, and the associated references, may go some way to successfully addressing the issues.

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