Effect of anti-mosquito midgut antibodies on development of malaria parasite, *Plasmodium vivax* and fecundity in vector mosquito *Anopheles culicifacies* (Diptera: culicidae)

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The effect of anti-mosquito-midgut antibodies on the development of the malaria parasite, *P. vivax* was studied by feeding the vector mosquito, *An. culicifacies* with infected blood supplemented with serum from immunized rabbits. In order to get antisera, rabbits were immunized with midgut proteins of three siblings species of *Anopheles culicifacies*, reported to exhibit differential vectorial capacity. The mosquitoes that ingested anti-midgut antibodies along with infectious parasites had significantly fewer oocysts compared to the control group of mosquitoes. The immunized rabbits generated high titer of antibodies. Their cross reactivity amongst various tissues of the same species and with other sibling species was also determined. Immunogenic polypeptides expressed in the midgut of glucose or blood fed *An. culicifacies* sibling species were identified by Western blotting. One immunogenic polypeptide of 62 kDa was exclusively present in the midgut of species A. Similarly, three polypeptides of 97, 94 and 58 kDa and one polypeptide of 23 kDa were present exclusively in species B and C respectively. Immunoelectron microscopy revealed the localization of these antigens on baso-lateral membrane and microvilli. The effects of anti-mosquito midgut antibodies on fecundity, longevity, mortality and engorgement of mosquitoes were studied. Fecundity was also reduced significantly. These observations open an avenue for research toward the development of a vector-based malaria parasite transmission-blocking vaccine.

**Keywords**: Anti-mosquito midgut antibodies, *Anopheles culicifacies*, *Plasmodium vivax*

The mosquito midgut represents one of the most challenging environments for the survival and development of *Plasmodium*¹. The sporogonic development of *Plasmodium*, from gamete to oocyst formation, takes place in the lumen and epithelium of the mosquito midgut. It therefore forms one of the most attractive sites for novel antigenic targets so as to draw malaria control strategies like transmission blocking vaccine (TBV).

Among members of the *Anopheles culicifacies* complex, only species A, B and C have been colonized so far. Various host-parasite interaction studies involving species A, B and C and different *Plasmodium* species unequivocally demonstrated that species A and C are highly susceptible while species B is the least susceptible to human²,³ and rodent malaria parasites⁵. The natural variability in malaria susceptibility because of differential genetic factors of mosquitoes is not yet been clearly understood. However, studies on *An. culicifacies* have shown correlation between different types of mosquito immune responses and differential susceptibility to various parasitic infections⁵. Vector internal organs (concealed antigens) can induce artificial immune responses in various species viz., *An. stephensi*²,⁶-⁹. Anti-midgut antibodies have already been postulated to cause deleterious effects on the reproductive capacity of mosquitoes and they simultaneously block parasite development in the mosquito midgut¹⁰,¹¹. Presence of some common antigenic peptides has already been indicated in different species of mosquitoes and also in various tissues (viz., salivary gland, midgut and haemolymph) of a given mosquito species⁷,¹². However, no such study appears to have been undertaken on any sibling species complex of mosquitoes.

Various studies unequivocally incriminated *An. culicifacies* as the major vector, responsible for 70% of malaria cases in India. *An. culicifacies* exists as a complex of five sibling species provisionally designated as A, B¹³, C¹⁴, D¹⁵ and E¹⁶. These sibling species are reported to have various biological differences viz., their distribution,
response to insecticides\textsuperscript{17} host preferences\textsuperscript{18} and vectorial capacity\textsuperscript{19}. \textit{An. culicifacies} A and C are primary vectors whereas, species B has very little role if at all, in the transmission of malaria\textsuperscript{20}.

The present study has been designed to determine the effect of anti-mosquito midgut antibodies on the development of malaria parasite and fecundity of the vector mosquito.

Materials and Methods

\textit{Mosquito rearing}—Cyclic colonies of \textit{An. culicifacies} Species A (Dhera Strain), B (Ladpur strain) and C (Raurkela strain) were maintained in an insectary at 28± 2°C and 70-80% RH and fitted with a simulated dawn and dusk machine to maintain a photoperiod of 14 h light and 10h dark. Adult mosquitoes were kept in 30 cubic cm cloth cages and were fed on 1% glucose solution and water soaked resins. Females were allowed to feed on rabbit blood for ovarian development. On the third day post-blood-feeding, females were allowed to lay eggs on filter paper lined in water filled plastic bowls. Larvae were reared in enamel trays and were fed on yeast extract and dog biscuits in the ratio 2:3 (w/w). The pupae were transferred to fresh bowl and kept in cloth cages for emergence to adult mosquitoes.

\textit{Antigen preparation and immunization}—Midguts from either glucose or blood fed \textit{An. culicifacies} sibling species mosquitoes were dissected out in ice cold phosphate buffered saline (PBS) containing 2 mM phenyl methyl sulfonly fluoride (PMSF). Antiseras were raised in New Zealand White rabbits by injecting subcutaneously with 100 µg protein/per kg body weight only once as described earlier\textsuperscript{10}. The control rabbits were injected with PBS and Freund’s adjuvant in the similar manner. Rabbits of either sex and irrespective of age were procured from H.A.U. Hisar after animal ethical clearance.

\textit{In vitro and in vivo ELISA}—Antibody titer was determined in dilution of antisera raised against midgut proteins from either glucose or blood fed mosquitoes from three sibling species of \textit{An. culicifacies} using \textit{in vitro} ELISA\textsuperscript{10}. Immunizing antigen (10 µg/ml) was used to coat the 96-wells ELISA plate (Tarsons, India). The antigen coated plate was incubated for 1 h at room temperature and then at 4°C overnight. This was followed by blocking for 1 h at room temperature with 5% dried skimmed milk (DSM) in PBS. Washing was done five times with PBS-Tween-20 (PBS-T) on the ELISA washer (Thermo Labsystem, USA) followed by incubation with alkaline phosphatase conjugated goat anti-rabbit IgG (1:5000) for 2 h. Anti-rabbit IgG was used because IgG was the primary antibody generated after infection. The immune complex was detected by p-nitrophenyl phosphate substrate system. Absorbance was read at 405 nm on ELISA reader (Thermo Scientific, USA).

The cross reactivity of the midgut antibodies with other tissues (salivary glands, hemolymph, ovary and midgut from other members of sibling species complex) was also examined by \textit{in vivo} ELISA as described previously\textsuperscript{4}. \textit{In vivo} ELISA, 20 mosquitoes were selected randomly after 24 h of blood feeding from immunized blood fed and non-immunized blood fed mosquitoes as control.

\textit{SDS PAGE}—Soluble proteins were separated by SDS-PAGE using thick slab gels (1 mm) containing 10% acrylamide and a tris-glycine (pH 8.6) buffer system\textsuperscript{10,20}. Equivalent amount of proteins from all the tissues were loaded in duplicates. One-half of the gel was silver stained\textsuperscript{21} and the other half was transferred electrophoretically to 0.45 nm nitrocellulose sheet for Western blotting\textsuperscript{22}.

\textit{Immunoblotting}—Nitrocellulose sheet with separated midgut proteins of glucose or blood fed mosquitoes from different members of \textit{An. culicifacies} species complex and other tissues (salivary glands, ovary and haemolymph of the same species) was blocked with 5% (w/v) dried skimmed milk (DSM) in PBS. Nitrocellulose membrane was incubated with hyper-immunized serum (primary antibody 1:100) in PBS for 1 h, washed with PBS containing tween-20 (0.1%) and then incubated with alkaline phosphatase (ALP) conjugated goat anti-rabbit antibodies (1:1000) in PBS for 1 h followed by washing. Antibody binding was visualized using BCIP-NBT substrate\textsuperscript{10}.

\textit{Immunized blood feeding, fecundity, mortality and longevity of mosquitoes}—Egg-laying pattern in \textit{An. culicifacies} mosquitoes fed on rabbits (immunized with midgut of either glucose or blood fed mosquitoes belonging to three sibling species) was examined. Different sets of 8-10 h starved mosquitoes were allowed to feed on sensitized rabbit for eight consecutive weeks after the last booster, i.e every week new set of mosquitoes were fed. Each set consisted of about 120 female mosquitoes. The procedure to evaluate the percentage reduction in fecundity, hatchability, mortality and longevity has been described in detail by Suneja \textit{et al}\textsuperscript{10}. Two days after feeding, 5 sub-sets were made from each group.
Total number of eggs laid, larvae hatched and the number of dead mosquitoes were observed. Ovaries of the females were also examined for the presence of any retained eggs after egg laying. Total number of egg production during first gonotrophic cycle was then calculated by adding up oviposited and retained eggs of females fed on blood from the sensitized and control rabbits. Fecundity, hatchability, engorgement and increase in mortality were calculated as under:

\[
\text{Fecundity} = \frac{\text{Total No. of eggs laid}}{\text{Total No. of females}}
\]

\[
\text{Mortality} = \frac{\text{Total No. of dead females}}{\text{Total No. of females}}
\]

\[
\text{Engorgement} = \text{Weight of mosquito after blood meal} - \text{Weight of mosquito before blood meal}
\]

\[
\text{Reduction in fecundity} (\%) = \frac{\text{No. of eggs laid/Control female} - \text{No. of eggs laid/Immunized Female}}{\text{No. of eggs laid/Control female}} \times 100
\]

\[
\text{Reduction in hatchability} (\%) = \frac{\text{No. of eggs hatched/control female} - \text{No. of eggs hatched/immunized female}}{\text{No. of eggs hatched/control female}} \times 100
\]

\[
\text{Increase in mortality} (\%) = \frac{\text{No. of mosquito died (immunized)}/\text{No. of mosquito died (control)}}{\text{No. of mosquito died (immunized)}} \times 100
\]

Transmission blocking (\%) = \frac{\text{Mean oocyst number in control-Mean oocyst number in mosquitoes fed on immunized blood} \times 100}{\text{Mean oocyst number in control}}

Mann-Whitney \text{\textit{u}}\text{ test (one-tailed) was applied by using the GRAPH-PAD prism software to evaluate the difference in oocyst count and also to determine significance of fecundity reduction between experimental and control groups.}

Immunoelectron microscopy—Localization of the midgut proteins was examined by immunoelectron microscopy as per Dinglasan et al.\textsuperscript{25}. Briefly, unfed midguts from 8- to 10-day-old \textit{An. culicifacies} mosquitoes were fixed in PBS containing 1% paraformaldehyde and 0.1% glutaraldehyde for 24 h at room temperature. Ultrathin sections of guts, embedded in LR-White resin were mounted on nickel grids and immunostained with PAbs raised against midgut proteins of \textit{An. culicifacies}. Antibody binding was visualized by using a 10-nm-colloidal gold-conjugated goat anti-mouse IgG. The grids were counter stained with uranyl acetate and examined with a Morgagni-268D Transmission electron microscope.

Results

The antibody titers in the rabbits (six sets immunized with the antigens of the midgut from \textit{An. culicifacies} species complex fed on either glucose or blood) reached at least $1:10^7$ during the third week after immunizations, as revealed by \textit{in vitro} ELISA. This level reached at peak during the third week after the last booster, then declined to the minimum level during the sixth week (Fig. 1).

Anti-midgut antibodies were capable of binding to various tissues viz., salivary glands, haemolymph and ovary and also to the midgut of other sibling species (Fig. 2 a and b). However, the highest cross-reactivity was observed between the salivary glands and midgut of the same species. These results exhibit that antibodies fed to the mosquitoes traverse through the midgut wall and reach other target tissues. The cross reactivity of these antibodies with various tissues and the midgut of other sibling species was also studied by Western blotting (Figs 3, 4 and 5). This demonstrated that species A and C (primary vectors) are more closely related than species B (poor vector), as antibodies raised against midgut of species A reacted with midgut proteins of species C and vice-versa but antibodies raised against midgut proteins of species B (especially glucose fed) exhibited minimal cross reactivity to the midgut of species A or C.
Species-specific polypeptides—Species specific anti-mosquito midgut antibodies helped in recognizing the following peptides of the midgut from both glucose and blood fed mosquitoes. One 66 kDa polypeptide was recognized in the midgut of all the three sibling species; 92 kDa polypeptide was recognized only in species A and C. One polypeptide (62 kDa) was exclusively present in the species A. Three polypeptides (97, 94 and 58 kDa) were found specifically in species B.

Blood and glucose meal induced polypeptides—After feeding the mosquitoes on blood, the following immunogenic polypeptides were recognized in their midgut. One polypeptide of 47 kDa could be recognized in the midgut of all the three sibling species. Two peptides of 80 and 70 kDa were
recognized only in the midgut of species A and C. Similarly, 40kDa peptide was present in species C and 76 kDa peptide in species B. However, one polypeptide (39 kDa) was observed only in the glucose fed An. Culicifacies A (Fig. 3b).

**Cross-reactivity in the midgut**—Antibodies raised against the midgut proteins of either glucose or blood fed mosquitoes of species A could commonly recognize two polypeptides (66 and 62 kDa) in the midgut of the other two sibling species B and C (Fig. 3a and b). However, 39 kDa polypeptide was revealed in all the three species with antibodies raised against the midgut of glucose fed mosquitoes of species A (Fig. 3b). Similarly three polypeptides (86, 52 and 47 kDa), were also recognized in the midgut of all three species by the immune sera raised against the midgut of blood fed An. Culicifacies A (Fig. 3a).

Antiserum against the midgut proteins of species A (glucose fed) could recognize 92 and 14 kDa polypeptides in the midgut of species- A and C. Similarly, the immune sera raised against the midgut proteins of blood fed species-A recognized two polypeptides i.e. 80 and 70 kDa in the species A and C.

Antibodies against the glucose fed midgut proteins of An. culicifacies C revealed 92 and 66 kDa (Fig. 3a and b). However, 39 kDa polypeptide was revealed in all the three species with antibodies raised against the midgut of glucose fed mosquitoes of species A (Fig. 3b). Similarly three polypeptides (86, 52 and 47 kDa), were also recognized in the midgut of all three species by the immune sera raised against the midgut of blood fed An. Culicifacies A (Fig. 3a).

Antiserum against the midgut proteins of species A (glucose fed) could recognize 92 and 14 kDa polypeptides in the midgut of species- A and C. Similarly, the immune sera raised against the midgut proteins of blood fed species-A recognized two polypeptides i.e. 80 and 70 kDa in the species A and C.

Antibodies against the glucose fed midgut proteins of An. culicifacies C revealed 92 and 66 kDa

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**Fig. 4**—Tissue and species specific immunogenic polypeptides recognized by antisera raised against the midgut proteins of (a) blood-fed, (b) glucose-fed An. culicifacies A mosquitoes. SG—Salivary glands; HL—Haemolymph; MG—Midgut; OV—Ovary.

**Fig. 5**—Tissue and species specific immunogenic polypeptides recognized by antisera raised against the midgut proteins of (a) blood-fed, (b) glucose-fed An. culicifacies B mosquitoes. SG—Salivary glands; HL—Haemolymph; MG—Midgut; OV—Ovary.
polypeptides in the midgut of all the three sibling species (Fig. 5b). Similarly three more polypeptides (66, 47 and 40 kDa) could be resolved in all the three sibling species when reacted with the antibodies against the blood fed midgut of species C (Fig. 5a).

It was interesting to note that the antibodies raised against the midgut of An. culicifacies B did not exhibit cross reactivity to the midgut of species-A or C (Fig. 4a).

Cross-reactivity among the various tissues was also determined by Western blots and is shown in Table 1.

**Effect on fecundity of mosquitoes**—Significant reduction in fecundity was observed in An. culicifacies mosquitoes after they had ingested anti-midgut antibodies as compared to the control. Maximum reduction (40%, \( P<0.01 \)) in fecundity was observed in the mosquitoes fed on rabbit immunized with midgut antigens of the glucose fed mosquitoes during the third week after immunization (Fig. 6b). Similarly, fecundity was reduced by 37% (\( P<0.05 \)) in mosquitoes after they had ingested polyclonal antibodies raised against the midgut proteins of blood fed mosquitoes (Fig. 6a). However, no statistically significant difference was noticed in engorgement, longevity and mortality of the females fed on immunized and non-immunized serum.

**Transmission blocking activity**—Significant reduction in parasite infection was observed in An. culicifacies mosquitoes that ingested anti-midgut polyclonal antibodies added to the P. vivax infected blood, as compared to the control. Statistical analysis showed that the reduction in infection rate and number of oocysts was more in glucose fed than in blood fed. The infection rate in An. culicifacies reduced by about 27% and the mean number of oocysts, per infected mosquito reduced by about 90% (\( P<0.01 \)) after they had ingested polyclonal antibodies raised against midgut proteins of glucose fed mosquitoes along with the blood infected with malarial parasite (Table 2). Similarly, infection rate

<table>
<thead>
<tr>
<th>Antibodies against midgut of</th>
<th>Cross reactive polypeptides (kDa) in various tissues recognized by Anti-midgut antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>An. culicifacies A (BF)</td>
<td>66, 62, 52, 47 and 43 66, 62, 60, 58, 52, 47 and 43</td>
</tr>
<tr>
<td>An. culicifacies A (GF)</td>
<td>92, 43 and 39 50, 39 -</td>
</tr>
<tr>
<td>An. culicifacies B (BF)</td>
<td>- 60, 58 and 56 47</td>
</tr>
<tr>
<td>An. culicifacies B (GF)</td>
<td>66 - -</td>
</tr>
<tr>
<td>An. culicifacies C (BF)</td>
<td>66, 60, 52 and 50</td>
</tr>
<tr>
<td>An. culicifacies C (GF)</td>
<td>66 and 43 - -</td>
</tr>
</tbody>
</table>

BF—Blood fed; GF—Glucose fed

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Table 1—Cross reactive peptides amongst various tissues in An. culicifacies species complex (glucose or blood fed)
reduced by 25% and the mean number of oocysts per infected mosquito by about 87% \((P<0.01)\) after they ingested antibodies raised against the midgut proteins of blood fed mosquitoes (Table 2).

**Immunolocalization**—PAbs recognize midgut antigens that are specifically located along the brush border which lines the apical side of the gut epithelial cells facing the lumen as observed by immunoelectron microscopy. Besides the microvillar region, PAbs labeled the basement membrane of the epithelial cells (Fig. 7 a, b, c).

**Discussion**

The present results demonstrated that antibodies raised against midgut proteins not only blocked parasite \((P. \text{vivax})\) development but concomitantly reduced the fecundity of \(\text{An. stephensi}\) mosquitoes as reported earlier \(^{11,10,27}\).

The reduced fecundity of \(\text{An. culicifacies}\) mosquitoes fed on immunized rabbit suggested that anti mosquito antibodies somehow interfere with the normal process of oogenesis. However, it did not seem to be related to any decline in the rate of oviposition. A few female mosquitoes from each feeding set were dissected after they had been fed on sensitized or control rabbit. All seemed to have laid their full complement of eggs. The mechanism of the anti-mosquito response is not yet known but could be one or combination of several factors i.e. fat body synthesis of vitellogenins may be down regulated, the uptake of circulatory vitellogenins may be inhibited or the content of some of the developing follicles may be reabsorbed \(^{10}\). Alternatively, the injected anti-mosquito antibodies may irritate the gut which in turn reduce the blood meal or reduce availability of nutrients. Moreover it is yet to identify the change in vitellogenesis \(^{26}\). It has also been observed that anti-micro villi antibodies bind to the midgut wall and can inhibit normal epithelial processes, such as enzyme or peritrophic membrane secretion \(^{1}\).

Previous observations on \(\text{Ae. aegypti}\) and \(\text{An. stephensi}\) mosquitoes, fed on similarly produced anti-mosquito antibodies, showed that the effects on mosquito mortality are small and variable \(^{2,10,27}\). However, the present investigations demonstrated that the ingestion of rabbit anti-\(\text{An. culicifacies}\) antibodies had no statistically significant effect on its mortality and longevity. Differences in the results could also be attributed to the amount of protein ingested, differences in antibody titers, biological activities (influenced by factor such as complement fixing ability and stability to proteases in the midgut) and specificity influenced by factors such as antigen being derived from blood and sugar fed mosquitoes.

The present study also indicated that anti-mosquito midgut antibodies when ingested with infected blood, adversely affect the development of oocysts in the midgut and/or translocation of sporozoites into the salivary glands. These results are in agreement with the findings of Suneja et al. \(^{10}\).

![Image](image_url)

**Fig. 7**—Immunoelectron microscopy of unfed \(\text{An. culicifacies}\) midgut. Transverse section of the midgut showing the association of antibodies with the (a) basolateral membrane of epithelial cell (b) extracellular microvilli (c) extracellular glycocalyx \((35,000\times)\).

<table>
<thead>
<tr>
<th>No. of mosquitoes</th>
<th>Fed/ group</th>
<th>Infected mosquitoes (%)</th>
<th>Mean no. of oocysts /mosquito</th>
<th>Transmission blocking (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Anti-MG</td>
<td>Control</td>
</tr>
<tr>
<td>Glucose fed</td>
<td>70</td>
<td>62</td>
<td>37</td>
<td>27</td>
</tr>
<tr>
<td>Blood fed</td>
<td>59</td>
<td>56</td>
<td>40</td>
<td>30</td>
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* Significant at \(P<0.001\)
The target molecule for the fecundity reduction and their relationship to the molecules mediating reduction in the infectivity of malarial parasite, if any, are yet to be determined. For the anti-mosquito antibodies to affect the parasite, they must either inhibit a physiological process essential to the parasite or block the normal ookinete-midgut interaction that may or may not be cell-specific. The reduced intensity of infections after ingestion of blood along with anti-midgut antibodies can be attributed to the inhibition of cellular interactions which are possibly directed at glycoproteins on the mosquito midgut cell surface. Antibodies generated in the present study recognized the antigens present at microvillar, glyocalyx and baso-lateral membrane. Oligosaccharides on gut microvillar glyco-conjugates have already been implicated as both receptors for microbial attachment and as a protective barrier against pathogens in both vertebrates and invertebrates. Midgut microvilli (MMV) glycoconjugates have been shown to play a role in establishment of parasite infections in the mosquitoes.

The disruption of the ookinete-to-oocyst transition may therefore occur either at the level of attachment to the glyocalyx or to the microvillus itself or at the level of more downstream events following invasion, e.g. interference of cell signaling and/or cell functioning. The reduction in the number of oocysts developed in the midgut may be due to two reasons. First, antibodies against midgut may have similar target sites for the parasite to recognize and invade, therefore may competitively inhibit the parasite. Second, these antibodies may adhere and lead to allosteric alteration in the receptor recognition site in the midgut and hence deny ookinetes to recognize and subsequently adhere to the target site.

Three immunogenic polypeptides (97, 94 and 58 kDa) exclusively present in the poor vector (An. culicifacies-B) in both glucose and blood fed mosquitoes may be involved in defence reactions directed against Plasmodium. Immunological superiority of An. culicifacies B refractory phenotype over susceptible strains has also been reported.

Presence of 62 kDa peptide in species-A, 97, 94 and 58 kDa in species-B and 23 kDa peptide in species-C may be used to target for designing the species-specific immunodiagnostic probes.

Extensive cross-reactivity of anti-mosquito midgut antibodies with various tissues viz., salivary glands, haemolymph and ovaries of same species vis-à-vis midgut of other sibling species, as observed by Western blots and in vivo ELISA may be attributed to the conserved antigens/epitopes in different tissues, or non-specific binding by low affinity antibodies. These experiments reiterated previous reports that anti-midgut antibodies could traverse through the midgut and reach other tissues.

Development of vector based malaria transmission blocking vaccines (TBVs) remains one such pragmatic approach that can complement or replace existing control methods. The results from the present study assertively implicated the midgut as the likely source for candidate antigens for malaria transmission blocking vaccine, however, the final formulation should be designed concocting multiple antigens from multiple tissues.

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References

2 Alger N E & Cabrera E J, An increase in death rate of Anopheles stephensi fed on rabbit immunized with mosquito antigen, J Eco Entomol, 65 (1972) 165.
10 Suneja A, Gulia M & Gakhar S K, Blocking of malaria parasite development in mosquito and fecundity reduction
22 Towbin H, Stachelin T & Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets, Procedure and some applications, PNAS, USA 76 (1979) 4350.