Development of antivenoms in South Africa

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Antivenoms to treat the bites of snakes and, later, of arthropods have been produced by the South African Institute for Medical Research for 70 years. The established technologies have stood the test of time remarkably well.

A myriad of venomous creatures co-inhabit the southern African subcontinent with man. Fortunately, the mortality of human envenomation remains low. In 1992, just 29 deaths due to venomous animals and plants were recorded.[1] Numerous remedies have been adopted to protect humans from these 'dreaded creatures'.

Four thousand years ago, Egyptian priests opened up the snake-bitten arm of their patients to let out evil spirits.[2] Today, a similar procedure (fasciotomy) is advocated by some for occasional complications of snakebites,[3] although the rationale, to relieve internal compartment pressure, is different. Many other folk remedies abound, but none is of proven value, and while anti-inflammatories and analgesics may alleviate symptoms, anti-venom, the serum of animals immunised with venom, remains the only specific treatment for envenomation.[4]

The development of anti-venomous sera was closely associated with the discoveries and inventions taking place in microbiology and immunology during the latter half of the nineteenth century.[5-6]

Stimulated by the discovery of neutralising antibodies to diphtheria and tetanus toxins, Calmette initiated similar studies on cobra venom.[6] In 1894, the possibility of anti-venomous serotherapy was demonstrated simultaneously by two groups of workers.[6] At the Pasteur Institute in Paris, Calmette reported that the serum from animals inoculated with his treated cobra venom was anti-venomous. In the same week, Phisalix and Bertrand, working at the Natural History Museum in Paris, showed that the serum of guinea pigs vaccinated with heated viper venom also provided protection against viper venom. Two years later, Calmette produced the first antivenom for therapeutic use, marketed as *Serum Antivenimeux anglicé Antivenine*.[6]

The specificity of these sera for the venom source soon became apparent, but the occurrence of adverse reactions to serotherapy was a cause for concern. Many problems had to be overcome in order to manufacture a safe, venom-neutralising serum. Extracted venoms had to remain stable and sterile for use as antigens in an animal host. To avoid toxicity of venom in host animals, methods of venom detoxification or inactivation were required. It was also imperative that these methods were developed for venoms from different (snake) species. Furthermore, mechanisms of purifying and concentrating plasma from immunised animals were necessary to reduce the incidence of adverse reactions to serum in recipients. Finally, information about antivenom efficacy was essential. The present article explores how former researchers in South Africa in collaboration with overseas scientists overcame these obstacles successfully to manufacture antivenom.

The development of snake antivenom

The use of snake venom as an antigen to raise an immune response in an animal host began as early as 1886,[7] and the production of snake antivenom in South Africa began in 1901 in a laboratory in Pietermaritzburg, carried out by Dr Watkins-Pitchford.[8] Serum was tested for therapeutic use as early as 1903.[9] However, until 1930 the only antivenom generally available for use in South Africa was provided by the Pasteur Institute in Paris, from animals immunised with venom from South African snakes.[10] This antivenom, effective against both cobras and adders, was supplied by Mr F.W. FitzSimons, director of the Port Elizabeth Museum and Snake Park.[11] Each 10 millilitres of Pasteur snake antivenom cost 15 shillings (current value about R125) per vial. A complete first-aid antivenene treatment outfit consisting of a lancet, ligature, syringe and two bottles of serum with instructions was sold at £3.5s (current value about R545).

Fig. 1. Dr Grasset in his laboratory standardising equine serum (1930).

Choice of animal. Experimental work, using rabbits and guinea pigs, had established the concept of raising neutralising antisera and large-scale production laboratories rapidly adopted the horse as the animal of choice. It was already accepted as the source of bacterial antitoxins, allowed large volumes (5–10 litres) of blood to be withdrawn without ill effects, was long lived, amenable to handling and lived in close association with humans. There was also an accepted protocol of ownership of these animals and legal protection for what would be a very precious asset, when fully immunised. Recently, workers have identified a number of shortcomings regarding the use of the horse and these will be discussed below.

Antivenom production began at the South African Institute for

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Table 1. Comparison of the pre-1946 and current horse immunisation schedules used at SAVP for the production of snake antivenom.

Pre-1946 schedule	Present schedule
Anavenoms used	Venoms not inactivated
1st (basal) immunisation over 8 weeks	1st immunisation over 10 weeks
No 2nd immunisation schedule	2nd immunisation over 7 weeks
Rest period of 4–6 weeks	Rest period of 4–6 weeks
Re-immunisation over 3 weeks	Re-immunisation over 2 weeks
Additive: tapioca	Adsorbent: bentonite
25–200 ml of venom injected	1–10 ml of venom injected
1% venom concentration	0.1–4% venom concentration
50 litres of blood/horse taken per year	75 litres of plasma/horse taken per year

Medical Research (SAIMR) in 1928, when Dr E. Grasset was appointed to the staff (Fig. 1). Several domestic animals were tested for the large-scale production of therapeutic sera but the horse proved to be the most suitable (Table 1).

Venom detoxification. Mitchell and Watkins-Pitchford, in 1915, had begun immunising horses with unmodified venom and hypochlorite.[8] This method proved unsatisfactory, causing unduly severe reactions at the injection site and produced symptoms of toxicity and thrombosis of injected veins. An inactivated (atoxic), but still antigenic, preparation of venom was consequently sought to immunise horses (Fig. 2). Several methods of venom detoxification were explored. Grasset and Zoutendyk (1933) used ox bile-detoxified venom and venom treated with an organic gold preparation (known as Solganol).[12] Ox bile had been credited with powers of neutralising venom.[13] While these detoxified venoms permitted larger doses of venom to be used for immunisation over shorter periods, their method still brought about unwelcome symptoms in the horses.

A formalin-detoxified venom (called anavenom) was introduced by Grasset in 1932, and proved to be a more practical method for the large-scale immunisation of horses[14] (Fig. 3). He included tapioca in the injection mixture, in an attempt to reduce toxicity and boost responses. Anavenoms were still in use in 1946, when Dr Poul Agerholm Christensen replaced Grasset as head of the Serum and Vaccine Department at the SAIMR. The large volumes of formol-precipitated venom and experimental use of Freund's adjuvant continued to cause reactions (abscesses, pyrexia and shock) in horses. Furthermore, strict economy of venom became necessary due to temporary shortages, and doubt had been cast on the antigenic value of formol-treated venom.[9] Based on some early work of Calmette (1894), Christensen began using bentonite-adsorbed (but not inactivated) venom,[15] without other adjuvants. This inert carrier, impregnated with venom, and injected subcutaneously, served to release small quantities of venom over a number of days, thereby stimulating the immune system without causing harm to the animal. These horse immunisations resulted in all animals responding with measurable amounts of antibody. Furthermore, much smaller quantities of active venom proved immunogenic than with the detoxified venom. Christensen's method is still used in present-day primary immunisation schedules at South African Vaccine Producers (Pty) Ltd (SAVP), a wholly owned subsidiary of the SAIMR, with the addition of lignocaine (Remicaine) to reduce local discomfort. Plasmaphoresis, introduced by Schrire in 1985, allowed the red blood cells suspended in saline to be transfused back into the horses after separation from the plasma by gravity sedimentation. This fact has enabled horses to be bled more frequently, at the peak of antibody output. In 1991, when Southern headed the Serum and Vaccine Department, new teams of polyvalent horses were immunised. Antigen-antibody diffusion assays were introduced to monitor horse responses to the immunisation schedules and ensure that only plasma with substantial levels of antibody was processed. This has reduced the number of *in vivo* tests on crude plasma and these essential neutralisation tests are now carried out only on the final bulk product.

Fig. 2. Grasset (*centre*), Zoutendyk (*right*) and their associate injecting a horse with antigen (1930).

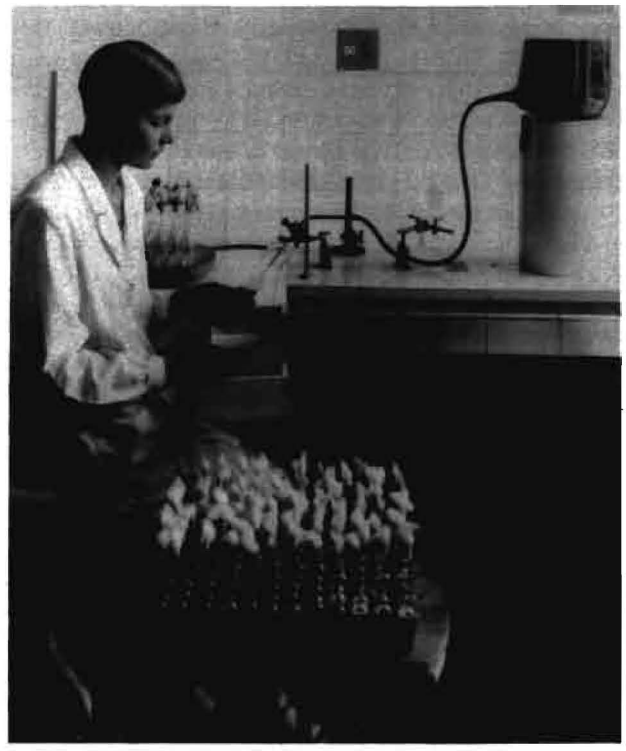

Fig. 3. Ampoules of serum being prepared from bulk equine serum (1930).

Development of polyvalent snake antivenom. The initial antivenoms produced by the SAIMR were limited to the Cape cobra (*Naja nivea*) and the Puff adder (*Bitis arietans*). These venoms

were readily available and there was a high reported incidence of bites by these snakes.[16] A bivalent antivenom was prepared by immunising horses with a combination of these detoxified venoms. Early reports indicated wide cross-neutralisation of the venoms from other snake species by this antivenom, but these reports were later proved to be unfounded.[17] This antivenom did not neutralise the venom of the Gaboon adder (*Bitis gabonica*) and in 1938 venom from this snake was incorporated into the immunisation schedule, widening the spectrum of activity of the antivenom.[18]

In 1941, as a result of the Second World War (1939–45), antivenom production had to be increased. Various African allies, who before the war were obtaining their antivenom from the Pasteur Institute in Paris, also required antivenom. As demands for venom could not be met, it became necessary temporarily to import Indian cobra (*Naja tripudians*) venom. During the period 1932 to 1943, a total of 46 horses was used for the production of antivenom (Fig. 4). During this time, the range of the polyvalent antivenom was expanded by incorporating venom from the rinkhals (*Hemachatus haemachatus*).[19]

During the 1950s and 1960s, several experimental monovalent and trivalent antivenoms to the southern African mamba species (*Dendroaspis polylepis*, *D. angusticeps* and *D. jamesoni*) were developed and tested,[20] and by 1971 the SAIMR Polyvalent Snake Antivenom incorporated these valencies.

Other venoms incorporated into the immunisation schedules during the 1970s were the Egyptian cobra (*Naja haje annulifera*), Forest cobra (*N. melanoleuca*) and the Spitting cobra (*N. mossambica/nigricollis*) until a single polyvalent antivenom was being produced.[21]

In view of the lack of neutralising action of available antivenom against boomslang (*Dispholidus typus*) venom, a specific antiserum against boomslang venom was developed during 1940.[22,23] Boomslang venom was shown to have a strong coagulant action, which was different from elapid and viper venoms. Methods of detoxification were analysed and again the formalin method proved most effective. The difficulty in obtaining sufficient quantities of boomslang venom, as well as the low incidence of bites, has made the incorporation of this valency into the polyvalent antivenom impractical.

Monovalent antivenom produced for the saw-scaled or carpet viper (*Echis carinatus/ocellatus*), not indigenous to South Africa, was developed at the urging of Moravian missionaries, and has been exported to North and Central Africa with particular success.[24]

Other venoms of South African snakes, which have been considered for production of antivenoms, include the berg adder (*Bitis atropos*), the Namibian variant of the Spitting cobra, known as the zebra snake (*Naja nigricollis nigricincta*) and the twig or vine snake (*Thelotornis* spp.). The low incidence of envenomation caused by the bites of these snakes has so far made it economically impossible to produce these antivenoms, or to incorporate them into the current polyvalent antivenom.[25]

Fig. 4. A snakebite kit from 1944 with two ampoules of polyvalent snake antivenom, syringe, tourniquet and instruction booklet.

SAVP presently manufactures three snake antivenoms. A polyvalent antivenom [a Schedule 2 medicine, in terms of the Medicines and Related Substances Control Act (101) of 1965] is produced against the venom of the Puff adder (*B. arietans*), Gaboon adder (*B. gabonica*), rinkhals (*H. haemachatus*), Green mamba (*Dendroaspis angusticeps*), Jameson's mamba (*Dendroaspis jamesoni*), Black mamba (*D. polylepis*), Cape cobra (*Naja nivea*), forest cobra (*N. melanoleuca*), Egyptian cobra (*N. haje annulifera*) and the black-necked Spitting cobra (*N. nigricollis*). A monovalent antivenom (a Schedule 4 medicine) effective only against the bite of the boomslang (*D. typus*), and an antivenom for the exotic saw-scaled viper (*E. carinatus/ocellatus*) are also produced.

South African arthropod antivenoms

Spiders. The earliest report of a spider bite in South Africa is from 1827.[26] The first series including six cases was published in 1929.[27] The most dreaded and certainly most dangerous of the southern African spiders is the button or widow spider ('*knopiespinnekop*') belonging to the genus *Latrodectus*.[28] This spider derives its name from its likeness to the spherical black shoe-button which was so popular at the beginning of the twentieth century.[29] Before the introduction of a specific antivenom in 1935 by Finlayson,[30] the treatment for *Latrodectus* bites was empirical and symptomatic.[31] *Latrodectus* antiserum, produced initially at the Union Health Department laboratory in Cape Town, was made from homogenised spider heads of various female black widow species. The lethal dose of black widow extract was found to be 2–4 times the lethal dose of cobra venom in mice,[30] even though the extraction procedures followed probably destroyed most of the activity of the neurotoxic latrotoxin.[32] Finlayson initially injected dried spider venom into goats. This goat anti-serum proved to be highly effective in counteracting the effects of the spider bite.[33–34] The actual production of *Latrodectus* antiserum from immunised horses at the SAIMR began in 1949.[35] Antivenom was, and today still is, prepared by a process identical to that used in the production of snake antivenom. The venom used for horse immunisations is now obtained from dissected venom glands from which a relatively pure venom is extracted and preserved by freeze-drying. A recent review of a series of 45 cases of envenomation by the button spider describes the successful use of this antivenom.[36] Other venomous southern African spiders are not considered life threatening and, to date, no attempt has been made to prepare antivenoms to these.

Scorpions. SAVP also currently manufactures an antivenom to treat serious stings from the *Parabuthus* scorpion. Investigations on scorpions were initiated by the SAIMR in 1940 in response to a request from the Union Defence Medical Services for the preparation of an anti-scorpion serum, owing to the presence of numerous scorpions in districts where army troops were camped.[37] Venom was collected from 338 *Parabuthus* specimens (with a total venom weight of 1.63 g), from 5 985 *Opisthophthalmus* scorpions (yielding 8.62 g of venom) and from 9 603 *Hadogenes* specimens (26.27 g of venom being obtained). The total number of scorpions used for all experiments was 16 946. Considering the satisfactory results obtained in the preparation of snake anavenoms, similar attempts were made in the preparation

of anti-scorpion equine serum. Neutralisation tests of hyperimmunised horse serum, conducted in mice and pigeons, culminated in therapeutic *Parabuthus transvaalicus* scorpion antivenom being prepared by the SAIMR. Initially, whole scorpion telsons were homogenised and detoxified for use as antigens. Today a colony of scorpions housed in the animal facility at SAVP permit routine venom collection by electro-stimulation. The venom is pooled and purified before horse immunisation. A review of 42 serious cases, some of which proved fatal, of scorpion sting questions the efficacy of this antivenom in the treatment of envenomation caused by *Parabuthus granulatus* in the Western Cape.[38]

Purification and concentration of antivenom

The incidence of adverse reactions following the administration of native equine serum motivated progress in the concentration and purification of the immune serum. In 1932 Grasset began purifying antivenom at the SAIMR with sodium sulphate fractionation.[39,40] This improved the potency, and consequently the volume of antivenom used for treating snake bites was reduced.

In 1947 a further improvement came about with the development of proteolytic enzyme purification, according to the method of Pope.[41,42] The increased antibody concentration, purity and stability[43] of enzyme-treated $F(ab)_2$ antivenom (Fig. 5) had a big therapeutic advantage in the treatment of snake bite.

Current purification of antivenom at SAVP is still based on Pope's method, although an ion-exchange step has allowed the establishment of higher potency limits for these products. The increased potency of enzyme-digested, refined equine immunoglobulin has enabled many more units of antivenom to be administered per gram of protein, thus reducing the risk of allergic reactions. The current potency standards are based on the capacity of a fixed volume of the antivenom to neutralise a set number of lethal doses of venom in mice (Table 2).

In 1948 Christensen set out to accumulate data relating to the efficacy of the antivenom being produced. He enclosed a questionnaire with each ampoule of serum issued by the SAIMR. From 1953 until 1979, a total of 2553 questionnaires were

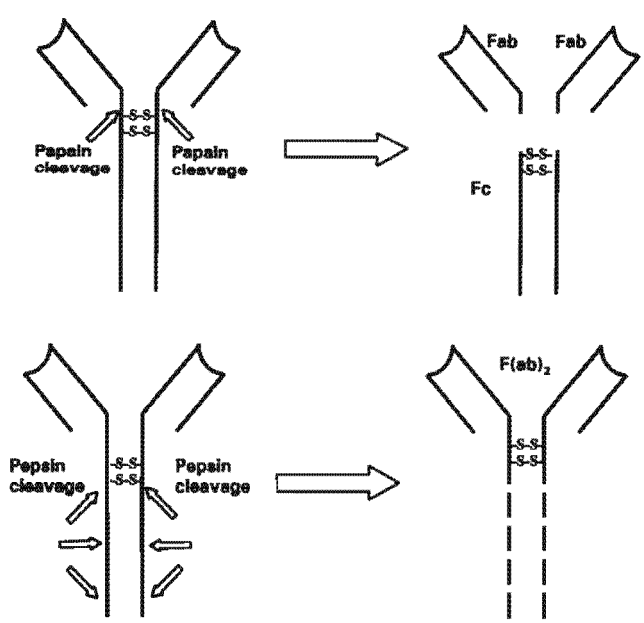

Fig. 5. A diagrammatic representation of an immunoglobulin molecule and the results of enzyme cleavage. [From: *Antibodies* by E. Harlow and D. Lane, Cold Spring Harbour; 1988]

Table 2. Minimum potency requirements for SAIMR Polyvalent Antivenom (expressed as mouse LD_{50} neutralised per 150 µl antivenom).

Venom source	Mouse LD_{50} neutralised
Cape cobra (*N. nivea*)	20
Puff adder (*B. arietans*)	12
Gaboon adder (*B. gabonica*)	12
Black mamba (*D. polylepis*)	15
Rinkhals (*H. haemachatus*)	7

Note: Due to cross-reactivity between species, it is not possible to differentiate antivenom potency for individual cobra or mamba species.

returned.[44] The low mortality (52 fatalities) from snake bites and reported low incidence of adverse reactions and anaphylaxis (<1%) to the antivenom therapy was felt to confirm the success of the antivenom being manufactured by the SAIMR. The majority of antivenom manufacturers worldwide continue to rely on a similar strategy for immunisation of horses and similar purification methods for these life-saving products.[45]

New developments in antivenom production. A clearer understanding of the mechanisms of raising and purifying antibodies and of the neutralisation of toxins has resulted in the development of a number of new approaches to antivenom production.

The acceptance of the horse as the host animal for antibody production has been challenged on a number of grounds.[46] They are expensive to maintain and require special handling and housing. Freund's adjuvant, which best promotes antibody production, causes unacceptable reactions in the horse, so that larger doses of expensive and rare venoms are required to boost antibody levels. Additionally, adverse reactions to horse proteins in antivenom recipients are reputedly more common than to those of other animal species.

However, these concerns must be balanced against the wealth of experience and knowledge, which has been generated by the use of horse antivenoms during the last century. Alternatives such as the goat or burro (mule) have been used on a limited scale, but the most promising successor to the horse is the sheep, which is economical to maintain and house, is not apparently badly affected by Freund's adjuvant and whose proteins have lower reactogenic potential in humans.[47]

The use of monoclonal antibodies has been seriously considered, particularly as these may be engineered to have the characteristics of human immunoglobulins, and may be produced in bulk *in vitro*. However, currently, their exquisite specificity remains a limiting factor, making their neutralising activity against all components and biological variants of a toxin or venom questionable and difficult to prove.[48]

Improved methods of processing and purification of immune serum have been developed which show great promise for reducing the two major forms of adverse reaction to immunotherapy. Anaphylaxis and anaphylactoid reactions are caused by the systemic release of histamines,[49] either by direct specific IgE mediation, or indirectly via complement activation by residual reactive parts of the administered IgG molecule (Fig. 5). The likelihood of these reactions can be reduced by removing as much of these foreign proteins as possible and by using enzymes, which remove the reactogenic portion of the immunoglobulin molecule.

Salt fractionation, ion-exchange chromatography and caprylic acid precipitation[50] have all been used during purification to improve the specific activity of antivenom by reduction of non-

specific proteins. The enzyme, papain, which cleaves the IgG molecule to individual Fab fragments, may also be used. These fragments are more able to penetrate extravascular parts of the body. These are also more rapidly excreted, which makes repeat dosing necessary in some instances, but may on the other hand have the advantage of reducing the incidence of serum sickness.[47] Antivenoms produced in this way have proved effective in comparative trials.[51] Affinity purification of the Fab fragments of neutralising antibody on columns containing venom coupled to a support medium, has been shown to enhance the relative potency of the antivenom threefold,[52] and is believed to be necessary to remove immunoglobulin Fc fragments (Fig. 5) responsible for adverse reactions seen in early trials.[51]

Manufacture in new facilities, increasingly complex production sequences, and the expense of development of these products has resulted in prices exceeding US$500 (about R3250) per ampoule. By comparison, 'old' products sell for US$20–50 per ampoule.

Serum sickness is a common and unpleasant sequel to treatment with foreign proteinaceous material and is caused by the processes by which the body eliminates this material. Purification processes which enhance the neutralising potency of the preparation relative to the total protein content, and which enhance excretion rates, will reduce this reaction.

Future production

It seems unlikely that products of the new technologies will replace immediately the equine antivenoms currently available, but it is possible that new preparations will be developed using these means. These products are likely to be monovalent, high-potency and -specificity antivenoms capable of neutralising and reversing the action of individual venoms and toxins. This in turn will drive the development of improved rapid and rugged diagnostic tests, to ensure that these effective and expensive therapies are used to the best advantage. The expense of these antivenoms may also result in a change in treatment strategies with the establishment of a few regional centres of expertise, to which victims are rapidly transported for the most effective treatment. The future role of South African manufacturers in the production and development of antivenoms for southern African use will depend on the availability of expertise, investment and the continued support of the community

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Colour generation in textile ink-jet printing

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Ink-jet printing systems have recently come into productive use where the design information is handled digitally and the application of ink is computer controlled. Printing cotton fabric with combinations of cyan, magenta and yellow reactive dye inks enabled the generation of a broad gamut of colours, which was ascertained by spectrophotometric means. The quantity of ink was controlled by varying the firing time within the duty cycle for drop-on demand electromagnetic valves. Although the print resolution using this technique was relatively low, the colours obtained indicated sufficient mixing of the primaries to produce subtractive mixing rather than partitive (additive) mixing.

Historical background

The earliest attempt at ink-jet printing for textiles is credited to the then East German firm, Textima, in Zittau, which patented a form of jet printing machine in 1970. That initial research soon sparked off interest in a host of competitors, notably in the US, Austria and Australia, some with varying degrees of achievement, particularly with respect to carpet printing. That notwithstanding, success in the ink-jet printing of textile fabrics itself still proved elusive. In 1991 a joint venture was announced between the Japanese firms of Canon, Kanebo and Toshin Kogyo to develop a bubble jet textile printing system and, also in that year, the Stork company in the UK launched the first commercial system for the ink-jet printing of textile samples. However, the low output rate of the Stork machine at 1 square metre per hour, whilst perfectly suitable for samples, renders it unviable for bulk production purposes. Thus, it is in this context that the present work was undertaken, to contribute to the development of an ink-jet printer capable of printing textiles at high speeds whilst maintaining high print resolution. It is hoped that, when fully functional, this technology will enable the printing of everyday consumer items such as clothes, bedlinen and curtains with high quality but at a much reduced cost.

The increasing demand for quick response times and short process runs in the textile printing industry has stimulated the development of a number of ink-jet printing technologies where the design information is handled digitally and the application of ink is computer controlled.[1] The advantages presented by ink-jet printing over conventional printing range from cost savings from printing without screens to achieving rapid pattern change with minimum wastage and speedily responding to the usually unpredictable changes in fashion. Furthermore, dyes used for conventional screen printing can also be used for ink-jet printing, which therefore permits both bulk and sample printing without having first to prepare pattern separations or screens.[2]

Of the several technologies of ink-jet printers available, the drop-on-demand (DOD) systems appear to offer the greatest potential for textile applications. The DOD printer produces an ink droplet when required and 'fires' it onto the substrate. The simplest systems are based on a pressurised supply of ink coupled via electromagnetic valves to a series of jet orifices. The DOD printer generates a print by directing one drop to a pixel location. Thus on any one pixel, there can either be an ink drop or no drop at all. Halftones are produced by a matrix of dots which form a super pixel, sometimes referred to as a dither pattern (Fig. 1).[3]

The pixel itself is defined as the smallest picture element.[4] Colour ink-jet printers produce a wide gamut of colours, either by using a matrix of dots to produce each pixel, or by jetting variable-sized dots of each primary coloured ink in a manner that enables them to mix on the substrate. The DOD electromagnetic solenoid ink-jet printers use the matrix pixel system because it is the easier to control and can be manipulated to produce dither patterns, as shown in the simplified 2 × 2 monochrome superpixel in Fig. 2.

The five grey levels possible can be 'dithered' to produce 16 variations for each of the primary colours, cyan (C), magenta

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